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**UDP-GLUCURONOSYLTRANSFERASES (UGTS) IN CHEMICALLY-INITIATED
TOXICITY**

by

PERRY M. KIM

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy,
Graduate Department of Pharmaceutical Sciences, University of Toronto

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UDP-Glucuronosyltransferases (UGTS) in chemically-initiated toxicity.

Doctor of Philosophy, 1998

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ABSTRACT

The majority of xenobiotics and endobiotics are eliminated through glucuronidation catalyzed by the UDP-glucuronosyltransferase (**UGT**) superfamily of isozymes. Glucuronidation competes with enzymatic (cytochromes P450, peroxidases) bioactivation which can form potentially toxic reactive intermediates. Since glucuronidation (i.e. elimination), unlike bioactivation, generally is a quantitatively major pathway, UGT deficiencies leading to a decrease in glucuronidation may significantly enhance xenobiotic bioactivation and thus potentially increase xenobiotic-initiated toxicity. This laboratory has previously shown, through *in vitro* and *in vivo* studies, that UGT deficiencies can increase susceptibility to both acetaminophen- and benzo[a]pyrene (**B[a]P**)-initiated toxicities (cytotoxicity, nephrotoxicity, hepatotoxicity, covalent binding, micronucleus formation). We have now provided the first evidence that *in vitro* B[a]P-initiated micronucleus formation, like carcinogenic initiation, is most likely due to enzymatic bioactivation of B[a]P to a reactive intermediate, and that the molecular lesion responsible may be oxidation of DNA and possibly protein (Free Rad. Biol. Med. 23: 579-596, 1997). Furthermore, we have shown for the first time through *in vitro* and *in vivo* studies that UGT deficiencies increase toxicological susceptibility to both the potent tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (**NNK**), and the teratogen phenytoin and its major para-hydroxylated metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin (**HPPH**). Micronucleus formation, which may involve reactive oxygen species formation (**ROS**) and DNA oxidation initiated by NNK, phenytoin and HPPH was enhanced in both heterozygous (+/j) and homozygous (j/j) UGT-deficient fibroblasts (Cancer Res. 56: 1526-1532, 1996; J. Pharmacol. Exp. Ther. 280: 200-209, 1997). Using salicylate hydroxylation as a measure of hydroxyl radical (**•OH**) formation, we demonstrated *in vivo* that peroxidase-catalysed bioactivation of phenytoin and possibly HPPH can initiate **•OH** formation (Mol. Pharmacol. 49: 172-181, 1996), and that **•OH** formation is increased in phenytoin

and HPPH-treated UGT-deficient rats, which are compromised in their ability to glucuronidate these compounds, suggesting a possible mechanism for increased toxicological susceptibility (Proc. 8th International Workshop on Glucuronidation, Abstr. No. 42). In these same rats we have shown that phenytoin-initiated embryotoxicity (*in utero* death) is increased in heterozygous (+/) UGT-deficient rats compared to UGT-normal (+/+) controls (Toxicol. Sci. 42, Abstr. No. 1288, 1998). These results show that UGT deficiencies can increase xenobiotic-initiated toxicity and therefore suggest that similar UGT deficiencies in the human population may prove to be important determinants of human susceptibility to carcinogenesis and teratogenesis.

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TABLE OF CONTENTS

	Page #
Abstract	ii
Acknowledgements	iv
List of Tables	iii
List of Figures	iv
List of Abbreviations	viii
List of publications and presentations arising from this thesis	x

SECTION 1: INTRODUCTION

1.1 RATIONALE AND RESEARCH OBJECTIVES	2
--	---

1.2 UDP-GLUCURONOSYLTRANSFERASES (UGTS)

1.2.1 INTRODUCTION	5
---------------------------------	---

1.2.2 BIOCHEMISTRY

1.2.2.1 Gene/Regulation.....	7
1.2.2.2 Protein/Topology.....	8
1.2.2.3 Induction.....	9
1.2.2.4 UGT Isozymes and Substrates.....	11

1.2.3 ANIMAL AND CELLULAR MODELS OF UGT DEFICIENCIES

1.2.3.1 Gunn/RHA rat.....	12
1.2.3.2 Glucuronidation as a Potentiator of Toxicity.....	13

1.2.4 HUMAN UGT DEFICIENCIES

1.2.4.1 Mutation.....	15
1.2.4.2 Clinical Manifestations.....	16
1.2.4.3 Altered Xenobiotic Metabolism and Related Toxicities.....	17

1.3 CYTOCHROMES P450

1.3.1. Constitutive P450s	20
--	----

1.3.2 Non-constitutive P450s	21
---	----

1.4 PROSTAGLANDIN H SYNTHASES

1.4.1 PHS-1.....	23
1.4.2 PHS-2.....	24
1.5 DNA DAMAGE	
1.5.1 MOLECULAR ASPECTS	
1.5.1.1 Oxidation.....	26
1.5.1.2 Covalent Binding.....	27
1.5.2. DNA REPAIR	
1.5.2.1 Oxidation/Strand breaks.....	28
1.5.2.2 Arylation /Methylation.....	29
SECTION 2: STUDIES	
2.1 STUDY #1: PEROXIDASE-DEPENDENT BIOACTIVATION AND OXIDATION OF DNA AND PROTEIN IN BENZO[A]PYRENE-INITIATED MICRONUCLEUS FORMATION.	
2.1.1 Abstract.....	33
2.1.2 Introduction.....	34
2.1.3 Materials and Methods.....	38
2.1.4 Results.....	48
2.1.5 Discussion.....	53
2.2 STUDY#2: GENOPROTECTION BY UDP-GLUCURONOSYLTRANSFERASES IN PEROXIDASE-DEPENDENT, REACTIVE OXYGEN SPECIES-MEDIATED MICRONUCLEUS INITIATION BY THE CARCINOGENS 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) AND BENZO[A]PYRENE.	
2.2.1 Abstract.....	61
2.2.2 Introduction.....	62
2.2.3 Materials and Methods.....	65
2.2.4 Results.....	68
2.2.5 Discussion.....	71
2.3 STUDY #3: UDP-GLUCURONOSYLTRANSFERASE-MEDIATED PROTECTION AGAINST <i>IN VITRO</i> DNA OXIDATION AND MICRONUCLEUS FORMATION INITIATED BY PHENYTOIN AND ITS EMBRYOTOXIC METABOLITE 5-(p-HYDROXYPHENYL)-5-PHENYLHYDANTOIN (HPPH)	
2.3.1 Abstract.....	77
2.3.2 Introduction.....	78
2.3.3 Materials and Methods.....	82
2.3.4 Results.....	89
2.3.5 Discussion.....	92
2.4 STUDY #4: PHENYTOIN-INITIATED HYDROXYL RADICAL FORMATION: CHARACTERISATION BY ENHANCED SALICYLATE HYDROXYLATION.	
2.4.1 Abstract.....	99

2.4.2	Introduction.....	100
2.4.3	Materials and Methods.....	104
2.4.4	Results.....	107
2.4.5	Discussion.....	110
2.5	STUDY #5: PHENYTOIN EMBRYOTOXICITY: PROTECTION BY UDP-GLUCURONOSYLTRANSFERASES.	
2.5.1	Abstract.....	118
2.5.2	Introduction.....	119
2.5.3	Materials and Methods.....	120
2.5.4	Results.....	125
2.5.5	Discussion.....	128
SECTION 3:		
3.1	SUMMARY AND CONCLUSION.....	132
3.2	FUTURE STUDIES	
3.2.1	Animal Studies.....	136
3.2.2	Human Studies.....	138
SECTION 4: APPENDICES		
4.1	HUMAN UGT1 PROMOTER (TATAA) DEFECT GENOTYPING	
4.1.2	Introduction.....	141
4.1.2	Methods.....	141
4.1.3	Results and Discussion.....	142
4.2	INVESTIGATION OF THE TOBACCO-SPECIFIC CARCINOGEN 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) FOR <i>IN VIVO</i> AND <i>IN VITRO</i> MURINE EMBRYOPATHY AND EMBRYONIC <i>ras</i> MUTATIONS.	
4.2.1	Abstract.....	145
4.2.2	Introduction.....	146
4.2.3	Methods.....	149
4.2.4	Results.....	153
4.2.5	Discussion.....	155
SECTION 5: REFERENCES.....		160

LIST OF TABLES

SECTION 1: INTRODUCTION	Page#
Table 1a. Location and modulation of some toxicologically important animal UGTs.....	9a
Table 1b. UGT isozymes in some animal cell lines.....	9c
Table 2a. Substrates for rat UGT1 and 2 families.....	10a
Table 2b. Substrates for human UGT1 and 2 families.....	11a
Table 3. Examples of the different classes of glucuronide conjugates.....	11c
Table 4. Compounds known to form acyl glucuronides.....	14c
Table 5. Characteristics of human bilirubin UGT deficiencies.....	15a
Table 6a. Location and modulation of some toxicologically important human UGTs.....	16a
Table 6b. UGT isozymes in some human cell lines.....	16c
Table 7. Toxicologically important human constitutive and non-constitutive..... hepatic P450s and known inducers.	20b
Table 8. Toxicologically important rat constitutive and non-constitutive hepatic P450s and known inducers.	20c
Table 9. Human cytochrome P450 isozymes and toxicologically important substrates....	22a
Table 10. Rat cytochrome P450 isozymes and toxicologically important substrates.....	22c
Table 11. Substrates bioactivated by peroxidases and lipoxigenase enzymes.....	24a
Table 12. Toxicologically important xenobiotics known to either oxidize..... and/or covalently bind to essential macromolecules.	26a
Table 13a. Enzymes involved in the repair of oxidative DNA damage.....	26c
Table 13b. Representative oxidized DNA bases.....	26c
 SECTION 4.2: APPENDIX	
Table 1. Fetal structural anomalies initiated by 4-methyl-(nitrosamino)-..... 1-(3-pyridyl)-1-butanone (NNK).	153e

LIST OF FIGURES

SECTION 1: INTRODUCTION

Figure 1a.	Molecular and biochemical determinants of toxicity.....	5a
Figure 1b.	Xenobiotic glucuronidation and sulfation	5b
Figure 2.	Xenobiotic glucuronidation versus bioactivation.....	5c
Figure 3.	UGT evolutionary divergence	7a
Figure 4.	Common UGT amino acid "signature sequence.".....	7b
Figure 5.	<i>UGT1</i> locus: processing, mutations and related syndromes.....	8a
Figure 6a.	A hypothetical topological model of UGTs in the endoplasmic reticulum.....	9d
Figure 6b.	Uptake, conjugation and secretion of bilirubin in the liver.....	9d
Figure 7.	Plasma bilirubin levels in the Gunn and RHA rats.....	12a
Figure 8a.	Representative acyl glucuronide and its isomers.....	14a
Figure 8b.	Nucleophilic displacement reaction of 1-O-acyl- β -glucuronide.....	14a
Figure 8c.	Distal metabolism of an arylamine glucuronide.....	14b
Figure 9.	Enzymatic bioactivation of a representative xenobiotic.....	20a
Figure 10.	Base excision repair pathway for oxidized and alkylated DNA.....	28a
Figure 11a.	Model of recombinant double-strand DNA break (DSB) repair.....	29a
Figure 11b.	Methyl-directed mismatch repair.....	29a
Figure 12.	Molecular mechanisms involved in nucleotide excision repair in humans..... and in <i>E. coli</i> .	29b

SECTION 2.1 (Study #1)

Figure 1.	Postulated roles of P450s and peroxidases such as prostaglandin H synthase and lipoxygenase in B[a]P bioactivation and micronucleus formation.....	34b
Figure 2.	Flow chart of culturing procedure and time involved.....	41b
Figure 3.	Effect of the P450 inhibitors SKF 525A and 1-aminobenzotriazole (ABT) on the covalent binding of benzo[a]pyrene (B[a]P).....	48b
Figure 4.	Effect of 1-aminobenzotriazole (ABT) on benzo[a]pyrene (B[a]P) covalent binding to bovine serum albumin protein catalysed by horseradish peroxidase.....	48d
Figure 5.	Detection of cytochrome P4501A1 (CYP1A1) and prostaglandin H	

	synthase (PHS) protein in cultured rat skin fibroblasts.....	49b
Figure 6.	Micronucleus formation in cultured Wistar rat skin fibroblasts.....	50b
Figure 7.	Effect of P450 and peroxidase inhibitors and inducers on benzo[a]pyrene (B[a]P)-initiated micronucleus formation.....	50d
Figure 8.	Comparison of mononucleated versus binucleated/cytochalasin B method.....	50f
Figure 9.	Effect of P450 and peroxidase inhibitors and inducers on benzo[a]pyrene (B[a]P)-initiated DNA oxidation in cultured skin fibroblasts.....	51b
Figure 10.	Effect of peroxidase inhibitors and inducers on benzo[a]pyrene (B[a]P)-initiated protein oxidation in cultured skin fibroblasts.....	51d

SECTION 2.2 (Study #2)

Figure 1.	Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to its major glucuronide metabolites.....	62b
Figure 2.	Effect of UDP-glucuronosyltransferase (UGT) deficiency and concentration of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on micronucleus formation.....	68b
Figure 3.	Comparison of the potencies of 10 μ M benzo[a]pyrene (B[a]P) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in initiating micronucleus formation.....	68d
Figure 4.	Effect of UDP-glucuronosyltransferase (UGT) deficiency, cytochromes P4501A1/2 (CYP1A1/2) and/or peroxidase induction and antioxidative cytoprotection on benzo[a]pyrene (B[a]P)-initiated micronucleus formation.....	68f
Figure 5.	Effect of UDP-glucuronosyltransferase (UGT) deficiency, cytochromes P4501A1/2 (CYP1A1/2) and/or peroxidase induction or inhibition and antioxidative cytoprotection on (methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-initiated micronucleus formation.....	69b

SECTION 2.3 (Study #3)

Figure 1.	Postulated genoprotective and cytoprotective roles of UDP-glucuronosyltransferases (UGTs) in peroxidase-catalysed phenytoin and 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) bioactivation and toxicity.....	78b
Figure 2.	Effect of UDP-glucuronosyltransferase (UGT) deficiency and concentration of 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) on micronucleus formation.....	89b
Figure 3.	Comparison of the potencies of phenytoin and 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) in initiating micronucleus formation.....	89d
Figure 4.	Effect of UDP-glucuronosyltransferase (UGT) deficiency on DNA oxidation initiated by 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH).....	90b

Figure 5.	Embryotoxicity of 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) in CD-1 mouse embryo culture.....	90d
Figure 6.	Collision activation spectra of urinary N3-glucuronide of phenytoin.....	90f

SECTION 2.4 (Study #4)

Figure 1.	Phenytoin bioactivation and subsequent salicylate hydroxylation by hydroxyl free radicals.....	100b
Figure 2.	Analysis of plasma concentrations of 2,3-dihydroxybenzoic acid (DHBA) and its glucuronide conjugate by high-performance liquid chromatography (HPLC).....	107b
Figure 3.	<i>In vivo</i> and <i>in vitro</i> characterization of 2,3-dihydroxybenzoic acid (DHBA) glucuronidation.....	107d
Figure 4.	Paraquat (PQ)-initiated hydroxyl radical formation as measured via 2,3-dihydroxybenzoic acid (DHBA) production.....	107f
Figure 5.	Phenytoin-initiated dose-dependent <i>in vivo</i> hydroxyl radical formation measured via 2,3-dihydroxybenzoic acid (DHBA) formation.....	108b
Figure 6.	Comparison of phenytoin-initiated <i>in vivo</i> hydroxyl radical formation when acetylsalicylic acid (ASA) was administered before or after phenytoin.....	108d
Figure 7.	Formation of the 2,5-isomer of dihydroxybenzoic acid (2,5-DHBA) in mice treated with acetylsalicylic acid followed by paraquat or phenytoin.....	108f
Figure 8.	Urinary concentrations of 2,3- and 2,5-dihydroxybenzoic acid (DHBA).....	108h

SECTION 2.5 (Study #5)

Figure 1.	Postulated protective roles of UDP-glucuronosyltransferases (UGTs) in phenytoin and 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) bioactivation and toxicity.....	119b
Figure 2.	Effect of UDP-glucuronosyltransferase (UGT) deficiency on phenytoin-initiated plasma 2,3-dihydroxybenzoic acid (DHBA) formation, as a measure of hydroxyl radical formation.....	125b
Figure 3.	Effect of UDP-glucuronosyltransferase (UGT) deficiency on phenytoin-initiated urinary 2,3-dihydroxybenzoic acid (DHBA) formation.....	125d
Figure 4.	Effect of UDP-glucuronosyltransferase (UGT) deficiency on 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH)-initiated plasma 2,3-dihydroxybenzoic acid (DHBA) formation.....	125f
Figure 5.	Effect of UDP-glucuronosyltransferase (UGT) deficiency on 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH)-initiated urinary 2,3-dihydroxybenzoic acid (DHBA) formation.....	125h
Figure 6.	Effect of UDP-glucuronosyltransferase (UGT) deficiency on phenytoin/HPPH-initiated lipid peroxidation.....	125j

Figure 7.	Autoradiogram of thin layer chromatographic plates.....	125b
Figure 8.	<i>In vitro</i> UGT1A1-catalysed bilirubin mono- and di-glucuronidation.....	126d
Figure 9.	Effect of 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) concentration and alamethicin activation on UGT1A1-catalysed glucuronidation of HPPH.....	126f
Figure 10.	High-performance liquid chromatography (HPLC)-UV chromatograms of 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) glucuronide.....	126h
Figure 11.	Phenytoin-initiated embryotoxicity in Gunn rats.....	126j
Figure 12.	Postpartum lethality of new born pups from phenytoin-treated Gunn rats.....	126l
Figure 13.	Phenytoin-initiated embryotoxicity in RHA rats.....	126n
Figure 14.	PCR digestion pattern of UGT1 mRNA.....	127b

SECTION 4.2: APPENDIX

Figure 1.	Postulated metabolic pathway mediating the toxicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).....	146b
Figure 2.	<i>In vivo</i> teratogenicity of NNK in CD-1 mice.....	153b
Figure 3.	<i>In vivo</i> effect of phenobarbital pretreatment on NNK teratogenicity in CD-1 mice.....	153d
Figure 4.	<i>In vitro</i> embryotoxicity of NNK in embryo culture.....	154b
Figure 5.	Mutational analysis of codon 12 of the <i>K-ras</i> gene using the PCR-PIREMA technique.....	154d

LIST OF ABBREVIATIONS

+/+	homozygous UGT-normal
+/j	heterozygous UGT-deficient
j/j	homozygous UGT-deficient
3-MC	3-methylcholanthrene
8-OH-2'-dG	8-hydroxy-2'-deoxyguanosine
AA	arachidonic acid
ABT	1-aminobenzotriazole
Ah	aromatic hydrocarbon
APAP	acetaminophen
ASA	acetylsalicylic acid
B[a]P	benzo[a]pyrene
CYP	cytochrome P450
CYP1A1	cytochrome P4501A1
DAPI	4',6-diamidino-2-phenylindole
DHBA	dihydroxybenzoic acid
DMSO	dimethyl sulfoxide
DRE	dioxin-responsive element
ETYA	5,8,11,14-eicosatetraenoic acid
GD	gestational day
H ₂ O ₂	hydrogen peroxide
HPLC	high-performance liquid chromatography
HPPH	5-(<i>p</i> -hydroxyphenyl)-5-phenylhydantoin
IL-1 α	interleukin-1 α
LPO	lipooxygenase
GSH	glutathione
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
•O H	hydroxyl radical
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PHS	prostaglandin H synthase
PG	prostaglandin
RHA	roman high avoidance strain
ROS	reactive oxygen species
SKF 525A	β -diethylaminoethyl-diphenylpropylacetate HCl
SOD	superoxide dismutase
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TPA	12-O-tetradecanoylphorbol-13-acetate
UDPGA	uridine-diphosphate glucuronic acid
UGT	UDP-glucuronosyltransferase

LIST OF PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS**REFEREED PAPERS:**

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- Wells, P.G., Kim, P.M., Laposa, R.R. Nicol, C.J., Parman, T. and Winn, L.M. (1997) Oxidative damage in chemical teratogenesis. *Mutat. Res.* 396: 65-78.

SUBMITTED PAPERS:

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SECTION 1: INTRODUCTION

1.1 RATIONALE AND RESEARCH OBJECTIVES

Human UDP-glucuronosyltransferase (UGT) deficiencies exist in the population with the phenotypically obvious bilirubin UGT deficiency being the most well characterized. Defects are in both the non-coding and coding regions of the *UGT1* gene complex which can lead to partial or total loss of UGT1 isozymes. A frameshift mutation in the common coding region of the *UGT1* gene complex in Gunn and RHA rats leads to a similar loss of the UGT1 isozyme family and thus these rats are an advantageous animal model for the study of human UGT1 deficiencies. UGT deficiencies in rats have been shown to increase xenobiotic-initiated toxicity (cytotoxicity, nephrotoxicity, hepatotoxicity, embryotoxicity, covalent binding, micronucleus formation) (de Morais and Wells, 1989, 1988; de Morais et al., 1992a; Wells et al., 1989a; Hu and Wells, 1992, 1993, 1994; Vienneau et al., 1995), and in humans, *in vivo* (de Morais et al., 1992b) and *in vitro* (Hu and Wells, 1988) studies suggest that individuals with decreased glucuronidation have increased acetaminophen (paracetamol) and B[a]P bioactivation, covalent binding and/or cell death.

Our studies were designed to further elucidate the toxicological relevance of UGTs in chemical carcinogenesis and teratogenesis, and to determine the potential molecular mechanism responsible for these toxicological sequelae. Our objectives were as follows:

Carcinogenesis:

- 1) To assess the carcinogenic relevance of micronucleus formation, since to our knowledge, no studies have addressed whether the molecular mechanism(s) responsible for xenobiotic-induced carcinogenic initiation are similar to those that initiate the formation of micronuclei. We determined whether reactive intermediate vs receptor-mediated mechanisms are responsible for the previously demonstrated, B[a]P-initiated micronucleus formation (Vienneau et al., 1995), and also determined whether the oxidation of essential macromolecules (e.g. DNA) is an important molecular mechanism (lesion) responsible for the formation of micronuclei (see Section 2.1).
- 2) To investigate the toxicological importance of concomitant chemical induction of enzymes

responsible for either xenobiotic bioactivation and cell cytoprotection. We determined whether a lack of UGT inducibility and concomitant P450 and/or peroxidase induction in fibroblasts cultured from UGT-deficient rats caused a significant enhancement in B[a]P-initiated micronucleus formation over that seen in similarly induced UGT-normal controls (see Section 2.2). This may prove to be particularly important since many chemicals, such as the class of chemicals known as polycyclic aromatic hydrocarbons, can induce numerous gene products which may or may not contribute to xenobiotic toxicity.

- 3) To investigate the potential clinical relevance of UGT deficiencies in predisposing individuals to toxicity initiated by the tobacco specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (**NNK**). We determined whether fibroblasts cultured from UGT-deficient rats have an increase in NNK-initiated micronucleus formation and further evaluated the bioactivating enzymes involved in the formation of micronuclei (see section 2.2).

Teratogenesis:

- 1) To assess whether UGT1 deficiencies in rats lead to a decrease in the glucuronidation of the anticonvulsant drug phenytoin and its major hydroxylated metabolite, 5-(p-hydroxyphenyl)-5-phenylhydantoin (**HPPH**) and to determine whether fibroblasts cultured from the skin of UGT-deficient rats have an increase in DNA damage (oxidation, micronucleus formation) versus those of UGT-normal cells (see section 2.3). Phenytoin is the most widely prescribed anticonvulsant drug in North America, and is teratogenic in humans. These studies are the first to address not only the potential broad toxicological implications of decreased phenytoin glucuronidation but also that of HPPH which was previously found to be non-teratogenic, although this latter result may be explained by efficient maternal glucuronidation and elimination of HPPH.
- 2) To determine whether phenytoin or HPPH can initiate *in vivo* hydroxyl radicals ($\bullet\text{OH}$) formation, and whether UGT-deficient rats have an increase in phenytoin- and/or HPPH-initiated $\bullet\text{OH}$ production (see Section 2.4 and 2.5).

- 3) To determine whether UGT-deficient rats are at an increased risk for phenytoin-initiated embryotoxicity or teratogenicity (see Section 2.5).

1.2 UDP-GLUCURONOSYLTRANSFERASES (UGTS)

1.2.1 INTRODUCTION

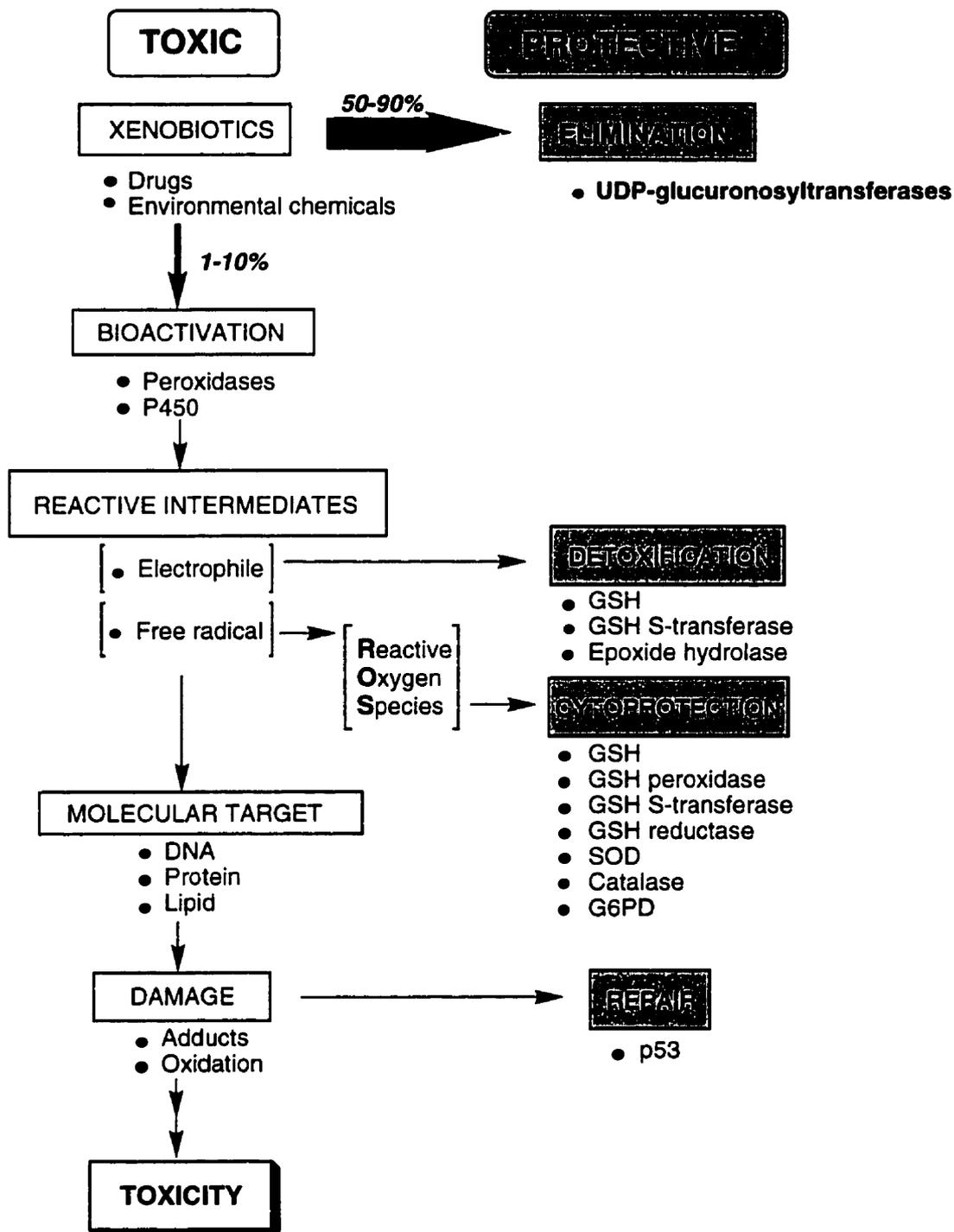
The toxicological outcome of xenobiotics often involves many different biochemical pathways, such as elimination, bioactivation, detoxification, cytoprotection and repair (**fig. 1a**). So-called, “phase II” conjugation reactions catalysed by various enzymes (UGTs, sulfotransferases, glutathione S-transferases) constitute major metabolic pathways by which the body eliminates numerous endogenous compounds (estrogen, bilirubin) and xenobiotics [acetaminophen, phenytoin, 5-(p-hydroxyphenyl)-5-phenylhydantoin (**HPPH**), benzo[a]pyrene (**B[a]P**), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (**NNK**)]. Conjugation involves attaching (conjugating) a water soluble compound [e.g. uridine-diphosphate glucuronic acid (**UDPGA**), sulfate, glutathione (**GSH**)] to the lipophilic molecule (**fig 1b**), thereby producing a highly hydrophilic product which facilitates its elimination and excretion in the urine or feces (Dutton, 1980).

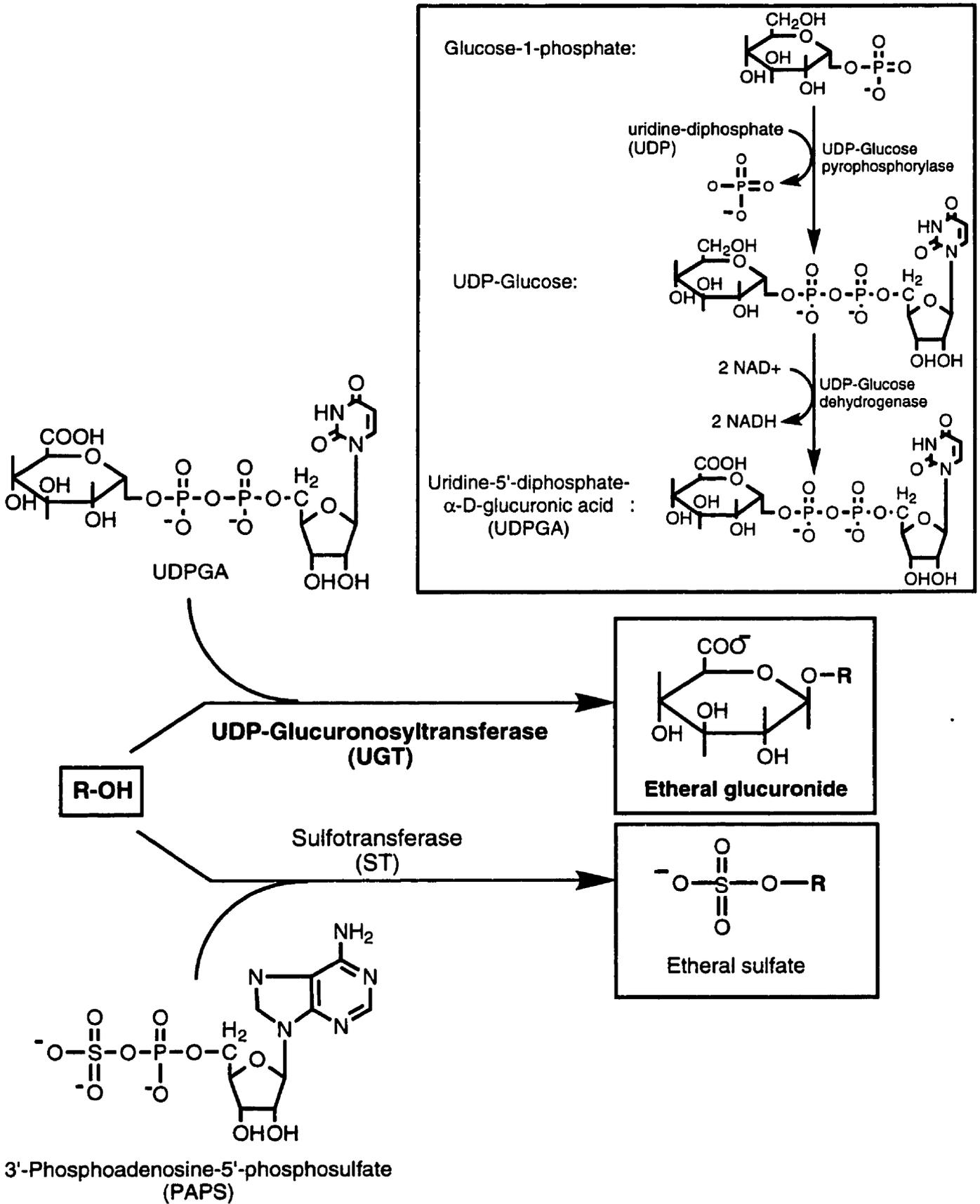
The UGT superfamily of membrane-bound isozymes exist as two families (UGT 1 and 2), and catalyzes the conjugation of most xenobiotics to UDPGA (Dutton, 1980; Bock et al., 1983; Burchell and Coughtrie, 1989; Burchell et al., 1991). The role of glucuronidation in both mitigating (acetaminophen, B[a]P, NNK) and in some cases possibly enhancing the toxicity of compounds (see Section 1.2.3.2), is considered to be an important modulator of toxicity. As discussed below, a deficiency in UGT isozyme protein and activity can reduce the quantitatively major pathway (>50%) of xenobiotic glucuronidation. This reduction can lead to impaired xenobiotic elimination which may substantially increase other pathways of biotransformation, such as bioactivation (**fig. 2**). Bioactivation under normal circumstances often constitutes a quantitatively minor metabolic pathway (1-10%) and thus relatively small decreases in major elimination pathways, such as glucuronidation, may significantly increase competing pathways of bioactivation and lead to increased toxicity.

Our lab has previously demonstrated that a reduction in glucuronidation can in fact increase xenobiotic-initiated toxicity. It has recently been demonstrated that UGT-deficiencies

FIGURE 1a

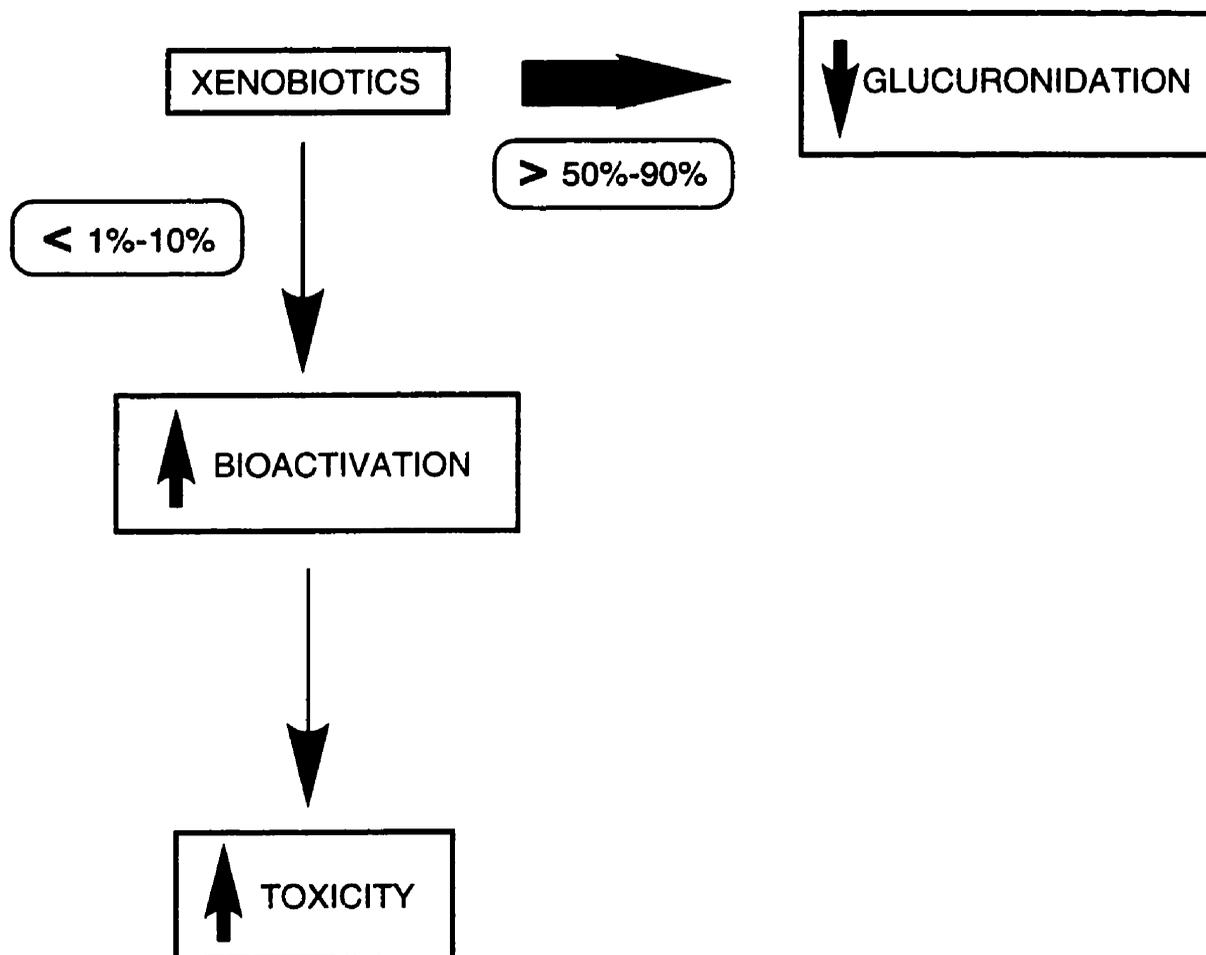
Molecular and Biochemical Determinants of Toxicity





Glucuronidation and sulfation of a representative xenobiotic with a hydroxyl functional group. UGTs and STs catalyse the conjugation of UDPGA and PAPS, respectively forming etheral conjugates. Inset figure shows the formation of UDPGA in the cytosol of a cell (Sipes and Gandolfi, 1993).

FIGURE 2



Xenobiotic glucuronidation versus bioactivation. UGT-catalyzed glucuronidation is a major (>50%-90%) route of elimination for most xenobiotics. Although enzymatic (e.g. peroxidases, cytochromes P450) bioactivation is a quantitatively minor pathway (<1%-10%), it is often responsible for the toxicity associated with many xenobiotics. Therefore, deficiencies in UGTs may decrease xenobiotic glucuronidation and thus increase bioactivation and toxicity.

can increase *in vitro* micronucleus formation initiated by B[a]P (Vienneau et al., 1995), the tobacco-specific nitrosamine NNK (Kim and Wells, 1996a; see Section 2.2) and the anticonvulsant drug phenytoin and its major hydroxylated metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin (**HPPH**) (Kim et al., 1997b; see Section 2.3). We have recently shown that heterozygous (+/J) UGT-deficient Gunn and RHA rats have an increased rate of phenytoin-initiated fetal resorptions (Kim and Wells, 1998; see Section 2.5) and earlier studies demonstrated that human and animal UGT-deficiencies can be important cytoprotective determinants in B[a]P-initiated embryotoxicity (Wells et al., 1989a), covalent binding and cytotoxicity (Hu and Wells, 1992, 1993, 1994) and in acetaminophen bioactivation (de Morais et al., 1992a,b), hepatotoxicity and nephrotoxicity (de Morais et al., 1992a).

The evidence suggests that UGT deficiencies may increase one's susceptibility to toxicity of many xenobiotics. However, since UGTs exist as a superfamily of isozymes, each with its own particular substrate profile, UGT deficiency must be viewed as a UGT isozyme deficiency (see Section 1.2.2.4). Conversely, toxicants also have isozyme specificity, and often have overlap. Therefore, the UGT isozyme profile of any single individual will be the ultimate determinant of whether he or she will experience toxicity, when exposed to a xenobiotic. The concepts of isozyme substrate-specificity and isozyme deficiency are clearly important with the recent knowledge that the *UGT1* gene complex is composed of numerous exons that are differentially spliced to produce all the different UGT1 isozymes (see Section 1.2.2). Human *UGT* mutations have been found in the *UGT1* gene complex and are responsible for both Gilbert's and Crigler-Najjar syndromes (see Section 1.2.4). These syndromes are a result of various gene mutations, which translate into functional deficiencies in at least the UGT1A1 isozyme that is responsible for the glucuronidation and elimination of bilirubin, a breakdown product of heme (Wolkoff et al., 1985; Owens and Evans, 1975).

It is now clear that, depending upon the substrate being studied, UGTs, and more specifically UGT isozymes, can mitigate or contribute to xenobiotic-initiated toxicity (see Section 1.2.3.3). Recent studies are utilizing various molecular techniques to investigate UGT isozyme-substrate specificity and their possible relevance to toxicity (Burchell et al., 1994a; Guengerich et

al., 1996). This process is now being aided by the commercial availability of specific human UGT isozymes expressed in various cell types, and with the construction of specific UGT isozyme (*UGT1a6*) knockout mice (Nebert and Duffy, 1997). UGT deficiencies, at least in the Gunn and RHA rat, as discussed have already been shown to be important determinants in chemically-initiated toxicities, including embryotoxicity/teratogenesis, cellular necrosis, preneoplastic DNA damage, and possibly carcinogenesis.

1.2.2 BIOCHEMISTRY

1.2.2.1 Gene/Regulation

A recent review by Mackenzie et al. (1997) has now placed UGTs within a larger cytosolic and membrane bound family of enzymes that may preferentially use a variety of sugars (e.g. UDP galactose, UDP glucose) other than UDPGA (**fig. 3**). A systematic nomenclature has been devised based upon a characteristic "signature sequence" of amino acids that now include 110 distinct cDNA/genes from animal, yeast, plant and bacteria (**fig. 4**). Since these proteins preferentially conjugate different sugars, it is now suggested that the acronym UGT stand for UDP-glycosyltransferase.¹ This systematic nomenclature is now similar to the way P450s are named, and thus provides a simple and consistent process for the naming of new UGT genes/proteins, which avoids the confusion often associated with trivial names based upon catalytic function (for a full review, see Mackenzie et al., 1997). The naming of the UGT gene now should follow an italicized root symbol (e.g. *UGT*), an Arabic number designating the family (e.g. *UGT2*), a letter to designate the subfamily (only if two or more subfamilies exist, e.g. *UGT2A*), and an Arabic number to designate the individual gene (e.g. *UGT2A1*). Families 1-50, 51-70, 71-100 and 101-200 are reserved for animals, yeast, plants and bacteria, respectively. For the gene products (cDNA, mRNA, protein, enzyme activity) the letters should not be italicized (e.g. UGT2A1).

The UGTs that we are interested in preferentially conjugate xenobiotics and endobiotics

¹ For this thesis UGT will continue to be used to designate UDP-glucuronosyltransferase, since this is the acronym used in all of the previously published work for this thesis.

FIGURE 4

rUGT2B1	FVAHGGTNGIYEAIYHGIPVIGIPLFADQ
hUGT2B11	FITHGGANGIYEAIYHGIPMVGVP LLADQ
lUGT2C1	FITHGGTNGLYEAIYHGVP MVGIP LFGDQ
rUGT2A1	FITHGGTNGIYEAIYHGIP MVGVPMFADQ
hUGT1A1	FITHAGSHGVYESICNGVPMVMPLFGDQ
UGT1B1	FLTHGGSHSVYEGICNAVPMMLP LFAEQ
rUGT8	FLSHGGLNSIFETMYHGVVVGIP LFGDH
UGT9	HVSHGGLNSVIESVYHGVVVGVP LTSRG
UGT10	FVTNGGMSVMEAVAHGVVIGVPLYGSN
UGT23	FVSHGGMNSVLETMYGVPMVIMPVFTDQ
UGT21B1	FITHAGYNSLMESAYAGVPIVILIPFMFDQ
UGT21A2	FITHSGYNSIVEAARAGVPLINIPFMFDQ
UGT15B1	FITHGGLGSVTELATMGKPAVVIP IFADQ
UGT19B1	FVTHCGQNSLLEAFNSGVRVLAVP LFGDQ
UGT19A1	FITHGGQNSLLET FHSNTRTLITP LFGDQ
UGT31D3	FITQGGLOSDEALEAGIPMVCLPMMGDQ
UGT31C1	FVTQAGVQSTDEAVENLVP LVGVPLMGDQ
UGT51	AVHHGSGTGTGASLRAGLP TVIKPFFGDQ
UGT77A1	FVTHAGWASVLEGVSSGVPMACRPF FGDQ
UGT74	FVTHCGWNSTLEALSEGVPMVAMALWTDQ
UGT75	FFTHCGWNSTLESICEEVP MVCRPFLADQ
UGT71A1	FVSHCGWNSVLES LWFVPIATWPMYAEQ
UGT71A2	LVSHSGWNSILES IWFVPIATWPMYAEQ
UGT78A1	FVTHCGWNSILES I VGGVPMICRPF FGDQ
UGT78C1	FVTHCGWNSILEGISEGVPMICRPF FGDQ
UGT77A2	FVTHAGWASVMEGVSSGVPMACRPF FGDQ
UGT102B1	AI THGGMNTVLDAINYRTP LLALPLAFDQ
UGT102A1	VITHGGLNTVLDALAAATPVLAVPLSFDQ
UGT101A2	FVTHAGAGGSQEGLATATP MIAVPQAADQ
UGT101A1	FVTHAGAGGSQEGLATATP MIAVPQAVDQ
Consensus	FhTHGGxxShxExhxxGVP h h x x P h x x D Q
	S A G T
	C

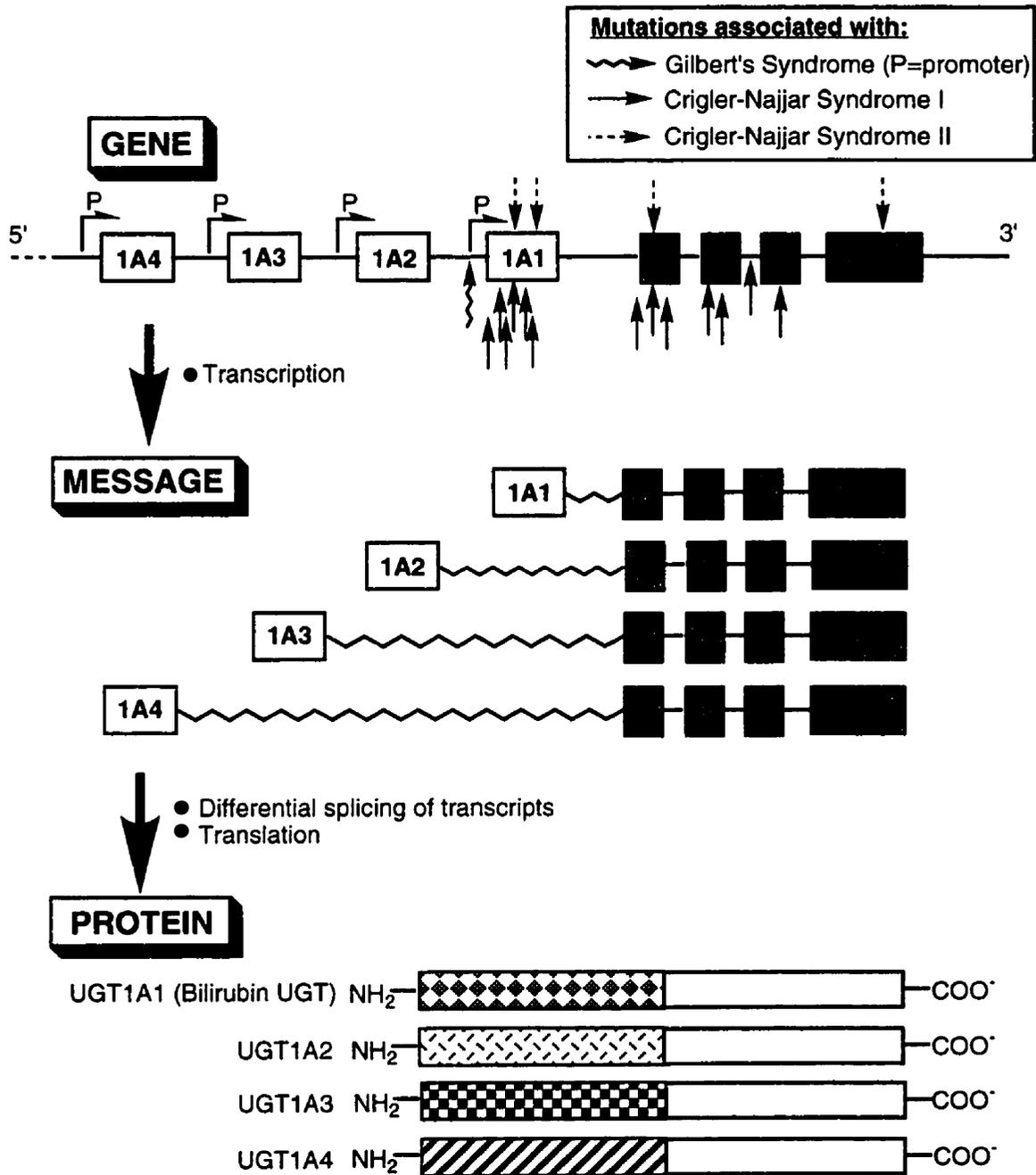
Common UGT amino acid "signature sequence" (i.e. consensus sequence; shown on bottom) (Mackenzie et al., 1997). The sequence is located in the carboxyl terminal half, thought to be responsible for binding to the UDP (uridine diphosphate) moiety of the nucleotide sugars (e.g. UDPGA; UDP-glucuronic acid) (also see fig. 5). Thirty representative sequences are shown. Abbreviations: h, hydrophobic residue; x, non-specific residue (i.e. any of > 3 amino acids).

with UDPGA, and are divided into two families (UGT1 and UGT2), with the UGT2 family being comprised of 2 subfamilies (Burchell et al., 1991). The human *UGT1* gene complex is found on chromosome 2 (Moghrabi et al., 1992), while at least some of the *UGT2* genes are located on chromosome 4 (Monaghan et al., 1992). The UGT1 family includes the dioxin- and phenobarbital-inducible isozymes and isozymes that conjugate bilirubin. The more than 100-kb-long *UGT1* gene complex consists of multiple isozyme-specific exons located at the 5'-variable region, and four common exons (numbered 2, 3, 4 and 5) at the 3'-common region (Brierley and Burchell, 1993; Iyanagi, 1991; Jansen et al., 1992) (**fig. 5**). The 5'-variable exon region contains specific exons that encode the amino-terminus of a particular UGT1 isozyme, which is thought to be responsible for substrate-specificity. The 3'-common exon region contains the coding region for the UDPGA binding site (exon 3 and 4) and the membrane anchoring area (exon 5) (Jansen, 1996). Each 5'-variable exon region contains its own promoter sequence facilitating the transcription of the *UGT1* gene complex (Owens and Ritter, 1995). Transcription produces numerous mRNA species that are processed via alternative splicing, creating mature mRNA, where one specific exon from the 5'-variable end is spliced together with the four common exons from the 3'-common region. This method of alternative splicing provides isozyme diversity and regulation of the *UGT1* gene, producing at least 9 rat UGT1 isozymes [*UGT1A4P* and *UGT1A9P* are pseudogenes (genes that are not transcribed and therefore have no phenotypic effect)]. Conversely in the rat and other species, UGT2 mRNA/protein are transcribed/translated from individual genes (e.g. 7 genes in the rat). The *UGT2* family include 2 olfactory-specific genes (*UGT2A1*) and genes for constitutively expressed isozymes (*UGT2B* subfamily) that are essential in the conjugation of endogenous compounds such as estrogen, steroids and bile acids (Iyanagi, 1991).

1.2.2.2 Protein/Topology

The UGT proteins contain an amino terminal domain that is responsible for the binding of the aglycone (280 to 290 amino acid residues), and an adjacent area responsible for the binding of the UDPGA molecule (Jansen et al., 1992; Burchell et al., 1994b; Meech et al.,

FIGURE 5

UGT1 Locus: Processing, Mutations and Related Syndromes

1996; Seppen et al., 1996). The carboxylic acid portion of the protein appears to be critical for UGT protein anchoring into the membrane of the endoplasmic reticulum (Meech et al., 1996).

The UGT proteins are found intracellularly in both the endoplasmic reticulum and the nuclear envelope (Dutton, 1980; Bansal et al., 1981), and similar to cytochromes P450 (**P450s**) (see Section 1.3), their presence in various organs (liver, lung, kidney, gut mucosa, brain and olfactory tissues) can be either constitutive or non-constitutive. **Tables 1a,b** list different UGT isozymes with respect to their constitutive or non-constitutive nature within various tissues and cells. Although certain UGT isozymes may be specifically located in a particular tissue (e.g. olfactory tissue), total UGT activity is greatest in the liver of most animals, including humans (Dutton, 1980; Kasper and Henton, 1980).

The subcellular localization of UGTs are such that the catalytic site of the protein is within the lumen of the endoplasmic reticulum (Tephly and Burchell, 1990; Yakota et al., 1992) (**fig. 6a**). No UGTs have been detected in other subcellular compartments such as mitochondria, golgi apparatus, peroxisome or the plasma membrane (Burchell and Coughtrie, 1989). Since the highly hydrophilic sugar molecule, UDPGA, is made in the cytosol, it therefore must cross the membrane of the endoplasmic reticulum in order to gain access to the UGT catalytic site and be conjugated with the aglycone (i.e. lipophilic endobiotics or xenobiotics) (Berry and Hallinan, 1976; Hauser et al., 1988; Jansen et al., 1992). Recent evidence suggests that UDPGA crosses into the lumen aided by transporter proteins located on the surface of the endoplasmic reticulum (**fig. 6b**) (Radominski et al., 1994; Jansen, 1996). As discussed by Burchell et al. (1994b), evidence from the TR rat, which is deficient in conjugate transport of both glucuronide and glutathione-conjugates (Jansen et al., 1987, 1993; Kitamura et al., 1990; Nishida et al., 1992), suggests that the conjugated hydrophilic product is released into the bile or blood by specific transporter proteins.

1.2.2.3 Induction

Chemical induction of UGT enzymes can often occur in conjunction with other phase II enzymes such as epoxide hydrolase and glutathione (**GSH**) S-transferase, or with phase I

Table 1a. Location and modulation of some toxicologically important animal UGTs

Species	Organ(s)	mRNA ^{a,b}	Protein/ Activity ^c	Modulator(s) ^{d,e}	Reference(s)
dog	liver		UGTDOG-PB ² UGTDOG-UT ³	PB ↑	Oguri et al., 1996 Oguri et al., 1996
monkey	liver	UGT2B18 ⁴ UGT2B19 ⁵			Belanger et al., 1996 Belanger et al., 1996
	prostate	UGT2B18 UGT2B19			Belanger et al., 1996 Belanger et al., 1996
mouse	liver	Ugt1a6		TCDD (2-3-fold) ↑	Lamb et al., 1994 ⁸
	skin		Ugt activity	β-naphthoflavone ↑	Coomes et al., 1983
rabbit	liver	UGT1A6 UGT2B13 UGT2B16		TCDD ↔ dexamethasone ↑	Lamb et al., 1994 ⁸ Nguyen et al., 1996 Nguyen et al., 1996
rat	brain	(UGT1A6) (UGT2B12)	UGT1A6 activity	TCDD ↑	Gradinaru et al., 1996 Munzel et al., 1994 ⁷ Green et al., 1995
	brain: • choroid plexus • astrocytes (cultured)		UGT1A6 activity UGT1A6 activity		Gradinaru et al., 1996 Gradinaru et al., 1996
	epididymis	UGT1A6		TCDD ↑	Munzel et al., 1994 ⁷
	heart	(UGT2B12)			Green et al., 1995 ¹¹
	Intestine	UGT1A6 UGT1A7	1-naphthol (-)-morphine α-naphthol 4-hydroxybiphenyl	low protein diet ↑ low protein diet ↑ oltipraz ↑ oltipraz (>2-fold) ↑	Koster et al., 1986 Bajjal & Fitzpatrick, 1996 ⁹ Grove et al., 1997 ¹⁰ Grove et al., 1997 ¹⁰
kidney	UGT1A6 UGT1A7 (UGT2B3) UGT2B12 (15%)	(morphine) (testosterone) bilirubin α-naphthol	high protein diet ↑ TCDD ↑ oltipraz ↔	Rush et al., 1983 Lucier & McDaniel, 1977 Foliot et al., 1977 Bajjal & Fitzpatrick, 1996 ⁹ Munzel et al., 1994 ⁷ Grove et al., 1997 ¹⁰ Green et al., 1995 ¹¹ Green et al., 1995 ¹¹	

(table continued)

rat	liver	UGT1A1	α -naphthol	low protein diet \uparrow	Bajjal & Fitzpatrick, 1996 ⁹ Ritter et al., 1996
		UGT1A6		β -naphthoflavone \uparrow	
		(UGT1A7)		oltipraz \uparrow	
		UGT2B1		TCDD (20-fold) \uparrow	
		UGT2B3 UGT2B4 UGT2B12		oltipraz \uparrow	
		UGT2B12		PB \uparrow	Lamb et al., 1994 ⁸ Grove et al., 1997 ¹⁰ Iyanagi et al., 1986 Munzel et al., 19947 Emi et al., 1995 Grove et al., 1997 ¹⁰ Mackenzie et al., 1996 Ritter et al., 1996 Green et al., 1995 ¹¹ Mackenzie et al., 1996 Green et al., 1995 ¹¹
			PB \uparrow		
			PB \downarrow		
			PB (2-fold) \uparrow		
	lung	UGT1A6		TCDD \uparrow	Coughtrie et al., 1985 Munzel et al., 19947 Grove et al., 1997 ¹⁰ Grove et al., 1997 ¹⁰
		UGT1A7		oltipraz \leftrightarrow	
	ovary	UGT1A6		TCDD \uparrow	Munzel et al., 19947 Grove et al., 1997 ¹⁰ Grove et al., 1997 ¹⁰
		UGT1A7		oltipraz \leftrightarrow	
				oltipraz \downarrow	
	spleen	UGT1A6 UGT1A7 (UGT2B12)		oltipraz \leftrightarrow	Grove et al., 1997 ¹⁰ Grove et al., 1997 ¹⁰ Green et al., 1995 ¹¹
				oltipraz \leftrightarrow	
	testes	UGT1A6 (UGT2B3) UGT2B12 (<15%)		TCDD \uparrow	Munzel et al., 19947 Green et al., 1995 ¹¹ Green et al., 1995 ¹¹

Abbreviations: PB, phenobarbital; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; UGT, UDP-glucuronosyltransferase; UT, untreated.

^a UGTs in parenthesis indicate undetectable levels of the UGT mRNA.

^b Percentages in parenthesis indicate levels of UGT mRNA relative to liver UGT mRNA.

^c UGTs in parenthesis indicates undetectable levels of the UGT protein or activity.

^d Up arrow (\uparrow), down arrow (\downarrow) and horizontal double arrow (\leftrightarrow) indicate induction, inhibition/suppression or no effect, respectively.

^e Numbers in parenthesis indicate fold induction of either UGT mRNA, protein or activity.

¹ Slight suppression of UGT2B3 mRNA.

² UGTDOG-PB (50 kDa) was found in PB-induced dog liver microsomes and the NH₂-terminal residue was highly homologous to UGT2B1, 2B8 and 2B15.

³ UGTDOG-UT protein was isolated from untreated animals and was 52 kDa with NH₂-terminal 15 residues similar to UGTDOG-PB.

⁴ UGT2B18 is 89% identical with the human UGT2B7 enzyme.

⁵ UGT2B19 is 87% identical with the human 2B4 enzyme.

⁶ UGT1A7 was decreased by oltipraz.

⁷ Although this paper uses the nomenclature UGT1A1, it is actually referring to the phenol UGT which is now considered UGT1A6 (Mackenzie et al., 1997). Hepatic but not extrahepatic tissue UGT1A6 was substantially induced by TCDD.

⁸ Mice were given 250 μ g/kg of 3-MC or 100 μ g/kg TCDD as a single ip injection and killed 18-24 hr later. Rats were given 100 μ g/kg TCDD as a single ip injection and killed 18-24 hr later. Rabbits were given 10 μ g/kg TCDD as a single ip injection and killed 48 hr later.

⁹ All rats were first fed a standard semipurified diet for 5 days and then received either a low (7.5%), standard (15%) or high (45%) protein diet. Rats were fed *ad libitum* for 7 or 14 days.

¹⁰ Rats were treated with oltipraz at a dose of 300 mg/kg/day for 2 days, or vehicle [30% (w/v) polyethylene glycol 8000 in water] and killed 24 hr after last dose.

¹¹ Phenobarbital (80 mg/kg) or saline vehicle was injected ip for 8 days.

Table 1b. UGT Isozymes in some animal cell lines.

Animal Cells/ Cell Line(s)	mRNA ^a	Protein/ Activity	Modulator(s) ^{b, c}	Reference(s)
Astrocytes (primary culture)		UGT1A6		Gradinaru et al., 1996
Class II (BP-) hepatoma cells (defect in Ah receptor translocation)	UGT1A6		TCDD ↔	Lamb et al., 1994 ¹
Hepa 1c1c7 (mouse hepatoma cell line)	Ugt1A6		TCDD ↑	Lamb et al., 1994 ¹
Oval cells (nonparenchymal liver epithelial cells, primary culture)	UGT1A1	UGT1A1	cell division ↑	Steinberg et al., 1994 ^{2, 3}
Ruber H4IIE cells (rat hepatoma cell line)	UGT1A6		TCDD (5-fold) ↑	Lamb et al., 1994 ¹
RALA25510G cells (LCS-3, SV-40tsA255- immortalized adult rat hepatocyte cell line)	UGT1A6 UGT1A7	B[a]P-7,8-diol	oltipraz (50%-140%) ⁴ ↑ oltipraz ⁵ ↔ oltipraz ⁶ ↑	Grove et al., 1996 Grove et al., 1996 Grove et al., 1996

^a UGTs in parenthesis indicate undetectable levels of the UGT mRNA .

^b Up arrow (↑), down arrow (↓) and horizontal double arrow (↔) indicate induction, inhibition/suppression or no effect, respectively.

^c Numbers in parenthesis indicate fold induction, when given, of either UGT mRNA , protein or activity.

¹ Cells were incubated with TCDD at a final concentration of 10 nM for 24 hr.

² UGT1A1 activity was assessed using 4-methylumbelliferone and 6-hydroxychrysene.

³ Exponentially growing cells had substantially higher levels of UGT1A1 mRNA versus confluent cells.

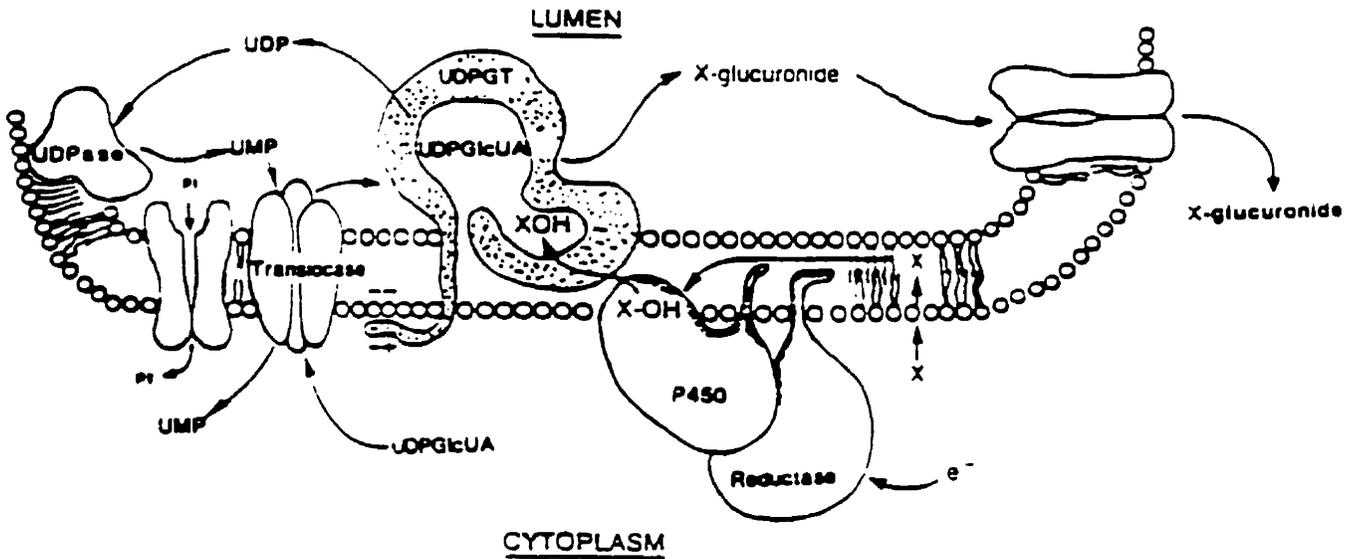
⁴ RALA255-10G cells were incubated for 72 hr with either vehicle (Me₂SO), or 25 μM or 50 μM of oltipraz.

⁵ RALA255-10G cells were incubated for 72 hr with either vehicle (Me₂SO), or 10 μM, 25 μM or 50 μM of oltipraz.

⁶ RALA255-10G cells were incubated with vehicle (Me₂SO), or 100 μM of oltipraz for 24 hr.

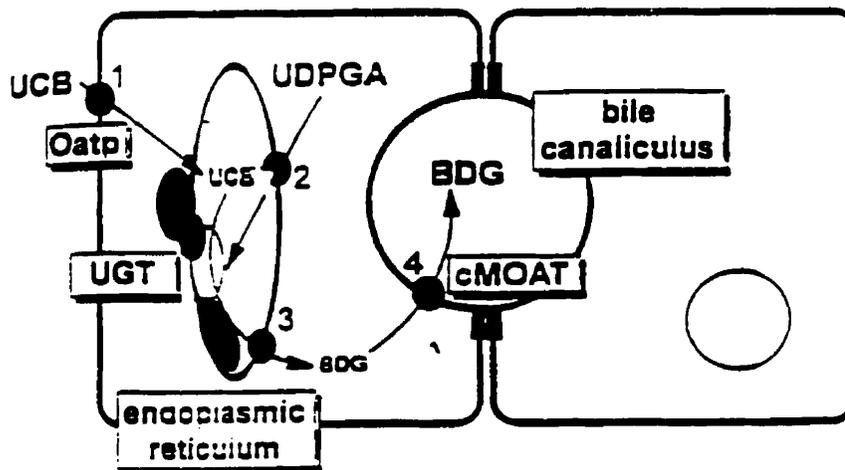
Figure 6a

9d



A hypothetical topological model of UGT in the endoplasmic reticulum and its relation to cytochromes P450 (P450) (Jansen et al., 1992). Abbreviations: Pi, phosphate; UDPGT, UDP-glucuronosyltransferase; UDPGlcUA, uridine diphosphate glucuronic acid; UDP, uridine diphosphate; UMP, uridine monophosphate; X, lipophilic compound; XOH, hydroxylated compound.

Figure 6b



Uptake, conjugation and secretion of bilirubin in the liver (Jansen, 1996). An organic anion transporting protein (Oatp) (1) transports unconjugated bilirubin (UCB) into the hepatocyte. UGTs located in the endoplasmic reticulum receive UDPGA from the cytosol, transported by protein (2) (not yet cloned). The bilirubin diglucuronide (BDG) is secreted into the bile by proteins (3) and (4). Protein (3) is uncharacterised, while protein (4) is an ATP-dependent carrier protein (cMOAT; canalicular multispecific organic anion transporter).

enzymes such as P450s (Franklin and Moody, 1992; Franklin et al., 1993; Slawson et al., 1996). This enzyme induction may, through a concerted effort, help eliminate and protect the cell from xenobiotic-initiated toxicity. Interestingly, UGTs can also be affected by high protein diets, although induction is dependent on the particular organ being examined (Baijal and Fitzpatrick, 1996). **Table 1a, b** lists UGT isozymes and known inducers and inhibitors.

Owens (1977) demonstrated that different Ah receptor mouse phenotypes (i.e. TCDD-responsive C57BL/6 mice, TCDD non-responsive DBA/2 mice) were differentially Ugt1-inducible (possibly Ugt1a6), suggesting that, similar to P450s, some UGT isozymes may be regulated by the aryl hydrocarbon (Ah) receptor via the dioxin/xenobiotic responsive element (DRE, XRE). Subsequent studies in rat liver demonstrated that UGT1A6, which can conjugate B[a]P hydroxylated metabolites (**table 2a**), is induced possibly through an Ah receptor mechanism (Bock et al., 1990; Emi et al., 1996), although the constitutive rabbit liver UGT1A6 was not inducible (Lamb et al., 1994). Grove et al. (1997) also demonstrated that oltipraz (a dithiol thione), a chemotherapeutic agent, can also induce UGT1A6. Further work has demonstrated that oltipraz and the polycyclic aromatic hydrocarbon β -naphthoflavone can, through an increase in gene transcription, induce the non-constitutive rat hepatic UGT1A7 isozyme that also can glucuronidate B[a]P-hydroxylated metabolites, including the proximal carcinogen, B[a]P-7,8,-diol (Emi et al., 1995; Grove et al., 1998; Metz and Ritter, 1998; Grove et al., 1997; Kessler and Ritter, 1996).

Fetal mouse hepatic Ugt inducibility measured by p-nitrophenol glucuronidation has been shown to occur in fetuses from C57BL/6 dams, but not in DBA/2 dams treated with β -naphthoflavone or 3-methylcholanthrene (polycyclic aromatic hydrocarbon) (Chauhan et al., 1991). However, although p-nitrophenol has been used as a substrate for 3-methylcholanthrene-type inducible Ugts (Bock et al., 1988), 3-hydroxy-B[a]P glucuronidation was not increased in fetuses from C57BL/6 dams. Earlier studies also have demonstrated that, in rats, dexamethasone (Wishart, 1978), but not TCDD (Lucier et al., 1979), can induce the glucuronidation of both p-nitrophenol and 4-methylumbelliferone, and in fetal mouse studies, hepatic glucuronidation was not increased by 3-methylcholanthrene induction (Owens, 1977).

Table 2a: Substrates for rat UGT1 and 2 families†

UGT ISOZYME(S)	TRIVIAL NAMES‡	SUBSTRATES (XENOBIOTICS)	NOT SUBSTRATES	INDUCER(S)	REFERENCE(S)
1 A 1	•Bilirubin UGT •1*0	•Bilirubin	•ND	•PB •clofibrate	•Iyanagi, 1991
1 A 6	•1.6 •1*06 •Phenol UGT •K39	•Acetaminophen •B[a]P: •5- & 12-OH-B[a]P •3-,6-quinol-B[a]P •2-AAF: •N-, 5- & 8-OH-2-AAF •Hydroxychrysene: •3- & 6- hydroxychrysene •3,6-Dihydroxychrysene •AminoBiPh: •N-OH- & 4-OH-AminoBiPh •4-OH-BiPh	•B[a]P: •All other OH-B[a]P •4,5- & 7,8-diol-B[a]P •2-AAF: •1-, 3-, 7-,9-OH-2-AAF	•TCDD •3MC •β-NF •Oltipraz	•Bock et al., 1988, 1992, 1993 •Jackson et al., 1988 •Mackenzie et al., 1993 •Munzel et al., 1994 •Gschaidmeier et al., 1995 •Grove et al., 1997
1 A 7	•1*7	•B[a]P: •3- & 9-OH-B[a]P •7,8-diol-B[a]P	•ND	•Oltipraz •PAH •Not PB	•Grove et al., 1997 •Grove et al., 1998
2 B 1	•UDPGTr-2	•B[a]P: •1- to 5-, 8- & 9-OH-B[a]P •4,5-diol-B[a]P •2-AAF: •N-OH-2-AAF •4-OH-BiPh	•B[a]P: •All other OH-B[a]P •7,8-diol-B[a]P •2-AAF: •1-, 3-, 5-, 7-, 8-, 9-OH-2-AAF	•PB	•Mackenzie, 1986a •Mackenzie, 1987 •Mackenzie et al., 1993
2 B 2	•UDPGTr-4 •rlug 23	•B[a]P: •1-, 3-, 4-, 5-, 7-, 9-, 11-, & 12-OH-B[a]P •4,5-diol-B[a]P •2-AAF: •N-, 1- & 3-OH-2-AAF	•B[a]P: •All other OH-B[a]P •7,8-diol-B[a]P •2-AAF: •5-, 7-, 8- & 9-OH-2-AAF	•ND	•Jackson & Burchell, 1986 •Mackenzie, 1986b •Mackenzie et al., 1993
2 B 3	•UDPGTr-3 •rlug38	•ND	•All OH-B[a]P •All OH-2-AAF	•PB	•Mackenzie et al., 1987 •Mackenzie et al., 1993 •Green et al., 1995 ¹
2 B 6	•UDPGTr-5	•ND	•All OH-B[a]P •All OH-2-AAF	•ND	•Mackenzie, 1990 •Mackenzie et al., 1993
2 B 12	•HBPA2 (cDNA)	•BiPh: •2-, 3- & 4-OH-BiPh	•Acetaminophen •m- & p-HPPH •Diethylstilbestrol	•PB	•Green et al., 1995

†: The UGT nomenclature it is in the process of being revised to be more indicative of the location for the variable exon region (Dr. Joseph Ritter, personal communication). ND: No data available. ‡: Trivial names are not indicative of UGT exon/gene location.

Abbreviations: 2-AAF, 2-acetylaminofluorene; 3-MC, 3-methylcholanthrene; β-NF, β-naphthoflavone; ASA, acetylsalicylic acid; BiPh, biphenyl; B[a]P, benzo[a]pyrene; DG, diglucuronide; m-HPPH, 5-(m-hydroxyphenyl)-5-phenylhydantoin; p-HPPH, 5-(p-hydroxyphenyl)-5-phenylhydantoin; OH, hydroxyl moiety; PAH, polycyclic aromatic hydrocarbons; PB, phenobarbital.

¹ Green et al. (1995) showed that mRNA levels are not elevated after PB treatment and in fact mRNA levels may be slightly depressed.

Conversely, bilirubin glucuronidation, which appears late in mouse fetal gestation (gestational day 18), was induced by phenobarbital (Burchell, 1973; Burchell and Dutton, 1975).

Evidence by Munzel et al. (1996) suggests that human UGT1A6 may be induced by TCDD via the Ah receptor, although Ritter et al. (1998) did not see UGT1A6 induction in primary cultured hepatocytes incubated with the inducers 3-methylcholanthrene, oltipraz or phenobarbital. Ritter et al. (1998) did demonstrate that the bilirubin UGT1A1 was induced by all three inducers and that UGT1A4 was induced by phenobarbital. However UGT1A9 was not induced by any of the above inducers. **Tables 2a and 2b** list various rat and human UGT isozymes and chemicals known or suspected to be inducers and some toxicologically important substrates.

1.2.2.4 UGT Isozymes and Substrates

Most xenobiotics are eliminated to some extent by UGT-catalysed conjugation with UDPGA. Glucuronidation can occur at various atoms such as O, N, S and C (**table 3**) and has been shown to be a major route of elimination for many compounds such as acetaminophen, B[a]P, NNK and phenytoin/HPPH (see Section 2). Although different UGT isozymes have a particular substrate specificity profile, there nevertheless is a certain amount of substrate overlap. More often than not, UGT1 isozymes have been classified for their ability to glucuronidate xenobiotics (e.g. simple versus complex phenols), whereas the UGT2 family are considered steroid conjugating UGTs. It is now apparent that such simple modes of classification are not appropriate since both families can glucuronidate both endogenous and exogenous compounds.

In the past various *in vitro* and *in vivo* systems have been used to study the glucuronidation of various chemicals, some of which relied upon UGT induction (see Section 1.2.2.3). More recently, different UGT isozyme-expression systems have facilitated the study of isozyme-specific glucuronidation of various compounds. **Tables 2a and 2b** list specific rat and human UGT isozymes and their xenobiotic substrates, for some of which their toxicity has been previously shown to be mitigated by UGT-catalysed elimination (see Section 2).

Table 2b: Substrates for human UGT1 and 2 families†

UGT ISOZYME(S)	TRIVIAL NAMES‡	SUBSTRATES (XENOBIOTICS)	NOT SUBSTRATES	INDUCER(S)	REFERENCE(S)
1 A 1	•Bilirubin UGT •HUG-Br1 •Hlug P3 •HP3 •1*1	•Bilirubin • p-HPPH •Naringenin •4-Nitrophenol • α -Naphthol	•Acetaminophen •ASA •Retinoic acid •Diethylstilbestrol •4-OH-BiPh	•Phenytoin •PB	•Kim and Wells, 1997 •Senafi et al., 1994 •Sutherland et al., 1993
1 A 4	•HUG-Br2 •Hlug P2 •HP2 •1*4	•Bilirubin •4-Aminobiphenyl	•ND	•Phenytoin •PB	•Ritter et al., 1991 •Sutherland et al., 1993 •Burchell et al., 1995
1 A 6	•phenol UGT •hlug P1 •HP1 •1.6 •1*6	•Acetaminophen • B[a]P : •4-,5-,8- & 12-OH-B[a]P •3,6-quinol-B[a]P • 2-AAF : •N-,1-,3-,5- & 8-OH-2-AAF •Hydroxychrysene: •3,6-Dihydroxychrysene •6-Hydroxychrysene •AminoBiPh: •N-OH- & 4-OH-AminoBiPH •4-OH-BiPh	•Bilirubin •ASA • 2-AAF : •7- & 9-OH •4-OH-BiPh	•ND	•Bock et al., 1993 •Burchell et al., 1995 •Ebner & Burchell, 1993 •Gschaidmeier et al., 1995 •Jin et al., 1993a •Orzechowski et al., 1994
1 A 9	•hlug P4 •HP4 •1*02 •1.7 •1*7	•Acetaminophen • B[a]P : •3,6-quinol-B[a]P •Hydroxychrysene: •3,6-Dihydroxychrysene •6-Hydroxychrysene • 2-AAF : •5- & 8-OH-2-AAF •4-OH-BiPh •Retinoic acid •13-cis-Retinoic acid	•Bilirubin •ASA	•ND	•Ebner and Burchell, 1993 •Gschaidmeier et al., 1995 •Burchell et al., 1995
2 B 7	•UDPGTh-2	•Valproic acid • B[a]P : •4,5- & 7,8-diol-B[a]P •1-,2-,4-,5-,6-,8-,9- & 10-OH-B[a]P • 2-AAF : •N-,1-,3- & 8-OH-2-AAF •4-OH-BiPh	•3-,7-,11- & 12-OH-B[a]P •3- & 9-OH-B[a]P ^V • p-HPPH •5-,7- & 9-OH-2-AAF •Diethylstilbestrol •Acetaminophen	•ND	•Ritter et al., 1990 •Jin et al., 1993a •Jin et al., 1993c
2 B 10	•ND	•ND	• B[a]P : •All OH-B[a]P •4,5- & 7,8-diol • 2-AAF : •N-,1-,3-,5-,7-,8- & 9-OH-2-AAF	•ND	•Jin et al., 1993a •Jin et al., 1993b
2 B 11	•ND	•4-OH-BiPh	• B[a]P : •All OH-B[a]P •4,5- & 7,8-diol • 2-AAF : •N-,1-,3-,5-,7-,8- & 9-OH-2-AAF	•ND	•Jin et al., 1993a •Jin et al., 1993b

Table 2b continued...

2 B 15	•HE8a (cDNA) •UDPGTh-3	•p- & m-HPPH • <u>OH-BiPh</u> : •2-, 4- & 4'-OH-BiPh	•Acetaminophen •Diethylstilbestrol •4-AminoBiPh	•ND	•Green et al., 1994
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†: The UGT nomenclature it is in the process of being revised to be more indicative of the location for the variable exon region (Dr. Joseph Ritter, personal communication). ‡: Trivial names are not indicative of UGT exon/gene location. ¥: Ritter et al., 1990 could not detect 9-OH-B[a]P-glucuronide. **ND**: No data available.

Abbreviations: 2-AAF, 2-acetylaminofluorene; ASA, acetylsalicylic acid; BiPh, biphenyl; B[a]P, benzo[a]pyrene; DG, diglucuronide; m-HPPH, 5-(m-hydroxyphenyl)-5-phenylhydantoin; p-HPPH, 5-(p-hydroxyphenyl)-5-phenylhydantoin; OH, hydroxyl moiety; PB, phenobarbital.

Table 3. Examples of the different classes of glucuronide conjugates (from Sipes and Gandolfi, 1993).

Acceptor		
Types of glucuronide	Functional Group	Example
<p>O-Glucuronide</p> $\begin{array}{c} \\ -\text{C}-\text{O}-\text{G} \\ \end{array}$ $\begin{array}{c} \text{O} \\ \\ -\text{C}-\text{O}-\text{G} \end{array}$ $\begin{array}{c} -\text{CH}=\text{C}-\text{O}-\text{G} \\ \end{array}$ $\begin{array}{c} -\text{N}-\text{O}-\text{G} \\ \end{array}$	<p>Alcohol</p> <ul style="list-style-type: none"> • Aliphatic • Alicyclic • Benzylic • Phenolic <p>Carboxylic acid</p> <ul style="list-style-type: none"> • Aliphatic • Aromatic <p>α,β-Unsaturated ketone</p> <p>N-hydroxy</p>	<ul style="list-style-type: none"> • Trichloroethanol • Hexobarbital • Methylphenylcarbinol • Estrone <ul style="list-style-type: none"> • α-Ethylhexanoic acid • <i>o</i>-Aminobenzoic acid <ul style="list-style-type: none"> • Progesterone <ul style="list-style-type: none"> • <i>N</i>-Acetyl-<i>N</i>-phenyl-hydroxylamine
<p>N-Glucuronide</p> $\begin{array}{c} -\text{O}-\text{C}-\text{N}-\text{G} \\ \quad \\ \text{O} \quad \text{H} \end{array}$ $\begin{array}{c} \text{Ar}-\text{N}-\text{G} \\ \\ \text{H} \end{array}$ $(\text{R}_3)-\text{N}^+-\text{G}$ $\begin{array}{c} \text{R}-\text{SO}_2-\text{N}-\text{G} \\ \\ \text{H} \end{array}$	<p>Carbamate</p> <p>Arylamine</p> <p>Aliphatic tertiary amine</p> <p>Sulfonamide</p>	<p>Meprobamate</p> <p>2-Naphthylamine</p> <p>Tripelennamine</p> <p>Sulfadimethoxine</p>
<p>S-Glucuronide</p> $\text{Ar}-\text{S}-\text{G}$ $\begin{array}{c} -\text{C}-\text{S}-\text{G} \\ \\ \text{S} \end{array}$	<p>Aryl thiol</p> <p>Dithiocarbamic acid</p>	<p>Thiophenol</p> <p><i>N,N</i>-Diethyldithiocarbamic acid</p>
<p>C-Glucuronide</p> $\begin{array}{c} \\ -\text{C}-\text{G} \\ \end{array}$	<p>1,3-Dicarbonyl system</p>	<p>Phenylbutazone</p>

Abbreviations: C, carbon; G, glucuronic acid; N, nitrogen; O, oxygen; S, sulfur.

1.2.3 ANIMAL AND CELLULAR MODELS OF UGT DEFICIENCIES

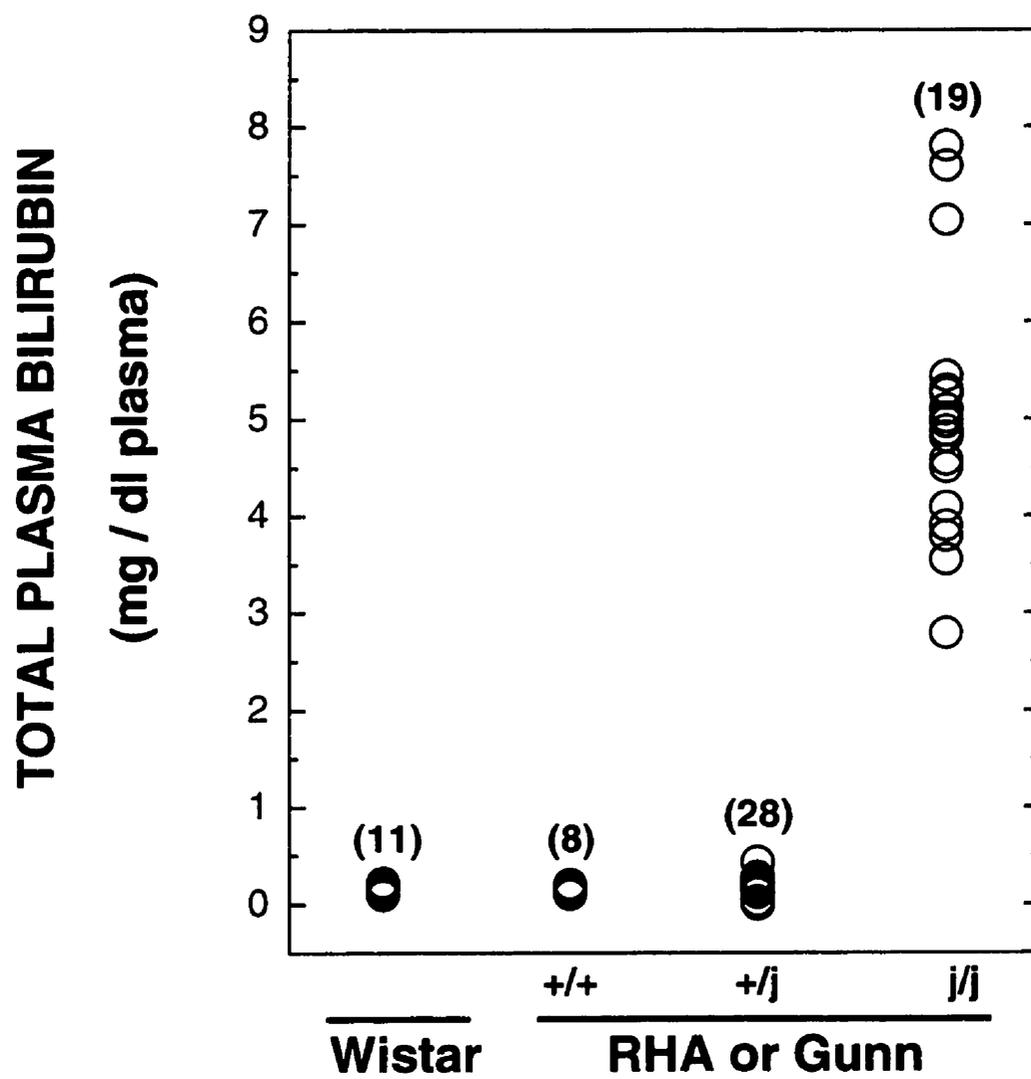
1.2.3.1 Gunn/RHA rats

The human *UGT1* gene complex appears to be similar to that of other mammalian species such as the rat. In 1938, C.H. Gunn discovered an icteric mutant strain of the Wistar rat, now called the Gunn rat, which is often used as a model for human UGT deficiencies. This icteric condition was described as a recessive trait, for which the phenotype was originally designated as *jj*, *Jj* and *JJ*, for homozygous UGT-deficient, heterozygous UGT-deficient and homozygous UGT-normal, respectively. The more recent nomenclature has kept the letter *j* for the designation of jaundice but has replaced the capital *J* with a plus (+) symbol to indicate a normal allele.

Newborn homozygous (*jj*) UGT-deficient rat pups (i.e. before weaning) are easily distinguishable from heterozygous (*+/j*) UGT-deficient and UGT-normal (*+/+*) pups due to their yellow coats. During adulthood, the yellow coat of the new born pup is lost. However analysis of the plasma bilirubin concentrations can clearly determine homozygous UGT-deficient rats. Homozygous UGT-deficient rats will often have total plasma bilirubin concentrations that are between 6-15 mg/dl (120-350 μ M), which is significantly greater than either *+/j* UGT-deficient or *+/+* UGT-normal rats (<1 mg/dl), which have total bilirubin concentrations that are indistinguishable from each other (Burchell et al., 1987). Results from our Gunn and RHA rat breeding colonies show similar bilirubin concentrations (**fig. 7**). Early studies showed that these animals had deficient hepatic UGT activity (Schmid et al., 1958; Lathe and Walker, 1958). Interestingly, heterozygous UGT-deficient and UGT-normal rats (pups or adults) cannot be distinguished based upon phenotype, and hence must be genotyped for the causal frameshift mutation (Huang et al., 1992). This would suggest that heterozygous rats have a bilirubin glucuronidating capacity sufficient to be phenotypically indistinguishable from UGT-normal rats. This is in sharp contrast to the *in vitro* and *in vivo* studies performed in our lab which show that xenobiotic-initiated toxicity is similarly increased in *+/j* and *jj* UGT deficient animals versus *+/+* UGT-normal controls (see Section 2.2, 2.3 and 2.5).

As a result of a frameshift mutation in exon 4 of the common exon region of the *UGT1* gene complex, RHA and Gunn rats are deficient in the entire UGT1 family of isozymes (Iyanagi, 1991;

Figure 7
Plasma Bilirubin Levels



Iyanagi et al., 1989; Roy Chowdhury et al., 1991). This mutation produces a premature stop codon which results in the production of truncated, inactive UGT1 protein. Therefore, although bilirubin glucuronidation and elimination is adequate in +/j heterozygous UGT-deficient rats, both j/j homozygous and most likely +/j heterozygous UGT-deficient rats may be unable to glucuronidate many toxicologically important compounds which may predispose them to increased xenobiotic toxicity (de Morais and Wells, 1988, 1989; de Morais et al., 1992a; El Awady et al., 1990; Chowdhury et al., 1991; Hu and Wells, 1992, 1994). The homozygous UGT-deficient animals can be used as a model for at least the severe form of the human UGT1 deficiency (Crigler-Najjar syndrome), while the heterozygous UGT deficient animals may be an effective model for the common and "relatively benign" human bilirubin UGT deficiency (Gilbert's syndrome) (see Section 1.2.4). As mentioned above, both *in vitro* and *in vivo* human and animal studies have demonstrated the importance of UGTs in xenobiotic (acetaminophen, B[a]P, NNK) bioactivation and toxicity (protein adducts, DNA oxidation, micronucleus formation, hepatotoxicity and nephrotoxicity).

1.2.3.2 Glucuronidation as a Potentiator of Toxicity

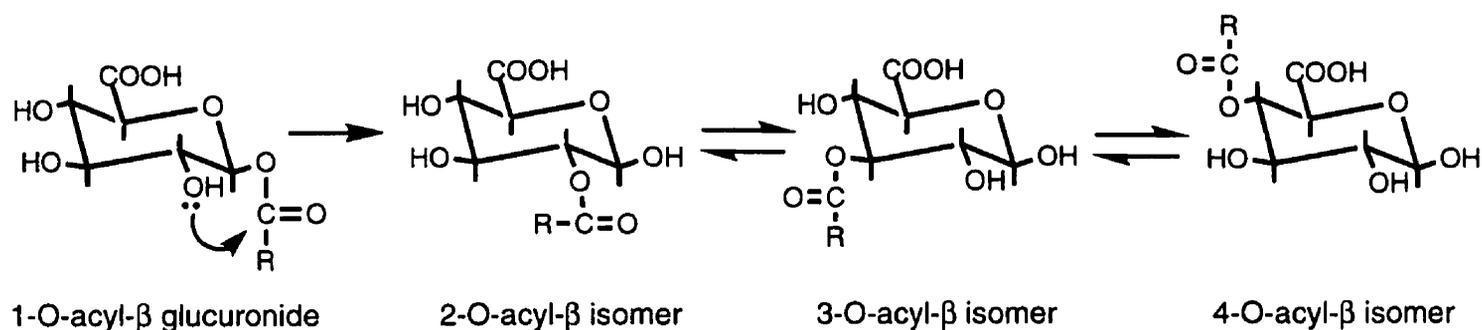
It has been hypothesized that enzyme-catalyzed conjugation, which is usually cytoprotective, can in some cases activate or bioactivate certain xenobiotics to either toxic reactive intermediates or pharmacologically active (or more active) metabolites, respectively (Mulder et al., 1986). Therefore, the under- or overactivity of a specific UGT isozyme responsible for the conjugation of a particular xenobiotic may directly mediate its toxicity.

The glucuronidation of drugs can be important in "classical" pharmacological receptor-mediated activity, for drugs such as morphine, the pharmacological efficacy of which is greatly enhanced by the formation of a morphine-glucuronide. Morphine-6-glucuronide is significantly more potent than the parent molecule (Pasternak et al., 1987; Osborne et al., 1988; Paul et al., 1989) and may be responsible for respiratory depression (Osborne et al., 1986; Hasselstrom et al., 1989; Bodd et al., 1990). Thus, theoretically, an absence of glucuronidation may significantly decrease drug response, while UGT overactivity may prove toxic.

Similarly, glucuronidation of drugs can in some cases constitute a bioactivation pathway, forming a potentially toxic reactive intermediate (Spahn-Langguth and Benet, 1992; Zia-Amirhosseini et al., 1994). The major route of elimination for many drugs that have carboxylic acid groups is usually through the formation of acyl glucuronides (Faced, 1984; Caldwell et al., 1988; Benet and Spahn, 1988) (**fig. 8a**). Acyl glucuronides are reactive electrophilic metabolites that can either undergo hydrolysis, rearrangement and/or covalent binding to protein (Dickinson et al., 1984; Hyneck et al., 1988a; Dickinson and King, 1989; Watt et al., 1991; Bradow et al., 1989) (**table 4, fig. 8a**). The molecular interaction of acyl glucuronides with protein may involve a transacylation mechanism with -SH, -OH or -NH₂ groups (Salmon et al., 1974; van Breeman and Fenselau, 1986; Dickinson and King, 1991) (**fig. 8b**). Figure 8b demonstrates a nucleophilic displacement reaction involving a cysteine thiol group of a protein (van Breeman and Fenselau, 1985). Other mechanisms of acyl glucuronide covalent binding have been proposed which involve an imine formation and an Amadori rearrangement (e.g. zomepirac glucuronide) (Smith et al., 1986). Interestingly covalent interactions do not always result in the loss of the glucuronide moiety (Tang and Abbott, 1996). It has been suggested that this covalent binding may induce immune-mediated reactions which may include anaphylactic or anaphylactoid reactions via hapten formation (Goldsmith, 1980; Bretza and Novey, 1985; Worrall and Dickinson, 1995).

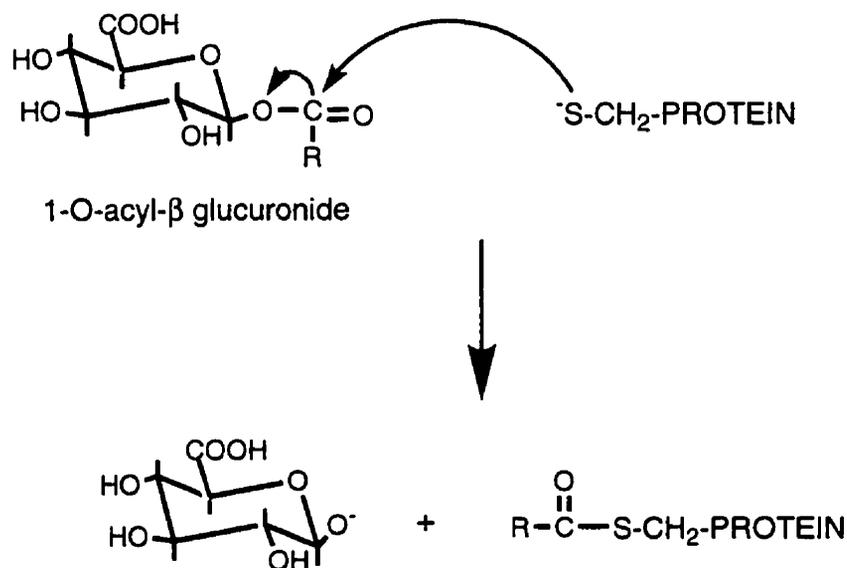
Non-steroidal anti-inflammatory drugs such as acetylsalicylic acid and diflunisal and the anticonvulsant drug valproic acid are predominantly cleared as acyl glucuronides in humans (Presle et al., 1996; Williams et al., 1992; Dickinson et al., 1996). Diflunisal and valproic acid have been shown to covalently bind to proteins such as albumin and the cytoprotective antioxidant glutathione (**GSH**) (Williams et al., 1992; Brouwer et al., 1993; Dickinson et al., 1996). Alternatively, it has been suggested that xenobiotic glucuronidation may in fact be a type of carrier system, whereby glucuronidation helps to transport a protoxin from the site of conjugation to a distal organ (lung, bladder) (**fig. 8c**) (Mulder et al., 1986; Bock, 1991). At this distal site, it has been hypothesized that the xenobiotic-conjugate may be enzymatically or chemically deconjugated reforming the protoxin, therefore allowing it to be bioactivated and initiate toxicity (Benet and Spahn, 1988; Sinclair and Caldwell, 1982; Meffin et al., 1983; Ruelius et al., 1986).

FIGURE 8a



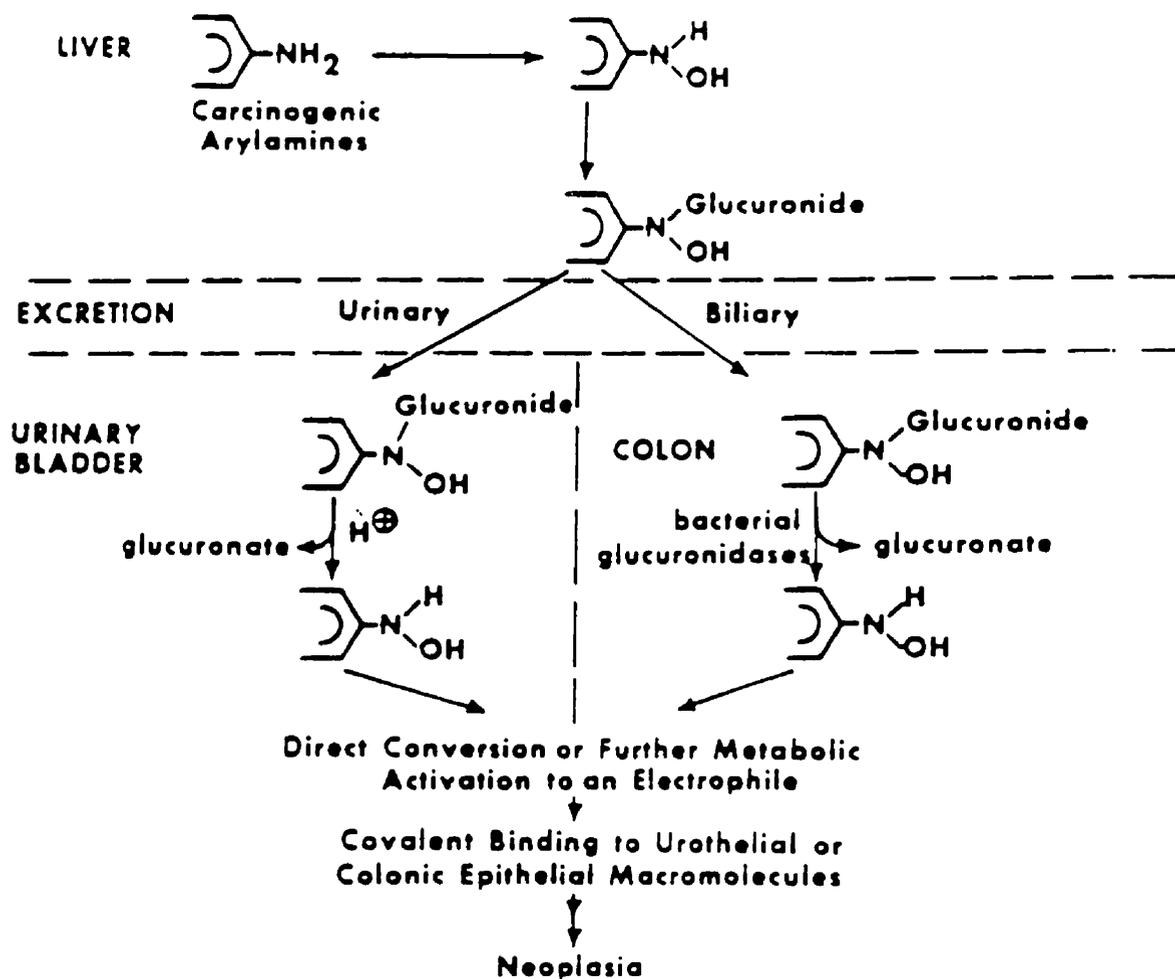
Representative glucuronide conjugate of a carboxylic acid drug (RCOOH) and its rearrangement from a β-glucuronidase-susceptible acyl glucuronide (1-O-acyl-β glucuronide) to β-glucuronidase-resistant 2-, 3- and 4-O-acyl-β isomers (Williams et al., 1992).

FIGURE 8b



Schematic representation of a nucleophilic displacement reaction of a 1-O-acyl-β glucuronide with a protein molecule and the resulting covalent bond (van Breeman and Fenselau, 1985).

FIGURE 8c



Hypothetical distal metabolism of a carcinogenic arylamine glucuronide in the bladder and colon (Mulder et al., 1986). Possible example may be the hydroxylation and subsequent N-glucuronidation of 2-naphthylamine in the liver. Biliary excretion (right side of figure) may lead to hydrolysis of the glucuronide forming the reactive hydroxylamine, which may then directly bind to essential macromolecules or be reduced to the amine. Similarly urinary excretion (left side of figure), due to the lower pH, may lead to the release of the glucuronide and formation of the hydroxylamine.

Table 4: Compounds known to form acyl glucuronides and irreversible covalent bonds.

Compound	Covalent Binding	Reference(s)
Anticonvulsant: • Valproic acid	• Albumin • GSH	Williams et al., 1992 Tang and Wang, 1996 ³
Antihyperlipidemic agent • Clofibrilic acid	• Albumin • Ethanethiol (small nucleophile)	Stogniew and Fenselau, 1982 Meffin et al., 1983 Rowe and Meffin, 1984 van Breeman and Fenselau, 1985
Bilirubin	• Albumin • Methanol (small nucleophile)	Salmon et al., 1974, 1975 Guatam et al., 1984 Benet and Spahn, 1988
Fenofibryl	• Albumin	Weil et al., 1988
NSAIDs: • acetylsalicylic acid • benoxaprofen ⁴ • diflunisal • flufenamic acid • flunoxaprofen • indomethacin • ketoprofen • oxaprozin • tolmetin ⁵ • zomepirac ⁴	• Albumin • Albumin • Albumin • Albumin • Albumin • Albumin • Albumin • Albumin • Albumin • Tubulin	Bradow et al., 1989 Dickinson et al., 1994 Bradow et al., 1989 Dickinson and King, 1989,1991 Watt et al., 1991 Worrall and Dickinson, 1995 ¹ Bradow et al., 1989 Benet and Spahn, 1988 van Breeman and Fenselau, 1985 Presle et al., 1996 Ruelius et al., 1986 Wells et al., 1987 Hyneck et al., 1988a, b Smith et al., 1985, 1986, 1990 Dickinson et al., 1996 ²
Oxaprozin	• Albumin	Ruelius et al., 1986

Abbreviations: GSH, glutathione; NSAID, non-steroidal anti-inflammatory drug.

¹ Rats injected with diflunisal acyl glucuronide-modified serum albumin developed anti-diflunisal antibodies (Worrall and Dickinson, 1995).

² Covalent binding inhibited tubulin polymerization, whereas the parent molecule had no effect.

³ A glucuronide/GSH diconjugate was detected.

⁴ Both zompirac and benoxaprofen were withdrawn from the market. Zompirac caused gastric irritation, nephritis and acute renal failure and also caused anaphylactic reactions (Clive and Stoff, 1984; Benet and Spahn, 1988).

⁵ Tolmetin, a structurally related drug to zompirac, may also cause anaphylactic and anaphylactoid reactions (Goldsmith, 1980; Bretza and Novey, 1985).

1.2.4 HUMAN UGT DEFICIENCIES

1.2.4.1 Mutations

Gilbert's syndrome, and the more severe case known as Crigler-Najjar syndromes type I and II, are phenotypically expressed as an increase in serum bilirubin levels and a jaundiced appearance (Wolkoff et al., 1985; Robertson et al., 1991). The molecular mechanisms responsible for these syndromes involve mutations in the coding and noncoding regions of the *UGT1* gene complex. At least 5-13% and less than 0.1% of the population have Gilbert's or Crigler-Najjar syndromes, respectively. It is generally believed that both Gilbert's and Crigler-Najjar syndromes type 1 and 2 are autosomal recessive disorders that result in jaundice due to decreased glucuronidation and accumulation of bilirubin (reviewed by Jansen, 1996) (**table 5**). While the more severe Crigler-Najjar syndromes are rare (about 0.1%), the incidence of Gilbert's syndrome in the population, once estimated to be about 6% based upon phenotype (plasma bilirubin concentrations), has been recently estimated, based upon genotype, to be as high as 15% (Monaghan et al., 1996; Jansen, 1996). As reviewed by Jansen (1996), Crigler-Najjar syndromes 1 and 2 are the result of one of twenty-one mutations in either exon 1*1 (*1A1*), or the common exons 2 to 5. These mutations have been reported in 24 type 1 and 4 type 2 Crigler-Najjar patients. The type 1 syndrome is due to various types of mutations, leading to either a truncated enzyme, full length inactive enzyme or no enzyme formation, which can lead in the most severe cases to complete absence of bilirubin-UGT activity, and thus total lack of bilirubin mono- and di-glucuronide formation. The 4 characterized type 2 patients reported in the literature were the result of missense mutations, one of which was peculiar in the sense that one allele expressed a truncated, totally inactive protein, while the second allele contained a missense mutation causing partial UGT-bilirubin protein inactivation. Clinically, the type 2 syndrome can be distinguished from type 1 since type 2 is less severe, includes the presence of bilirubin mono- and di-glucuronides in the bile, and bilirubin glucuronidation is inducible by phenobarbital.

The molecular mechanism of Gilbert's syndrome in at least some cases involves an abnormality in the promoter region at the 5' end of the *UGT1*1* (*1A1*) exon (Bosma et al., 1995; Monaghan et al., 1996). This area normally contains a TATAA element with six TA repeats

Table 5: Characteristics of Human Bilirubin UGT Deficiencies.

	Gilbert's Syndrome	Crigler Najjar Syndrome Type II	Crigler Najjar Syndrome Type I	Reference(s)
Severity	Least	Intermediate	Most	Burchell and Coughtrie, 1988
Pathological Consequence(s)	None (benign)	Usually benign	Kernicterus	Roy Chowdhury et al., 1995
Treatment	<ul style="list-style-type: none"> Phenobarbital 	<ul style="list-style-type: none"> Phenobarbital (30% reduction in serum bilirubin) Clofibrate Barbiturate Glutethimide 	<ul style="list-style-type: none"> No response to phenobarbital Photo therapy (photodegradation) Plasmaphoresis Liver transplant (bilirubin > 450 to 500 $\mu\text{mol/L}$) 	Burchell and Coughtrie, 1988 Jansen et al., 1992 Moghrabi et al., 1993
Phenobarbital Inducibility	Yes	Yes (limited)	No	
Serum Bilirubin Concentration (Normal=17 μM, 1 mg/dl)	<60 μM (<3.5 mg/dl)	60-340 μM (3.5mg/dl-20 mg/dl)	>340 μM (>20 mg/dl)	Fevry et al., 1977 Burchell and Coughtrie, 1988 Sinaasappel and Jansen, 1991 Bosma et al., 1992 Moghrabi et al., 1993
Bile Composition (Mono- or Diglucuronides)	<ul style="list-style-type: none"> Increased monoglucuronide Decreased diglucuronide 	<ul style="list-style-type: none"> 57% \pm 14% Monoglucuronide 10% \pm 2% Diglucuronide 	<ul style="list-style-type: none"> 9% \pm 11% Monoglucuronide 1% \pm 1% Diglucuronide 	Burchell and Coughtrie, 1988 Jansen et al., 1992
Decreases In Other UGT Activities	<ul style="list-style-type: none"> Acetaminophen Tolbutamide Rifamycin Josamycin Menthol 	<ul style="list-style-type: none"> 1-Naphthol o-Aminophenol 4-Methylumbelliferone 	<ul style="list-style-type: none"> Acetaminophen Menthol 1-Naphthol 4-Nitrophenol Propofol 4-Methylumbelliferone 5-Hydroxytryptamine 	Arias, 1962 Burchell and Coughtrie, 1988 de Morais et al., 1992b Jansen et al., 1992 Moghrabi et al., 1993
Mechanism(s) of Syndrome	Mutation in promoter region of <i>UGT1*1 (UGT1A1)</i>	Mutation in coding region (exons 1A1 and exons 2 and 5)	Mutations in coding regions (exons 1A1 and exons 2 to 5).	Jansen et al., 1992 Bosma et al., 1992 Moghrabi et al., 1993 Jansen, 1996 Monaghan et al., 1996

[(TA)₆TAA], while in Gilbert's patients this promoter region contains an extra TA. Monaghan et al. (1996) found that individuals heterozygous for an extra TA were phenotypically normal, while subjects identified as having Gilbert's syndrome with decreased bilirubin glucuronidation were homozygous for the extra TA [(TA)₇TAA]. It has been speculated that this extended promoter area may be less efficient in binding to regulatory proteins responsible for gene transcription. In contrast, Aono et al. (1995) have suggested that Gilbert's syndrome also may be due to a heterozygous mutation in either the *UGT1*1* (*1A1*) exon or the common exon 4, the homozygous equivalent of which would cause Crigler-Najjar syndrome. However, there is some controversy as to whether this latter molecular mechanism can produce Gilbert's syndrome (Jansen, 1996). Heterozygous and homozygous mutations in other specific *UGT* exons, such as *UGT1*4* (*UGT1A4*), have been reported with respective frequencies of 16% and 6%, but their toxicological importance is yet unknown (Burchell et al., 1994b). This would suggest that mutations in other *UGT* genes (exons) are likely to occur and be toxicologically important, depending upon the substrate and organ specificity (**table 6a, b**).

1.2.4.2 Clinical Manifestations

Bilirubin is eliminated as an ester acyl glucuronide via the biliary system, in a mono- or di-conjugated form (Billing et al., 1957; Schmid, 1956; Talafant, 1956). Thus a deficiency in bilirubin *UGT* activity would cause an accumulation of both plasma- and tissue-bilirubin in deficient individuals, the severity of which will determine the pathological outcome.

John F. Crigler and Victor A. Najjar in 1952 published a report of a syndrome descriptively termed congenital familial nonhemolytic jaundice and kernicterus, referred to as Crigler-Najjar syndromes. Their first report described 7 children with extremely elevated bilirubin levels and associated neurological dysfunction. Most of the children died before the age of one. Although the serum levels of bilirubin can vary among patients with the Crigler-Najjar syndromes, Arias et al. (1969) categorized the syndrome phenotypically into type I (>340 μ M or 20 mg/dl) and type II (<340 μ M), based upon unconjugated serum bilirubin levels (**table 5**).

Table 6a. Location and modulation of some toxicologically important human UGTs

Organ(s)	mRNA ^{a, b}	Protein/ Activity ^c	Modulator(s) ^{d, e}	Reference(s)
Buccal Mucosa	(UGT1A1) (UGT1A4)			McDonnell et al., 1996 McDonnell et al., 1996
Gut:				
• colon (sigmoidal)	UGT1A1 (7% ± 5%) UGT1A4 (15% ± 7%)	UGT antibody ¹² UGT1A1		Peters et al., 1987 ⁶ McDonnell et al., 1996 McDonnell et al., 1996
• esophagus	(UGT1A1) UGT1A4 (19% ± 10%)			McDonnell et al., 1996 McDonnell et al., 1996
• duodenum	UGT1A1 (97% ± 34%) UGT1A4 (38% ± 24%) UGT1A6	bilirubin glucuronidation UGT1A1 (UGT1A4) ⁴	smokers ¹ ↔	McDonnell et al., 1996 ⁵ Munzel et al., 1996 McDonnell et al., 1996 Munzel et al., 1996
• small intestine		UGT antibody ¹¹		Peters et al., 1987 ⁶
• stomach body	(UGT1A1) (UGT1A4)	UGT antibody ¹⁰		Peters et al., 1987 ⁶ McDonnell et al., 1996 McDonnell et al., 1996
• stomach antrum	(UGT1A1) (UGT1A4)	(bilirubin glucuronidation) (UGT1A1) (UGT1A4)		McDonnell et al., 1996 ⁵ McDonnell et al., 1996
Kidney	(UGT1A1) (UGT1A4) UGT1A6 UGT1A9	UGT antibody ⁹		Peters et al., 1987 ⁶ Sutherland et al., 1993 Ritter et al., 1992 ¹³ Sutherland et al., 1993 Sutherland et al., 1993 Ritter et al., 1992 ¹³ Munzel et al., 1996 Sutherland et al., 1993
Liver	UGT1A1 UGT1A4 UGT1A6 (UGT1F) UGT1A9	UGT antibody ⁷ UGT1A1 UGT1A4	phenobarbital ↑	Peters et al., 1987 ⁶ Sutherland et al., 1993 Munzel et al., 1996 Ritter et al., 1992 ¹³ Sutherland et al., 1993 McDonnell et al., 1996 ⁵ Ritter et al., 1992 ¹³ Sutherland et al., 1993 Munzel et al., 1996 Sutherland et al., 1993
Lung	UGT1A6			Munzel et al., 1996

(table continued)

Pharyngeal Mucosa • neoplastic • leukoplakia (preneoplastic) • normal	UGT1A6 UGT1A6 UGT1A6		smokers ² ↔	Ullrich et al., 1997 ³ Ullrich et al., 1997 ³ Ullrich et al., 1997 ³
Skin	(UGT1A1) (UGT1A4) UGT1A6	UGT antibody ⁸		Peters et al., 1987 ⁶ Ritter et al., 1992 ¹³ Ritter et al., 1992 ¹³ Ritter et al., 1992 ¹³

Abbreviations: UGT, UDP-glucuronosyltransferases; TCDD, 2,3,7,8-tetracholorodibenzo-p-dioxin.

^a UGTs in parenthesis indicate undetectable levels of the UGT mRNA .

^b Percentages in parenthesis indicate levels of UGT mRNA relative to liver UGT mRNA.

^c UGTs in parenthesis indicated undetectable levels of the UGT protein or activity.

^d Up arrow (↑), down arrow (↓) and horizontal double arrow (↔) indicate induction, inhibition/suppression or no effect, respectively.

^e Numbers in parenthesis indicate fold induction of either UGT mRNA , protein or activity.

¹ Smokers did not have higher UGT1A6 mRNA levels.

² The mean and median UGT activity measured by 4-methylumbelliferone and 1-naphthol was not significantly higher in smokers.

³ The mean and median levels of UGT1A6 expression and UGT activity measured by 4-methylumbelliferone and 1-naphthol were similar in all tissues.

⁴ HUG-Br-2 reactivity in duodenal samples was below the level of sensitivity of detection.

⁵ Bilirubin-UGT activity as measured by bilirubin-monoglucuronide and bilirubin-diglucuronide was detected in liver and duodenal samples, but was absent in stomach antral samples.

⁶ UGT monoclonal antibody produced against human liver UGTs inhibits 4-nitrophenol, 4-methylumbelliferone and bilirubin human UGT activity, but does not inhibit phenolphthalein, testosterone or estrone human UGT activity, or 4-nitrophenol rat UGT activity. It appears to block the utilization of UDPGA.

⁷ Immunoreactivity in human liver cryostat sections of the UGT monoclonal antibody was exclusively localized to the parenchymal cells and no immunoreactivity was seen in either the bile ducts, endothelium or Kupffer cells, and no zonal distribution was evident

⁸ Immunoreactivity of the UGT monoclonal antibody, in human skin cryostat sections was seen in both the stratum corneum and in the cells of the deeper stratum spinosum, with the former staining more intensely. The stratum basale had no detectable UGT immunoreactivity.

⁹ Immunoreactivity was found in the proximal tubular cells, whereas glomerulus, distal tubular cells and collecting tubules showed no staining.

¹⁰ Immunoreactivity was only seen in surface mucosa and not in parietal, chief or neck cells.

¹¹ Immunoreactivity was seen in the mucosa (maximal staining in the villous tips), with lesser staining of the epithelial crypt cells. The exact tiox of small intestine was not indicated in the original publication.

¹² The lining epithelial cells showed reactivity, while colon polyps showed decreased or no staining. Colon carcinoma showed no reactivity.

¹³ UGT1F, UGT1D and UGT1A (Ritter et al., 1992) were designated, using the new nomenclature (Mackenzie et al., 1997), as UGT1A1, UGT1A4 and UGT1A6, respectively.

Table 6b. UGT isozymes in some human cell lines.

Human Cell/Cell Line(s)	mRNA ^a	Protein/ Activity ^c	Modulator(s) ^{e,f}	Reference(s)
A549 cells (Lung carcinoma)	UGT1A6		TCDD ³ ↔	Munzel et al., 1996
Caco-2 cells (colon carcinoma)	UGT1A6	4-methylumbelliferone glucuronidation	TCDD (2.7-fold) ¹ ↑	Abid et al., 1995 Munzel et al., 1996
Hepatocytes (primary culture)	UGT1A6	4-methylumbelliferone glucuronidation	TCDD (2.4-fold) ² ↑	Munzel et al., 1996
LNCaP (testicular) cells	UGT2B10 UGT2B15			Belanger et al., 1995 Belanger et al., 1995

Abbreviations: UGT, UDP-glucuronosyltransferases; TCDD, 2,3,7,8-tetracholorodibenzo-p-dioxin.

^a UGTs in parenthesis indicate undetectable levels of the UGT mRNA .

^b Percentages in parenthesis indicate levels of UGT mRNA relative to liver UGT mRNA.

^c UGTs in parenthesis indicates undetectable levels of the UGT activity.

^d Percentages in parenthesis indicate levels of UGT activity relative to liver UGT activity.

^e Chemicals in parenthesis have been shown to either have no effect, downregulate or inhibit UGT isozyme gene expression or activity.

^f Numbers in parenthesis indicates fold induction of either UGT mRNA or activity.

^{1,2} Both mRNA and activity (i.e. 4-methylumbelliferone glucuronidation, non-specific for UGT1A6) was induced. Fold increases refer to activity.

³ A549 cells had constitutively elevated levels of UGT1A6 mRNA versus Caco-2 cells and were not induced.

In contrast to the Crigler-Najjar syndromes, Gilbert's syndrome, as discussed earlier, is thought to be relatively benign and is often undiagnosed since contributing conditions such as fasting and/or stress must often be present to reveal the underlying condition (Monaghan et al., 1996). Gilbert's syndrome is generally clinically characterized as a serum total bilirubin level of greater than 17 $\mu\text{mol/L}$. Due to the potential misdiagnosis, confirmation of the syndrome can be established by a 24 to 48 hr fast, which should increase total serum bilirubin in the range of 25-50 $\mu\text{mol/L}$ (Lascelles and Donaldson, 1989), or raise the percent of unconjugated bilirubin to greater than 90% (Owens and Sherlock, 1973).

Most textbooks of medicine do not associate Gilbert's syndrome with any pathological complications. However, as discussed below, it is still unclear whether patients with Gilbert's syndrome are more susceptible to xenobiotic-initiated toxicities, which may be particularly important for substrates that rely upon bilirubin UGT that are known to be deficient in Gilbert's patients. The fact that a large portion of the population is afflicted with this disorder suggests the need for further investigation.

1.2.4.3 Altered Xenobiotic Metabolism and Related Toxicities

In most cases, molecular studies have demonstrated that deficient substrate glucuronidation is due to decreased enzymatic activity secondary to gene alterations, rather than impairment of other processes such as uptake, transport or secretion (Burchell and Coughtrie, 1989; Robertson et al., 1991). As discussed previously, hereditary UGT deficiencies are common. It has been known for some time that people with Gilbert's or Crigler-Najjar syndromes have decreased glucuronidation of various substrates (**table 5**), other than bilirubin (Burchell and Coughtrie, 1989; Burchell et al., 1994b). Unlike the rare Crigler-Najjar syndromes, Gilbert's syndrome is not thought to be associated with any remarkable pathology, other than a high bilirubin plasma concentration (jaundice) (Wolkoff et al., 1985). However, most if not all textbooks in discussing syndromes of bilirubin-UGT deficiencies do not consider the potential toxicological implications of other UGT deficiencies which may only manifest itself when exposed to a potentially toxic xenobiotic which relies on UGTs, other than bilirubin UGT, for its elimination

(table 3). Furthermore, past reviews (Burchell and Coughtrie, 1989; Tephly and Burchell, 1990; Bock, 1991) have only speculated whether genetic variations in UGTs might lead to adverse drug effects, including chemically-initiated carcinogenesis.

Our lab has demonstrated that the glucuronidation of the analgesic drug acetaminophen in subjects with Gilbert's syndrome was impaired (de Morais et al., 1989, 1992b). Furthermore, recent evidence suggests that individuals with normal bilirubin-UGT have decreased glucuronidation of the ubiquitous environmental carcinogen B[a]P (Hu and Wells, 1993, 1998). Thus, in the absence of Gilbert's or Crigler-Najjar syndromes, hereditary deficiencies in other UGT isozymes and in particular the UGT isozymes for acetaminophen and B[a]P-metabolites, may be more common than Gilbert's syndrome. For example, in one human study, 1 of 5 subjects with normal bilirubin glucuronidation had deficient glucuronidation of acetaminophen (de Morais et al., 1992b), while in another study, 2 out of 12 subjects had virtually no glucuronidation of benzo[a]pyrene metabolites, and at least 1 other subject had deficient glucuronidation (Hu and Wells, 1993).

Thus, it appears that the incidence of UGT deficiencies for xenobiotics in subjects with normal bilirubin glucuronidation is at least 10%. Since Gilbert's syndrome is well-documented to be at least 5%, it is likely that the overall incidence in hereditary UGT deficiencies is at least 10%. Although limited data are available, it is becoming apparent that the readily recognizable bilirubin-UGT deficiency is not the only UGT deficiency in humans. Our lab has already found a 200-fold variation in lymphocytic-UGT activity from normal subjects (no bilirubin UGT deficiency) for the glucuronidation of hydroxylated B[a]P metabolites (Hu and Wells, 1993). Other studies (Carmella et al., 1993; Hecht et al., 1994) looking at human urinary metabolites of NNK have demonstrated a bimodally distributed, 100-fold variability in the levels of the NNAL (major carbonyl reduced metabolite of NNK) glucuronide, suggesting the potential clinical importance of UGT-catalyzed glucuronidation of this carcinogen. As mentioned above, UGT-deficient cultured fibroblasts have already been shown to be more susceptible to NNK-initiated genotoxicity (Kim and Wells, 1996a) (see Section 2.2).

Therefore, because UGTs play a critical role in the elimination of many xenobiotics, and

especially due to the way the *UGT1* gene is transcribed and translated (see above), it is now becoming more apparent that, depending on the molecular lesion(s) responsible, a deficiency of bilirubin glucuronidation could be a phenotypic marker for increased susceptibility to chemically-initiated toxicities. For this reason, we have postulated that hereditary deficiencies in UGTs could result in alternate, enhanced bioactivation of xenobiotics to toxic reactive intermediates (de Morais and Wells, 1988). This would result in a diverse spectrum of potential toxicological sequelae, including cellular necrosis, teratogenesis and carcinogenic initiation, depending upon which UGT isozyme was deficient, the severity of deficiency, the type and amount of xenobiotic exposure, and other genetic and environmental factors. We would not expect such an association to be revealed by traditional epidemiological studies, given the above modulating factors and the fact that both the types of UGT isozymes deficient and the severity of the deficiencies vary remarkably in Gilbert's syndrome (de Morais et al., 1992b; Robertson et al., 1991); hence, people with Gilbert's syndrome would not all respond the same. Of greater concern would be people with deficiencies in UGT isozymes other than bilirubin UGT1A1 (*UGT1*1*) (de Morais et al., 1992b; Hu and Wells, 1993), since at least the bilirubin UGT-deficient group are often visibly recognizable by their jaundice.

1.3 CYTOCHROMES P450

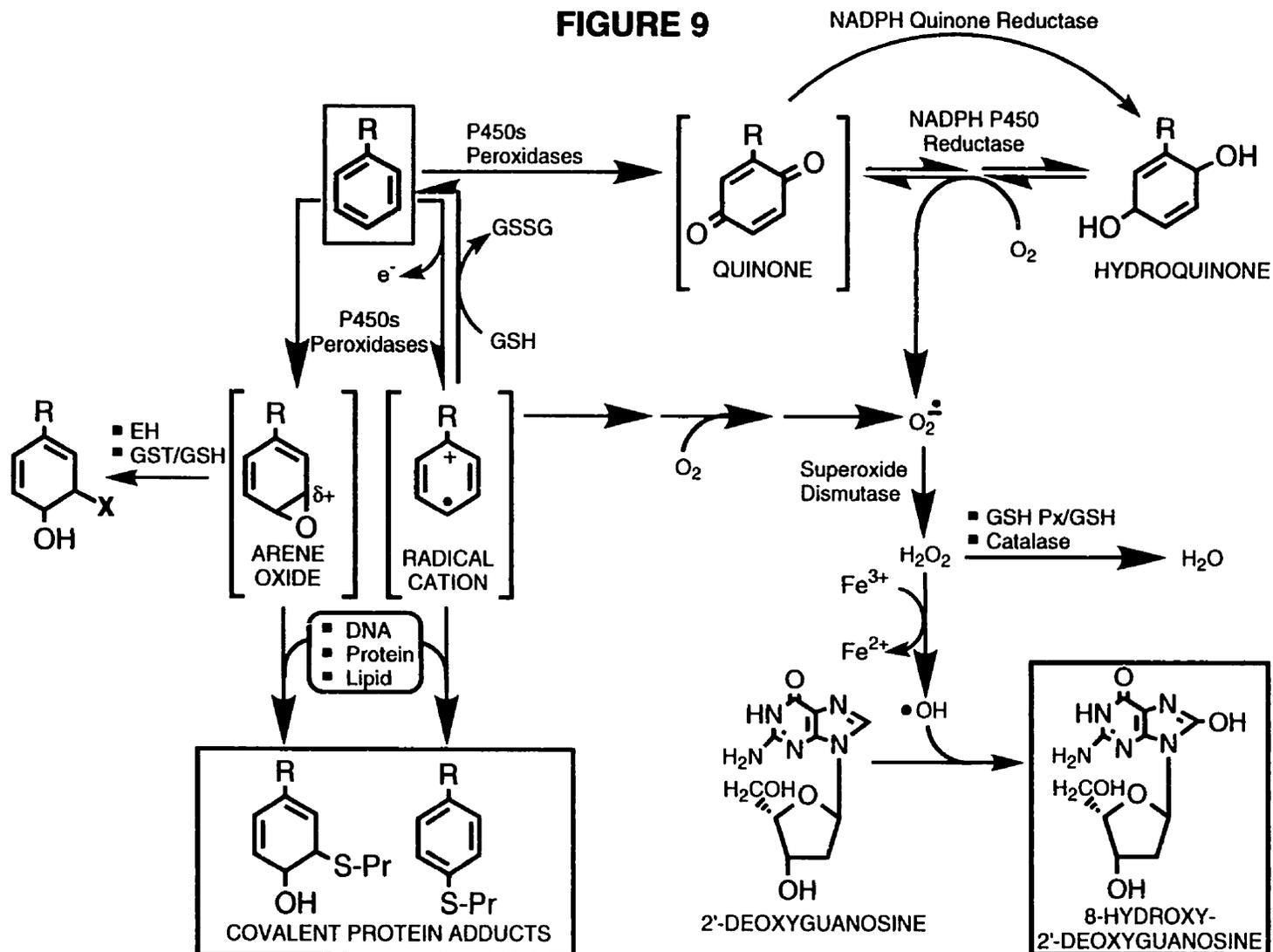
1.3.1. Constitutive P450s

Cytochromes P450 (**P450s**) are a superfamily of membrane-bound isozymes that are involved in the oxidation (e.g. hydroxylation) of numerous endobiotics and xenobiotics (Ortiz de Montellano, 1995). P450-catalyzed oxidation directly increases the hydrophilicity of lipophilic compounds, and provides a functional group for subsequent conjugation to more highly water soluble compounds, such as glucuronic acid. This increase in hydrophilicity facilitates rapid excretion of the previously lipophilic compound from the body. Oxidation of xenobiotics can also lead to the formation of various reactive intermediates which can damage essential macromolecules, leading to toxicity (**fig. 9**)

P450s can be categorized based on whether the P450 protein is normally expressed in a particular tissue (i.e. constitutive), or protein expression depends upon chemical induction (i.e. non-constitutive). However, categorizing P450s as simply constitutive or non-constitutive is in some ways an oversimplification since, similar to UGTs, P450 isozyme expression is variable depending on the organ examined, and whether an adult or embryo/fetus is being considered. A key example is the P4501A family of isozymes, and in particular cytochrome P4501A1 (**CYP1A1**) which is non-constitutive in human liver (see below), as opposed to CYP1A2, which is a constitutive hepatic isozyme with very low expression in extrahepatic tissue (Kaminsky and Fasco, 1992; Farin and Omiecnski, 1993). **Tables 7 and 8** list, based upon hepatic tissue, constitutive and non-constitutive P450 isozymes.

As discussed by Guengerich (1995), although total P450 levels only vary a few fold among individuals, specific constitutive P450 isozyme expression can vary many fold more, often as the result of P450 induction by environmental agents. Not surprisingly, the genetic makeup of an individual is a significant determinant of P450 expression and therefore toxicological susceptibility. Similar to UGT deficiencies, variations in the levels and activities of P450s have been measured in the human population, which is often referred to as P450 isozyme polymorphisms, where a significant (>1%) percentage of the population will have a dramatic increase or decrease in P450 activity/ levels. These polymorphisms can often occur in different

FIGURE 9



Enzymatic bioactivation of a representative xenobiotic (shaded box). Xenobiotics can be bioactivated to a number of reactive intermediary metabolites, including arene oxides/epoxides, radicals and quinones, which can covalently bind to essential macromolecules (DNA, protein, lipid), thereby initiating toxicity. Formation of toxic reactive oxygen species (ROS) such as superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$), can be initiated by xenobiotic radicals, or via the P450 reductase-dependent redox cycling of quinones. ROS can oxidise proteins, lipids and DNA, exemplified in the latter case by the oxidation of 2'-deoxyguanosine (a DNA base) to its 8-hydroxylated derivative, a mutagenic lesion. Xenobiotic reactive intermediates are variously detoxified by epoxide hydrolase (EH) and/or glutathione (GSH) S-transferase (GST) (arene oxides/epoxides, quinones), while xenobiotic radicals can be non-enzymatically detoxified (reduced) by GSH, forming the parent compound. X represents -OH for EH-catalysed reactions, and -SG for GST-catalysed reactions. For ROS, $O_2^{\cdot-}$ can be converted by superoxide dismutase to H_2O_2 , which in turn can be detoxified by GSH peroxidase or catalase, forming water.

Table 7: Toxicologically Important human constitutive and non-constitutive hepatic P450s and known inducers.

Cytochrome P450 Isozyme (CYP)	Constitutive/ Non-constitutive	Inducer(s)	Reference(s)
CYP1A1	Non-constitutive	TCDD, 3-MC, B[a]P	McManus et al., 1990 Okey et al., 1994
CYP1A2*	Constitutive	TCDD, 3-MC, B[a]P, Omeprazole	Shimada et al., 1989a,b Kaminsky and Fasco, 1992 Farin and Omiecinski, 1993 Quattrochi et al., 1994 Diaz et al., 1990 Quattrochi and Tukey, 1993 Rost and Roots, 1993
CYP1B1	Constitutive	TCDD	Sutter et al., 1994 Savas et al., 1994
CYP2A6†	Constitutive	-	Yun et al., 1991
CYP2C9	Constitutive	Barbiturates	Zilly et al., 1977
CYP2E1	Constitutive	Ethanol	Perrot et al., 1989
CYP3A4‡	Constitutive	α-naphthoflavone Barbiturates, Glucocorticoids	Shimada and Guengerich, 1989 Shimada et al., 1989a,b Watkins et al., 1985 Mimura et al., 1993
CYP3A5	Constitutive	-	McManus et al., 1990 Wrighton et al., 1989
CYP3A7‡	Non-constitutive	-	Komori et al., 1990 Nebert et al., 1991 Wrighton et al., 1988 Wrighton and VanBranden, 1989 Yang et al., 1994

Abbreviation: 3-MC, 3-methylcholanthrene; B[a]P, benzo[a]pyrene; CYP, cytochrome P450; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

† CYP2A6 is a minor form comprising only 1% of the total hepatic P450 content (Gonzalez and Gelboin, 1994).

‡ P4503A family of isozymes is the most abundant hepatic P450 (Guengerich, 1990).

‡ CYP3A7 is a major constitutive P450 isozyme in human fetuses (Nebert et al., 1991; Wrighton et al., 1988; Wrighton and VanBranden, 1989), and is not expressed in human adult liver (Wrighton et al., 1989; Wrighton et al., 1990). However, Schuetz et al. (1994) did show CYP3A7 mRNA in adult human liver tissue (7 out of 13 adults).

* CYP1A2 appears to be strictly a hepatic enzyme (i.e. no extrahepatic expression).

Table 8: Toxicologically important rat constitutive and non-constitutive hepatic P450s and known inducers.

Cytochrome P450 Isozyme (CYP)	Constitutive/ Non-constitutive	Inducer(s)	Reference(s)
CYP1A1	Non-constitutive*	TCDD, 3-MC, β -NF	Lau and Strobel, 1982 Thomas et al., 1983 Ayrton et al., 1988, 1990 Ryan and Levin, 1990 Okey et al., 1984
CYP1A2	Constitutive	TCDD, 3-MC, β -NF	Lau and Strobel, 1982 Thomas et al., 1983 Ayrton et al., 1988, 1990
CYP1B1	Non-constitutive \forall	TCDD	Pottenger et al., 1991 Sutter et al., 1994 Walker et al., 1995
CYP2A1	Constitutive	TCDD, 3-MC, β -NF	Lau and Strobel, 1982 Thomas et al., 1983 Ryan and Levin, 1990 Ryan et al., 1979 Okey et al., 1984
CYP2B1	Non-constitutive	Phenobarbital	Thomas et al., 1983 Ryan and Levin, 1990 Adesnik and Atchison, 1985
CYP2B2	Constitutive	Phenobarbital	Thomas et al., 1983 Ryan and Levin, 1990 Adesnik and Atchison, 1985
CYP2C11	Constitutive \dagger	Hormonal regulation	Legraverend et al., 1992 Ryan and Levin, 1990
CYP2C12	Constitutive \ddagger	Hormonal regulation	Strom et al., 1987 Ryan and Levin, 1990
CYP2E1	Constitutive	Ethanol	Gonzalez and Gelboin, 1994 Koop and Tiery, 1990 Yang et al., 1992 Ryan et al., 1986

Abbreviation: 3-MC, 3-methylcholanthrene; B[a]P, benzo[a]pyrene; CYP, cytochrome P450; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

\dagger Male rat specific P450 isozyme (Legraverend et al., 1992).

\ddagger Female rat specific P450 isozyme (Strom et al., 1987).

* CYP1A1 is either non-constitutive or expressed at very low levels in the liver of experimental animals (Ryan and Levin, 1990).

\forall CYP1B1 mRNA appears to be non-constitutive in the male rat liver, and at very low levels in female rat liver (Walker et al., 1995). CYP1B1 appears to be constitutive primarily in the rat adrenal glands.

racial populations, and have been shown to involve the CYP2D6 and CYP2C19 isozymes (Shimada et al., 1994; Nakamura et al., 1985; Relling et al., 1991; Keeney and Waterman, 1993; Nakamura et al., 1985; Wedland et al., 1984; Kupfer and Preisig, 1984; de Morais et al., 1994). Although controversial (Roots et al., 1993; Ritter et al., 1986; Drakoulis et al., 1986; Spiers et al., 1990; Ladero et al., 1991; Caporaso et al., 1992), it has been hypothesized that the underactivity or overactivity of the xenobiotic bioactivating enzyme P450 (e.g. CYP2D6) may be a significant contributing factor to xenobiotic-initiated toxicities. For example, an early study demonstrated an association between high CYP2D6 activity (extensive metabolizer phenotype) and lung cancer (Ayesh et al., 1984) and interestingly, the tobacco-specific carcinogen NNK is known to be bioactivated by CYP2D6 (Crespi et al., 1991) and thus may explain the possible association of CYP2D6 activity to lung cancer. However, conflicting results do exist (Sugimura et al., 1990, Caporaso et al., 1989, 1990; Boobis and Davies, 1990; Law et al., 1989; Spiers et al., 1990), and may be related to the fact that NNK is only one of many carcinogens found in cigarette smoke (e.g. B[a]P), NNK is bioactivated by many other P450 isozymes, and NNK may be a poor substrate for CYP2D6 (Islam et al., 1991). Conversely, it appears that a deficiency in the constitutive CYP2D6 isozyme may be protective against lung cancer, although the mechanism for this finding is still unknown (Idle et al., 1992; Caporaso et al., 1991; Ingelman-Sundberg, 1992). Likewise, a CYP2D6 isozyme deficiency may predispose certain people to Parkinson's disease (Barbeau et al., 1985, 1987; Armstrong et al., 1992; Fonne-Pfister et al., 1987), and deficiencies in the P450s (CYP21, CYP17, CYP11B1) mediating steroid hydroxylation (i.e. steroidogenesis) can lead to congenital adrenal hyperplasia (Miller and Morel, 1989; White et al., 1991; Higashi et al., 1991; Keeney and Waterman, 1993; Donohoue et al., 1995). Thus, it appears that overexpression/overactivity or underexpression/underactivity of P450s may, at least in part, play a role in disease which may not necessarily be related to xenobiotic exposure.

1.3.2 Non-constitutive P450s

Non-constitutive P450 isozymes must be induced by endogenous or exogenous compounds in order for significant activity to be present. As discussed above, P450s can be constitutive or non-constitutive depending on the tissue examined. For example, CYP1A1 is

expressed at very low levels (i.e. non-constitutive) in human liver but is at significantly higher levels in extrahepatic tissues such as the lung (Crestiel and Eisen, 1988; Shimada et al., 1992; Schweikl et al., 1993). This P450 isozyme has been extensively investigated in both humans and animals since it plays a major role in the bioactivation of carcinogens, such as B[a]P (Gelboin, 1980; see section 2.1). Overactivity of the CYP1A1 isozyme has been associated with enhanced bioactivation of tobacco carcinogens such as B[a]P and lung cancers have recently been linked to mutations in the *p53* tumor suppressor gene, possibly caused by B[a]P (Denissenko et al., 1996). Extensive epidemiological work has been conducted in order to associate CYP1A1 with lung cancer, and it appears that absolute levels of CYP1A1 do significantly correlate with lung cancer (Kouri et al., 1982; McLemore et al., 1990), although the association of CYP1A1 inducibility and cancer is still unclear (Kellerman et al., 1973a,b; Paigen et al., 1981; Leboeuf et al., 1981; Kouri et al., 1984). **Table 9** and **10** lists some toxicologically important xenobiotics and the P450 isozymes involved in their bioactivation.

Table 9: Human cytochrome P450 isozymes and important toxicological substrates.

Human Cytochrome P450 Isozyme (CYP)	Substrate(s)	Reference(s)
CYP1B1	2-Aminofluorene Polycyclic aromatic hydrocarbons: • B[a]P-7,8-diol • Dimethylbenz(a)anthracene • Chrysene	Shimada et al., 1996
CYP1A1	Polycyclic aromatic hydrocarbons: • B[a]P • B[a]P-7,8-diol • Dimethylbenz(a)anthracene	Guengerich and Shimada, 1991 Guo et al., 1995 Roberts-Thomas et al., 1993 Shimada et al., 1989a,b Yun et al., 1992 Bauer et al., 1995 Gonzalez and Gelboin, 1994 Roberts-Thomas et al., 1993
CYP1A2	• Arylamines/Heterocyclic amines • Acetaminophen • Aflatoxin • B[a]P • B[a]P-7,8-diol • Caffeine • NNK • Steroids	Guengerich and Shimada, 1991 McManus et al., 1990 Butler et al., 1992, 1989 Crespi et al., 1990, 1991 Patten et al., 1993 Sandhu et al., 1994 Fisher et al., 1992 Bauer et al., 1995 Gonzalez and Gelboin, 1994 Roberts-Thomas et al., 1993
CYP2A6	• Aflatoxin B ₁ • Nitrosamines • NNK • NNAL • NNN • DMN • DEM	Guengerich and Shimada, 1991 Yun et al., 1991 Crespi et al., 1990, 1991a,b Yamazaki et al., 1992 Smith et al., 1992 Gonzalez and Gelboin, 1994
CYP2C8	• Retinoic acid • Retinol • B[a]P†	Leo et al., 1989 Cosme et al., 1994 Bauer et al., 1995
CYP2C9	• B[a]P† • Phenytoin • Warfarin	Veronese et al., 1991 Rettie et al., 1992 Bauer et al., 1995
CYP2C10	• B[a]P†	Bauer et al., 1995
CYP2C18	• B[a]P†	Bauer et al., 1995
CYP2C19	• S-Mephenytoin	Wrighton et al., 1993 Goldstein et al., 1994
CYP2D6	• Codeine • Debrisoquine • Sparteine • NNK	Dayer et al., 1988 Gonzalez et al., 1987 Tucker et al., 1977 Distlerath and Guengerich, 1984 Gonzalez and Gelboin, 1994

(table continued)

CYP2E1	<ul style="list-style-type: none"> • Acetaminophen • B[a]P† • Benzene • Ethanol • Nitrosamines <ul style="list-style-type: none"> • NNK • NNAL • NNN • DMN • DEM 	Guengerich et al., 1991 Guengerich and Shimada, 1991 Crespi et al., 1990, 1991b Yamazaki et al., 1992 Smith et al., 1992 Bauer et al., 1995 Gonzalez and Gelboin, 1994 Seaton et al., 1994
CYP3A4	<ul style="list-style-type: none"> • 6-Hydroxychrysene • Acetaminophen • Aflatoxin B₁ & G₁ • B[a]P • B[a]P-7,8-diol • Codeine • Warfarin 	Shimada et al., 1989a,b Guengerich and Shimada, 1991 Patten et al., 1993 Guengerich et al., 1994 Pellinen et al., 1994 Brian et al., 1990 Shimada and Guengerich, 1989 Bauer et al., 1995 Gonzalez and Gelboin, 1994 Roberts-Thomas et al., 1993
CYP3A5	<ul style="list-style-type: none"> • B[a]P • B[a]P-7,8-diol 	Roberts-Thomas et al., 1993
CYP4A11	Suspected substrates: <ul style="list-style-type: none"> • Prostaglandins • Leukotrienes 	Kawashima et al., 1992 Nelson et al., 1993 Roman et al., 1993
CYP5	<ul style="list-style-type: none"> • Prostaglandin H₂ 	Haurand et al., 1985 Nusing et al., 1990 Ullrich and Graf, 1984 Hecker and Ullrich, 1989

Abbreviations: B[a]P, benzo[a]pyrene; CYP, cytochrome P450 isozyme; DEN, N,N-diethylnitrosamine; DMN, N,N-dimethylnitrosamine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, nornitrosocotine.

† CYP2C8, CYP2C10, CYP2E1, and CYP2C18 metabolized minor amounts (e.g. 7,8-diol, <1 pmol/min) of B[a]P versus CYP1A1 (e.g. 7,8-diol, 594 nmol/min) (Bauer et al., 1995).

Table 10: Rat cytochrome P450 isozymes and toxicologically important substrates.

Rat Cytochrome P450 Isozyme (CYP)	Substrate(s)	Reference(s)
CYP1B1	• Benzantracene	Otto et al., 1991, 1992
CYP1A1	• 2-Acetylaminofluorene • 7,12-Dimethylbenz[a]anthracene • Benzo[a]pyrene • Benzo[a]pyrene-7,8-diol • Ethoxyresorufin	Guengerich, 1988 Ellard et al., 1991 Nebert, 1982 Lake, 1983 Thakker et al., 1985 Kadlubar and Hammons, 1987
CYP1A2	• 2-Acetylaminofluorene • Aflatoxin B ₁ • Benzo[a]pyrene	Guengerich, 1988 Nebert, 1982
CYP2B1	• 2-Acetylaminofluorene • Aflatoxin B ₁ • Benzo[a]pyrene • Pentoxyresorufin	Guengerich, 1988 Lake, 1983 Lubet et al., 1985 Doehmer et al., 1988
CYP2C11†	• Aflatoxin B ₁ • Benzo[a]pyrene	Guengerich, 1988 Todorovic et al., 1991‡
CYP2C12‡	• Aflatoxin B ₁	Guengerich, 1988
CYP2E1	• N,N-Dimethylnitrosamine • Benzene • Acetaminophen • Ethanol	Guengerich, 1988 Koop et al., 1989 Nakajima et al., 1989 Ryan and Levin, 1990 Yang et al., 1990 Schrenk et al., 1992 Gut et al., 1993, 1996

† Male rat specific P450 isozyme (Legraverend et al., 1992)

‡ Female rat specific P450 isozyme (Strom et al., 1987).

‡ CYP appears to metabolize B[a]P to the 9,10- and 7,8-diol but does not convert B[a]P-7,8-diol to tetrols.

1.4 PROSTAGLANDIN H SYNTHASE

1.4.1 PHS-1

Prostaglandin H synthase-1 (**PHS-1**), which is also referred to by one of its components as cyclooxygenase-1 (**COX-1**) is the constitutive isoform found in various cell types and organs (Otto and Smith, 1995), whereas PHS-2 is the nonconstitutive and highly inducible isoform (Smith et al., 1996). PHS-1 is a developmentally regulated “housekeeping” protein that is encoded by a 22 kilobase gene, consisting of 11 exons (Kraemer et al., 1992). Although PHS-1 is constitutive in most tissues, it is not expressed in every cell of a particular organ (Smith and DeWitt, 1996). PHS-1 levels appear to change throughout development (Brannon et al., 1994) and during cell differentiation (Smith et al., 1993; Hoff et al., 1993; Nusing et al., 1995; Takahashi et al., 1994), although its expression is constant throughout the cell cycle (DeWitt and Meade, 1993). As reviewed by Smith et al. (1996), regulation of the PHS-1 gene is not yet well characterized, and it appears that similar to other so-called “housekeeping” genes, the TATA box sequence is not involved in gene expression. However, similar to PHS-2, PHS-1 can be induced by compounds such as phorbol esters, and hence is both a constitutive and inducible isoform (Ueda et al., 1997).

As reviewed by Smith et al. (1996), PHSs are pharmacologically important as targets for non-steroidal antiinflammatory agents, such as aspirin (acetylsalicylic acid), which inhibit PHS activity. Aspirin plays an important role in reducing the risk of mortality from cardiovascular disease by blocking PHS-1-catalysed production of platelet thromboxane A_2 (Patrono, 1994). PHSs are toxicologically important due to their ability to bioactivate xenobiotics to highly toxic reactive intermediates, usually short-lived radicals (**fig. 9**). Both PHS-1 and -2 are glycosylated, heme-containing, homodimeric proteins that have a cyclooxygenase and a hydroperoxidase catalytic component, which respectively convert arachidonic acid (**AA**) to prostaglandin G_2 (**PGG₂**), and PGG₂ to prostaglandin H_2 (**PGH₂**) (Smith et al., 1996). AA is further converted to

many different PGs and thromboxanes. It is the latter hydroperoxidase step, catalysed by both PHS isoforms, that can use endobiotics and xenobiotics as reducing equivalents, in the process often producing toxic reactive intermediates (Smith et al., 1991). **Table 11** lists some toxicologically important xenobiotics that are known or suspected to be bioactivated to reactive intermediates by peroxidases such as PHSs.

1.4.2. PHS-2

PHS-2 is encoded by an 8 kilobase long gene consisting of 10 exons, and is located on a separate chromosome from that of PHS-1 (Kujubu and Herschman, 1992). Upon induction, PHS-2 protein appears to be expressed at higher levels in the nuclear envelope when compared to the endoplasmic reticulum, whereas PHS-1 is equally distributed (Morita et al., 1995; Ueda et al., 1997). Since PHS-2 is considered the nonconstitutive PHS isoform, it is not surprising that its pattern of expression and biology differ from that of PHS-1, which shares 60% amino acid similarity (Smith et al., 1996). Although PHS-2 is often referred to as a non-constitutive inducible isoform, it is actually constitutively present in a number of different organs, including the brain (Yamagata et al., 1993), testes (Simmons et al., 1991), trachea (epithelial cells) (Walenga et al., 1996) and the kidney (macula densa) (Harris et al., 1994). Nevertheless, as reviewed by Smith et al. (1996) PHS-2 is undetectable in most mammalian tissue and has been shown *in vitro* to be quickly induced (2-6 hr) by various stimuli (e.g. growth factors, tumor promoters, hormones, bacteria, endotoxin, cytokines) in various cells such as fibroblasts (Evetts et al., 1993; DeWitt and Meade, 1993; Kujubu et al., 1993; Hans et al., 1990), endothelial cells (Jones et al., 1993), monocytes (O'Sullivan et al., 1992) and ovarian follicles (Sirios et al., 1992).

PHS-2 can be induced by numerous stimuli such as endotoxins, cytokines and mitogens (Lee et al., 1992; Xie et al., 1992; Thiemermann, 1994). Induction of PHS-2 mRNA levels can be due to either increased gene expression (DeWitt and Meade, 1993) or post-transcriptional regulation (Ristimaki et al., 1996), both of which are inhibited by anti-inflammatory steroids such as cortisol and dexamethasone (Kujubu and Herschman, 1992; Masferrer et al., 1994; Crofford et al., 1994). As reviewed by Mitchell et al. (1995), it is believed that the PHS-2, through the active

Table 11: Substrates bioactivated by peroxidase and lipoxygenase enzymes.

Substrate(s)	Enzyme(s)	Reference(s)
2-Naphthylamine	Prostaglandin H synthase	Boyd and Eling, 1987
2-Acetylaminofluorene	Horseradish peroxidase Prostaglandin H synthase	Boyd et al., 1983 Boyd and Eling, 1984
Acetaminophen	Prostaglandin H synthase	Moldeus and Rahimtula, 1980 Mohandas et al., 1981 Zenser and Davis, 1984 Moldeus et al., 1985 Potter and Hinson, 1987 Ben-Zvi et al., 1990 Keller and Hinson, 1991
Aflatoxin B ₁	Lipoxygenase Prostaglandin H synthase	Battista and Marnett, 1985 Liu et al., 1990 Datta and Kulkarni, 1994
Benzene	Myeloperoxidase	Smith et al., 1989 Smith, 1996
Benzidine	Horseradish peroxidase Prostaglandin H synthase	Joseph et al., 1983a,b
Benzo[a]pyrene • parent compound	Horseradish peroxidase Lipoxygenase Prostaglandin H synthase	Marnett et al., 1977 Nemoto and Takayama, 1984 Cavalieri et al., 1988a,b Cavalieri and Rogan, 1992a,b Eling et al., 1990 Kim et al., 1997 Winn and Wells, 1997
• 7,8-diol	Lipoxygenase Prostaglandin H synthase	Marnett et al., 1975 Panthanickal et al., 1983 Byczkowski and Kulkarni, 1989 Hughes et al., 1989 Eling et al., 1990 Joseph et al., 1994
Cyclophosphamide	Lipoxygenase Prostaglandin H synthase	Kanekal and Kehrer, 1993
Diethylstilbestrol	Prostaglandin H synthase	Degen et al., 1982
Dimethadione	Prostaglandin H synthase	Wells et al., 1989b Liu and Wells, 1995a Parman et al., 1996 Parman et al., 1998
HPPH	Prostaglandin H synthase	Parman et al., 1998
Mephenytoin (l-, d-isomers)	Prostaglandin H synthase	Liu and Wells, 1995a Parman et al., 1996 Parman et al., 1998

(table continued)

Nirvanol (l-, d-isomers)	Prostaglandin H synthase	Liu and Wells, 1995a Parman et al., 1996 Parman et al., 1998
NNK	Prostaglandin H synthase	Bilodeau et al., 1995 ¹ Kim and Wells, 1996
Phenobarbital	Prostaglandin H synthase	Parman et al., 1998
Phenytoin	Horseradish peroxidase Lipoxygenase Myeloperoxidase Prostaglandin H synthase Thyroid peroxidase	Utrecht and Zahid, 1988 Kubow and Wells, 1989 Miranda et al., 1994 Liu and Wells, 1995b Yu and Wells, 1995 Parman et al., 1996 Parman et al., 1998
Retinoic acid	Prostaglandin H synthase	Samokyszyn et al., 1984
Tetramethylhydrazine	Prostaglandin H synthase	Kalyamaraman et al., 1983
Thalidomide	Prostaglandin H synthase	Wells et al., 1989b Liu and Wells, 1995a Arlen and Wells, 1996 Parman et al., 1996 Parman et al., 1998
Trimethadione	Prostaglandin H synthase	Wells et al., 1989b Liu and Wells, 1995a Parman et al., 1996 Parman et al., 1998

Abbreviations: HPPH, 5-(p-hydroxyphenyl)-5-phenylhydantoin; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

1. Bilodeau et al. (1995) did not show an inhibition of NNK-initiated DNA oxidation by nonsteroidal anti-inflammatory agents.

production of PGs, plays a central role in the process of inflammation.

1.5 DNA DAMAGE

1.5.1 MOLECULAR ASPECTS

1.5.1.1 Oxidation

Cellular damage via the oxidation of essential macromolecules, such as DNA, protein and lipid is now recognized as a major factor in both the normal aging process (Harman, 1981; Randerath et al., 1996), and in xenobiotic-initiated diseases which are not necessarily associated with aging (e.g. carcinogenesis, atherosclerosis, teratogenesis) (Cathcart et al., 1984; Adelman et al., 1988; Ames, 1983; Heinecke, 1987; Berlett and Stadtman, 1996; Wells et al., 1997). Xenobiotic reactive intermediates are not only capable of covalently binding to macromolecules (see below), but also can initiate oxidative damage, a molecular mechanism potentially mediating toxicity. Since DNA encodes all of the cellular components, it is thought that DNA damage may be the critical lesion responsible for carcinogenesis (Cavalieri and Rogan, 1992) and possibly teratogenesis (Wells et al., 1997). DNA oxidation has been extensively investigated in various animal and cellular models resulting in the discovery of numerous types of oxidized DNA lesions (i.e. oxidized DNA bases), such as 8-hydroxy-2'-deoxyguanosine (**8-OH-2'-dG**) (**fig. 9, tables 13a, b**) (Breen and Murphy, 1995; Cadet et al., 1997; Infante et al., 1973; Nishimoto et al., 1983; Dizdaroglu and Simic, 1985; Berger and Cadet, 1985; Dizdaroglu, 1985). These oxidative lesions can both induce DNA strand breaks, as well as cause DNA point mutations, both of which may be factors in chemically-initiated carcinogenesis (Floyd, 1990). **Table 12** lists some toxicologically important agents/xenobiotics which have been shown to initiate oxidative damage.

As discussed above (see P450 and PHS sections), enzymatic bioactivation of xenobiotics is often required for the production of highly reactive intermediary metabolites (Wells et al., 1996), including free radicals that can secondarily produce various reactive oxygen species (**ROS**) such as superoxide anions ($\text{O}_2^{\cdot-}$), hydroxyl radicals ($\cdot\text{OH}$) and hydrogen peroxide (H_2O_2) (**fig. 9**) (Halliwell and Gutteridge, 1989; Fridovich, 1997). As discussed above, ROS may either directly damage macromolecules, or further interact with cellular transition metals (e.g. iron, copper) or lipids to produce further reactive species, such as peroxy radicals (**ROO \cdot**),

Table 12: Toxicologically important xenobiotics known to either oxidize and/or covalently bind to essential macromolecules.

Xenobiotics	Molecular Damage		References
	Oxidation	Covalent Binding	
Acetaminophen	• GSH	• GSH • Protein	Dahlin et al., 1984 Albano et al., 1985 Jaeschke, 1990 Bruno et al., 1991 Roberts et al., 1991
Aflatoxin B ₁	• Lipid	• GSH • DNA • Protein	Booth et al., 1981 Battista and Marnett, 1985 Toskulkao and Glinsukon, 1988 Liu et al., 1990 Walsh et al., 1992 Hsieh and Hsieh, 1993
Benzene	• DNA • Protein	• DNA • Protein	Snyder et al., 1978 Lutz and Schlatter, 1979 Irons, 1985 Kahn et al., 1990 Leanderson and Tagesson, 1990 Lagorio et al., 1994 Soucek et al., 1994 Gut et al., 1996 Smith, 1996 Snyder and Hedli, 1996
Benzo[a]pyrene	• DNA • Protein	• GSH • DNA • Protein	Osborne and Crosby, 1987 Frenkel, 1989 Kim and Wells, 1997 Winn and Wells, 1997
Cyclophosphamide	• DNA • Lipid	• DNA	Benson et al., 1988 Pilans et al., 1989 Lear et al., 1992
HPPH	• DNA		Kim et al., 1997
Mephenytoin	• DNA		Liu and Wells, 1995a
Nirvanol	• DNA		Liu and Wells, 1995a
NNK	• DNA	• DNA • Protein	Ronai et al., 1993 Peterson et al., 1993 Bilodeau et al., 1995 Hecht, 1996

(table continued)

phenytoin	<ul style="list-style-type: none"> • GSH • DNA • Lipid • Protein 	<ul style="list-style-type: none"> • DNA • Protein 	Martz et al., 1977 Liu and Wells, 1994, 1995a Winn and Wells, 1995, 1997 Yu and Wells, 1995 Kim et al., 1997
Thalidomide	<ul style="list-style-type: none"> • GSH • DNA • Protein 		Liu and Wells, 1995a Arlen and Wells, 1996
Trimethadione	<ul style="list-style-type: none"> • DNA 		Liu and Wells, 1995a
Valproic acid		<ul style="list-style-type: none"> • GSH • Protein 	Porubek et al., 1989 Tang and Abbott, 1996

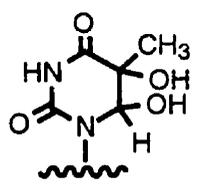
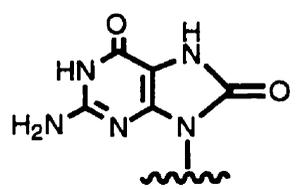
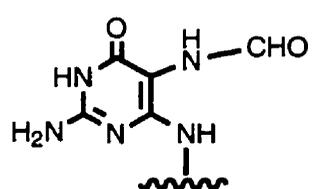
Abbreviations: HPPH, 5-(p-hydroxyphenyl)-5-phenylhydantoin; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

Table 13a. Enzymes involved in the repair of oxidative DNA damage (from Demple and Harrison, 1994) (also see fig. 10).

<i>E. coli</i> enzymes	Specificity	Eukaryotic Counterpart
Exonuclease III	AP endo (II); 3'-repair Inducible (<i>katF/rpos</i>)	<ul style="list-style-type: none"> • Ape (human) • Apex (mouse) • Bap1 (bovine)
Endonuclease IV	AP endo (II); 3'-repair Inducible (<i>soxRS</i>)	<ul style="list-style-type: none"> • Apn1 (yeast)
Endonuclease III (Thymine glycol glycosylase)	TG glycosylase; β -lyase	<ul style="list-style-type: none"> • UV endonuclease I & II (mouse, human) • Activity found in yeast & bovine cells
FAPy glycosylase (Fpg/MutM)	FAPy/8oxoG glycosylase	<ul style="list-style-type: none"> • Activity detected in HeLa cells
MutY protein	Adenine glycosylase (8oxoG: A preference)	<ul style="list-style-type: none"> • G:A mismatch endonuclease activity detected in human cells

Abbreviations: AP endo (II), class II (hydrolytic) AP endonuclease; 3'-repair, 3'-PGA diesterase/3'-phosphatase; TG glycosylase, thymine glycol glycosylase; β -lyase, class I AP endonuclease; FAPy, formamidopyrimidine; 8oxoG, 8-oxo-7,8-dihydroguanine (8-hydroxyguanine); dRPase, 5'-deoxyribosephosphodiesterase.

13b. Representative oxidised DNA bases repaired by the above proteins (from Demple and Harrison, 1994).

Oxidised DNA Base	Structure	Possible Effects
Thymine glycol		<ul style="list-style-type: none"> • Replicative block • Poorly mutagenic (?)
8-Oxoguanine		<ul style="list-style-type: none"> • Miscoding • Mutagenic
Formamidopyridine		<ul style="list-style-type: none"> • Replicative block • Cytotoxic (?)

which also are known to damage macromolecules, possibly through the direct epoxidation of xenobiotics, such as B[a]P-7,8-diol (Marnett et al., 1978; Dix et al., 1985) and aflatoxin B₁ (Battista and Marnett, 1985).

Cytoprotective mechanisms exist within the cell to mitigate the toxicity associated with ROS production. Enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase all work together to detoxify or eliminate both O₂^{•-}, H₂O₂ and ROOH (**fig. 9**) (Halliwell and Gutteridge, 1989; Mannervik, 1985; El-Hage and Singh, 1990; Fridovich, 1986; Anderson, 1996). The cell also has molecules such as glutathione (GSH; also a co-factor for GSH peroxidase) and vitamins C and E, and β-carotene, that can directly act as antioxidants by quenching or terminating free radicals (Kosower and Kosower, 1978; Packer, 1991; Halliwell and Gutteridge, 1989).

1.5.1.2 Covalent Binding

Xenobiotics, such as B[a]P, 7,12-dimethylbenz[a]anthracene and NNK, can be bioactivated to electrophilic reactive intermediates that can irreversibly or covalently bind to essential macromolecules (i.e. adduct formation) (Osborne and Crosby, 1989; Devanesan et al., 1993) (**fig. 9**). Electrophilic intermediates capable of adduct formation can either be an arene oxide, epoxide, free radical or radical cation (O'Brien, 1985; Cavalieri and Rogan, 1992a,b; Wells et al., 1996) (see above) (**table 12**). Similar to oxidative DNA damage, DNA adduct formation may be particularly important since the latter also can cause DNA sequence mispairings, and mutations potentially leading to cancer (Williams, 1992; Groopman and Kensler, 1993; Groopman et al., 1994; Hemminki et al., 1994).

The cell has developed cytoprotective enzymes which help to protect the cell from electrophilic reactive intermediates. The enzyme epoxide hydrolase catalyzes the insertion of a hydroxide ion into the arene oxides and epoxides to form a stable (non-toxic) trans-dihydrodiol, which can either be directly excreted or further conjugated with glucuronic acid. GSH can either directly reduce xenobiotic free radicals and radical cations back to the parent molecule, or act as a co-substrate for the enzyme GSH S-transferase (**GST**). GSH S-transferase catalyze the

conjugation of arene oxides and other electrophiles with GSH (Larsson et al., 1983).

If the cytoprotective mechanisms fail to prevent macromolecular adduct formation or oxidation, various cellular repair mechanisms exist (see below). These repair processes are critical, since both oxidation and adduct formation occur naturally from normal cellular processes and hence the cell must protect itself from both endogenous and exogenous insult (Marnett and Burchman, 1993; Gupta and Spencer-Beach, 1996). Interestingly, DNA adducts may be stable or they may be relatively unstable and thus cause depurination of DNA bases, which also must be repaired (Todorovic et al., 1991; Devanesan et al., 1996).

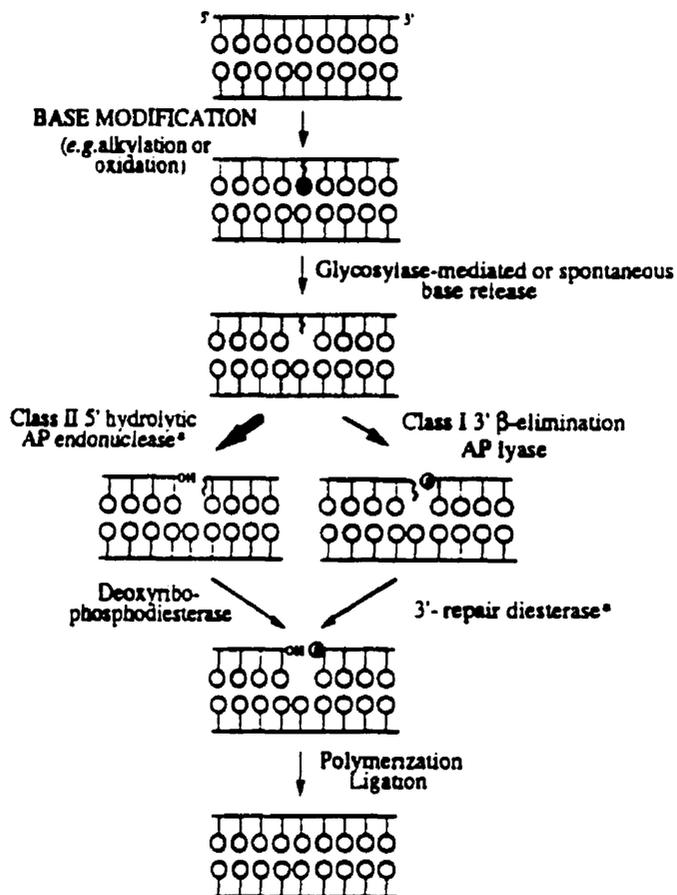
1.5.2 DNA REPAIR

1.5.2.1 Oxidation/Strand breaks

The repair of DNA is carried out by numerous different enzymes, all of which have evolved to handle specific types of DNA damage. The importance of these repair processes is highlighted by the fact that DNA repair enzymes are highly conserved among species (Ramotar and Masson, 1996). Furthermore, although DNA repair deficiencies are relatively rare, the essential nature of these enzymatic pathways is evident by the prevalence of diseases caused by DNA repair deficiencies, such as xeroderma pigmentosum (Friedberg, 1985) and certain types of cancer (Moderich and Lahue, 1996).

As discussed above, since aerobic organisms are constantly bombarded with ROS, it is critical that oxidized DNA repair mechanisms exist. Although not specific for oxidized DNA bases, the class of enzymes termed glycosylases or endonucleases are important in hydrolysing the base-sugar (N-C glycosidic) bond of modified (oxidative and non-oxidative) bases and thus forming abasic sites (Friedberg, 1985, Sancar and Sancar, 1988) (**fig. 10, tables 13a, b**). The abasic sites are then cleaved by endonucleases, and DNA synthesis and ligation fill in the gaps (Graves et al., 1992, Price and Lindahl, 1991). This repair process is termed base excision repair, not to be confused with nucleotide excision repair (see below). An example of base excision repair is the formamidopyrimidine glycosylase (Fpg) enzyme, also known as MutM, which is critical in the repair of oxidized guanosine (8oxoG or 8-OH-2'-dG) from DNA. It has been

FIGURE 10



The base excision repair pathway for oxidized and alkylated DNA. Repair is initiated by cleavage of the modified DNA base from the sugar backbone. The abasic site is then hydrolyzed further by endonucleases, followed by repair synthesis (from Memisoglu and Samson, 1996).

demonstrated that the loss of Fpg in bacteria causes approximately a 5-fold increase in the rate of spontaneous GC to TA transversions, indicating the importance of the Fpg enzyme (Cabrera et al., 1988). In addition to the glycosylase enzymes, exonucleases and general excision repair also can help repair oxidized DNA lesions although the latter is a secondary defense (Demple and Harrison, 1994; Sancar and Tang, 1993; Czczot et al., 1991).

The production of xenobiotic reactive intermediates (e.g. radical, quinones) and associated ROS can often result in single and double strand DNA breaks. However, unlike single strand breaks, double strand DNA breaks pose a particularly challenging problem of repair, since no template strand is available for the repair process. Recombinant repair carried out by specific enzymes (e.g. Rec enzymes) catalyze the process by which two opposing DNA ends are realigned, and joined together using a homologous sequence from a donor DNA strand (West, 1992; Peters et al., 1991; Nickoloff and Little, 1997) (**fig. 11a, b**). In conjunction with recombination, enzymatic mismatch repair is critical for the final proofreading of the new recombinant DNA by correcting any base mismatches (Modrich and Lahue, 1996). Interestingly, mismatch repair enzymes can stop recombination if the DNA sequences are too divergent. Similar to other repair pathways, recombination appears to be highly conserved, and deficiencies in recombination can lead not only to hypersensitivity to DNA strand breaking agents, but also can lead to immune dysfunction, such as that seen in the severe combined immunodeficient (SCID) mouse (Sancar and Sancar, 1988, West, 1992, Friedberg et al., 1991; Petes et al., 1991; Nickoloff and Little, 1997).

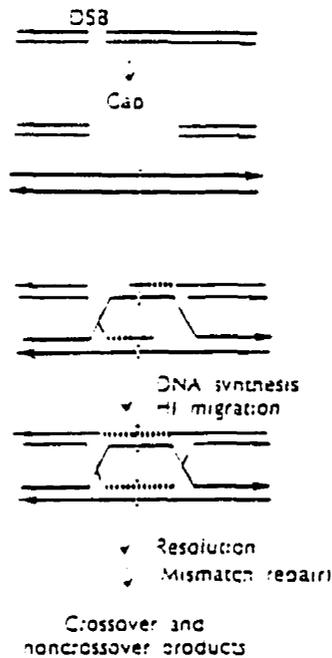
1.5.2.2 Arylation/Methylation

Nucleotide excision repair is similar to base excision repair, but the former does not involve the initial removal of the modified DNA base. DNA damage associated with bulky adducts such as B[a]P are repaired by nucleotide excision. Nucleotide excision repair involves the hydrolysis of phosphodiester bonds upstream and downstream of the damaged DNA base, resulting in an excised stretch of nucleotides between 12 to 13 nucleotides (prokaryotes) or 27 to 29 nucleotides (eukaryotes) (Sancar, 1994; Ramotar and Masson, 1996) (**fig. 12**).

FIGURE 11a

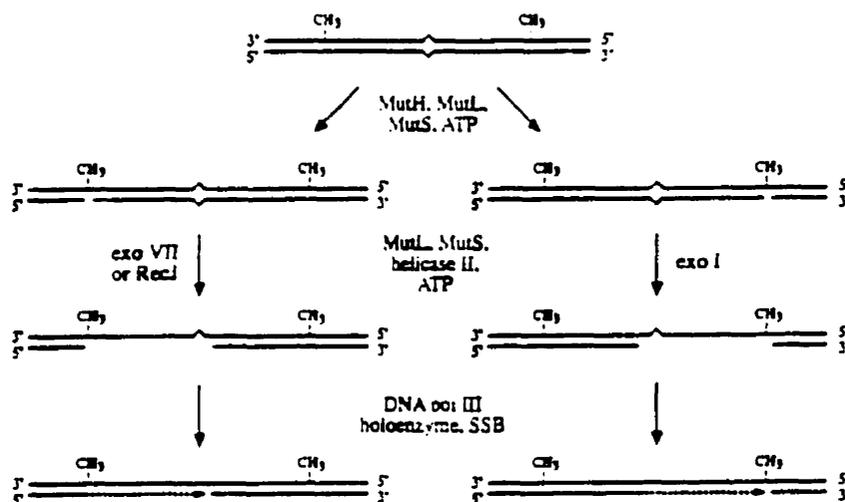
DSB/gap repair model

29a

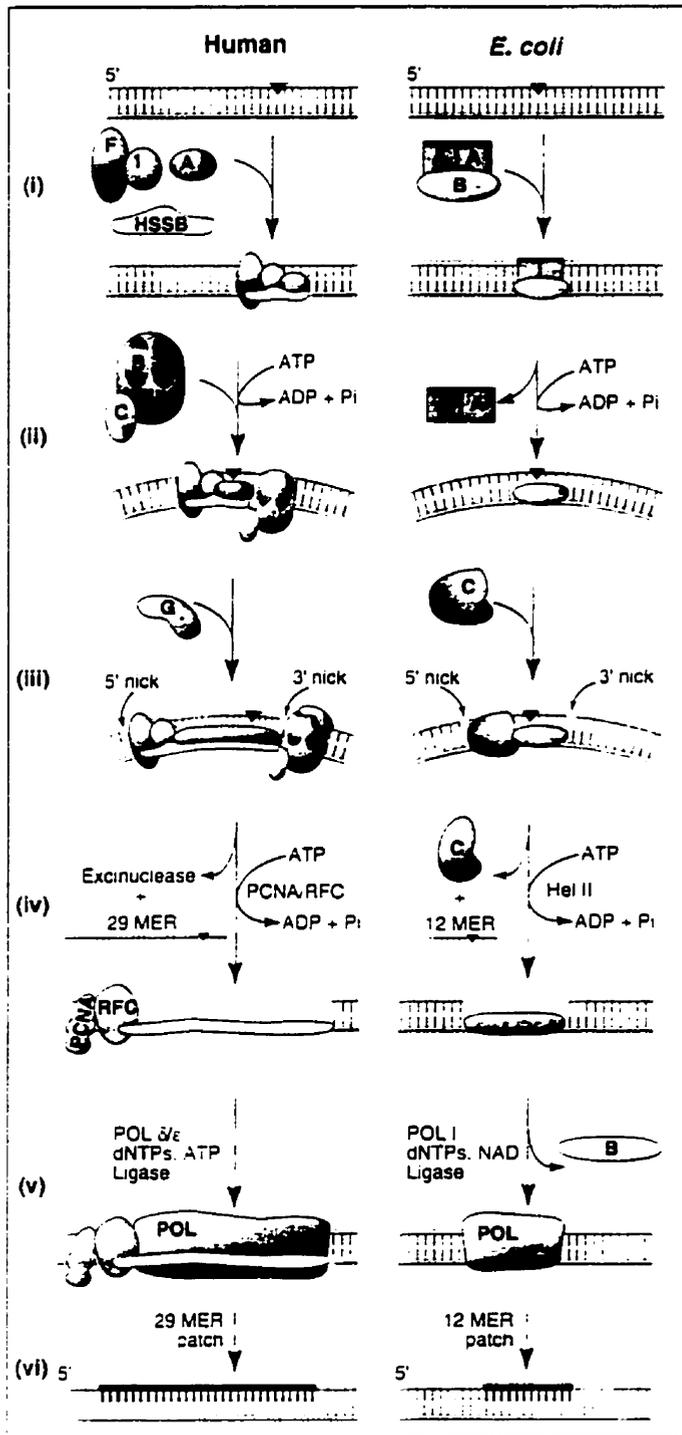


Model of recombinant double-strand break (DSB) repair. The DSB/gap repair model involves the enlargement of the gap (DSB) and the invasion of a single DNA strand into an undamaged allele. This then initiates primer repair synthesis, producing two Holliday junctions (HJ) that are cleaved and then DNA ends are annealed (resolution). DNA mismatched repair then follows (see below). (from Nickoloff and Little, 1997).

FIGURE 11b



The figure above depicts methyl-directed mismatch repair. Mismatch repair can follow recombinant DNA repair (Modrich and Lahue, 1996).



Molecular mechanisms involved in nucleotide excision repair in humans and *E. coli* (from Sancar, 1994). In *E. coli* two UvrA (A) proteins and one UvrB (B) protein form a complex and bind to the damaged DNA strand, which unwinds and kinks the DNA (step i). UvrB undergoes a conformational change which releases the UvrA proteins (step ii), and then UvrC binds to UvrB which make 3'- and 5'-nicks in the DNA, respectively (step iii). UvrD (Helicase II, Hel II) releases both UvrC and the excised oligomer (12-mer) (step iv). DNA polymerase I then releases UvrB and fills in the excision gap and the patch is ligated (steps v and vi). A similar mechanism occurs in humans.

Similar to other DNA lesions, the methylation of DNA, caused by carcinogens such as NNK, can induce transition mutations (G to T) and thus erroneous methylation of DNA has been associated with cancer (Laird and Jaenisch, 1994). Interestingly, both abnormal hyper- or hypomethylation of DNA may play a role in cancer. Unlike the repair processes above, DNA methylation is repaired without the need for DNA excision, resynthesis and ligation (Memiscglu and Samson, 1996). The enzyme, DNA methyltransferase directly reverses the damaged (i.e. methylated) DNA base by transferring the methyl group (as well as larger alkyl groups) on DNA bases to an active cysteine residue within the methyltransferase protein. In so doing, the methyltransferases are inactivated, hence they are often termed, "suicide enzymes" (Friedberg et al., 1995; Lindahl et al., 1982, Pegg and Byers, 1992, Samson, 1992).

SECTION 2: STUDIES

2.1 STUDY #1: PEROXIDASE-DEPENDENT BIOACTIVATION AND OXIDATION OF DNA AND PROTEIN IN BENZO[A]PYRENE-INITIATED MICRONUCLEUS FORMATION^{1,2}

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1. Preliminary reports of this research were presented at the annual meetings of the Society of Toxicology USA (Toxicologist 14:223, 1994) and the American Association for Cancer Research (Proc. Am. Assoc. Cancer Res. 36: 600, 1995). The evidence demonstrating TCDD induction of PHS was first reported at the March 1996 meeting of the Society of Toxicology USA (Fundam. Appl. Toxicol. 30 (Suppl. 1): 246, 1996). This research was supported by a grant to PGW from the Medical Research Council of Canada.
2. Published in Free Rad. Biol. Med. 23(4): 579-596, 1997.
3. All studies were conducted by Perry Kim.

2.1.1 ABSTRACT

Micronucleus formation initiated by benzo[a]pyrene (B[a]P) and related xenobiotics is widely believed to reflect potential carcinogenic initiation, yet neither a dependence upon bioactivation nor the critical enzymes have been demonstrated. Using rat skin fibroblasts, protein oxidation (carbonyl formation) and content of prostaglandin H synthase (PHS) and cytochrome P4501A1 (CYP1A1) protein were determined by Western blot/immunodetection with enhanced chemiluminescence. DNA oxidation as 8-hydroxy-2'-deoxyguanosine formation was quantified using high-performance liquid chromatography with electrochemical detection. Fibroblast CYP1A1 activity assessed as ethoxyresorufin-O-deethylase was not detectable, and even CYP1A1 protein was measurable only after induction with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). However, TCDD additionally induced prostaglandin H synthase (PHS), which also was detectable constitutively. B[a]P 10 μ M initiated the oxidation of DNA and protein, and the formation of micronuclei, all of which were enhanced over 2-fold by the dual CYP1A1/PHS inducer TCDD 10 nM, as well as by other PHS inducers, 12-O-tetradecanoylphorbol-13-acetate 1 μ M and interleukin-1 α 0.625 or 1.25 ng/ml, that do not induce CYP1A1 ($p < 0.05$). Conversely, B[a]P target oxidation and micronucleus formation were abolished by 1-aminobenzotriazole 1mM ($p < 0.05$), which was a potent inhibitor of both peroxidases and P450. These results provide the first direct evidence that B[a]P-initiated micronucleus formation, like carcinogenic initiation, requires enzymatic bioactivation, and that peroxidase-dependent, reactive oxygen species-mediated oxidation of DNA, and possibly protein, constitutes a molecular mechanism of initiation in uninduced cells. Induction of either CYP1A1 or peroxidases such as PHS substantially enhances this genotoxic initiation, which may reflect cancer risk.

2.1.2 INTRODUCTION

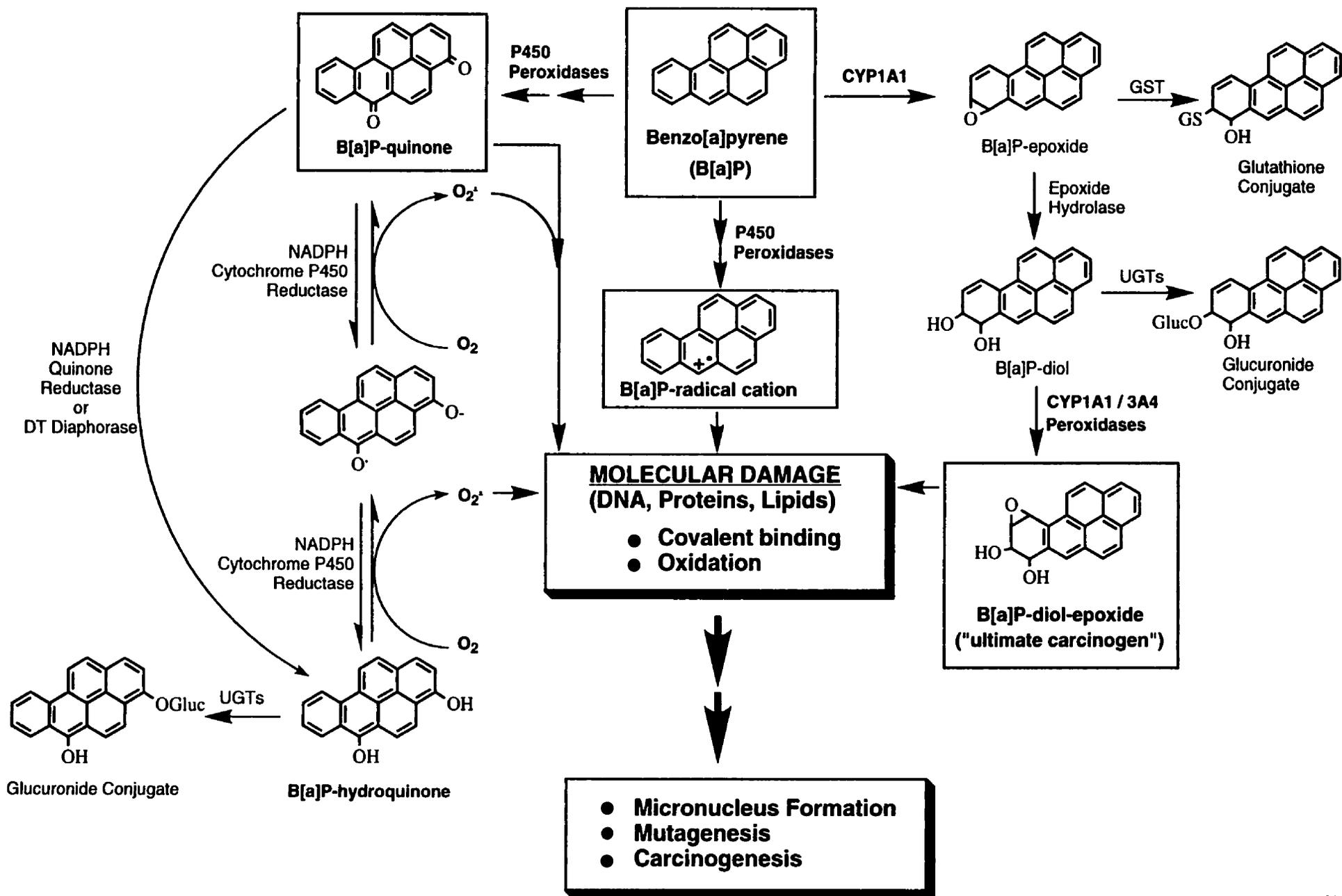
DNA damage by chemicals, including covalent binding and oxidation, is thought to constitute an essential molecular mechanism in the initiation of cancer (Miller, 1970; Kehler, 1993) and possibly teratogenesis (Manson, 1980; Nicol et al., 1995). Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon (PAH) formed from the incomplete combustion of organic matter, is both a human and animal carcinogen (Osborne and Crosby, 1987), and a teratogen in animals (Nicol et al., 1995). While nontoxic itself, B[a]P is metabolized *in vivo* to reactive intermediates that can covalently bind to DNA and protein (Osborne and Crosby, 1987), and initiate oxidative stress and the formation of reactive oxygen species (ROS), resulting in the oxidation of DNA, protein and lipid (Frenkel, 1989). The mutagenicity, carcinogenicity and teratogenicity of B[a]P are thought to depend upon its enzymatic bioactivation to reactive intermediates, and the subsequent irreversible damage to molecular targets (Nicol et al., 1995; Dipple et al., 1984). Several potential bioactivating pathways exist, catalysed by cytochromes P450 (P450) (Osborne and Crosby, 1987), in particular P4501A1 (CYP1A1), and peroxidases such as prostaglandin H synthase (PHS) (Marnett, 1990a) (fig. 1). In addition to the detoxification of reactive intermediates by epoxide hydrolase and glutathione S-transferase (Osborne and Crosby, 1987), the UDP-glucuronosyltransferases (UGTs) appear to be cytoprotective and genoprotective by catalyzing the glucuronidation and elimination of B[a]P, thereby avoiding bioactivation (Vienneau et al., 1995).

The electrophilic reactive intermediate B[a]P-7,8-diol-9,10-epoxide (BPDE), which binds covalently to DNA and protein, is believed to be a primary proximate mediator of toxicity, or "ultimate carcinogen". In humans and rats, CYP1A1 catalyses the initial epoxidation of B[a]P. The epoxide product then serves as a substrate for epoxide hydrolase producing B[a]P-7,8-diol, which is subsequently epoxidized via a second cytochrome P450-catalysed reaction to BPDE (fig. 1).

BPDE also can be produced by the reaction of B[a]P-7,8-diol and peroxy radicals (ROO•). ROO• are carbon-centered radicals coupled to molecular oxygen (Ingold, 1969). They

Figure 1. Postulated roles of P450s and peroxidases such as prostaglandin H synthase and lipoxygenases in B[a]P bioactivation and micronucleus formation. B[a]P may be bioactivated to the electrophile B[a]P-7,8-diol-9,10-epoxide ("ultimate carcinogen"), catalyzed by the isozyme CYP3A4 in humans and CYP1A1 in rats. Peroxidases also may catalyze the formation of peroxy radicals (ROO•) that react with B[a]P forming B[a]P-quinones. B[a]P-quinones can covalently bind or redox cycle producing reactive oxygen species (e.g. superoxide anion, O₂⁻). Both P450s and peroxidases also can bioactivate B[a]P via a one-electron oxidation to a free radical cation. Both electrophiles and free radicals can damage DNA and proteins via covalent binding and oxidation. Abbreviations: P450, cytochromes P450; CYP1A1, cytochrome P4501A1; GST, glutathione S-transferase; -SG, glutathione conjugates; UGT, UDP-glucuronosyltransferases; OGluc, glucuronide conjugate.

FIGURE 1



are relatively long-lived (0.1-10 sec) (Pryor, 1986), and can epoxidize isolated double bonds found in retinoic acid (Marnett, 1990b), aflatoxin B₁ (Battista and Marnett, 1985) and B[a]P-7,8-diol (Marnett et al., 1978; Dix et al., 1985). ROO• can be intermediates in the generation of hydroperoxides and are products of their metabolism (Marnett, 1990b). Lipid hydroperoxides are produced from polyunsaturated fatty acids, catalysed both by the cyclooxygenase component of PHS, and by lipoxygenases (LPOs) (Pace-Asciak and Asotra, 1989; Chamulitrat and Mason, 1989). The lipid hydroperoxides are then converted to prostaglandins and other eicosanoids by the hydroperoxidase activity associated with these enzymes (Pace-Asciak and Asotra, 1989). Thus, B[a]P can be bioactivated by a three-step mechanism involving CYP1A1 followed by epoxide hydrolase, with the third step catalysed by either P450, and/or a peroxidase (ROO•)-mediated oxidation, producing the BPDE (Marnett, 1987) (**fig. 1**).

A third potential mechanism of B[a]P toxicity is through its oxidation to toxic quinones (Marnett et al., 1977) (**fig. 1**). B[a]P can be oxidized by P450s and during prostaglandin biosynthesis to form isomeric B[a]P quinones (Marnett et al., 1977; Marnett et al., 1975; Joseph and Jaiswal, 1994). The oxidants responsible for peroxidase-catalysed quinone formation are likely the lipid-derived ROO• (Reed, 1988), catalysed by both native and heat-denatured PHS, as well as by methemoglobin, metmyoglobin and monomeric hemein (Marnett and Reed, 1979). Acyl and alkyl peroxy radicals also have been shown to convert B[a]P to quinones (Mahoney et al., 1982). Depending upon the molecule, quinones can both redox cycle, forming highly toxic reactive oxygen species (ROS), and can covalently bind to cellular macromolecules (Reed, 1988; Monks et al., 1992). B[a]P quinones are capable of producing superoxide anions (O₂⁻) and hydrogen peroxide (H₂O₂) (Lorentzen and T'so, 1977) (**fig. 1**). O₂⁻ can be enzymatically converted to H₂O₂, which if not detoxified (i.e. converted to H₂O) by catalase or glutathione (GSH) peroxidase, can produce other ROS such as hydroxyl radicals (HO•) via the Fenton reaction (Halliwell and Gutteridge, 1989).

B[a]P also may be bioactivated by a one-electron oxidation reaction, catalysed by

P450s and peroxidases, to a B[a]P radical cation which eventually can either form B[a]P quinones (Cavalieri et al., 1988), or directly covalently bind to DNA (Cavalieri et al., 1988; Cavalieri and Rogan, 1992a,b) (**fig. 1**). This array of toxic reactive intermediates (i.e. epoxides, quinones, radicals) all are potentially capable of initiating genotoxicity, teratogenesis and carcinogenesis (Gutteridge, 1993; Halliwell and Cross, 1994; Cerutti, 1994; Wells and Winn, 1996).

A widely used and simple measure, or “biomarker”, of genotoxicity and potential carcinogenic initiation is the formation of micronuclei. These structures contain chromosomal fragments or whole chromosomes that fail to be incorporated into the daughter nucleus upon cell division (Heddle et al., 1988). Micronuclei have been studied in many cell types including human skin fibroblasts (Hennig et al., 1988) and buccal cells (Tolbert et al., 1992).

B[a]P can initiate the formation of micronuclei containing either chromosomal fragments at low doses (1-5 µg/ml, 4-20 µM), or whole chromosomes (kinetochore-positive micronuclei) at higher doses (5-10 µg/ml, 20-40 µM), in XEM2 cells (genetically engineered V79 Chinese hamster lung cells expressing rat liver CYP1A1) (Ellard and Parry, 1993). Recent studies using cultured skin fibroblasts from RHA rats showed that B[e]P, a non-carcinogenic isomer of B[a]P, fails to initiate micronucleus formation, whereas B[a]P caused a significant increase (Vienneau et al., 1995). However, the molecular mechanism of B[a]P-initiated micronucleus formation remains to be elucidated. Although Vienneau et al. (1995) demonstrated enhanced B[a]P-initiated micronucleus formation in UGT-deficient RHA rat skin fibroblasts, the mechanism remains unknown, since CYP1A1 is non-constitutive (Gonzalez, 1988), and the fibroblasts were not induced. It was not shown whether B[a]P was bioactivated by CYP1A1 or other enzymes such as peroxidases, or indeed whether bioactivation was required for micronucleus formation. Furthermore, it remains to be determined whether enzyme induction of either CYP1A1 or peroxidases causes a further increase in B[a]P-initiated micronucleus formation in rat skin fibroblasts, as would be expected with carcinogenic initiation. Accordingly, while enzymatic bioactivation of B[a]P and irreversible molecular target damage are thought to be essential for carcinogenic initiation, their role in micronucleus formation has not been demonstrated, raising a basic question as to the carcinogenic relevance of micronucleus formation.

This hypothesis and its potential relevance to carcinogenic initiation was evaluated in cultured Wistar rat skin fibroblasts incubated with CYP1A1 and peroxidase inhibitors and inducers. These studies provide the first evidence that the molecular mechanism of B[a]P-initiated micronucleus formation involves ROS-mediated oxidation of DNA, and possibly protein, which in uninduced cells is dependent upon constitutive peroxidase activity, and is substantially enhanced by induction of either peroxidases or CYP1A1.

2.1.3 MATERIALS AND METHODS

Animals

Mice. Male C57BL/6 mice weighing 22-26 g (Charles River Canada Ltd., St. Constant, Quebec) were housed five per plastic cage with ground corn cob bedding (Beta Chip; Northeastern Products Corp., Warrensburg, NY).

Rats. Male Wistar rats, 180-200 g (Charles River Canada Ltd., St. Constant, Quebec) were housed in separate cages with a wire mesh floor.

All animals were kept in a temperature-controlled room with a 12-hr light-dark cycle automatically maintained. Food (Laboratory Rodent Chow 5001, PMI Feeds Inc.; St. Louis, MO) and tap water were provided *ad libitum*. Animals were acclimatized for a minimum of one week.

All animal studies were approved by the University of Toronto Animal Care Committee in accordance with the standards of the Canadian Council on Animal Care.

Chemicals

The CYP1A1 inducer 2,3,7,8-[1,6-³H]tetrachlorodibenzo-p-dioxin (1 dpm=1 x 10⁻¹⁴ moles TCDD) was a gift from Dr. Allan B. Okey (University of Toronto, Toronto, Ontario). The P450 inhibitor 1-aminobenzotriazole (ABT) was a gift from Dr. Jack P. Uetretch (University of Toronto, Toronto, Ontario). The P450 inhibitor β -diethylaminoethyl-diphenylpropylacetate HCL (SKF 525A) was a gift from Smith, Kline and French Canada Ltd. (Toronto, Ontario). 5,8,11,14-Eicosatetraynoic acid (ETYA) was a gift from Hoffmann-La Roche Ltd. (Etobicoke, Ontario). B[a]P, uridine diphosphate glucuronic acid (UDPGA), formaldehyde, horseradish peroxidase (type VI-A), 12-O-tetradecanoylphorbol-13-acetate (TPA; phorbol-12-myristate-13-acetate), interleukin-1 α (IL-1 α), 4',6-diamidino-2-phenylindole (DAPI), cytochalasin B, ribonuclease A, ribonuclease T₁, *Escherichia coli* alkaline phosphatase, hydrogen peroxide (H₂O₂), bovine serum albumin (BSA), 2,4-dinitrophenylhydrazine, anti-dinitrophenyl antisera raised in rabbit, anti-goat IgG whole molecule peroxidase conjugate antibody were purchased from Sigma Chemical Co. (St. Louis, MO). [7,10-¹⁴C]B[a]P (specific activity, 50 mCi/mmol), mouse IgG horseradish

peroxidase linked whole antibody, and chemiluminescence reagents were obtained from Amersham Canada Ltd. (Oakville, Ontario), and NADPH from Boehringer Mannheim Canada Ltd. (Dorval, Quebec). Mouse monoclonal anti-rat CYP1A1 primary antibody was a gift from Dr. Harry Gelboin (National Cancer Institute, U.S.A.). Pure PHS-1 and goat polyclonal anti-ovine PHS-1 primary antibody was purchased from Oxford Biomedical Research, Inc. (Oxford, MI). Acrylamide, sodium dodecyl sulfate (SDS), N-N-methyl-bis-acrylamide, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), and mercaptoethanol were purchased from ICN Biochemical, Inc. (Aurora, Ohio). 8-hydroxy-2'-deoxyguanosine (8-OH-2'-dG) was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). All other reagents used were of analytical or HPLC grade.

Dulbecco's modified eagle medium (D-MEM), fetal bovine serum (FBS), lyophilized penicillin/streptomycin (pen/strep), Hanks' balanced salt solution (HBSS) (without calcium chloride, magnesium chloride and magnesium sulphate), and 0.25% trypsin were purchased from Gibco BRL (Toronto, Ontario)

***In vivo* CYP1A1 induction**

Radiolabelled TCDD was used in these studies to allow precise quantitation of the TCDD injected into each mouse. To prepare the stock [³H]TCDD solution in benzene for injection, the benzene was evaporated under nitrogen and the [³H]TCDD was redissolved in corn oil/acetone (95/5, v/v). The exact amount of TCDD in solution was determined by counting radioactivity in a liquid scintillation spectrometer (model LS 5000TD, Beckman Instruments, Toronto, Ontario). Mice were treated with a single injection of TCDD, 1 µg/kg ip, 3 days prior to preparation of hepatic microsomes. This dose of TCDD is known to induce hepatic CYP1A1 activity in C57BL/6 mice (Nicol et al., 1995).

Preparation of mouse hepatic microsomes

Whole livers from TCDD-induced mice were perfused with 5 ml of cold 1.5% KCl, weighed, and homogenized on ice in 4 volumes of 1.15% KCl for 20 sec using a probe

homogenizer (Ultra-Turrax^R, Janke and Kunkel, Terochem Laboratories, Toronto, Ontario). The homogenates were centrifuged at 9,000 x g for 25 min at 4°C (Model J2-21M, Beckman). The supernatants were centrifuged at 100,000 x g for 1 hr at 4°C (Model L5-65B, Beckman). Microsomal pellets were resuspended in 1 ml of cold 1.15% KCl and aliquots frozen in liquid nitrogen were stored at -80°C. Protein content of the microsomal samples was determined by the method of Bradford (1976) using a standard kit (Protein Assay Kit, Bio-Rad Laboratories, Richmond, CA).

Hepatic microsomal covalent binding studies

TCDD-induced liver microsomes (final protein concentration, 1 mg/ml) were incubated with [¹⁴C]B[a]P (0.25 µCi/4.81 nmol; final B[a]P concentration, 16 µM) in 0.1 M phosphate buffered saline (PBS), pH 7.4, in glass borosilicate test tubes. Uridine diphosphate glucuronic acid (UDPGA) 10 mM was added to test tubes examining UGT protection. Reactions were initiated by adding NADPH at a final concentration of 5 mM and incubated at 37°C for 1 hr in a shaking water bath (final incubation volume, 300 µl). Reactions were stopped by adding 300 µl of ice-cold methanol. Samples were then centrifuged at 1000 x g for 20 min at 4°C. The supernatants were discarded and 5 ml of hot methanol were added to each sample to resuspend the pellet. Each pellet was transferred to a 0.45 µm membrane filter (SPE Ltd., Concord, Ontario) mounted in a vacuum drum well (Millipore Ltd., Mississauga, Ontario) by washing the incubation tube 5 times each with 5 ml of hot methanol. Samples in the drum wells were washed 5 times with 5 ml of hot methanol under suction filtration. Filters were then transferred to scintillation vials containing 500 µl of BTS-500 tissue solubilizer (Beckman) and placed in a warm oven overnight to dissolve the protein. Ten ml of standard liquid scintillation cocktail (Ready Protein, Beckman) were added to each vial and samples were left for 2-3 hr in darkness.

Inhibition of covalent binding. SKF 525A 1 mM was preincubated with hepatic microsomes and NADPH 10 mM for 5 min prior to addition of [¹⁴C]B[a]P and NADPH. ABT, 1 mM or 10 mM, was preincubated with hepatic microsomes and NADPH 10 mM for 30 min. Previous studies showed 81% loss of P450 protein within the 1st 10 min and a further 5% loss in the

subsequent 20 min (Ortiz de Montellano and Mathews, 1981). Microsomes were then washed by adjusting volume to 1 ml with PBS and centrifuged at 1000 x g for 20 min (Mathews et al., 1985). The pellet was washed by resuspension in 1 ml PBS and recentrifuged. The supernatant was removed and the pellet was resuspended prior to addition of [¹⁴C]B[a]P and NADPH. [¹⁴C]B[a]P covalent binding for both groups were determined as described above.

Horseradish peroxidase-catalysed covalent binding studies

Pure horseradish peroxidase 1 mg/ml with or without ABT 1 mM was incubated at 25°C with H₂O₂ 160 µM and BSA 5 mg/ml for 1 hr at a final incubation volume of 300 µl. ABT was preincubated with horseradish peroxidase for 5 min. Incubations were stopped with 1 ml of ice cold methanol. Samples were then treated as above.

Cell culture studies

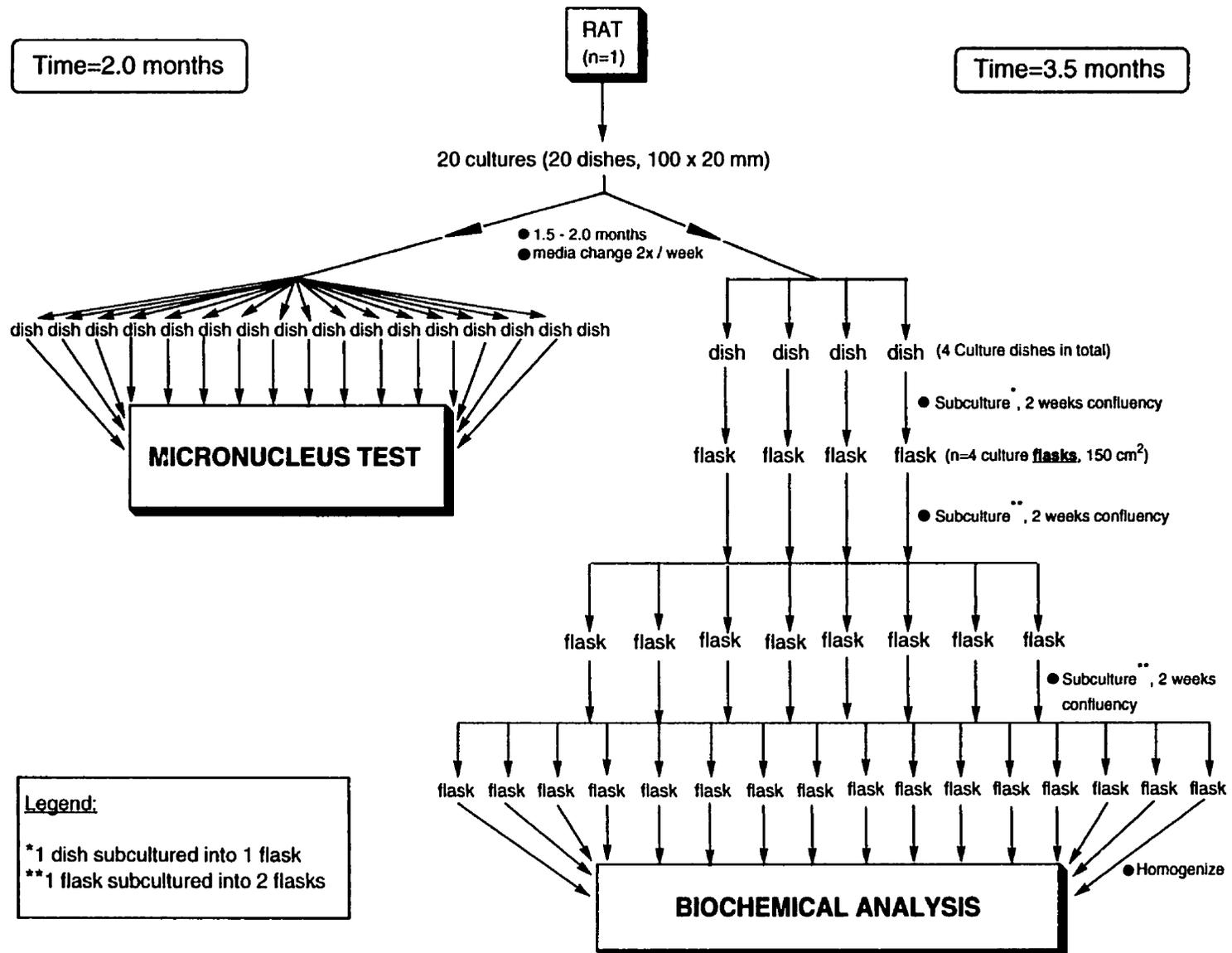
The various methods used in these studies have been described in length elsewhere (Vienneau et al., 1995).

Cell culture method. Briefly, rats were sacrificed by CO₂ asphyxiation, bathed in 70% ethanol and two 2 x 2 cm pieces of shaved skin were removed from the dorsal surface and placed in HBSS with 2% pen/strep. Skin was cultured immediately. **Figure 2** diagrammatically depicts the culturing procedure and the time involved (also see Diana Vienneau's thesis, 1993 for further details on the culturing procedure).

All following steps were conducted in a laminar flow hood. The skin was minced into 1 mm³ pieces, stored in 20 ml of HBSS (2% pen/strep) and transferred to sterile 100 mm polystyrene tissue culture dishes and arranged to fit under square 18 mm coverslips. Medium (D-MEM 500 ml with 75 ml FBS and 5 ml pen/strep) was added at the margin of the coverslip, allowing it to move across by capillary action, and then a further 5 ml of medium was added to the dish. All dishes were incubated at 37°C in a humidified incubator with 5% CO₂ in air and left undisturbed for 10 days. The dishes were then examined with an inverted phase contrast

Figure 2. Diagrammatic representation of the cell culture method to perform both the micronucleus assay and biochemical endpoints such as enzyme activities and DNA damage.

FIGURE 2



microscope to confirm the formation of a monolayer of fibroblasts at the margins of the skin pieces. Thereafter the medium was changed twice a week. After 2 months, the cultures were confluent, defined as a single layer of cells covering the bottom of the dish.

Subculture method. The medium was aspirated off and the petri dishes were washed 3 times with fresh 5 ml HBSS. To detach cells, 3 ml of 0.25% trypsin (Gibco) were added to dishes and incubated at 37°C for 4-6 min. Microscopic examination confirmed the detachment of cells. The trypsin action was then stopped by adding 3 ml of FBS. The cells were transferred to sterile polyethylene test tubes and centrifuged at 1000 x g at 4°C for 10 min. The supernatant was aspirated off and 5 ml of medium were added. The cells were resuspended and transferred to a 150 cm² culture flask containing 20 ml of medium. Flasks were incubated for 1-2 weeks until cultures became confluent.

Preparation of fibroblast homogenates. Once the cultures in flasks reached confluency, the cells were harvested as above. To detach cells, 12 ml of trypsin were used for 4-6 min at 37°C. Trypsin action was stopped with 12 ml of FBS. The cells were transferred to sterile 50 ml polyethylene test tubes, pelleted by centrifugation at 1000 x g for 10 min at 4°C, and stored on ice. The pellets from each individual flask were resuspended in 1 ml of PBS, combined in a 17 x 100 mm sterile test tube, and centrifuged at 1000 x g at 4°C for 10 min. The final pellet (approximately 6 to 8 cultures) was resuspended in 1 ml of PBS. Cells were hand-homogenized using a glass 5 ml tissue grinder (Mandel Scientific Ltd., Guelph, Ontario) and the homogenate was separated into 100 µl aliquots, frozen in liquid nitrogen, and stored at -80°C until DNA could be isolated and microsomes prepared as described above.

Micronucleus Formation. For studies using potential inhibitors of bioactivation, skin fibroblasts from Wistar rats were preincubated with medium containing either the P450 inhibitor 1 mM ABT for 2 hr, or the PHS/LPO inhibitor 40 µM ETYA for 24 hr, prior to incubation with 10 µM B[a]P for 5 hr. In a further coincubation study, fibroblasts were incubated with medium containing both 40 µM ETYA and 10 µM B[a]P for 5 hr. The concentration of ETYA was chosen since it is well above the K_i for tissue homogenates (Klein et al., 1984). The vehicle for ETYA and B[a]P was DMSO, and PBS was the vehicle for ABT.

For studies using inducers of bioactivation, skin fibroblasts were preincubated with medium containing either the CYP1A1 inducer 10 nM [³H]TCDD, or the PHS inducers TPA 1 μM or IL-1α [0.625 (0.0367 nM) or 1.25 (0.073 nM) ng/ml] for 24 hr and 4 hr respectively, prior to incubation with 10 μM B[a]P for 5 hr. *In vitro* studies using cultured human epithelial, breast carcinoma and mouse hepatoma cells demonstrated maximal induction of ethoxycoumarin O-deethylase (Hudson et al., 1983) and AHH activities (Jaiswal et al., 1985) with 10 nM TCDD for 24 hr. Similarly, the concentrations and times for both TPA and IL-1α were chosen since these were shown to be effective in inducing prostaglandin E₂ synthesis (Dinarelli, 1994), and increasing cyclooxygenase protein and activity in cultured human dermal fibroblasts (Seibert et al., 1990; Herschman et al., 1994). The vehicle for [³H]TCDD and TPA was DMSO, and the vehicle for IL-1α was PBS.

To determine whether or not B[a]P and/or DMSO alters the cellular kinetics (i.e. cell division), which can increase or decrease micronucleus formation and thereby confound interpretation of B[a]P-initiated micronucleus formation, a study was performed using the cytokinesis blocking agent cytochalasin B. Cells were incubated with either 10 μM B[a]P or the DMSO vehicle for 5 hr, and then incubated with new medium containing cytochalasin B at either 1, 3 or 6 μg/ml until the end of one mitotic cycle. The mitotic cycle for Wistar rat skin fibroblasts was determined previously to be 38 hr (Vienneau et al., 1995).

In all studies, the cells were washed 3 times with 5 ml of HBSS after chemical/vehicle incubation, 5 ml of fresh medium was added, and cells were allowed to undergo one complete mitotic cycle. The 5 hr B[a]P or DMSO incubation was included as part of the mitotic cycle. Upon the completion of one mitotic cycle, the medium was aspirated off and the cells were washed 3 times with 5 ml of HBSS to remove all residual medium. To fix the cells, 5 ml of formalin solution (37% formaldehyde solution:PBS = 1:9 v/v) was added to the cells. After 30 min, the formalin solution was aspirated off and cells were washed 3 times with 5 ml of PBS.

Once fixed, the cells were stained with 5 ml of DAPI fluorescent stain 2 μg/ml in water, and 2000 mononucleated or a minimum of 500 binucleated cells (standardised to 1000) were counted

for the formation of micronuclei, using an inverted microscope with a 40X objective.

DNA and protein oxidation in live fibroblasts

To determine the potential role of DNA oxidation as a molecular mechanism in B[a]P-initiated micronucleus formation, fibroblasts were incubated with inhibitors and inducers of P450s and peroxidases, as described above in the micronucleus studies, except that after the 5 hr B[a]P incubation, cells were harvested and homogenized. Microsomal protein was prepared as described previously and DNA from cellular homogenates was obtained as follows.

Fibroblast DNA Isolation. A modified method of Gupta (1984) was used to isolate DNA from Wistar rat skin fibroblasts. Briefly, fibroblast homogenates were incubated overnight with proteinase K at 55°C. Tris-HCl 1 mM at a volume of 25 µl was added and DNA extracted with one volume of chloroform:isoamyl alcohol:phenol (CIP, 24:1:25) and two successive extractions of one volume chloroform:isoamyl alcohol (CI, 24:1). At each stage mixtures were vortexed for 30 sec and microcentrifuged at 18,000 X g for 1 min (model E, Beckman) to separate extraction phases. The DNA was then precipitated with 500 µl of 100% ice cold (-20°C) ethanol and pelleted by microcentrifugation for 1 min. The DNA pellet was dissolved in 500 µl phosphate buffer (pH 7.4), and incubated at 37°C on a rocker, with ribonuclease A (100 µg/ml) and ribonuclease T₁ (50 units/ml) to digest residual RNA. One volume of CI (24:1) was used to reextract the dissolved DNA and then microcentrifuged for 1 min. The DNA was reprecipitated as above. The pellet was redissolved in 500 µl of 20 mM Na-acetate buffer (pH 4.8) and quantified using a UV/Vis spectrophotometer (model Lamda 3, Perkin Elmer Canada Ltd.) at a wavelength of 260 nm with calf thymus DNA as the standard. The DNA was then digested to nucleotides by incubation with nuclease P₁ (67 µg/ml) at 37°C for 30 min, followed by a 60 min incubation with *Escherichia coli* alkaline phosphatase (0.37 units/ml) at 37°C. The mixture of nucleosides was syringe tip filtered (0.22 µm) and analysed via HPLC coupled with electrochemical detection (Shigenaga and Ames, 1991).

DNA oxidation analysis. DNA oxidation was quantified by measuring the formation of 8-OH-2'-dG using an isocratic HPLC (Scientific Systems, Inc., USA) equipped with an

electrochemical detector (Model 5100A, Coulochem, ESA, CA, USA), a reverse-phase C18 column (Jones Chromatography, Lakewood, CO, USA), and an integrator (Model CR501 Chromatopac, Shimadzu, Japan). Samples were eluted using a mobile phase consisting of 50 mM KH_2PO_4 buffer (pH 5.5) and methanol (90:10, v/v) at a flow rate of 0.8 ml/ min and an oxidation potential of +0.7 V.

Protein oxidation analysis. Immunochemical detection of oxidized protein was determined by the method of Keller et al. (Keller et al., 1993). Briefly, microsomal protein (prepared above) was immediately incubated for 1 hr at room temperature with equal volume of 0.5 mM 2,4-dinitrophenylhydrazine in 0.1 M sodium phosphate buffer (pH 6.3). Proteins were separated and transferred as described below. Oxidized protein bands were detected by incubating membranes for 3 hr with anti-dinitrophenyl antisera (1:5,000) followed by a 1 hr incubation with peroxidase-labelled goat anti-rabbit IgG (1:50,000) and detected using enhanced chemiluminescence.

P450 activities in hepatic microsomes and cellular homogenates/microsomes

Cytochromes P450 activities were measured as described by Burke et al. (1985) using ethoxyresorufin and pentoxyresorufin as substrates. Ethoxyresorufin O-deethylase (EROD) and pentoxyresorufin O-dealkylase (PROD) respectively reflect activities of CYP1A1 and CYP2B1/2.

Cellular microsomal CYP1A1 and PHS protein using Western Blot/Immunodetection

Microsomal protein from cultured rat skin fibroblasts treated as above (oxidation studies) was analysed using Western blot/immunodetection with enhanced chemiluminescence. Briefly, protein was separated under reducing conditions with sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) using a Mini-PROTEIN® II electrophoresis cell (Bio-Rad, CA). Protein was separated using a 10% acrylamide gel made up as follows: 8 ml 30% acrylamide (60 g acrylamide, 1.6 Bis, final volume to 100 ml with deionized H_2O), 8.96 ml 1M Tris pH 8.8, 6.8 ml

deionized H₂O, 240 µl 10 % SDS, 16 µl TEMED, 80 µl ammonium persulphate stock (10 mg/100 µl deionized H₂O). Gels were allowed to polymerize for 45-60 min before the stacking gel was poured. The stacking gel was made as follows: 1.67 ml 30% acrylamide, 1.25 ml 1M Tris pH 6.8, 6.98 ml deionized H₂O, 100 µl 10% SDS, 10 µl TEMED, 50 µl ammonium persulfate stock. Gels were allowed to polymerize for 15 min before samples were loaded. Each sample was diluted with at least 1/5 volume of 5X sample buffer (0.2 ml 10% SDS, 0.1 ml glycerol, 0.1 ml 1M Tris, pH 6.8, 10 µl mercaptoethanol, 100 µl/ml final volume of 10% bromophenol blue). Samples were boiled for 3 min in a boiling water bath and loaded onto the gel. A commercial set of pre-stained protein molecular weight marker standards 10 µl (Bio-Rad) also was loaded onto the gel. Proteins were then separated for 40 min using running buffer (0.025 M Tris, 0.192 M glycine, 1% SDS) at a constant voltage of 200 volts (power supply, Model 1000/500, Bio-Rad).

Gels were then removed and cellular microsomal protein was transferred to nitrocellulose membranes (Amersham) using a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad). A constant voltage of 100 for 1 hr was used with a transfer buffer consisting of 25 mM Tris, 192 mM glycine, 20% v/v methanol at a pH of 8.3.

After the transfer, membranes were rinsed with deionized H₂O and then incubated overnight with 20 ml skim milk/TNT buffer [2 g of skim milk powder, 5 ml 10X TNT (24.22 g Tris, 175.32 g NaCl, 40 g Tween 20, final volume 2 litres), 10-20 mg of thiomerosal (preservative), final volume 50 ml with deionized H₂O] to block non-specific binding sites.

The following morning, membranes were analysed for either CYP1A1 or PHS-1/2 protein bands. The membrane for CYP1A1 was incubated with primary monoclonal anti-rat CYP1A1 antibody for 4 hr at a dilution of 1:250 (antibody:skim milk/TNT) washed for 30 min, alternating every 5 min with either H₂O or 1X TNT. The membrane was then incubated for 1 hr with the secondary anti-mouse IgG horseradish peroxidase-linked whole antibody (Sigma), at a dilution of 1:2000 (secondary antibody:skim milk/TNT). The membrane was then washed as described above and CYP1A1 protein detected using enhanced chemiluminescence (Amersham).

Similarly, PHS-1/2 protein was analysed as described above, using the primary

antibody, goat polyclonal anti-ovine PHS-1 (Oxford) which recognizes both PHS-1 and -2 from rodents. A dilution of 1:200 (primary antibody:skim milk/TNT) was used. The primary antibody was recognized using an anti-goat IgG (whole molecule) peroxidase-conjugated secondary antibody (Sigma) at a dilution of 1:2000 (secondary antibody:skim milk/TNT).

Stripping and reprobing membranes

Membranes were stripped and reprobed using a modified method from Amersham's Western blotting protocols (1994). Membranes were incubated with stripping buffer (100 mM 2-mercaptoethanol, 62.5 mM Tris-HCL pH 6.7, 2% SDS) for 40 min at 50°C in a shaking water bath, washed twice with 25 ml of 1X TNT for 15 min and once with 25 ml of H₂O for 15 min at room temperature. Membranes were then blocked overnight with skim milk and immunodetection was performed with new primary and secondary antibodies.

Statistical analysis

Statistical significance of differences between treatment groups in each study was determined by Student's t test or one factor analysis of variance (ANOVA) as appropriate using a standard, computerized statistical program (Statview, Abacus Concepts, Inc.) The level of significance was $p < 0.05$.

2.1.4 RESULTS

Characterization of chemical probes

P450 inhibition. To determine the optimal selection and concentration of P450 inhibitor for subsequent cellular studies, an *in vitro* hepatic murine microsomal system was used to characterize P450 inhibition by ABT and SKF 525A.

Preincubation of hepatic microsomes from TCDD-induced C57BL/6 mice with either the classical P450 inhibitor SKF 525A, or ABT, decreased B[a]P covalent binding when incubated with NADPH ($p < 0.05$) (**fig. 3**). When comparing the two P450 inhibitors, ABT was considerably more effective than SKF 525A (**fig. 3, insert**). At equimolar concentrations (1 mM), SKF 525A inhibited B[a]P covalent binding by only 40%, compared to 80% inhibition by ABT.

The UGT co-substrate UDPGA, reduced B[a]P covalent binding in hepatic microsomes incubated with either no P450 inhibitor or SKF 525A, confirming previous studies showing that glucuronidation of B[a]P metabolites reduces bioactivation ($p < 0.05$) (Vienneau et al., 1995) (**fig. 3**). UDPGA did not reduce B[a]P covalent binding in ABT incubations, possibly because of maximal inhibition by ABT. This was corroborated by a lack of further inhibition of B[a]P covalent binding with higher ABT concentrations (10 mM). Alternatively, ABT competition for UGT elimination (conjugation with UDPGA) (Town et al., 1993) may contribute to these results. Absolute amounts of B[a]P covalent binding in the SKF 525A study were three times higher than that in the ABT study (**fig. 3**), likely due to the use of washed microsomes in the latter study (see Methods).

Peroxidase inhibition. An *in vitro* horseradish peroxidase system was used to characterize the inhibitory efficacy of ABT on peroxidase-catalysed B[a]P covalent binding to bovine serum albumin (BSA) (**fig. 4**). At a concentration of 1 mM, ABT inhibited B[a]P covalent binding by 81%, indicating that ABT is an effective inhibitor of peroxidases that catalyze B[a]P bioactivation.

Figure 3. Effect of the P450 inhibitors SKF 525A and 1-aminobenzotriazole (ABT) on the covalent binding of benzo[a]pyrene (B[a]P) to hepatic microsomal protein from TCDD-induced (1 $\mu\text{g}/\text{Kg}$) C57BL/6 mice. Insert compares P450 inhibitory potencies for ABT and SKF 525A. Incubation conditions: [^{14}C] benzo[a]pyrene, 0.25 μCi , 16 μM ; SKF 525A, 1 mM (preincubated for 5 min); ABT, 1 or 10 mM (preincubated for 30 min); NADPH, 10 mM; UDPGA, 10 mM (if included); microsomal protein, 1 mg/ml; final volume 300 μl ; incubated at 37°C for 60 min. The number of samples is given in parentheses. Asterisks indicate a difference from respective controls, and the plus symbols indicate a difference from respective groups with NADPH alone ($p < 0.05$).

COVALENT BINDING OF B[a]P

(picomoles/mg protein/hr, mean + SD)

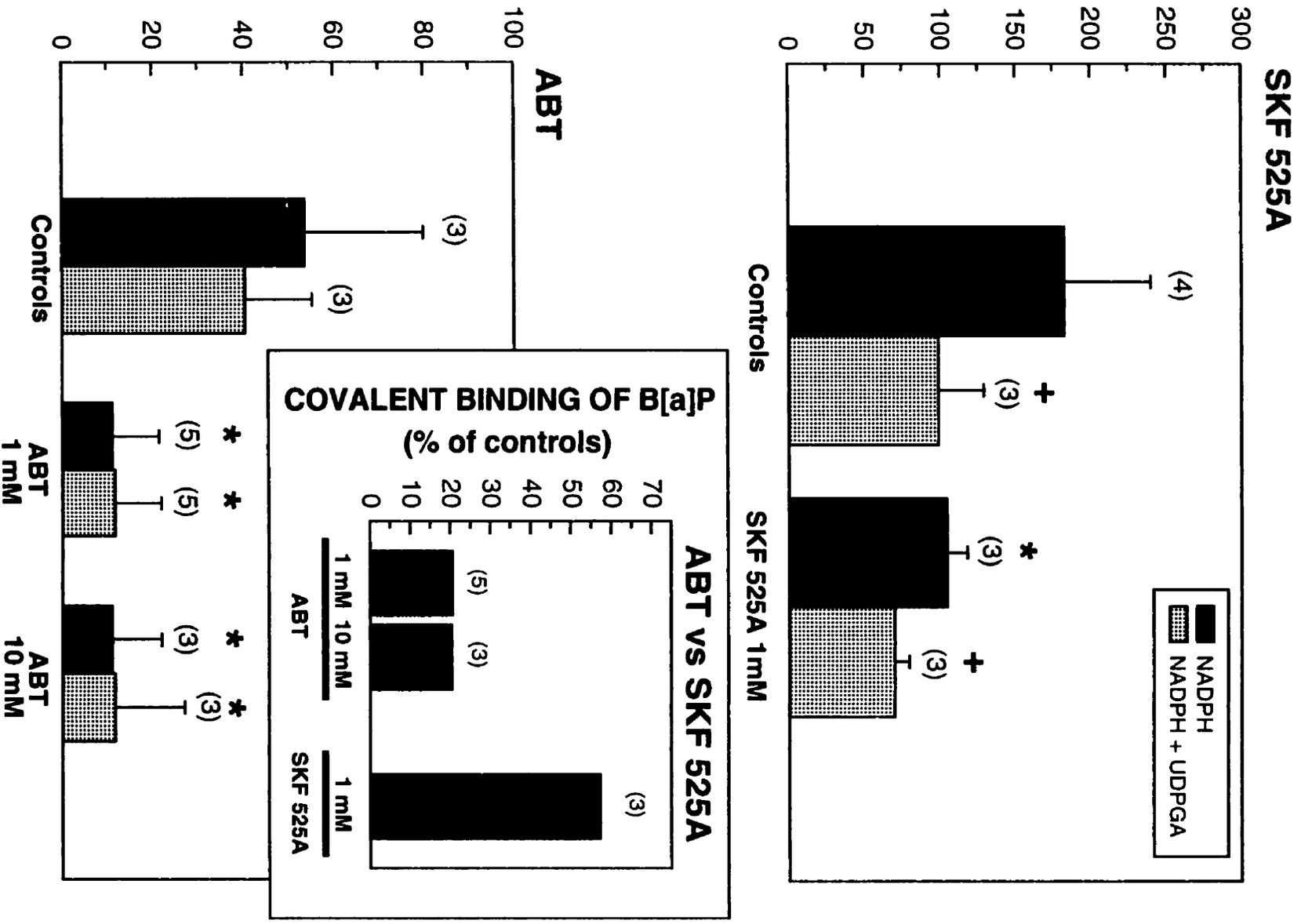
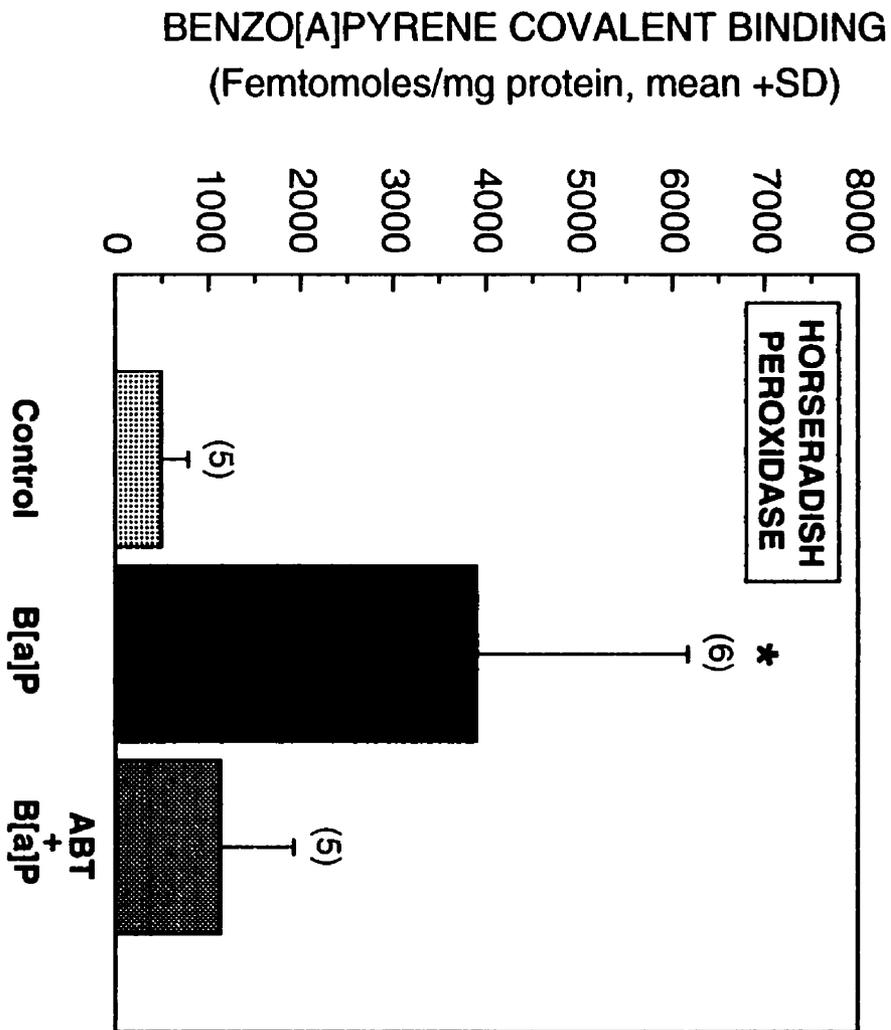


FIGURE 3

Figure 4. Effect of 1-aminobenzotriazole (ABT) on benzo[a]pyrene (B[a]P) covalent binding to bovine serum albumin protein catalyzed by horseradish peroxidase. ABT 1 mM was preincubated with horseradish peroxidase and hydrogen peroxide (H₂O₂) for 5 min at 25°C, and further incubated with B[a]P for an additional 55 min. H₂O₂ was not added in the control incubations. The asterisk indicates a significant difference from both control and ABT-treated groups (p<0.05).

FIGURE 4



Characterization of the fibroblast cell line

P450 activity and content. Cellular homogenates and microsomes from both control (up to 679 μg protein) and TCDD-treated (up to 137 μg protein) fibroblasts failed to show measurable EROD or PROD activities (lower limit of detection=4.0 pmol/min/mg protein). Western blotting/immunodetection with enhanced chemiluminescence was used to detect both CYP1A1 and PHS protein. Initially, a commercially available Western blot/enhanced chemiluminescence CYP1A1 kit (Amersham) supplied with a primary polyclonal antibody was used to detect CYP1A1 in cultured rat skin fibroblasts treated as above (oxidation studies). A number of experiments were conducted in which primary antibody concentration and incubation time and time of film exposure were varied to optimize conditions for our study. All trials were unsuccessful in detecting CYP1A1 protein bands without the appearance of lane-specific secondary bands. Therefore the validity of the CYP1A1 protein band could not be assured.

Subsequently, an alternative primary monoclonal CYP1A1 antibody was used to detect CYP1A1 from similarly treated cells. Cells treated with either B[a]P or TCDD alone showed measurable CYP1A1 protein, with substantially greater amounts in the TCDD-treated fibroblasts (**fig. 5, panel A**).

One week later the membrane from the experiment using the CYP1A1 kit (Amersham) was stripped of both primary and secondary CYP1A1 antibody, blocked overnight and reprobed for PHS protein. Microsomal protein from fibroblasts treated with TCDD alone showed a distinct PHS protein band which was not present in the other lanes (**fig. 5, panel B**). To ensure that the band seen was not a result of residual chemiluminescence from the previous study (one week prior), the membrane was stripped the following morning as described previously, blocked for only 1 hr and incubated for 1 hr with the secondary antibody (1:2000). The membrane was then subjected to enhanced chemiluminescence to detect any chemiluminescence present. No chemiluminescence was detectable, even when film was exposed to membranes for up to 30 min. This would suggest that the band seen was specific to the PHS primary antibody, which recognizes both PHS-1 and PHS-2.

Microsomal protein from untreated fibroblasts showed constitutive PHS protein, as

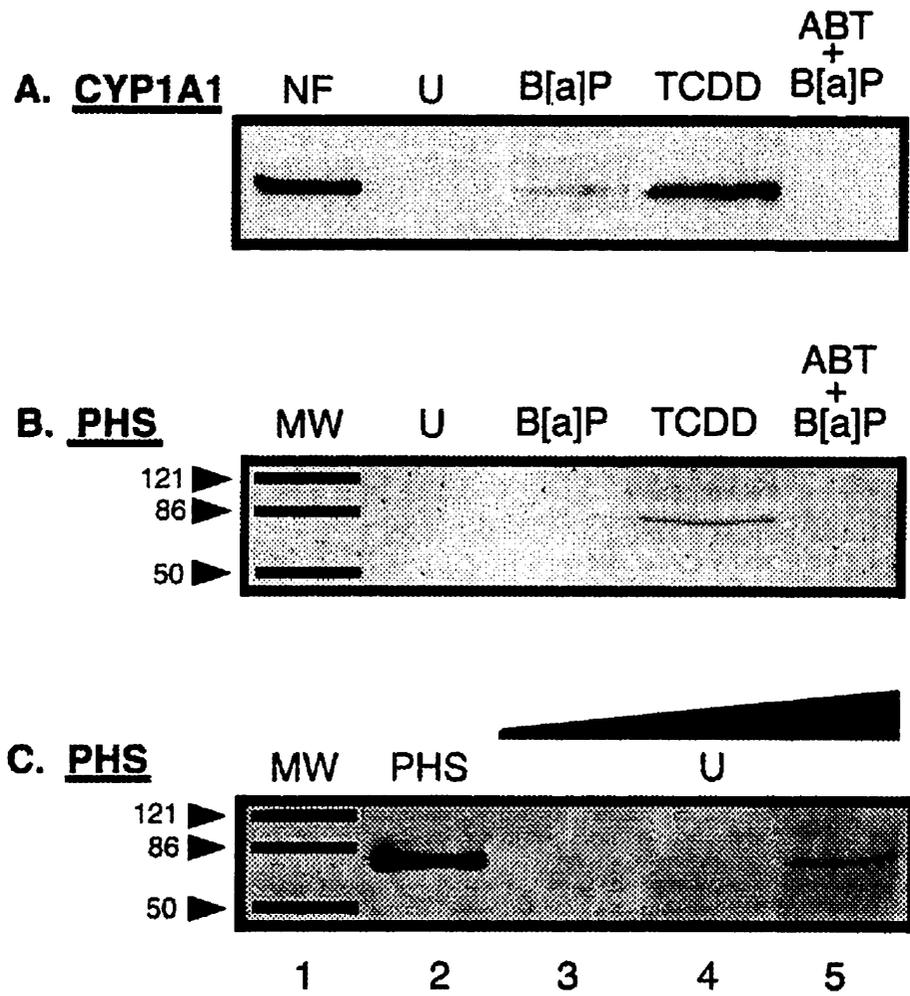
Figure 5. Detection of cytochrome P4501A1 (CYP1A1) and prostaglandin H synthase (PHS) protein in cultured rat skin fibroblasts.

Panel A. CYP1A1 in microsomal protein from cultured skin fibroblasts and hepatic microsomes from β -naphthoflavone (NF)-treated rats (positive control) (CYP1A1 Amersham kit). Live fibroblasts were cultured either untreated (U), or with 10 μ M benzo[a]pyrene (B[a]P) or 10 nM TCDD alone, or preincubated with the P450 inhibitor ABT 1 mM prior to B[a]P. Cells were harvested 5 hr after B[a]P incubation. Each lane was loaded with 110 μ g of protein, except lane NF which had 1.25 μ g. Protein was separated using SDS/PAGE and transferred to a nitrocellulose membrane. Membranes were incubated for 4 hr with anti-CYP1A1 monoclonal antibody (1:250) followed by a 1 hr incubation with mouse IgG horseradish peroxidase-linked whole antibody (1:2000). Bands were detected using enhanced chemiluminescence.

Panel B. PHS in microsomal protein from fibroblasts shown above. Each lane was loaded with 110 μ g of protein except for lane one, which was loaded with molecular weight markers (MW), shown in kD. The membrane was stripped of all CYP1A1 primary and secondary antibodies by incubating membrane for 40 min at 50°C in stripping buffer. Membranes were then blocked overnight at 4°C with skim milk and reprobed with goat polyclonal anti-ovine PHS-1 (recognizes both PHS-1 and -2) (1:200) for 4 hr, followed by a 1 hr incubation with anti-goat IgG peroxidase conjugate secondary antibody (1:2000) and detected as above.

Panel C. PHS in microsomal protein from untreated fibroblasts. Lanes 1 and 2 contain respectively, molecular weight markers (MW), shown in kD and 0.35 μ g of pure PHS-1 (>95% pure) protein (positive control). Lanes 3, 4 and 5 were loaded with 200, 300, 400 μ g of microsomal protein. PHS was detected as described above.

FIGURE 5



evidenced in the lane loaded with 400 µg of protein (**fig. 5, panel C**). Lower amounts of microsomal protein did not show either PHS or CYP1A1 protein.

B[a]P-initiated micronucleus formation in rat skin fibroblasts

P450 inhibition/induction. Since both concentrations of ABT were equally efficacious in inhibiting P450 activity in hepatic microsomes, 1 mM ABT was used in the fibroblast studies to avoid any potential cytotoxicity caused by the higher concentration of ABT. No cytotoxicity was evident in cultures treated with 1 mM ABT. **Figure 6** shows micronucleus formation in cultured rat skin fibroblasts stained with the DNA DAPI fluorescent stain.

The baseline incidence of micronucleus formation in fibroblasts exposed to the buffer plus DMSO (essentially DMSO alone) was approximately 10 micronuclei per 2000 cells (**fig. 7**). Previous studies by Vienneau et al. (1995) using a similar fibroblast model showed that DMSO did not induce micronucleus formation over untreated controls, indicating that the 10 micronuclei per 2000 cells counted was intrinsic to rat skin fibroblasts in culture. This was confirmed in subsequent studies (**fig. 8**). Fibroblasts exposed to buffer plus B[a]P (essentially B[a]P alone) demonstrated over a 2-fold increase in micronucleus formation ($p < 0.05$). Preincubation with the P450/peroxidase inhibitor ABT decreased micronucleus formation by 76% when compared to the B[a]P control group ($p < 0.05$).

Preincubation with the CYP1A1 inducer TCDD increased B[a]P-initiated micronucleus formation by a further 2-fold when compared to the B[a]P alone group ($p < 0.05$) (**fig. 7**). TCDD itself did not affect micronucleus formation, in that the TCDD plus DMSO group was not different from the DMSO alone group.

Peroxidase inhibition/induction. Coincubation with the dual PHS/LPO inhibitor ETYA produced a 50% reduction in B[a]P-initiated micronucleus formation compared to the B[a]P alone group ($p < 0.05$) (**fig. 7**). Preincubation with ETYA failed to demonstrate a protective effect. As indicated above, inhibition also was observed with the dual P450/peroxidase inhibitor ABT ($p < 0.05$). Coincubation of ETYA with DMSO showed no increase in micronucleus formation when compared to the DMSO control group.

Figure 6. Micronucleus formation in cultured Wistar rat skin fibroblasts stained with the fluorescent DNA DAPI stain.

Figure 7. Effect of P450 and peroxidase inhibitors and inducers on benzo[a]pyrene (B[a]P)-initiated micronucleus formation. Skin fibroblasts were obtained from male Wistar rats. Cells were preincubated with either P450 or PHS inhibitors (ABT 1 mM, ETYA 40 μ M) or inducers (TCDD 10 nM, TPA 1 μ M, IL-1 α 0.625 or 1.25 ng/ml) prior to B[a]P 10 μ M. ABT is a dual P450/peroxidase inhibitor (figs. 3 &4), and TCDD induces PHS as well as CYP1A1 (fig. 4, panel B). After addition of B[a]P, cells were left for one mitotic cycle, at which time the cells were fixed and stained, and micronuclei were counted. Bars indicate the mean of a minimum of 3 fibroblast cultures minus baseline micronucleus formation (buffer plus DMSO=10 micronuclei/2000 cells). Asterisks indicate a difference from controls treated with B[a]P ($p < 0.05$).

FIGURE 7

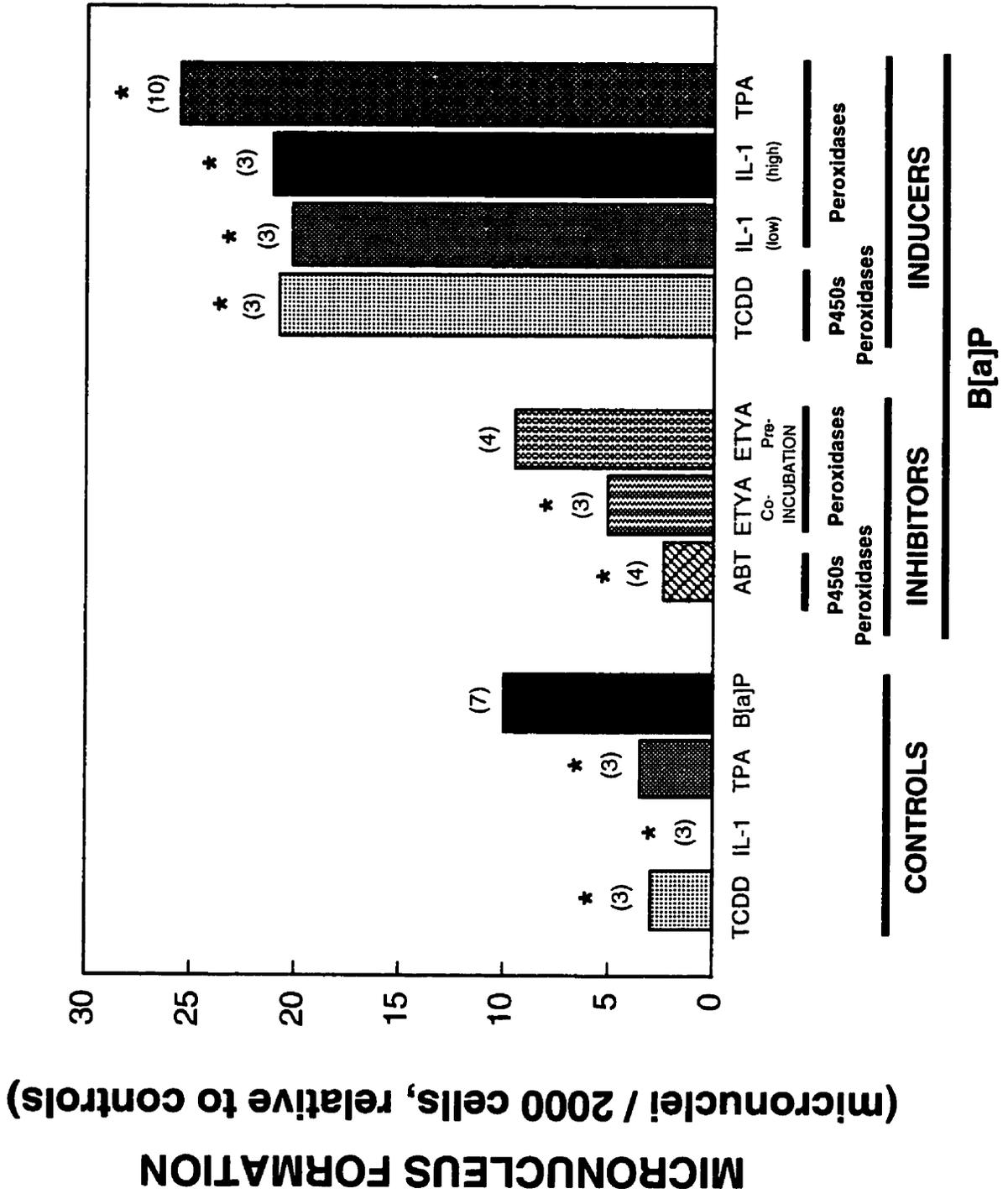
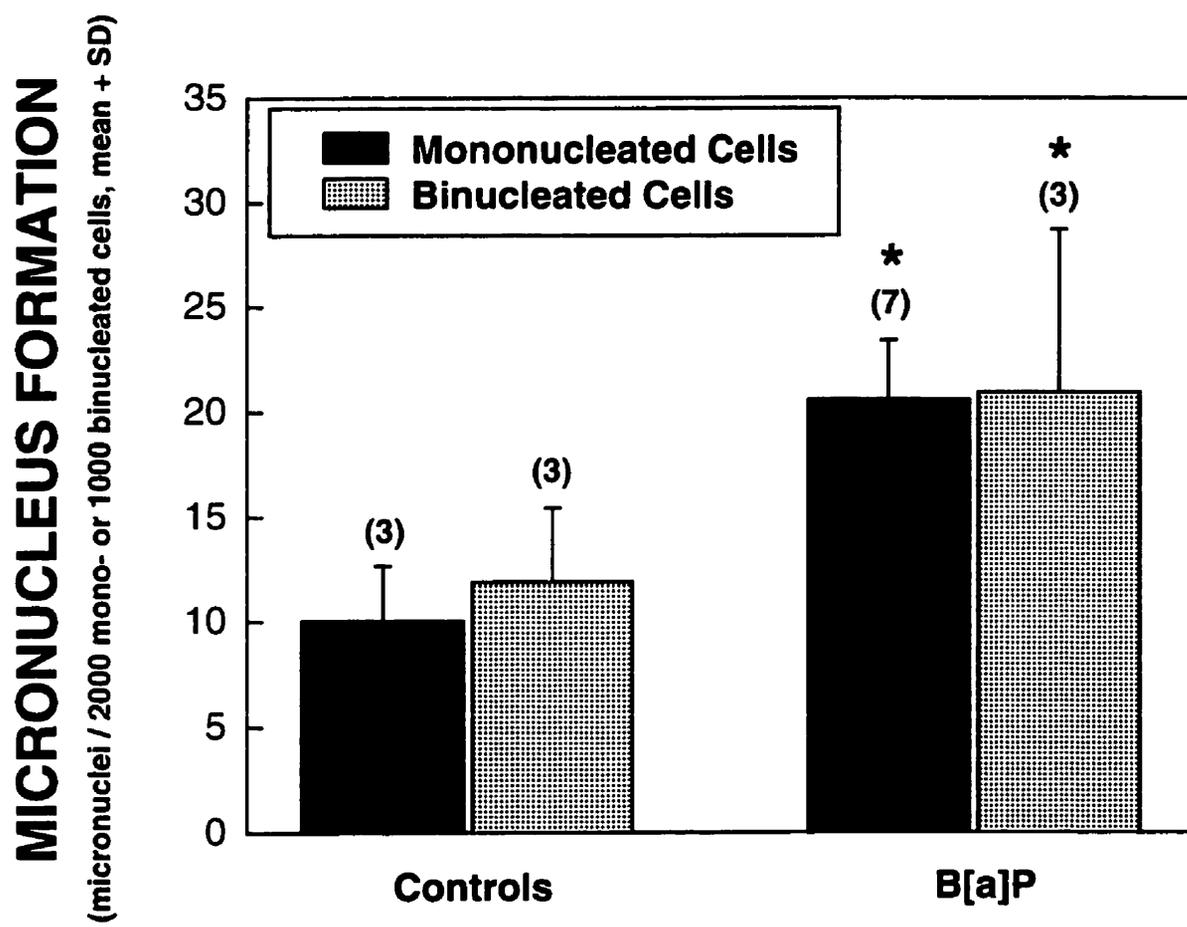


Figure 8. Comparison of mononucleated versus binucleated/cytochalasin B methods for assessing benzo[a]pyrene (B[a]P)-initiated micronucleus formation. Methodological details for the mononucleated method are described in fig. 5. For the binucleated/cytochalasin B method, fibroblasts were incubated with either B[a]P 10 μ M or its vehicle DMSO for 5 hr, after which cytochalasin B 3 μ g/ml was added, and cells were incubated for one mitotic cycle (see methods). The cells were fixed and stained, and micronuclei were counted per a minimum of 500 binucleated cells. Values were standardised to 1000 binucleated cells (equivalent to 2000 mononucleated cells). Asterisks indicate a difference from respective DMSO controls ($p < 0.05$).

FIGURE 8



Preincubation with the peroxidase inducers TPA or IL-1 α increased B[a]P-initiated micronucleus formation a further 2-fold above that seen in the B[a]P alone group ($p < 0.05$) (**fig. 7**). As discussed above, TCDD induced PHS (**fig. 5, panel B**) as well as CYP1A1, which may contribute to the enhancement by TCDD of B[a]P-initiated DNA/protein oxidation (below) and micronucleus formation.

Binucleate (cytochalasin B-mediated cytokinesis block) method

For cells treated with either B[a]P or its DMSO vehicle, there was no difference in micronucleus formation between 1000 (standardized) binucleated and 2000 mononucleated cells (**fig. 8**), corroborating previous studies in human blood lymphocytes (Lippoli, 1995). Both methods showed a significant increase in B[a]P-initiated micronucleus formation over DMSO controls, demonstrating similar degrees of sensitivity using either method, with the added benefit of the mononucleated method avoiding the introduction of additional chemicals which potentially may confound the interpretation of results ($p < 0.05$) (**fig. 8**).

In our hands cytochalasin B at a concentration of 6 $\mu\text{g/ml}$, previously used in human lymphocytes studies (Vian et al., 1993) was highly cytotoxic, and lower concentration of 1 $\mu\text{g/ml}$ failed to produce binucleated cells above the level seen in non-treated groups. These and other problems have been discussed by Norppa et al. (1990).

B[a]P-initiated DNA and protein oxidation in fibroblasts

P450 inhibition/induction. As measured by the formation of 8-OH-2'dG, B[a]P-treated skin fibroblasts had a 2.5-fold increase in DNA oxidation ($p < 0.05$) and a substantial increase in protein oxidation compared to untreated controls (**figs. 9 & 10**). Preincubation with the dual P450/peroxidase inhibitor ABT completely inhibited both B[a]P-initiated DNA ($p < 0.05$) and protein oxidation to levels observed in untreated controls.

Preincubation with the CYP1A1 inducer TCDD, which also induces PHS (**fig. 5, panel B**), enhanced B[a]P-initiated DNA oxidation by a further 1.9-fold above that seen in the B[a]P-treated group ($p < 0.05$) (**fig. 9**). Fibroblasts treated with TCDD alone did not have increased

Figure 9. Effect of P450 and peroxidase inhibitors and inducers on benzo[a]pyrene (B[a]P)-initiated DNA oxidation in cultured skin fibroblasts. DNA oxidation was measured via the formation of 8-hydroxy-2'-deoxyguanosine (8-OH-2'-dG) in skin fibroblasts cultured from male Wistar rats. Live fibroblasts were cultured with either B[a]P alone, or preincubated with either P450 or peroxidase inhibitors (ABT 1 mM) or inducers (TCDD 10 nM, TPA 1 μ M, IL-1 α 0.625 ng/ml) prior to B[a]P 10 μ M. Untreated fibroblasts also were compared to freshly isolated skin samples. Cells were harvested 5 hr post B[a]P incubation. The asterisk indicates a difference from treated controls, and the plus symbol indicates a difference from B[a]P-treated cells ($p < 0.05$).

FIGURE 9

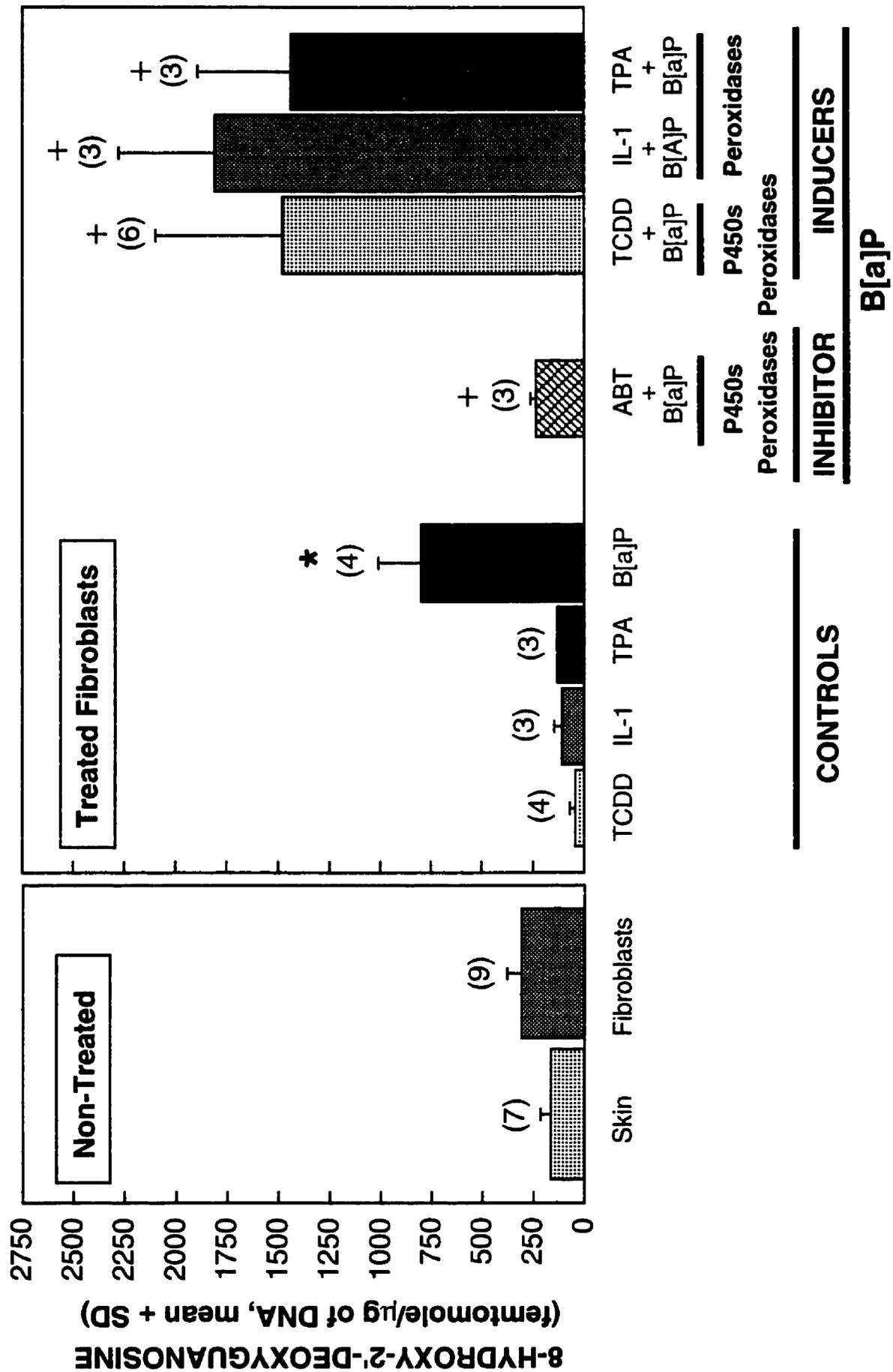
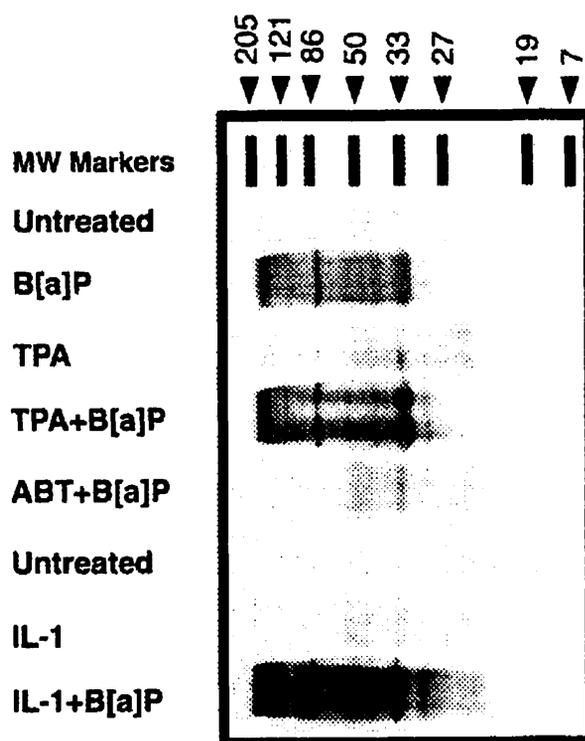


Figure 10. Effect of peroxidase inhibitors and inducers on benzo[a]pyrene (B[a]P)-initiated protein oxidation in cultured skin fibroblasts. The top lane was loaded with molecular weight markers (MW), shown in kD. All other lanes were loaded with 32 μ g of microsomal protein from either untreated fibroblasts or cells treated with B[a]P, a peroxidase inhibitor (ABT) and/or peroxidase inducers (TPA, IL-1 α) alone or in combination, using concentrations detailed in fig. 7. Microsomal protein was derivatized by incubation for 1 hr with an equal volume of 0.5 mM 2,4-dinitrophenylhydrazine. Proteins were separated and transferred as described in fig. 4. Oxidized protein bands were detected by incubating membranes for 3 hr with anti-dinitrophenyl antisera (1:5000), followed by a 1 hr incubation with peroxidase-labelled goat anti-rabbit IgG (1:50,000) and detection by enhanced chemiluminescence.

FIGURE 10



DNA oxidation.

Peroxidase induction. Preincubation with the cyclooxygenase inducers IL-1 α and TPA increased B[a]P-initiated DNA oxidation by a further 2.3- and 1.8-fold, respectively above that seen in the B[a]P-treated control ($p < 0.05$) (**fig. 9**). B[a]P-initiated protein oxidation also was substantially increased when cells were pretreated with either peroxidase inducer (**fig. 10**). IL-1 α caused a greater enhancement in B[a]P protein oxidation compared to cells pretreated with TPA (**fig. 10**). Incubation with the inducers plus DMSO did not increase DNA or protein oxidation. As discussed above, TCDD also may enhance target oxidation at least in part via its induction of PHS.

2.1.5 DISCUSSION

B[a]P is relatively nontoxic unless bioactivated by nonconstitutive CYP1A1 or constitutive peroxidases, such as PHS, to toxic reactive intermediates, which are thought to irreversibly damage cellular macromolecules and initiate carcinogenesis, teratogenesis and cytotoxicity. However, previous studies including that of Vienneau et al. (1995) have not demonstrated that B[a]P-initiated micronucleus formation is dependent upon enzymatic bioactivation to a reactive intermediate, as distinct from other mechanisms such as binding to the Ah receptor. Hence, the relation of micronucleus formation to carcinogenic and teratogenic initiation remains unclear.

In this study, inhibitors and inducers of both P450s and peroxidases were used in a Wistar rat skin fibroblast culture model to demonstrate that enzymatic bioactivation is essential for B[a]P-initiated micronucleus formation, and that peroxidase-catalyzed bioactivation of B[a]P can produce a toxic reactive intermediate that oxidizes DNA and protein, and initiates micronucleus formation. The substantial enhancement in B[a]P-initiated micronucleus formation by peroxidase inducers, in addition to CYP1A1 inducers, indicates a potentially important new determinant of mutagenic susceptibility. The discovery that the so-called CYP1A1 inducer TCDD also induces PHS in this fibroblast model emphasizes the potential role of peroxidase-catalyzed bioactivation in toxicologic initiation, and reveals an interpretive dilemma for TCDD-based studies. This observation is consistent with the work by Kraemer et al. (1992; 1996) who recently have shown that a dioxin-responsive element (DRE) exists in the 5'-flanking region of the PHS-2 gene, and that TCDD can induce a 25-fold increase in PHS-2 protein, which is apparently a function of increased PHS-2 gene transcription. Our results further indicate that ROS-mediated oxidation of DNA, and possibly protein, constitute a potential molecular mechanism of B[a]P-initiated micronucleus formation, which may be relevant to carcinogenicity and teratogenicity.

The role of P450-catalyzed bioactivation in B[a]P-initiated micronucleus formation was investigated using ABT. Both ABT, a newer suicide substrate or mechanism-based inhibitor of both rat hepatic and renal P450 (Mugford et al., 1992), and SKF 525A, the classical P450 inhibitor,

caused a significant inhibition of B[a]P covalent binding to hepatic microsomal protein from TCDD-induced mice, indicating P450-catalyzed bioactivation. However, 1 mM ABT was twice as effective as 1 mM SKF 525A in inhibiting B[a]P covalent binding, suggesting important differences in the relative inhibitions of P450 isoforms bioactivating B[a]P. Thus, ABT was used in all subsequent cellular studies of micronucleus formation and DNA and protein oxidation. However, ABT also inhibited peroxidase-catalyzed B[a]P covalent binding, indicating that the enhanced effectiveness of ABT could be due in part to its inhibition of substantial peroxidase-catalyzed B[a]P bioactivation and initiation of micronucleus formation, which is consistent with the apparent toxicological importance of the observed induction of PHS by TCDD.

In uninduced fibroblasts, B[a]P initiated both the oxidation of DNA and protein, and the formation of micronuclei, indicating substantial bioactivation of B[a]P in uninduced cells with little or no CYP1A1. Preincubation with the dual CYP1A1/PHS inhibitor ABT prior to B[a]P exposure reduced the formation of B[a]P-initiated micronuclei. The loss of CYP1A1 protein with ABT suggests a degradative mechanism for ABT inhibition of CYP1A1, as previously described in *in vivo* studies (Mathews et al., 1985). Likewise, ABT preincubation reduced B[a]P-initiated DNA and protein oxidation down to untreated control levels. However, although ABT is a known P450 inhibitor which did reduce micronucleus formation and DNA and protein oxidation in uninduced fibroblasts, the involvement of CYP1A1-catalyzed B[a]P bioactivation is questionable, since CYP1A1 is a nonconstitutive enzyme. In fact, an early study by van Pelt et al. (1991) showed that pretreatment of cultured human keratinocytes with the broad spectrum P450 inducer Aroclor 1254 (possibly also a PHS inducer) was needed for cyclophosphamide-initiated micronucleus formation. We could not detect CYP1A1 protein in uninduced cells, and saw barely detectable protein (panel A) and undetectable activity in B[a]P-exposed cells. Undetectable P450 activity may be due in part to assay sensitivity (lower limit of detection=4.0 pmol/min/mg protein), since previous studies in murine fibroblasts from several strains showed that both TCDD and benz[a]anthracene increased the activity of aryl hydrocarbon hydroxylase (AHH) activity (Hosomi et al., 1983), a measure of B[a]P hydroxylation reactions thought to reflect CYP1A1 activity. However, AHH activity at least in part may also reflect PHS activity, and, as mentioned

earlier, B[a]P can be bioactivated by constitutive peroxidases to various toxic reactive intermediates, including B[a]P-7,8-diol-9,10-epoxide (Marnett, 1990a,b; Dix et al., 1985), B[a]P-quinone (Joseph and Jaiswal, 1994) and B[a]P radical cation (Cavalieri and Rogan, 1992a).

Enzymes such as PHS and LPOs catalyze the formation of eicosanoids and the intermediate product ROO^{*}, the latter of which can react with B[a]P-7,8-diol or B[a]P to form respectively BPDE and B[a]P-quinone, which can either covalently bind, or redox cycle producing toxic ROS (Reed, 1988). Prior studies have suggested that ABT may be a potential inhibitor of heme-containing peroxidases such as PHS that bioactivate B[a]P (Smith and Kehrer, 1991; Ortiz de Montellano, 1992).

In this study, horseradish peroxidase, a heme-containing enzyme, was used to show that ABT can inhibit peroxidase-catalysed B[a]P bioactivation and covalent binding. Thus, the inhibition of B[a]P-initiated micronucleus formation in uninduced fibroblasts by ABT may be due to inhibition of constitutive peroxidases rather than CYP1A1. This suggests that B[a]P in uninduced fibroblasts may be bioactivated by peroxidases to reactive intermediates that initiate micronucleus formation. When increasing amounts of microsomal protein from uninduced fibroblasts were probed for PHS, constitutive PHS protein was detected when analysed via Western blot/immunodetection (**fig. 5, panel C**). Conversely, uninduced fibroblasts failed to show detectable levels of CYP1A1 protein (**fig. 5**).

Similarly, coincubation with ETYA, a dual inhibitor of PHS and LPOs (Downing et al., 1972), substantially reduced B[a]P-initiated micronucleus formation (**fig. 7**), indicating that these enzymes can catalyze the bioactivation of B[a]P to genotoxic reactive intermediates in uninduced fibroblasts. Since preincubation, unlike coincubation, with ETYA failed to protect against micronucleus formation (**fig. 7**), the competitive rather than the irreversible inhibitory effects of ETYA on peroxidase activity appear to be critical in inhibiting B[a]P bioactivation.

TCDD is a potent, Ah receptor-mediated inducer of the nonconstitutive CYP1A1 isozyme in hepatic microsomes of many animal species (Okey et al., 1994). TCDD preincubation of Wistar rat skin fibroblasts prior to B[a]P exposure enhanced B[a]P-initiated micronucleus formation (**fig. 7**) and DNA oxidation (**fig. 9**). TCDD also substantially increased CYP1A1 protein, which

was not evident in the control group (**fig. 5**). B[a]P-treated cells showed a faint CYP1A1 protein band that was not evident in cells pretreated with ABT. This suggests that TCDD increased CYP1A1 content and activity, and hence the bioactivation of B[a]P to reactive intermediates that initiate DNA oxidation and micronucleus formation. However, as discussed below, this interpretation is confounded by the discovery that TCDD also induced PHS (**fig. 5**).

Increased transcription of other genes responsible for cell growth and differentiation may be important in TCDD toxicity and carcinogenicity, since CYP1A1 induction is not necessary for TCDD toxicity (Okey et al., 1994). Our study provided evidence for TCDD-initiated induction of PHS protein as a molecular mechanism for the enhancement of B[a]P bioactivation, molecular target oxidation and genotoxic sequelae. Microsomal protein from TCDD-treated fibroblasts showed a distinct PHS protein band that was not evident in the other groups (**fig. 5, panel B**). At this point it is not clear whether PHS-1 and/or PHS-2 is induced by TCDD, since the primary antibody used recognizes both types. PHS-1 is constitutively expressed in tissues such as the kidney and vascular smooth muscle (Smith et al., 1994), and appears to be maintained at a constant level with minor two- to four-fold induction by hormones and growth factors (DeWitt, 1991). On the other hand, PHS-2 is inducible by as much as ten- to eighty-fold by compounds such as cytokines (IL-1) and phorbol esters (TPA) (Smith et al., 1994). Accordingly, due to its high inducibility, the protein band in our fibroblast model may be PHS-2. Recently, Kraemer et al. (1996) have shown that, in Mardin Darvey canine kidney cells, TCDD greatly increases PHS-2, but not PHS-1 gene expression and protein. This suggests that TCDD incubation in our fibroblast model also is inducing PHS-2 protein via a similar mechanism. However, in the Kraemer study, PHS-2 protein induction was short-lived (3-4 hr) and appeared as a doublet of 69 and 72 kDa, which is somewhat inconsistent with our findings of PHS protein induction after 24 hr and a single protein band. These discrepancies may be related to the 10-fold higher TCDD concentration used in our study and the possible inherent differences in cell type (fibroblasts vs kidney cells) and species (dog vs rat).

DREs have been found in the 5'-flanking region of the mouse and canine PHS-2 gene (Kraemer et al., 1992, 1996), which would suggest a mechanism involving the Ah receptor.

However, it has been shown that the Ah receptor/ARNT complex was insufficient to account for the induction of the PHS-2 gene. Therefore the pathway leading to PHS-2 expression may differ from the Ah receptor/DRE pathway responsible for the expression of CYP1A1 (Kraemer et al., 1996). An early study conducted by Hassid and Levine (1977) demonstrated that 1 $\mu\text{g/ml}$ (3.96 μM) B[a]P incubated with canine kidney cells caused a significant increase in the release of radioactive eicosanoid, prostaglandin $F_{2\alpha}$, and microsomes prepared from similarly treated cells showed over a 2-fold increase in prostaglandin $F_{2\alpha}$, which was inhibited by indomethacin, a PHS inhibitor, suggesting Ah receptor-mediated PHS induction by B[a]P. Induction of PHS and potentially other peroxidases by TCDD may indicate a potential mechanism of *in vivo* TCDD-initiated oxidative stress (Stohs, 1990; Stohs et al., 1990), since peroxidase activity is associated with free radical production (Marnett, 1990b). This raises a question as to the mechanism of xenobiotic bioactivation in previous studies where TCDD pretreatment was employed. Although ROS are capable of initiating DNA damage, our results show that, at the concentrations employed, TCDD without B[a]P (i.e. TCDD plus DMSO control group) did not increase either micronucleus formation (**fig. 7**) or DNA oxidation (**fig. 9**) when compared to their respective control groups. This indicates that 10 nM TCDD itself does not directly or indirectly initiate DNA oxidation or micronucleus formation, at least in cultured Wistar rat skin fibroblasts. The lack of TCDD-initiated micronucleus formation also corroborates the hypothesis that micronuclei initiated by B[a]P occur via bioactivation and formation of DNA- and potentially protein-damaging reactive intermediates, rather than via binding to the Ah receptor, since both TCDD and B[a]P bind to this receptor, but only B[a]P is bioactivated by CYP1A1 to a reactive intermediate (Okey et al., 1994).

The role of peroxidase induction in B[a]P bioactivation, DNA and protein oxidation and micronucleus formation in skin fibroblasts was examined using the cytokine IL-1 α and the tumor promoter TPA. Both IL-1 α and TPA *in vitro* can induce the synthesis of prostaglandins (Dinarello, 1994; Kiss, 1991). Additionally, TPA has also been shown to increase the production of lipoxygenase products such as 12-hydroxyeicosatetraenoic acid, as well as increasing 8-lipoxygenase activity (Kiss, 1991). The increase in IL-1 α -induced prostaglandin synthesis has

been associated with increased de novo synthesis of cyclooxygenase in human dermal fibroblasts (Seibert et al., 1990). Similarly, TPA has been shown in Swiss 3T3 cells to induce the *tis10* gene that encodes for PHS-2, the cytokine-inducible form of PHS (Herschman et al., 1994). IL-1 α also can increase phospholipase A₂ activity in human embryonic fibroblasts, which has been associated with increased gene expression in the form of increased mRNA production (Dinarello, 1994; Levine and Xiao, 1985). Likewise, both IL-1 α and TPA can stimulate protein kinase C activity which, through the production of second messengers (diacylglycerol), can directly increase phospholipase A₂ activity (Dinarello, 1994; Kiss, 1991). Phospholipase A₂ can liberate AA from membrane phospholipids, which is a substrate for both PHS and LPOs. Many of the effects of TPA can be associated with increased AA release (Das, 1991).

In this study, both IL-1 α and TPA in Wistar rat skin fibroblasts increased B[a]P-initiated micronucleus formation (**fig. 7**) and oxidation of DNA (**fig. 9**) and protein (**fig. 10**). Neither micronucleus formation nor oxidation of DNA or protein were increased in fibroblasts treated with IL-1 α or TPA alone, demonstrating that the previously reported increase in ROS initiated by either TPA (0.06 μ M) in granulocytes (Floyd et al., 1986) or IL-1 α (280 nM) in human skin fibroblasts (Meier et al., 1989) was insufficient to produce the genotoxic consequences observed in cultured rat skin fibroblasts. In the former case, the different cell type (granulocytes) may be more sensitive to TPA activation, since a lower TPA concentration was used. In the latter case, however, the concentration of IL-1 α was about 10,000-fold higher than that used in our study (0.037 nM or 0.073 nM), which likely accounts for their results.

The induction of PHS by cytokines (IL-1 α) and xenobiotics (TPA, TCDD), and its substantial enhancement of B[a]P bioactivation and mutagenicity, as evidenced by increased micronucleus formation, reveals a further dimension in the question of carcinogenic and teratogenic susceptibility. Cytokines including IL-1 α are released in a multitude of physiological and pathophysiological conditions (Yoshida, 1994), most obviously during infection with viruses, bacteria and other foreign organisms and molecules (Biron, 1994; Jung et al., 1995). Such conditions, and/or exposure to environmental chemicals with peroxidase-inducing activity similar to that observed with TPA and TCDD, would be expected to enhance xenobiotic bioactivation

and toxicologic initiation for procarcinogens and proteratogens. The clinical significance of peroxidase induction remains to be determined.

In summary, these results provide the first direct evidence that B[a]P-initiated micronucleus formation, like carcinogenic and teratogenic initiation, requires enzymatic bioactivation, and that peroxidase-dependent, ROS-mediated oxidation of DNA, and possibly proteins, constitutes a molecular mechanism of initiation in uninduced cells. The substantial enhancement of this genotoxic initiation by induction of peroxidases such as PHS, as well as by CYP1A1 induction, reveals a further dimension of carcinogenic and teratogenic risk assessment.

2.2 STUDY #2: GENOPROTECTION BY UDP-GLUCURONOSYLTRANSFERASES IN PEROXIDASE-DEPENDENT, REACTIVE OXYGEN SPECIES-MEDIATED MICRONUCLEUS INITIATION BY THE CARCINOGENS 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) AND BENZO[A]PYRENE^{1,2}

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2.2.1 ABSTRACT

UDP-glucuronosyltransferases (UGTs) catalyze the glucuronidation and elimination of putative tobacco carcinogens such as benzo[a]pyrene (B[a]P) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which may reduce competing bioactivation and toxicity. B[a]P-initiated cytotoxicity and micronucleus formation, believed to reflect carcinogenic initiation, are enhanced in UGT-deficient rat fibroblasts, and UGTs may provide similar genoprotection against NNK. Using skin fibroblasts from wild type UGT-normal (+/+), and congenic heterozygous (+/j) and homozygous (j/j) UGT-deficient rats, this study evaluated NNK in relation to B[a]P with respect to the mechanism of genotoxicity, evidenced by micronucleus formation, and genoprotection by UGTs. Molecular mechanisms were determined by changes in B[a]P- and NNK-initiated micronucleus formation when cells were incubated with the antioxidative enzyme superoxide dismutase (SOD, 1680 IU/ml), inhibitors of cytochrome P450 (CYP) [1 mM 1-aminobenzotriazole, ABT] and peroxidases [ABT; 40 μ M eicosatetraynoic acid, ETYA], and inducers of CYP1A1/2 [10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD] and peroxidases [TCDD; 0.625 ng/ml (0.0367 nM) interleukin-1 α , IL-1 α ; 1 μ M 12-O-tetradecanoylphorbol-13-acetate, TPA]. In +/+ fibroblasts, NNK and B[a]P initiated concentration-dependent, respective maximal 2.7-fold and 1.7-fold increases over DMSO controls in micronucleus formation ($p < 0.05$), with 10 μ M NNK being 2.4-fold more genotoxic than B[a]P ($p < 0.05$). In both +/j and j/j UGT-deficient cells, micronuclei initiated by NNK and B[a]P each were over 2-fold higher than that in +/+ UGT-normal cells ($p < 0.05$). Both NNK- and B[a]P-initiated micronuclei were decreased by SOD and P450/oxidase inhibitors, while only that initiated by B[a]P was enhanced, up to 2.4-fold, by inducers, of which only IL-1 α was effective in all UGT-phenotypes ($p < 0.05$). These results provide the first evidence that: (1) UGTs may be genoprotective for NNK, with even heterozygous UGT-deficiencies being toxicologically critical; and, (2) oxidase-catalyzed bioactivation, reactive oxygen species, and molecular target oxidation may contribute differentially to the genotoxicity of both NNK and B[a]P.

2.2.2 INTRODUCTION

The elimination of endogenous chemicals and xenobiotics, including the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Adams et al., 1983; Hecht et al., 1994; Hoffman et al., 1991) and the ubiquitous environmental carcinogen benzo[a]pyrene (B[a]P) (Osborne and Crosby, 1987), are catalyzed by the superfamily of membrane-bound isozymes known collectively as UDP-glucuronosyltransferases (UGTs) (Dutton, 1980; Burchell and Coughtrie, 1989; Burchell et al., 1991). UGTs catalyze the conjugation of xenobiotics with the hydrophilic molecule UDP-glucuronic acid (UDPGA), allowing for excretion in urine or feces (**fig. 1**). Recent evidence indicates that human deficiencies in UGTs may lead to increased susceptibility to xenobiotic bioactivation and toxicity (de Morais and Wells, 1988; Hu and Wells, 1993), including carcinogenesis (Vienneau et al., 1995) and teratogenesis (Well et al., 1989).

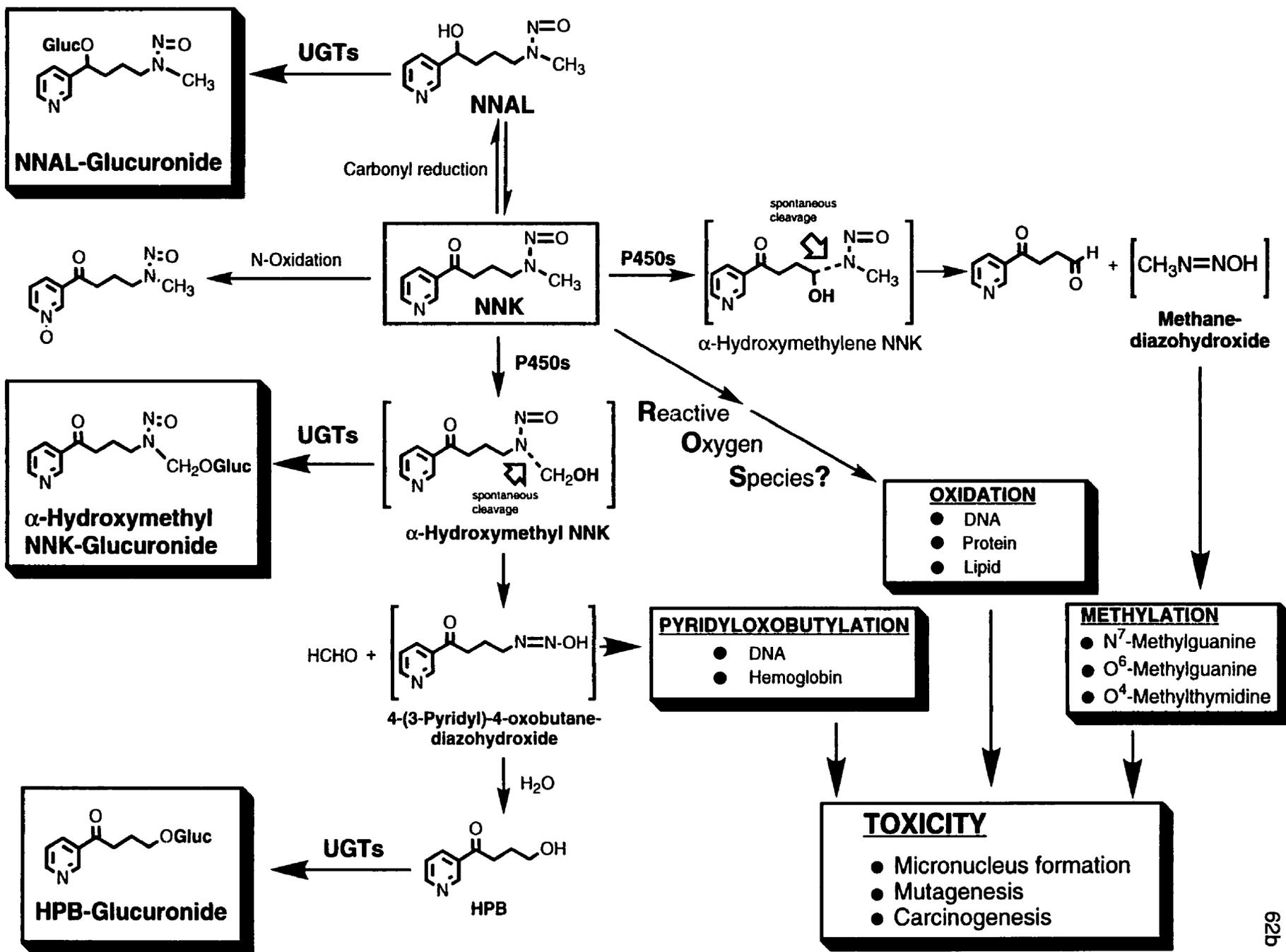
UGTs exist as two families (UGT1 and 2) located on separate chromosomes (Moghrabi et al., 1992; Monaghan et al., 1992) that are regulated by distinctly different mechanisms. The different UGT1 isozymes are produced from a single gene complex consisting of multiple isozyme-specific exons located at the 5'-variable/specific region, which are alternatively spliced with exons at the 3'-constant region (Brierly and Burchell, 1993; Jansen et al., 1992). In contrast, UGT2 isozymes are produced from the transcription of separate and complete genes.

Approximately 2 to 5% of the human population have hereditary deficiencies in at least the bilirubin UGT1A1 (UGT1*1) isozyme (Gilbert's and Crigler-Najjar types I and II syndromes) (Odell and Childs, 1980), which results from various mutations (Jansen et al., 1992; Bosma et al., 1992, 1993; Moghrabi et al., 1993a,b). These mutations lead to the loss of bilirubin UGT protein or activity, which is phenotypically expressed as jaundice due to abnormally elevated bilirubin blood concentrations (Burchell and Coughtrie, 1989; Jansen et al., 1992; Moghrabi et al., 1993a,b).

UGT-deficient Gunn and RHA rats have decreased acetaminophen glucuronidation, resulting in enhanced bioactivation, hepatotoxicity and nephrotoxicity (de Morais and Wells, 1988, 1989; de Morais et al., 1992a), and humans with Gilbert's syndrome have decreased

Figure 1. Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to its major glucuronide metabolites. NNK is nonenzymatically converted to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and also is α -hydroxylated by various cytochromes P450 (P450s) to α -hydroxymethyl NNK, the precursor of the putative reactive intermediate thought to pyridyloxobutylate DNA. NNK also is hydroxylated by P450s at the methylene carbon, forming a reactive intermediate that can methylate DNA. Finally, NNK initiates DNA oxidation, possibly via the formation of reactive oxygen species, but the bioactivating enzymes have not been characterized. Abbreviations: UGTs, UDP-glucuronosyltransferases; OGluc, glucuronide conjugate; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone.

FIGURE 1



acetaminophen glucuronidation and increased acetaminophen bioactivation (de Morais et al., 1992b). Similarly, *in vitro* and *in vivo* studies using UGT-deficient Gunn and RHA rats showed decreased glucuronidation of B[a]P metabolites, with enhanced B[a]P bioactivation and covalent binding to protein and DNA (Hu and Wells, 1992), and enhanced embryotoxicity (Wells et al., 1989a). Further studies showed that lymphocytes obtained from UGT-deficient rats accurately reflected the decreased glucuronidation and enhanced bioactivation and covalent binding of B[a]P in hepatic microsomes from the same animals (Hu and Wells, 1994). Lymphocytes from normal human volunteers demonstrated a 200-fold variation in UGT activity for B[a]P metabolites, and decreased glucuronidation correlated with enhanced cytotoxicity, suggesting that human UGT deficiencies may be an important determinant of toxicological susceptibility to B[a]P and related xenobiotics (Hu and Wells, 1993, 1998). A recent *in vitro* study demonstrated that B[a]P-initiated micronucleus formation in skin fibroblasts cultured from UGT-deficient rats was greater than that in UGT-normal cells, suggesting enhanced carcinogenic initiation (Vienneau et al., 1995). As with carcinogenic initiation, this B[a]P-initiated micronucleus formation subsequently was shown in Wistar rat skin fibroblasts to be dependent upon peroxidase- and cytochromes P450 (P450)-catalysed B[a]P bioactivation, and possibly mediated by DNA oxidation (Kim and Wells, 1994, 1995) (see Section 2.1).

Similar to B[a]P, NNK is bioactivated by various isozymes of the cytochrome P450 superfamily (CYP1A1, CYP1A2, CYP2A6, CYP2B1, CYP2B7, CYP2D6, CYP2E1) to reactive intermediates (Crespi et al., 1991a,b; Guo et al., 1992; Smith et al., 1992; Lacroix et al., 1993; Nesnow et al., 1994). NNK can initiate micronucleus formation (Alaoui-Jamali et al., 1989), as well as DNA oxidation (Bilodeau et al., 1995), methylation (Ronai et al., 1993, Van Benthem et al., 1994), arylation (Peterson et al., 1993, Liu et al., 1993), and strand breaks (Weitberg and Corvese, 1993, Jorquera et al., 1994) (**fig. 1**). UGTs not only help eliminate NNK through the glucuronidation of the carbonyl reduced metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), but also directly eliminate or detoxify the α -methyl-hydroxylated NNK metabolite that is a proximate DNA arylating agent (Murphy et al., 1995) (**fig. 1**). In this study, we investigated NNK-initiated micronucleus formation in skin fibroblasts cultured from UGT-normal (+/+), and

heterozygous (+/j) and homozygous (j/j) UGT-deficient Gunn rats to determine the potential genoprotective role of UGTs in NNK carcinogenesis. We further evaluated the role of bioactivation and reactive oxygen species (ROS) in NNK-initiated micronucleus formation, in comparison to B[a]P, using inducers and inhibitors of potential bioactivating enzymes (CYP1A1 and peroxidases), and the antioxidative enzyme superoxide dismutase (SOD).

These results constitute the first evidence that UGTs may be critical in protecting against NNK genotoxicity, with even heterozygous UGT deficiencies being important. The mechanism of NNK genotoxicity, like that for B[a]P, appears to involve peroxidase- as well as P450-dependent bioactivation, although the relative contributions may differ, with ROS ultimately contributing to micronucleus formation.

2.2.3 MATERIALS AND METHODS

Animals

Male HsdBlu:Gunn rats, 180-200 g (Harlan Sprague Dawley Inc., Indianapolis, Indiana) were housed in separate plastic cages. All animals were kept in a temperature-controlled room with a 12-hr light-dark cycle automatically maintained. Food (Laboratory Rodent Chow 5001, PMI Feeds Inc.; St. Louis, MO) and tap water were provided *ad libitum*. Animals were acclimatized for a minimum of one week. All animal studies were approved by the University Animal Care Committee in accordance with the standards of the Canadian Council on Animal Care.

Chemicals

The CYP1A1 inducer 2,3,7,8-[1,6-³H]tetrachlorodibenzo-p-dioxin (1 dpm=1 x 10⁻¹⁴ moles TCDD) was a gift from Dr. Allan B. Okey (University of Toronto, Toronto, Ontario). The P450 inhibitor 1-aminobenzotriazole (ABT) was a gift from Dr. Jack P. Uetrecht (University of Toronto). 5,8,11,14-Eicosatetraynoic acid (ETYA) was a gift from Hoffmann-La Roche Ltd. (Etobicoke, Ontario). Benzo[a]pyrene (B[a]P), formaldehyde, 12-O-tetradecanoylphorbol-13-acetate (TPA; phorbol-12-myristate-13-acetate), interleukin-1 α (IL-1 α), superoxide dismutase (SOD) 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of an analytical grade.

Dulbecco's modified eagle medium (D-MEM), fetal bovine serum (FBS), lyophilized penicillin/streptomycin (pen/strep), Hanks' balanced salt solution (HBSS) (without calcium chloride, magnesium chloride and magnesium sulfate) and 0.25% trypsin were purchased from Gibco BRL (Toronto, Ontario)

Cell culture studies

The various methods used in these studies have been described in length elsewhere (Vienneau et al., 1995).

Cell culture method. Briefly, rats were sacrificed by CO₂ asphyxiation, bathed in 70%

ethanol and two 2 x 2 cm pieces of shaved skin were removed from the dorsal surface and placed in HBSS with 2% pen/strep. Skin was cultured immediately.

All following steps were conducted in a laminar flow hood. The skin was minced into 1 mm³ pieces, stored in 20 ml of HBSS (2% pen/strep) and transferred to sterile 100 mm polystyrene tissue culture dishes and arranged to fit under square 18 mm coverslips. Medium (D-MEM 500 ml with 75 ml FBS and 5 ml pen/strep) was added at the margin of the coverslip, allowing it to move across by capillary action, and then a further 5 ml of medium was added to the dish. All dishes were incubated at 37°C in a humidified incubator with 5% CO₂ in air and left undisturbed for 10 days. The dishes were then examined with an inverted phase contrast microscope to confirm the formation of a monolayer of fibroblasts at the margins of the skin pieces. Thereafter the medium was changed twice a week. After 2 months, the cultures were confluent, defined as a single layer of cells covering the bottom of the dish.

Micronucleus Formation

For studies using potential inhibitors of bioactivation, skin fibroblasts from HsdBlu:GUNN rats were preincubated with medium containing either the P450 inhibitor 1 mM ABT for 2 hr, or the PHS/LPO inhibitor 40 µM ETYA, which were coincubated with 10 µM B[a]P for 5 hr. The rationale for the concentrations of each chemical used are discussed by Kim et al. (1997a) (see Section 2.1). The vehicle for ETYA, NNK and B[a]P was DMSO, and PBS was the vehicle for ABT.

For studies using inducers of bioactivation, skin fibroblasts were preincubated with medium containing either the CYP1A1 and prostaglandin H synthase (PHS) inducer TCDD 10 nM, or the PHS inducers TPA 1 µM or IL-1α [0.625 ng/ml (0.0367 nM)] for 24 hr, prior to incubation with 10 µM B[a]P for 5 hr. The rationale for the concentrations of each chemical used was discussed by Kim et al. (1997a). The vehicle for TCDD and TPA was DMSO, and the vehicle for IL-1α was PBS.

For studies evaluating the cytoprotective role of SOD in both B[a]P- and NNK-initiated micronucleus formation, SOD 1680 IU/ml (500 µg/ml) was preincubated with the cells for 24 hr

before addition of either B[a]P or NNK. This concentration of SOD was shown in embryo culture to be embryoprotective against phenytoin-initiated DNA oxidation and embryotoxicity (Winn and Wells, 1995a).

In all studies, the cells were washed 3 times with 5 ml of HBSS after chemical/vehicle incubation, 5 ml of fresh medium was added, and cells were allowed to undergo one complete mitotic cycle, which previously was shown to be 26 hr (Vienneau et al., 1995). The 5 hr B[a]P, NNK or DMSO incubation was included as part of the mitotic cycle time. Upon the completion of one mitotic cycle, the medium was aspirated off and the cells were washed 3 times with 5 ml of HBSS to remove all residual medium. To fix the cells, 5 ml of formalin solution (37% formaldehyde solution:PBS = 1:9 v/v) was added to the cells. After 30 min, the formalin solution was aspirated off and cells were washed 3 times with 5 ml of PBS.

Once fixed, the cells were stained with 5 ml of DAPI fluorescent stain 2 µg/ml in water, and 2000 mononucleated were counted for the formation of micronuclei, using an inverted microscope with a 40X objective.

Statistical analysis

Statistical significance of differences between treatment groups in each study was determined by Student's t test or one-factor analysis of variance (ANOVA) as appropriate using a standard, computerized statistical program (Statview, Abacus Concepts, Inc., Berkley, CA) The level of significance was $p < 0.05$.

2.2.4 RESULTS

Concentration- and UGT phenotype-dependent increases in NNK-initiated micronucleus formation

NNK-initiated micronucleus formation showed a concentration-dependent response in all cell phenotypes, with maximal increases between 4- and 8-fold with in 100-500 μ M NNK (**fig. 2**).

Micronucleus formation was not only NNK concentration-dependent, but also dependent upon UGT-phenotype (**fig. 2**). At all NNK concentrations, both +/j and j/j UGT-deficient cells were more susceptible to micronucleus formation than similarly treated +/+ cells ($p < 0.05$). NNK-initiated micronucleus formation in +/j and j/j cells was similar at 10 and 100 μ M NNK, whereas at 500 μ M, j/j cells had a lower incidence than +/j cells ($p < 0.05$), likely due to enhanced NNK cytotoxicity in j/j cells. In untreated cells, the incidence of micronucleus formation was not different among the UGT phenotypes.

Comparative genotoxicity of NNK and B[a]P

When the effectiveness of 10 μ M B[a]P and NNK were compared in the +/+ UGT-normal cells, increased micronucleus formation initiated by NNK was 2.4-fold higher than that by B[a]P ($p = 0.002$) (**fig. 3**). Although NNK also appeared to be more effective than B[a]P in +/j and j/j UGT-deficient cells, these differences were not statistically significant.

Modulators of B[a]P-initiated micronucleus formation

As previously reported (Vienneau et al., 1995, Kim and Wells, 1994, 1995; Kim et al., 1997a), 10 μ M B[a]P initiated substantial micronucleus formation compared to DMSO controls ($p < 0.05$), the magnitude of which was genotype-dependent (**figs. 3 and 4**). B[a]P-initiated micronucleus formation was increased respectively by 3.1- and 3.6-fold in cells cultured from both +/j heterozygous ($p = 0.06$) and j/j homozygous UGT-deficient ($p = 0.0003$) Gunn rats over +/+ UGT-normal congenic controls (**fig. 3**). There was no difference in micronucleus formation between B[a]P-treated +/j and j/j UGT-deficient cells.

Figure 2. Effect of UDP-glucuronosyltransferase (UGT) deficiency and concentration of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on micronucleus formation. Skin fibroblasts were cultured from either homozygous UGT-normal (+/+), or heterozygous (+/j) or homozygous (j/j) UGT-deficient Gunn rats. Cells were incubated with the DMSO vehicle or NNK 10, 100 or 500 μ M for 5 hr, washed and cultured for the rest of one mitotic cycle (26 hr). Symbols indicate the mean of 4 fibroblast cultures. Asterisks indicate a difference from respective NNK-treated +/+ UGT-normal groups ($p < 0.05$). All NNK concentrations were significantly different from controls.

FIGURE 2

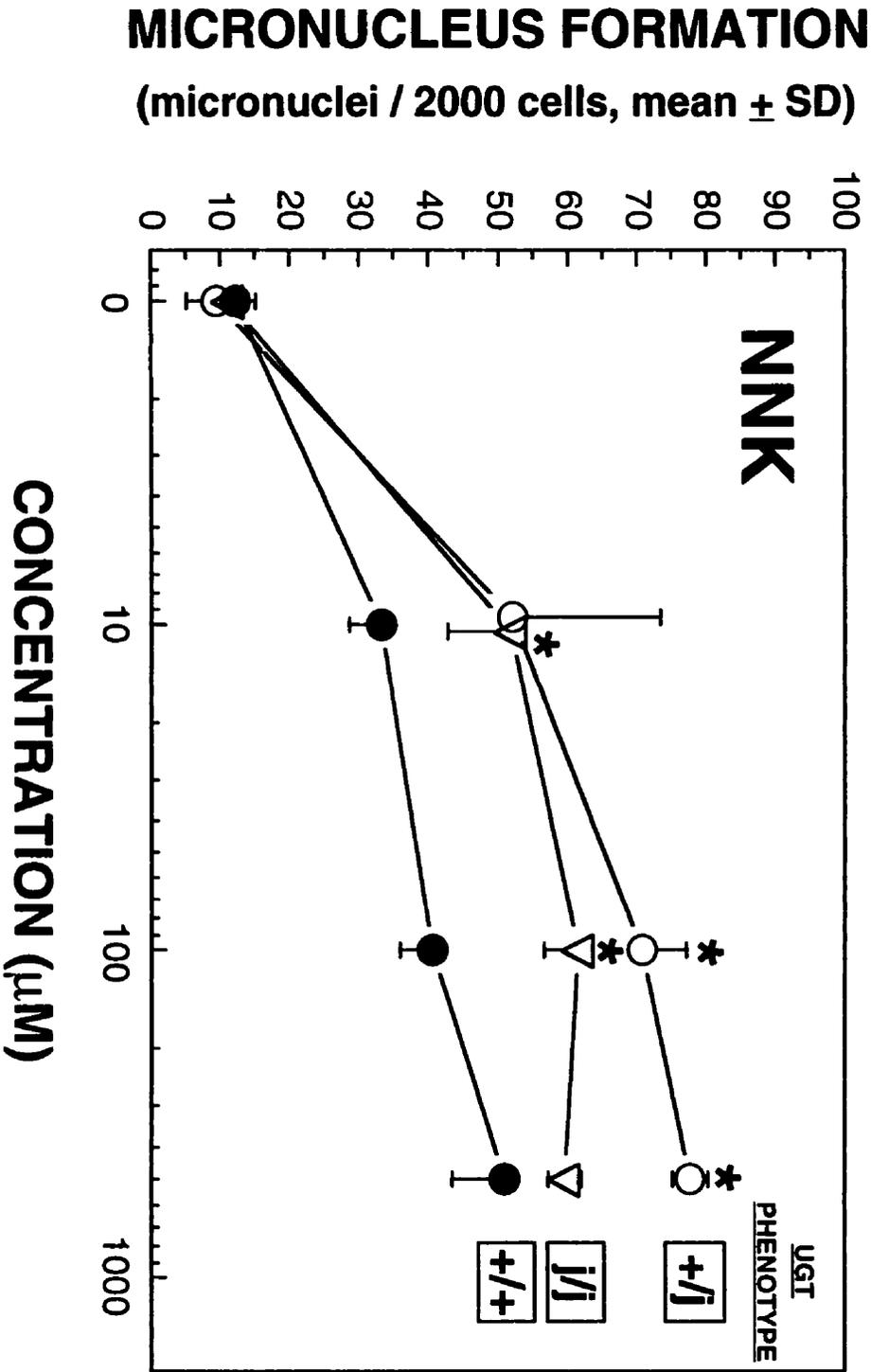


Figure 3. Comparison of the effectiveness of 10 μ M benzo[a]pyrene (B[a]P) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in initiating micronucleus formation. Bars indicate the mean of 4 fibroblast cultures. Micronuclei initiated by both B[a]P and NNK in all cell phenotypes were greater than for respective DMSO controls ($p < 0.05$). Asterisks indicate a difference from the respective treatment in +/+ UGT-normal cells ($p < 0.05$), and the plus symbol indicates a difference from B[a]P-treated +/+ cells ($p < 0.05$).

FIGURE 3

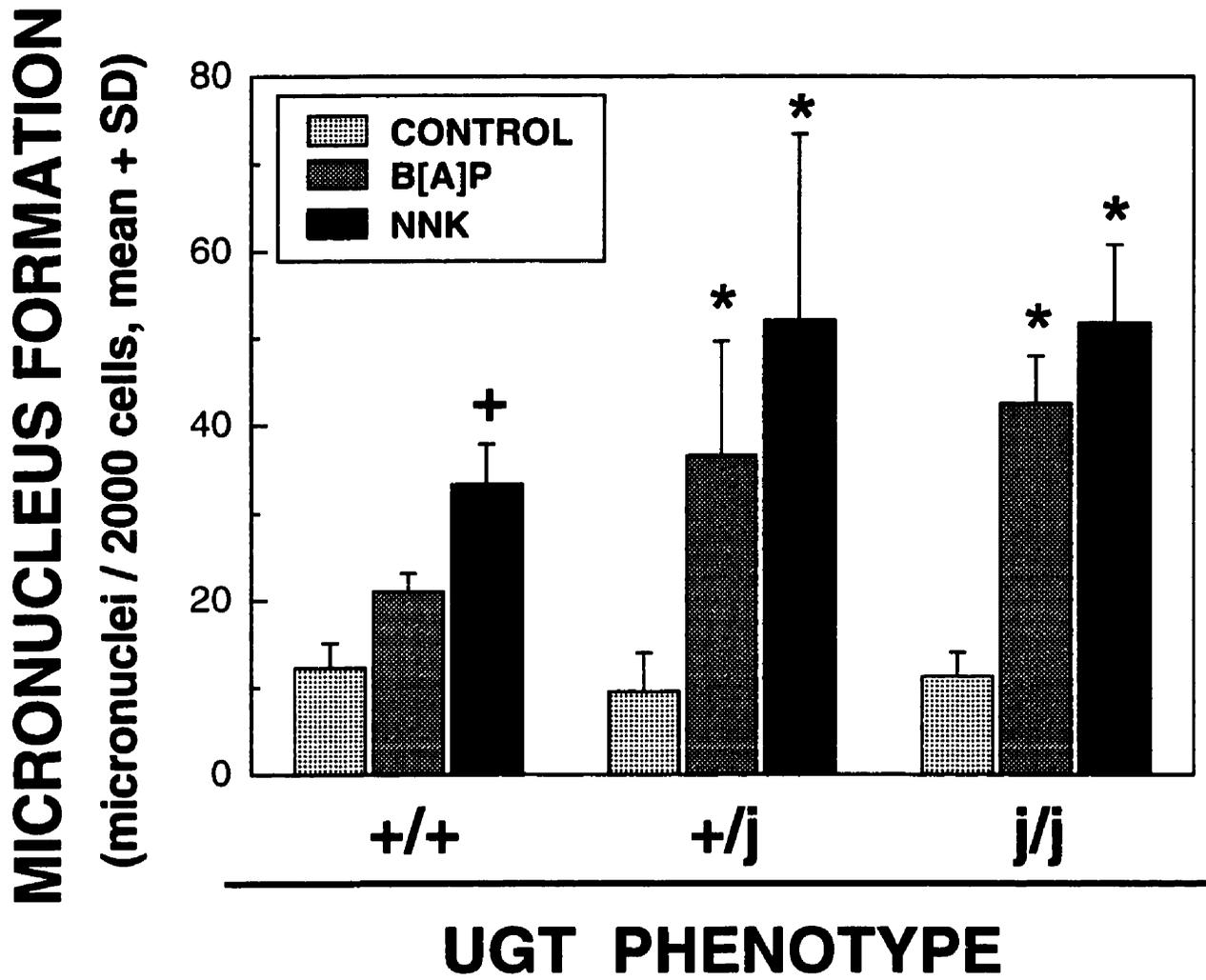
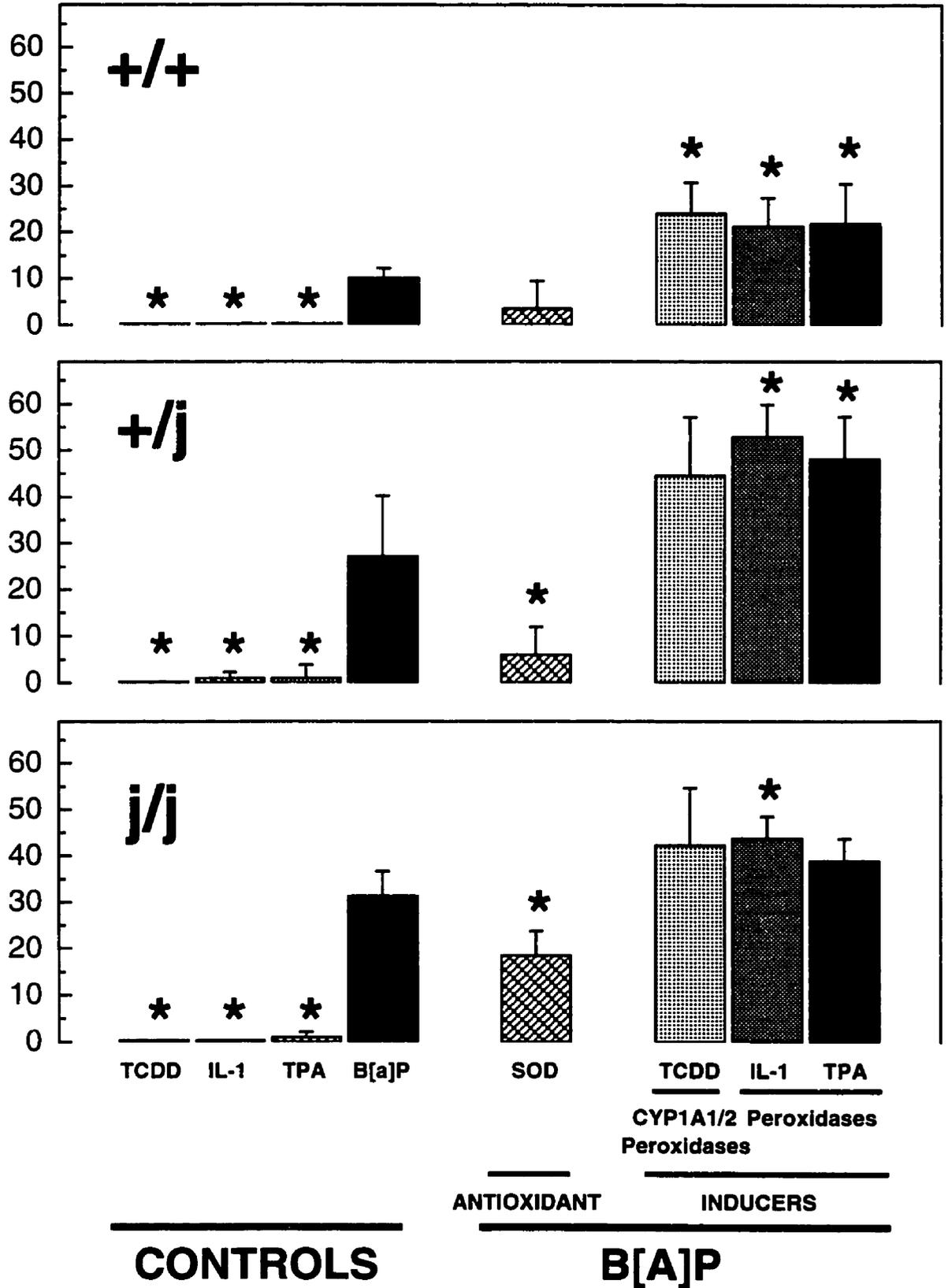


Figure 4. Effect of UDP-glucuronosyltransferase (UGT) deficiency, cytochromes P4501A1/2 (CYP1A1/2) and/or peroxidase induction and antioxidative cytoprotection on benzo[a]pyrene (B[a]P)-initiated micronucleus formation. Skin fibroblasts were cultured from either homozygous UGT-normal (+/+), or heterozygous (+/j) or homozygous (j/j) UGT-deficient Gunn rats. Cells were preincubated with either the dual CYP1A1/2/peroxidase inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD 10 nM), the peroxidase inducers (IL-1 α 0.625 ng/ml, 0.0367 nM) or 12-O-tetradecanoylphorbol-13-acetate (TPA 1 μ M), or the antioxidative enzyme superoxide dismutase (SOD 1680 IU/ml, 500 μ g/ml) for 24 hr prior to B[a]P 10 μ M for 5 hr. Bars indicate the mean of 4 fibroblast cultures after subtracting the respective mean values for the DMSO controls for each UGT phenotype (+/+, 12.25 \pm 2.87; +/j, 9.5 \pm 4.43; j/j, 11.25 \pm 2.75) (mean \pm SD). Asterisks indicate a difference from B[a]P-treated cells ($p < 0.05$).

FIGURE 4

MICRONUCLEUS FORMATION

(micronuclei / 2000 cells, mean + SD; relative to DMSO controls)



Preincubation with the antioxidative enzyme SOD decreased B[a]P-initiated micronucleus formation by 78% ($p=0.027$) and 41% ($p=0.015$) respectively in +/j and j/j UGT-deficient cultured skin fibroblasts (**fig. 4**). The apparent 74% decrease by SOD in +/+ UGT-normal cells was not statistically significant ($p=0.087$).

As previously reported (Kim and Wells, 1994, 1995, Kim et al., 1997a), preincubation with either TCDD, IL-1 α or TPA increased B[a]P-initiated micronucleus formation ($p<0.05$) (**fig. 4**). In +/+ UGT-normal cells, TCDD ($p=0.008$) increased B[a]P-initiated micronucleus formation by 2.6-fold over B[a]P alone, and both IL-1 α ($p=0.014$) and TPA ($p=0.04$) caused 2.3-fold increases. There were respective 1.7-, 2.0- and 1.8-fold increases in B[a]P-initiated micronucleus formation in +/j UGT-deficient cells preincubated with either TCDD ($p=0.10$), IL-1 α ($p=0.014$) or TPA ($p=0.04$). In j/j UGT-deficient cells, preincubation with IL-1 α caused a 1.4-fold increase ($p=0.014$) in B[a]P-initiated micronucleus formation over cells incubated with B[a]P alone, whereas the respective apparent 1.25- and 1.4-fold increases by TPA ($p=0.09$) and TCDD ($p=0.16$) were not statistically significant.

Modulators of NNK-initiated micronucleus formation

NNK (10 μ M)-treated cells cultured from +/+ UGT-normal, +/j and j/j UGT-deficient Gunn rats showed respective 2.7- ($p=0.0001$), 5.5- ($p=0.008$) and 4.6-fold ($p=0.0001$) increases in micronucleus formation compared to DMSO vehicle controls (**figs. 3, 5**).

Preincubation with SOD caused respective 65%, 56% and 49% decreases in NNK-initiated micronucleus formation in cells cultured from +/+ UGT-normal ($p=0.001$), and +/j ($p=0.08$) and j/j ($p=0.02$) UGT-deficient Gunn rats (**fig. 5**).

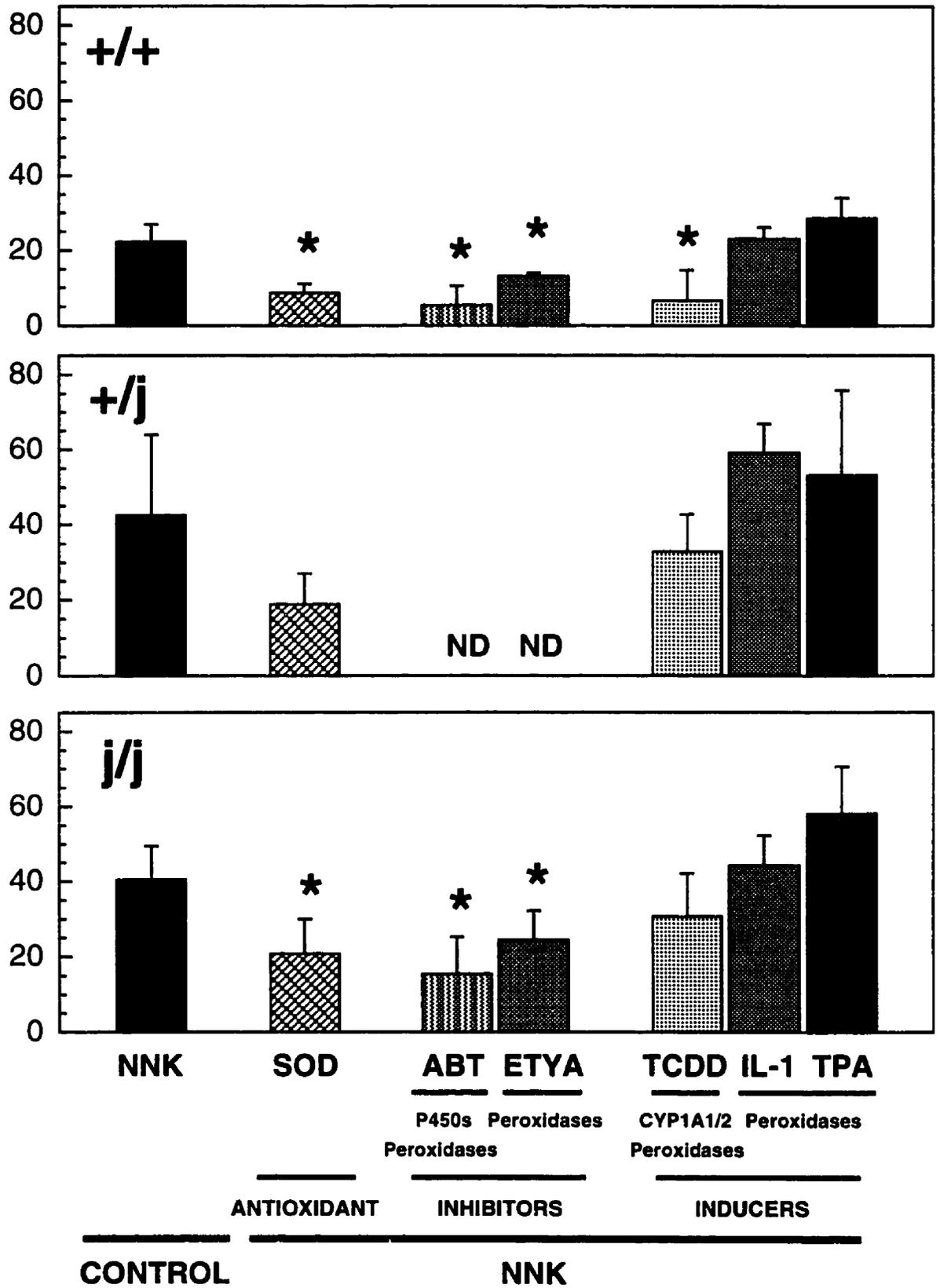
When cells were preincubated with 1-aminobenzotriazole (ABT), a known P450 inhibitor, NNK-initiated micronucleus formation was respectively decreased by 81% and 62% in +/+ UGT-normal ($p=0.001$) and j/j UGT-deficient ($p=0.0095$) cells (**fig. 5**). The dual prostaglandin H synthase (PHS) and lipoxygenase (LPO) inhibitor, ETYA, reduced NNK-initiated micronucleus formation respectively by 44% and 40% in +/+ UGT-normal ($p=0.006$) and j/j UGT-deficient ($p=0.04$) cells. ABT and ETYA were not tested in +/j cells.

Figure 5. Effect of UDP-glucuronosyltransferase (UGT) deficiency, cytochromes P4501A1/2 (CYP1A1/2) and/or peroxidase induction or inhibition and antioxidative cytoprotection on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-initiated micronucleus formation. Skin fibroblasts were cultured from either homozygous UGT-normal (+/+), or heterozygous (+/j) or homozygous (j/j) UGT-deficient Gunn rats. Cells were preincubated with either the dual CYP1A1/2/peroxidase inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD 10 nM), peroxidase inducers interleukin-1 α (IL-1 α 0.625 ng/ml, 0.0367 nM) or 12-O-tetradecanoylphorbol-13-acetate (TPA 1 μ M), the dual P450/peroxidase inhibitor 1-aminobenzotriazole (ABT 1 mM), the selective peroxidase inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA 40 μ M), or antioxidative enzyme superoxide dismutase (SOD 1680 IU/ml, 500 μ g/ml) for 24 hr prior to 10 μ M NNK for 5 hr. Bars indicate the mean of 4 fibroblast cultures, after subtracting the respective mean values for the DMSO controls for each UGT phenotype (+/+, 12.25 \pm 2.87; +/j, 9.5 \pm 4.43; j/j, 11.25 \pm 2.75) (mean \pm SD). Asterisks indicate a difference from NNK-treated cells ($p < 0.05$), and ND denotes treatment groups not determined.

FIGURE 5

MICRONUCLEUS FORMATION

(micronuclei / 2000 cells, mean + SD; relative to DMSO controls)



Surprisingly, TCDD reduced NNK-initiated micronucleus formation in +/+ UGT-normal cells compared to cells treated with NNK alone ($p=0.008$), whereas TCDD had no effect in +/j or j/j UGT-deficient cells (**fig. 5**). Both IL-1 α and TPA failed to increase micronucleus formation in either +/+ UGT-normal, +/j or j/j UGT-deficient cells.

2.2.5 DISCUSSION

UGTs catalyze the elimination of most xenobiotics, including both B[a]P (Osborne and Crosby, 1987) and NNK (Hecht et al., 1994; Murphy et al., 1995; Carmella et al., 1993; Morse et al., 1990), which can reduce competing bioactivation and thus decrease or eliminate toxicity (**fig. 1**). Similar to a previous report on B[a]P by Vienneau et al. (1995), skin fibroblasts cultured from +/j and j/j UGT-deficient rats showed increased NNK- and B[a]P-initiated micronucleus formation with increasing UGT deficiencies, which in the present study was reduced by the antioxidative enzyme SOD. Results from these and previously reported studies of the toxicological relevance of UGT-deficiencies in animals (de Morais and Wells, 1988, 1989; de Morais et al., 1992a; Hu and Wells, 1992, 1994) and humans (Hu and Wells, 1993, de Morais et al., 1992b, Hu and Wells, 1998) consistently have demonstrated a reduction in glucuronidation, with enhanced bioactivation and toxicity, for heterozygous UGT-deficiencies that in several cases, including the present study, were equivalent to the toxicologic predisposition of the homozygous UGT-deficient group. These observations suggest that hereditary heterozygous UGT-deficiencies, which are common in the human population, may be an important clinical determinant of toxicological susceptibility. With homozygous UGT-deficiencies, studies in pregnant UGT-deficient Gunn rats suggests that *in utero* exposure to xenobiotics like B[a]P may be embryolethal (Wells et al., 1989a).

The tobacco-specific carcinogen NNK initiated micronucleus formation in skin fibroblasts cultured from Gunn rats (**figs. 2, 3, 4**). Micronucleus formation initiated by NNK at 10, 100 and 500 μ M demonstrated a concentration-response relationship in cells cultured from +/+ Gunn rats, and a similar pattern was seen in both the +/j and j/j UGT-deficient cells (**fig. 2**). Interestingly, micronucleus formation in j/j UGT-deficient cells incubated with 500 μ M NNK was lower than that of +/j UGT-deficient cells, which may have resulted from increased NNK-mediated cytotoxicity in j/j UGT-deficient cells. This is supported by the fact that 100 μ M B[a]P is cytotoxic to cultured rat skin fibroblasts (Vienneau et al., 1995), and since the effectiveness of 10 μ M NNK in the +/+ cells in initiating micronucleus formation is over 2-fold higher than that of B[a]P (**fig. 3**), both 100 and 500 μ M NNK would be expected to be cytotoxic. Furthermore, previous studies found that an

NNK concentration of 200 $\mu\text{g/ml}$ (190 μM) in transformed C3H/10T1/2CL8 mouse embryo fibroblasts was severely cytotoxic (Nesnow et al., 1994).

Similar to previous reports (Kim and Wells, 1994, 1995, Kim et al., 1997a), results from this study in cells cultured from $+/j$ and j/j UGT-deficient Gunn rats showed that preincubation with the respective CYP1A1 and peroxidase inducers TCDD and/or IL-1 α and TPA significantly enhanced B[a]P-initiated micronucleus formation (**fig. 4**). Although P450s (Okey et al., 1994), UGTs (Owens, 1977, Roy Chowdhury et al., 1991) and PHS (DeBoni et al., 1996; see Section 2.1) all are known to be induced by TCDD, we would expect preincubation with either TCDD, IL-1 α or TPA to induce bioactivation pathways (P450, PHS) of B[a]P, while leaving UGT-catalysed elimination at deficient or basal levels in $+/j$ and j/j UGT-deficient cultured cells. We hypothesized that this differential induction of the two competing pathways would cause an imbalance favoring bioactivation and a substantial enhancement in B[a]P-initiated micronucleus formation in $+/j$ and j/j UGT-deficient cells to congenic normal controls. However, preincubation with TCDD, IL-1 α or TPA in $+/j$ and j/j UGT-deficient cells, prior to B[a]P caused a similar fold enhancement in micronucleus formation to that seen in $+/+$ UGT-normal controls (**fig. 4**). This lack of enhanced differential susceptibility to B[a]P-initiated micronucleus formation may be explained by the broad spectrum of gene induction known to occur from TCDD, IL-1 α or TPA exposure, and such broad induction may significantly complicate interpretation of the results (Okey et al., 1994, Dinarello, 1994). However, since induction of both CYP1A1 and peroxidases are major determinants in B[a]P bioactivation and micronucleus formation, as seen by significant enhancement upon CYP1A1 and peroxidase induction (Kim and Wells, 1994, 1995, Kim et al., 1997a) (**fig. 5**), a more likely explanation for the lack of enhancement may be that increased cytotoxicity of $+/j$ and j/j UGT-deficient cells to enhanced B[a]P bioactivation. A similar effect likely would explain the decrease in NNK-initiated micronucleus formation in j/j UGT-deficient versus $+/j$ UGT-deficient cells at the highest NNK concentration (**fig. 2**).

In this study, preincubation with SOD protected against B[a]P-initiated micronucleus formation, thus suggesting a role for ROS-mediated DNA damage as a molecular mechanism of initiation (**fig. 4**). These results are supported by a previous report that ROS-mediated DNA

oxidation is a likely molecular mechanism in B[a]P-initiated micronucleus formation (Kim and Wells, 1995). Interestingly, although SOD was protective against micronucleus formation initiated by B[a]P, the level of micronuclei in *j/j* UGT-deficient cells was not reduced down to DMSO controls, indicating significantly increased B[a]P bioactivation and ROS production, above the protection afforded by exogenous SOD (**fig. 4**). Conversely, the lack of total SOD protection may indicate that severe UGT deficiencies may enhance micronucleus formation by allowing for greater peroxidase- and/or CYP1A1-catalyzed B[a]P bioactivation and formation of the electrophilic reactive intermediate, B[a]P-7,8-diol-9,10-epoxide, which can covalently damage DNA. However, the above evidence and the study by Kim and Wells (1995) suggests that DNA oxidation initiated by B[a]P is the predominant mechanism in B[a]P-initiated micronucleus formation in *+/+* UGT-normal and *+/j* UGT-deficient cultured rat skin fibroblasts, and at least a major contributor in *j/j* UGT-deficient cells.

Evidence to date has linked various P450s (CYP1A1, CYP1A2, CYP2A6, CYP2B1, CYP2B7, CYP2D6, CYP2E1) in the bioactivation of NNK and its major metabolite NNAL to reactive intermediates that can oxidize, methylate and pyridyloxobutylate DNA (Crespi et al., 1991; Guo et al., 1992; Smith et al., 1992; Lacroix et al., 1993; Nesnow et al., 1994; Bilodeau et al., 1995). A P450-catalysed bioactivation pathway is supported by the reduction in NNK-initiated micronucleus formation by ABT, a known P450 inhibitor (Ortiz de Montellano and Mathews, 1981; Mugford et al., 1992) (**fig. 5**).

Interestingly, when cells were preincubated with TCDD, there was a significant reduction in NNK-initiated micronucleus formation in *+/+* UGT-normal cells, with no effect in *+/j* or *j/j* UGT-deficient cells (**fig. 5**). TCDD preincubation may have induced UGT isozymes responsible for NNK elimination, thereby avoiding NNK bioactivation. This is supported by the fact that NNK-initiated micronucleus formation in *+/j* and *j/j* UGT-deficient cells, which respectively are less and non-UGT-inducible compared to *+/+* UGT-normal cells, was not reduced by TCDD preincubation (**fig. 5**). Alternative mechanisms, such as TCDD induction of CYP1A1, PHS (DeBoni et al., 1996) or other enzymes that might produce less toxic NNK metabolites, are possible, although such effects also would be expected in UGT-deficient cells.

Since many xenobiotics such as B[a]P (Marnett et al., 1977; Gelboin, 1980; Eling et al., 1990) and the teratogen phenytoin (Winn and Wells, 1995b) are bioactivated by both P450s and peroxidases, we investigated the role of peroxidase-catalysed NNK bioactivation. ETYA, a dual inhibitor of both PHS and LPOs, significantly reduced NNK-initiated micronucleus formation, suggesting peroxidase-mediated NNK bioactivation. However, this bioactivation pathway remains questionable, since the peroxidase inducers IL-1 α or TPA failed to enhance micronucleus formation (**fig. 5**). In fact others have found that NNK-initiated DNA oxidation was not reduced by various non-steroidal anti-inflammatory drugs (Bilodeau et al., 1995), which are known to inhibit peroxidases such as PHS.

Similar to B[a]P, exogenous SOD significantly reduced NNK-initiated micronucleus formation, indicating the involvement of NNK-mediated ROS production (**fig. 5**). SOD or catalase also have been shown to inhibit NNK-initiated DNA strand breaks in MRC-5 fetal human lung fibroblasts (Weitberg and Corvese, 1993), which suggests that, in our study, DNA damage may be a direct mechanism in NNK-initiated micronucleus formation. However, unlike with B[a]P, SOD in all three UGT phenotypes was only marginally protective against micronucleus formation initiated by NNK. As discussed above, the inability of exogenous SOD to totally protect against micronucleus formation may be due to excessive ROS production initiated by NNK which may result in the saturation of exogenous SOD. Conversely, unlike with B[a]P, micronucleus formation initiated by NNK in +/+ UGT-normal, +/j and j/j UGT-deficient cells may involve not only ROS-mediated DNA damage, but also damage in the form of both methylation and pyridyloxobutylation.

In summary, these results demonstrate that UGT-catalyzed NNK elimination in cultured rat skin fibroblasts may be an important cytoprotective determinant in NNK-initiated micronucleus formation, and suggest that the involvement of ROS-mediated damage to essential macromolecules such as DNA may constitute a potential molecular mechanism. These results also show that, similar to NNK carcinogenicity, NNK effectiveness in initiating micronucleus formation at least in cells cultured from +/+ UGT-normal rats is greater than that of B[a]P, and that UGTs also may be critical determinants in peroxidase-dependent, B[a]P-initiated ROS production

and micronucleus formation. Further research on the toxicological relevance of even heterozygous human hereditary UGT-deficiencies is warranted.

2.3 STUDY #3: UDP-GLUCURONOSYLTRANSFERASE-MEDIATED PROTECTION AGAINST *IN VITRO* DNA OXIDATION AND MICRONUCLEUS FORMATION INITIATED BY PHENYTOIN AND ITS EMBRYOTOXIC METABOLITE 5-(p-HYDROXYPHENYL)-5-PHENYLHYDANTOIN (HPPH)^{1,2}

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2. Published in Journal of Pharmacology and Experimental Therapeutics 280: 200-209, 1997.
3. All studies were conducted by Perry Kim except for the following experiments. Embryo culture studies were conducted by Louise M. Winn. Toufan Parman was responsible for the original mass spectrometry work that helped identify the phenytoin and HPPH glucuronide in rat urine. For this study, Toufan was instrumental in analyzing the mass spectrometry data.

2.3.1 ABSTRACT

UDP-glucuronosyltransferases (UGTs) are important in the elimination of most xenobiotics, including 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH), the reputedly nontoxic, major metabolite of the anticonvulsant drug phenytoin. However, HPPH alternatively may be bioactivated by peroxidases such as prostaglandin H synthase (PHS) to a reactive intermediate that initiates DNA oxidation (reflected by 8-hydroxy-2'-deoxyguanosine [8-OH-2'-dG]), genotoxicity (reflected by micronuclei) and embryopathy. This hypothesis was evaluated in skin fibroblasts cultured from heterozygous (+/j) and homozygous (j/j) UGT-deficient Gunn rats, and in mouse embryo culture, with confirmation of direct N3-glucuronidation of phenytoin in Gunn rats *in vivo*. HPPH 80 μ M increased micronuclei by 2.0-, 4.8- and 4.6-fold respectively in +/+ UGT-normal cells ($p=0.03$), and in +/j and j/j UGT-deficient cells ($p=0.0001$). HPPH-initiated micronucleus formation was increased 3.0-fold and 3.4-fold respectively in +/j ($p=0.02$) and j/j ($p=0.04$) UGT-deficient vs +/+ UGT-normal cells. Micronuclei were not initiated by 10 μ M HPPH in +/+ UGT-normal cells, but were increased respectively by 4-fold and 3.8-fold in +/j and j/j UGT-deficient cells ($p=0.0001$), and were respectively increased 2.7-fold and 3.0-fold in +/j ($p=0.007$) and j/j ($p=0.0002$) UGT-deficient vs +/+ UGT-normal cells. 8-OH-2'-dG was increased in j/j UGT-deficient, but not +/+ UGT-normal, cells treated with 80 μ M HPPH ($p<0.05$). The embryotoxicity of 80 μ M HPPH in embryo culture, reflected by decreases in anterior neuropore closure, turning, yolk sac diameter and crown-rump length ($p<0.05$), was equivalent to that reported for phenytoin. Phenytoin 80 μ M enhanced micronucleus formation 1.7-fold, 4.4-fold and 3.8-fold respectively in +/+ cells ($p=0.03$), and +/j and j/j UGT-deficient cells ($p=0.0001$). Phenytoin-initiated micronucleus formation was increased about 4-fold in both the +/j ($p=0.006$) and j/j ($p=0.009$) UGT-deficient vs +/+ UGT-normal cells, providing the first evidence that the bioactivation and oxidative toxicity of phenytoin itself may be avoided by direct N-glucuronidation, which was confirmed by tandem mass spectrometry. These results further indicate that, with UGT deficiencies, HPPH potentially is a potent mediator of phenytoin-initiated genotoxicity and embryopathy, which may be relevant to teratogenesis and other adverse effects of phenytoin.

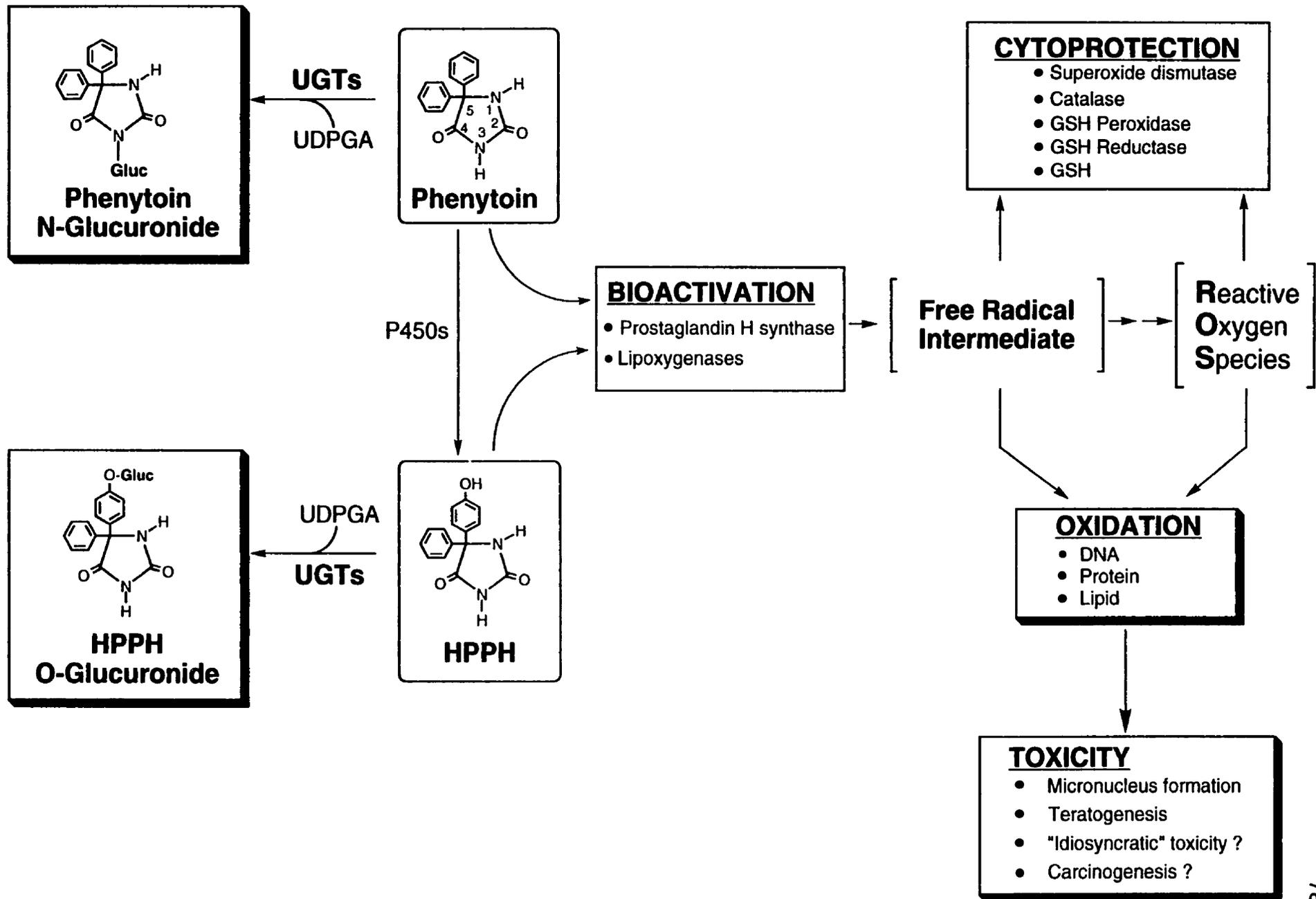
2.3.2 INTRODUCTION

The glucuronidation and elimination of endogenous compounds (e.g. bilirubin) and xenobiotics, including 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH), the major para-hydroxylated metabolite of the anticonvulsant drug phenytoin (diphenylhydantoin) (Butler, 1957), is catalysed by a superfamily of membrane-bound isozymes known collectively as UDP-glucuronosyltransferases (UGTs) (Dutton, 1980). UGTs catalyze the conjugation of xenobiotics to UDP-glucuronic acid (UDPGA), allowing the conjugated product to be excreted in the urine and feces. The teratogenicity of phenytoin and related xenobiotics in animals and humans is thought to be due to their bioactivation to embryotoxic reactive intermediates (reviewed in: Hansen, 1991; Juchau et al., 1992; Winn and Wells, 1995b; Wells and Winn, 1996). UGT-catalysed glucuronidation and elimination may prevent competing bioactivation of such xenobiotics to toxic reactive intermediates that can initiate a spectrum of toxicological sequelae (**fig. 1**). In animals and humans, UGTs have been shown to be important cytoprotective modulators in: (1) benzo[a]pyrene (B[a]P)-initiated micronucleus formation (Vienneau et al., 1995), embryotoxicity (Wells et al., 1989a), molecular damage and cytotoxicity (Hu and Wells, 1992, 1993, 1994); (2) micronucleus formation initiated by the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Kim and Wells, 1996a); and, (3) *in vivo* bioactivation (de Morais et al., 1992a,b), hepatotoxicity and nephrotoxicity (de Morais et al., 1992a) of the analgesic drug acetaminophen.

UGTs exist as two families, UGT1 and UGT2, which are located on separate chromosomes (Moghrabi et al., 1992; Monaghan et al., 1992) and are regulated by distinctly different mechanisms. UGT1 isozymes are produced by alternative splicing of the UGT1 gene complex (Brierly and Burchell, 1993). The UGT1 gene complex exists as multiple isozyme-specific exons located at the 5'-variable/specific region, which are spliced with a group of four exons at the 3'-constant region, the latter being common to all UGT1 isozymes. Conversely, UGT2 isozymes are produced from separate and complete genes located on various chromosomes.

Figure 1. Postulated genoprotective and cytoprotective roles of UDP-glucuronosyltransferases (UGTs) in peroxidase-catalyzed phenytoin and 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) bioactivation and toxicity. Hereditary UGT-deficiencies may allow greater bioactivation to free radical reactive intermediates that can initiate the formation of reactive oxygen species (ROS). These reactive intermediates and ROS can irreversibly damage DNA, proteins and lipids via covalent binding and oxidation, potentially initiating teratogenesis and other toxicities. Similarly, UGTs also may protect against cytochrome P450-catalyzed bioactivation of phenytoin to an electrophilic, arene oxide reactive intermediate (not shown), which particularly postnatally may result in hepatotoxicity and other toxicities (Winn and Wells, 1995b; Wells and Winn, 1996). The contribution of molecular target oxidation to idiosyncratic drug reactions and reversible lymphoma initiated by phenytoin is speculative. Abbreviations: Gluc, glucuronide conjugate.

FIGURE 1



Gilbert's syndrome, a moderate hereditary bilirubin-UGT deficiency, is estimated to occur in 6% of the population (Odell and Childs, 1980). The Crigler Najjar syndromes (type I and II), which are more severe forms of bilirubin-UGT deficiency, have been suggested to occur in 0.1% of the population in a heterozygous form (Bosma et al., 1995). UGT deficiencies in both humans (Bosma et al., 1992; Moghrabi et al., 1993a,b) and rats (Iyanagi, 1991) are due to various mutations in either the variable or constant exon regions. People who have deficient UGTs for catalyzing the glucuronidation and elimination of bilirubin (i.e. UGT1A1 or UGT1*1) phenotypically express abnormally elevated bilirubin blood concentrations, and thus appear jaundiced (Moghrabi et al., 1993a,b). Heterozygous and homozygous mutations in other specific UGT exons, such as *UGT1*4 (UGT1A4)*, have been reported with respective frequencies of 16% and 6% (Burchell et al., 1994b).

Hereditary UGT deficiencies in rats and humans have been shown to decrease the glucuronidation of the analgesic drug acetaminophen and the environmental carcinogen/teratogen B[a]P, resulting in enhanced bioactivation, molecular target damage and various toxicities. With acetaminophen, enhanced bioactivation was evident in UGT-deficient humans (de Morais et al., 1992b), and enhanced hepatotoxicity and nephrotoxicity in several strains of UGT-deficient rats (de Morais et al., 1992a). In human lymphocytes, decreased UGT activity for B[a]P metabolites correlated with enhanced cytotoxicity (Hu and Wells, 1993), while *in vivo* and *in vitro* studies with UGT-deficient rats showed reduced glucuronidation of B[a]P metabolites, resulting in enhanced B[a]P bioactivation, molecular target damage and, in pregnant animals, embryotoxicity (Wells et al., 1989a; Hu and Wells, 1992, 1994).

Recent *in vitro* studies have shown that B[a]P- and NNK-initiated micronucleus formation, a form of genotoxicity thought to reflect carcinogenic initiation, was higher in cells cultured from UGT-deficient RHA or Gunn rats compared to UGT-normal congenic controls (Vienneau et al., 1995; Kim and Wells, 1996a). A similar study in cultured Wistar rat skin fibroblasts, using inducers and inhibitors of both cytochromes P450 (P450) and peroxidases, and exogenous addition of superoxide dismutase, suggested that reactive oxygen species (ROS)-mediated DNA oxidation produced by peroxidase- and/or P450-catalysed B[a]P bioactivation was a potential

molecular mechanism in micronucleus formation (Kim and Wells, 1994, 1995, 1996a), which is thought to reflect the potential for the initiation of cancer, and may similarly reflect teratological initiation.

Similar to B[a]P, a number of studies suggest that both phenytoin and HPPH are bioactivated by peroxidases such as prostaglandin H synthase (PHS) to free radical intermediates, the former of which can oxidize lipids, proteins and DNA (Winn and Wells, 1995a; Parman et al., 1996) (**fig. 1**). Phenytoin also has been shown *in vivo* to initiate the production of hydroxyl radicals measured by salicylate hydroxylation (Kim and Wells, 1996b). Although HPPH was reported to be nonteratogenic in pregnant mice after *in vivo* administration (Harbison and Becker, 1974), this may have been due to maternal glucuronidation preventing HPPH from reaching the embryo. Since UGTs catalyze the glucuronidation and elimination of HPPH (Vore et al., 1979), we hypothesized that UGT deficiencies may increase susceptibility to various phenytoin toxicities via HPPH-initiated genotoxicity, reflected in this study by micronucleus formation. This mechanism might be relevant not only to the teratogenic effects of phenytoin (Winn and Wells, 1995a,b), but also to other potential consequences of phenytoin genotoxicity (**fig. 1**). For example, the mechanisms underlying the idiosyncratic drug reactions (fever, rash, etc.) and reversible lymphoma caused by phenytoin (Porter, 1989) have yet to be established.

In this study, we evaluated the potential for UGT-catalysed genoprotection against phenytoin and HPPH-initiated micronucleus formation in skin fibroblasts cultured from heterozygous (+/j) and homozygous (j/j) UGT-deficient Gunn rats versus UGT-normal controls (+/+). While *in vivo* metabolism for most pathways occurs primarily in the liver, this *in vitro* skin fibroblast system has proven useful in characterizing the genoprotective role of UGTs for other teratogens and carcinogens such as benzo[a]pyrene (Vienneau et al., 1995) and NNK (Kim and Wells, 1996a). The embryopathic potential of HPPH was determined directly in a mouse embryo culture model that has been well-characterized for phenytoin embryopathy (Winn and Wells, 1995a), and avoids the confounding effect of maternal glucuronidation. The increased genotoxicity of phenytoin in UGT-deficient cells provides the first evidence that direct N3-glucuronidation of phenytoin, confirmed in this study *in vivo* by tandem mass spectrometry,

appears to constitute an important and heretofore unrecognized cytoprotective reaction in addition to the O-glucuronidation anticipated for and observed with HPPH. The direct and potent embryopathic effects of HPPH in mouse embryo culture, and the enhanced genotoxicity of HPPH, as well as phenytoin, even in heterozygous UGT-deficient cells, suggests that human UGT deficiencies may be important determinants of susceptibility to the toxicity and teratogenicity initiated by phenytoin and related xenobiotics.

2.3.3 MATERIALS AND METHODS

Animals

Male HsdBlu:Gunn rats, 180-200 g (Harlan Sprague Dawley Inc., Indianapolis, Indiana), and age-matched Wistar rats, 200-250 g (Charles River Canada Ltd., St. Constant, Quebec), the UGT-normal parent strain of the Gunn rat, were housed in separate plastic cages. Virgin female CD-1 mice (Charles River Canada) were housed in plastic cages with ground corn cob bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY, USA). Three females were housed with one male breeder from 1700 to 0900 hr. The presence of a vaginal plug in a female mouse was considered as gestational day 1, and these females were separated from the colony and housed together in groups of five or fewer animals per cage.

All animals were kept in a temperature-controlled room with a 12-hr light-dark cycle automatically maintained. Food (Laboratory Rodent Chow 5001, PMI Feeds Inc.; St. Louis, MO) and tap water were provided *ad libitum*. Animals were acclimatized for a minimum of one week. All animal studies were approved by the University Animal Care Committee in accordance with the standards of the Canadian Council on Animal Care.

Chemicals

Phenytoin, HPPH, 4',6-diamidino-2-phenylindole (DAPI), ribonuclease A, ribonuclease T₁, and *Escherichia coli* alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO). 8-Hydroxy-2'-deoxyguanosine (8-OH-2'-dG) was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). All other reagents used were of analytical or HPLC grade.

Dulbecco's modified eagle medium (D-MEM), fetal bovine serum (FBS), lyophilized penicillin/streptomycin (pen/strep), Hanks' balanced salt solution (HBSS) (without calcium chloride, magnesium chloride and magnesium sulphate), Waymouth's MB 752/1, sodium bicarbonate solution, HEPES, L-glutathione and 0.25% trypsin were purchased from Gibco BRL (Toronto, Ontario)

Cell culture studies

The cell culture methods have been described in length elsewhere (Vienneau et al., 1995).

Cell culture method. Briefly, rats were sacrificed by CO₂ asphyxiation, bathed in 70% ethanol and two 4 x 4 cm pieces of skin were removed from the dorsal surface and placed in HBSS with 2% pen/strep. Skin was cultured immediately.

All following steps were conducted in a laminar flow hood. The skin was minced into 1 mm³ pieces, stored in 20 ml of HBSS (2% pen/strep) and transferred to sterile 100 mm polystyrene tissue culture dishes (Corning) and arranged to fit under a 18 mm² coverslips. Medium (D-MEM 500 ml with 75 ml FBS and 5 ml pen/strep) was added at the margin of the coverslip, allowing it to move across by capillary action and then a further 5 ml of medium was added to the dish. All dishes were then incubated at 37°C in a humidified incubator with 5% CO₂ in air and were left undisturbed for 10 days. The dishes were examined with an inverted phase contrast microscope to confirm the formation of a monolayer of fibroblasts at the margins of the skin pieces. Thereafter 5 ml of new medium was changed twice a week. After 2 months, the cultures were confluent, defined as a single layer of cells covering the bottom of the dish.

Subculture method. Briefly, medium was removed from the dishes and the cells were washed 3 times with fresh 5 ml HBSS. Cells were detached with 3 ml of 0.25% trypsin (Gibco) and incubated at 37°C for 4-6 min. The trypsin action was then stopped by adding 3 ml of FBS. The liquid was transferred to a sterile polyethylene test tube and centrifuged at 1000 x g at 4°C for 10 min. The supernatant was removed, cells were resuspended in 5 ml medium and transferred to a 150 cm² culture flask containing 20 ml of medium. Flasks were incubated for 1-2 weeks until cultures became confluent.

Preparation of fibroblast homogenates. Briefly, to detach cells, confluent cultures (6 to 8/treatment group) were incubated with 12 ml of trypsin for 4-6 min at 37°C and then stopped with 12 ml of FBS. The cells were pelleted by centrifugation as above, resuspended in 1 ml of phosphate buffered saline (PBS) and hand-homogenized using a glass 5 ml tissue grinder

(Mandel Scientific Ltd., Guelph, Ont.). Homogenates were separated into 100 μ l aliquots, frozen in liquid nitrogen, and stored at -80°C until DNA was isolated.

Micronucleus Formation.

The cells were incubated with either phenytoin 80 μM or HPPH at either 10 or 80 μM for 5 hr, at which point the cells were washed 3 times with 5 ml of HBSS. Fresh medium 5 ml was then added, and cells were allowed to undergo one complete mitotic cycle (26 hr) (Vienneau et al., 1995), at which point the medium was aspirated off and the cells were washed 3 times with 5 ml of HBSS to remove all residual medium. The 5 hr phenytoin or HPPH incubation was included as part of the mitotic cycle. To fix the cells, 5 ml of formalin solution (37% formaldehyde solution:PBS = 1:9 v/v) was added to the cells. After 30 min, the formalin solution was aspirated off and cells were washed 3 times with 5 ml of PBS. Control cells were treated with the HPPH and phenytoin vehicle dimethyl sulfoxide (DMSO).

Once fixed, the cells were stained with 5 ml of DAPI fluorescent stain 2 $\mu\text{g}/\text{ml}$ in water, and 2000 mononucleated cells were counted for the formation of micronuclei, using an inverted microscope with a 40X objective.

DNA oxidation

To determine the potential role of DNA oxidation as a molecular mechanism in HPPH-initiated micronucleus formation, +/+ or +/j fibroblasts were incubated with or without HPPH 80 μM , as described above in the micronucleus studies. The cells were harvested and homogenized after one mitotic cycle, stored at -80°C , prepared and analyzed as described below.

Fibroblast DNA Isolation. A modified method of Gupta (1984) was used to isolate DNA from rat skin fibroblasts. Briefly, fibroblast homogenates were incubated overnight with proteinase K at 55°C . Tris-HCl 1 mM at a volume of 25 μl was added and DNA extracted with one volume of chloroform:isoamyl alcohol:phenol (CIP, 24:1:25), and two successive extractions of one volume chloroform:isoamyl alcohol (CI, 24:1). At each stage mixtures were vortexed for 30 sec and microcentrifuged at 18,000 X g for 1 min (model E, Beckman) to separate extraction

phases. The DNA was then precipitated with 500 μ l of 100% ice cold (-20°C) ethanol and pelleted by microcentrifugation for 1 min. The DNA pellet was dissolved in 500 μ l phosphate buffer (pH 7.4), and incubated at 37°C on a rocker platform, with ribonuclease A (100 μ g/ml) and ribonuclease T₁ (50 units/ml) to digest residual RNA. One volume of Ci (24:1) was used to reextract the dissolved DNA, and the sample was microcentrifuged for 1 min. The DNA was reprecipitated as above. The pellet was redissolved in 500 μ l of 20 mM Na-acetate buffer (pH 4.8) and quantified using a UV/Vis spectrophotometer (Lamda 3, Perkin Elmer Canada Ltd.) at a wavelength of 260 nm with calf thymus DNA as the standard. The DNA was then digested to nucleotides by incubation with nuclease P₁ (67 μ g/ml) at 37°C for 30 min, followed by a 60 min incubation with *Escherichia coli* alkaline phosphatase (0.37 units/ml) at 37°C. The mixture of nucleosides was syringe tip-filtered (0.22 μ m) and analyzed via high-performance liquid chromatography (HPLC) coupled with electrochemical detection (Shigenaga and Ames, 1991).

DNA oxidation analysis. DNA oxidation (8-OH-2'-dG) was quantified using an isocratic HPLC (Scientific Systems, Inc., USA) equipped with an electrochemical detector (Model 5100A, Coulochem, ESA, CA, USA), a reverse-phase C18 column (Jones Chromatography, Lakewood, CO, USA), and a recording integrator (Model CR501 Chromatopac, Shimadzu, Japan). Samples were eluted using a mobile phase consisting of 50 mM KH₂PO₄ buffer, pH 5.5, and 10% methanol at a flow rate of 0.8 ml/ min and an oxidation potential of +0.4 V.

Embryo culture

The embryo culture method has been described in length elsewhere (Winn and Wells, 1995a). Male rat serum (MRS) contains undefined nutrients and factors required by murine embryos for survival and growth and therefore it was used as the medium in which the embryos were cultured. Blood was obtained as described elsewhere (Winn and Wells, 1995a) from retired CD-1 male rat breeders (Charles River, Quebec). The blood was centrifuged for 5 min at 1000 x g at 4°C (Model TJ-6, Beckman Instruments, Toronto, Ontario) and kept on ice until blood was obtained from all animals. All blood samples were then centrifuged for 30 min at 1900 x g at 4°C

(Model J2-21M, Beckman Instruments). To evaporate residual protein-bound ether, pooled serum was heat-inactivated for 1 hr at 58°C and gassed (5% CO₂ in air, Cannox Canada, Toronto, Ontario) for 30 min . The heat-inactivated MRS was aliquoted and stored at -80°C.

On gestational day 9.5 pregnant murine dams were sacrificed by cervical dislocation and embryos were explanted according to the method of New (1978). Briefly, the uterus was removed from the dam and rinsed in warmed HBSS and the individual implantation sites were exposed using a No. 5 watchmaker's forceps (Dumont and Fils, Montignez, Switzerland). The decidua, trophoblast, parietal endoderm and the outermost membrane, Reichert's membrane, were then removed, leaving the amnion, visceral yolk sac and ectoplacental cone intact. Explanted embryos were kept at 37°C in a holding bottle which contained pre-gassed (5% CO₂ in air, Cannox Canada) "holding medium" (50 ml Waymouth's MB 752/1, 14 mM NaHCO₃, 2.5 mM HEPES, 1.0 mM L-glutamine and 17 ml MRS) until all embryos from all dams were explanted.

Embryos at a similar stage of development (4-6 somite pairs) were pooled and cultured in 25-cm² sterile cell culture flasks (Corning Glasswork Inc., Corning, NY, USA) which contained 10 ml of CO₂ saturated embryo culture medium [50 ml holding-medium, penicillin (50 units/ml) and streptomycin (50 mg/ml)]. Flasks were incubated at 37°C (Forma Scientific, Toronto, Ontario) on a platform rocker (Bellco Biotechnology, Vineland, NJ). Embryonic morphological and developmental parameters were observed after 24 hr using a dissecting microscope (Carl Zeiss, FRG) as described below.

Developmental parameters. Developmental parameters included dorsal-ventral flexure (turning), anterior neuropore closure and somite development. Since somite development can be correlated with discrete and distinct developmental events, and is directly related to the growth and development of the embryo, somite development in each embryo was assessed. Somite development of individual embryos was determined by subtracting the number of somites present at the termination of the culture from the somite count noted at the beginning of each culture. The final somite count was determined by counting from the location of the anterior limb bud (13th somite) in a cranial-to-caudal direction. This technique was employed because somites

cranial to the 13th somite begin to disperse in preparation for future morphological development, making accurate somite determination difficult.

Embryos were also examined for dorsal-ventral flexure, or turning. Gestational day 9.5 embryos are S-shaped with the hindbody lying in the same plane as the head. After 24 hr of culture (day 10.5), under normal conditions the embryo will turn assuming a C-shaped position (fetal position) with the tail lying on the right side of the head.

To assure proper development of the nervous system and cranial tissues, sufficient neural tube growth and neuropore closure are essential. The cranial end of the developing neural tube, from which the central nervous system develops, is called the anterior neuropore. Each embryo was examined for anterior neuropore closure since anterior neuropore closure can be a potentially important measure of embryotoxicity as indicated by the evidence that phenytoin can cause congenital central nervous system dysfunction in humans and animals (Winn and Wells, 1995b). Anterior neuropore closure occurs at the same time as the development of the 16th somite pair. Therefore, embryos that had reached the 16th somite stage or greater without anterior neuropore closure were classified as having an open anterior neuropore.

Morphological parameters. Morphological assessment included yolk sac diameter (mm) and crown-rump length (mm). The measurement of yolk sac diameter was made at the widest point perpendicular to the ectoplacental cone. Measurements were made at either 3.2X or 4.0X magnification with an eye piece reticle micrometer. The crown-rump length was defined to be the distance from the mesencephalon to the lumbar-sacral region in embryos that had turned and was not measured in embryos that had not turned.

Mass Spectrometry

Urine Sample Preparation. UGT-normal Wistar and +/j and j/j UGT-deficient Gunn rats were treated with a teratogenic dose of phenytoin (150 mg/ kg ip) and housed separately in metabolic cages (Nalgene, Sybron Corp., Rochester, NY) and urine was collected over a 4 hr period. The urine samples were diluted with 10 volumes of methanol (pre-cooled to -20°C) and were kept at -20°C for 20 min to precipitate all protein in the urine. The samples were then

centrifuged (Model TJ-6, Beckman Instruments, Toronto, Ontario) at 1000 x g for 20 min at 4°C. A 1 ml aliquot of the supernatant was passed through a 0.22 µm syringe tip filter (Millex-GS, Millipore Corp., Bedford, MA), reduced to 50 µl under a stream of nitrogen gas, and 20 µl was then injected to an HPLC in line with a tandem mass spectrometer (MS).

Sample Analysis. HPLC-MS (Perkin-Elmer Sciex, API III, Concord, Ontario) was employed in the ion spray mode. An isocratic pump equipped with a 15 cm-ODS IIC-18 column, particle size of 5 µm (Jones Chromatography, Lakewood, Colorado) was used with a mobile phase composition of 40% acetonitrile, 59% deionized water and 1% acetic acid at a flow rate of 1 ml/min. The collision activation spectra of the phenytoin and HPPH glucuronides were obtained using HPLC-MS/MS with argon as the target gas at an energy of 80 eV. The mean mass \pm SE was calculated from the multiply charged ions by the software Mass spec. (version 3.3).

Statistical analysis

Statistical significance of differences between treatment groups was determined by Student's t test or one-factor analysis of variance (ANOVA) as appropriate using a standard, computerized statistical program (Statview, Abacus Concepts, Inc.) The level of significance was $p < 0.05$.

2.3.4 RESULTS

Cell Culture Studies

Concentration- and UGT phenotype-dependent increases in HPPH-initiated micronucleus formation. HPPH-initiated micronucleus formation exhibited a concentration-dependent response in all cell phenotypes, although the UGT-deficient phenotypes were substantially more susceptible (**fig. 2**). In *+/+* UGT-normal cells, enhanced micronucleus formation required 80 μ M HPPH ($p=0.03$), while with both *+/j* and *j/j* UGT-deficient cells, near maximal micronucleus formation was initiated with only 10 μ M HPPH. The magnitude of micronucleus formation initiated by 10 μ M HPPH was equivalent in *+/j* and *j/j* UGT-deficient cells. These *+/j* and *j/j* UGT-deficient cells treated with 10 μ M HPPH showed respective 4.0-fold and 3.8-fold increases in micronucleus formation compared to respective DMSO-treated phenotypes ($p=0.0001$), and respective 2.7-fold and 3.0-fold enhancements compared to the increase observed in comparable HPPH-treated *+/+* UGT-normal cells ($p=0.007$, $p=0.0002$). Compared to respective DMSO-treated phenotypes, micronucleus formation initiated by 80 μ M HPPH was increased 2.0-fold, 4.8-fold and 4.6-fold respectively in *+/+* UGT-normal ($p=0.03$), *+/j* and *j/j* UGT-deficient cells ($p=0.0001$) (**fig. 2**). There also were respective 3.0-fold and 3.4-fold enhancements in micronucleus formation initiated by 80 μ M HPPH in *+/j* ($p=0.02$) and *j/j* ($p=0.04$) UGT-deficient compared to the respective increase in HPPH-treated *+/+* UGT-normal cells (**fig. 2**). In DMSO-treated cells, micronucleus formation was not different among the UGT phenotypes.

Comparative genotoxicity of phenytoin and HPPH. At equimolar concentrations (80 μ M), phenytoin and HPPH initiated similar increases in micronucleus formation (**fig. 3**). Compared to respective DMSO-treated controls, micronucleus formation initiated by 80 μ M phenytoin was increased 1.7-fold, 4.4-fold and 3.8-fold respectively in *+/+* UGT-normal ($p=0.03$), and *+/j* and *j/j* UGT-deficient ($p=0.0001$) cells. There was over a 3.9-fold increase in phenytoin-initiated micronucleus formation in both *+/j* ($p=0.006$) and *j/j* ($p=0.009$) UGT-deficient cells compared to the increase observed in phenytoin-treated *+/+* UGT-normal cells.

Figure 2. Effect of UDP-glucuronosyltransferase (UGT) deficiencies and concentration of 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) on micronucleus formation. Skin fibroblasts were cultured from either homozygous $+/+$ UGT-normal, or heterozygous $+/j$ or homozygous j/j UGT-deficient Gunn rats. Cells were incubated with HPPH, 10 or 80 μM for 5 hr, washed and cultured for the rest of one mitotic cycle (26 hr). Cells were fixed, stained and micronuclei counted. Symbols indicate the mean of 4 fibroblast cultures. Asterisks, plus and cross symbols respectively indicate a difference from DMSO controls, similarly treated $+/+$ cells, and 10 μM HPPH-treated $+/+$ cells ($p < 0.05$).

FIGURE 2

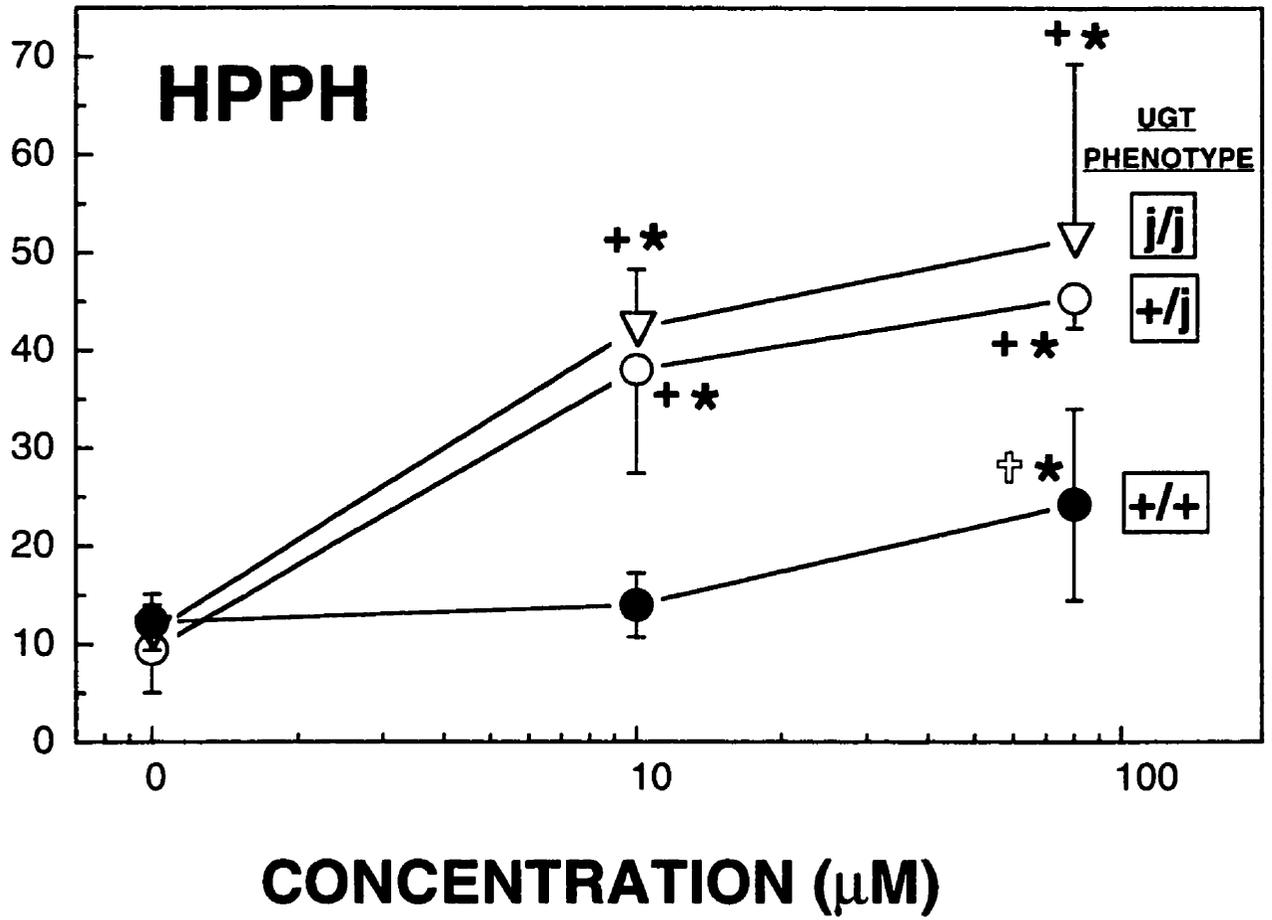
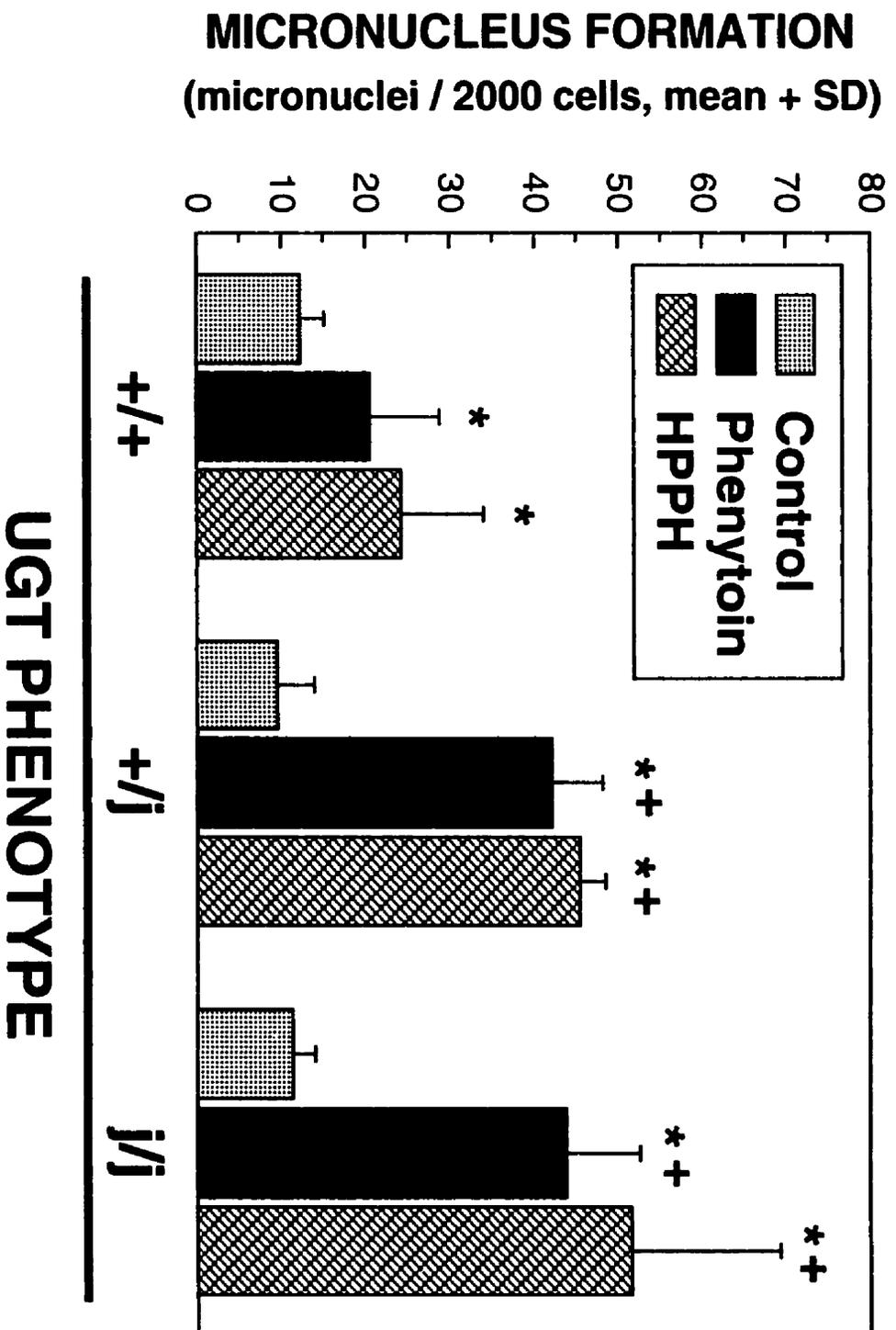
MICRONUCLEUS FORMATION**(micronuclei / 2000 cells, mean \pm SD)**

Figure 3. Comparison of the effectiveness of phenytoin and 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) in initiating micronucleus formation. Skin fibroblasts were cultured from either homozygous $+/+$ UGT-normal, or heterozygous $+/j$ or homozygous j/j UGT-deficient Gunn rats. Cells were incubated with $80\mu\text{M}$ phenytoin or HPPH for 5 hr, washed and cultured for the rest of one mitotic cycle (26 hr). Cells were fixed, stained and micronuclei counted. Bars indicate the mean of 4 fibroblast cultures. Asterisks indicate a difference from DMSO controls, and plus symbols a difference from respective $+/+$ UGT-normal cells ($p < 0.05$).

FIGURE 3



HPPH-initiated DNA oxidation. 8-OH-2'-dG was increased in *j/j* UGT-deficient cells treated with 80 μ M HPPH compared to both HPPH-treated and DMSO-treated *+/+* UGT-normal cells (**fig. 4**) ($p < 0.05$).

Embryo Culture Studies

Similar to results from cell culture/micronucleus studies, mouse embryos exposed for 24 hr to 10 μ M HPPH did not demonstrate embryotoxicity when compared to vehicle controls (**fig. 5**). However, upon incubation with 80 μ M HPPH, there was significant dysmorphogenesis, as evidenced by decreases in anterior neuropore closure (45%), turning (35%), yolk sac diameter (8%) and crown-rump length (9%) ($p < 0.05$) (**fig. 5**). These embryopathic effects of 80 μ M HPPH were equivalent to those reported with phenytoin at an identical concentration (Winn and Wells, 1995a), which also is within the therapeutic range of phenytoin in maternal plasma (Winn and Wells, 1995a). Interestingly, unlike phenytoin (Winn and Wells, 1995a), HPPH did not significantly reduce somite development.

HPLC-MS/MS

Analysis of urine samples from UGT-normal Wistar and *+/j* UGT-deficient Gunn rats by HPLC-MS/MS showed a parent ion with an m/z of 429 and a retention time of 1.87 min. This compound was designated as the N3-glucuronide of phenytoin (**fig. 1**) based upon a number of experimental observations. MS analysis of this parent ion resulted in the fragmentation pattern shown in **fig. 6**. An N3-glucuronide conjugate of phenytoin in bile extract from Wistar rats previously has been reported (Smith et al., 1977). In that study, ions that appeared with m/z values of 322 and 378 were said to arise from retro Diels-Alder rearrangements of the glycone ring. Our studies are consistent with the previously reported fragmentation patterns of an N3-glucuronide of phenytoin, with the exception of an m/z value of 337 (378 in the study by Smith et al.). This discrepancy likely was due to a different method of ionization (ion sprayTM) used in our mass spectrometer. Importantly, the N3-glucuronide of phenytoin was not detected in the urine of *j/j* UGT-deficient Gunn rats.

Figure 4. Effect of UDP-glucuronosyltransferase (UGT) deficiency on DNA oxidation initiated by 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH). DNA oxidation was quantified by the formation of 8-hydroxy-2'-deoxyguanosine (8-OH-2'-dG). Skin fibroblasts were cultured from either homozygous *+/+* UGT-normal or homozygous *j/j* UGT-deficient Gunn rats. Cells were incubated with 80 μ M HPPH for 5 hr, washed, harvested and analyzed for 8-OH-2'-dG. Bars indicate the mean of 4 cultures. The asterisk indicates a difference from HPPH-treated *+/+* cells ($p < 0.05$).

FIGURE 4

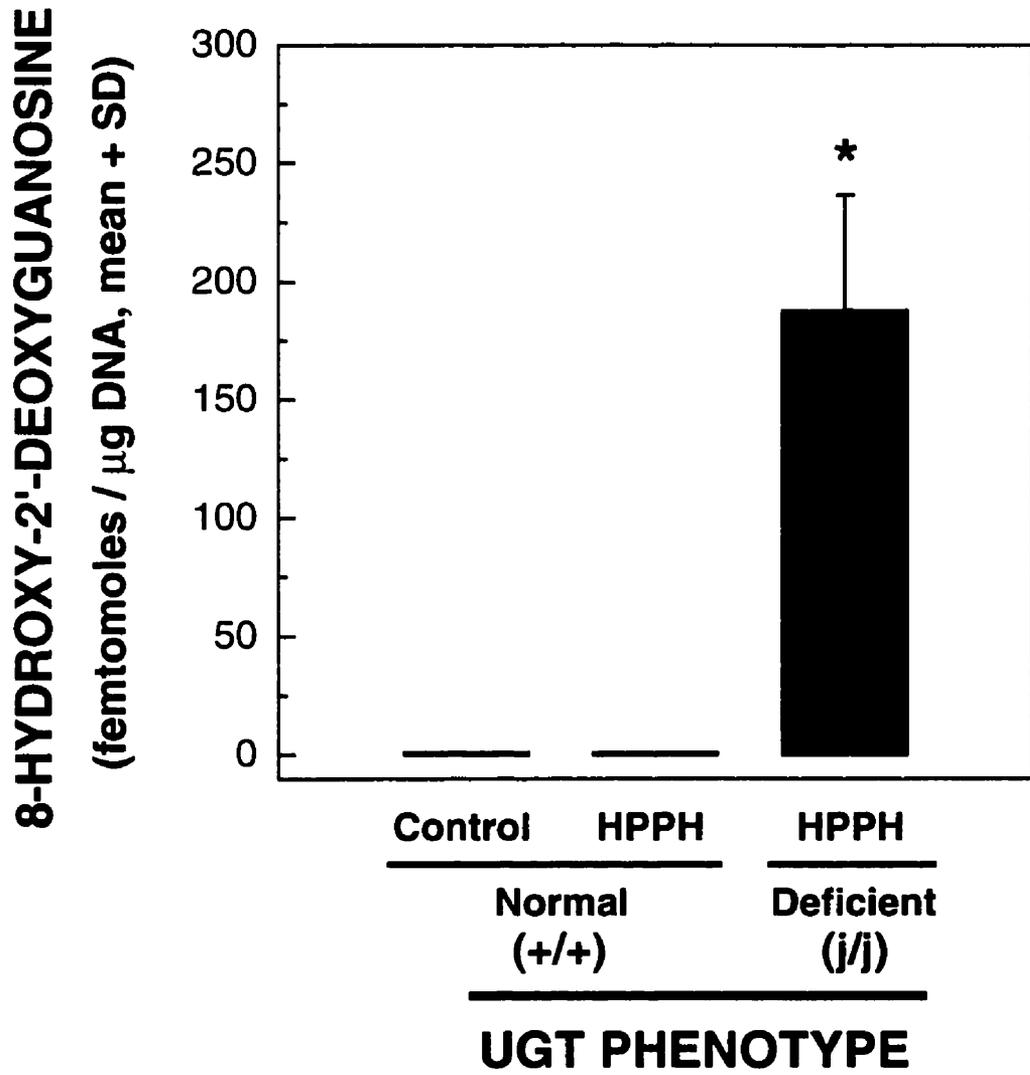


Figure 5. Embryotoxicity of 5-(p-hydroxyphenyl)-5-phenylhydantoin in CD-1 mouse embryo culture. Embryos were cultured on gestational day 9.5 in the presence of either HPPH (2.5 µg/ml, 10 µM or 20 µg/ml, 80 µM) or the vehicle (0.002 N NaOH) for 24 hr. The concentration of 80 µM is equimolar both to that for phenytoin known to be embryopathic in mouse embryo culture (Winn and Wells, 1995a), and to the therapeutic concentration of phenytoin to maternal plasma. The number of embryos is given in parentheses. Asterisks indicate a difference from vehicle controls ($p < 0.05$).

FIGURE 5

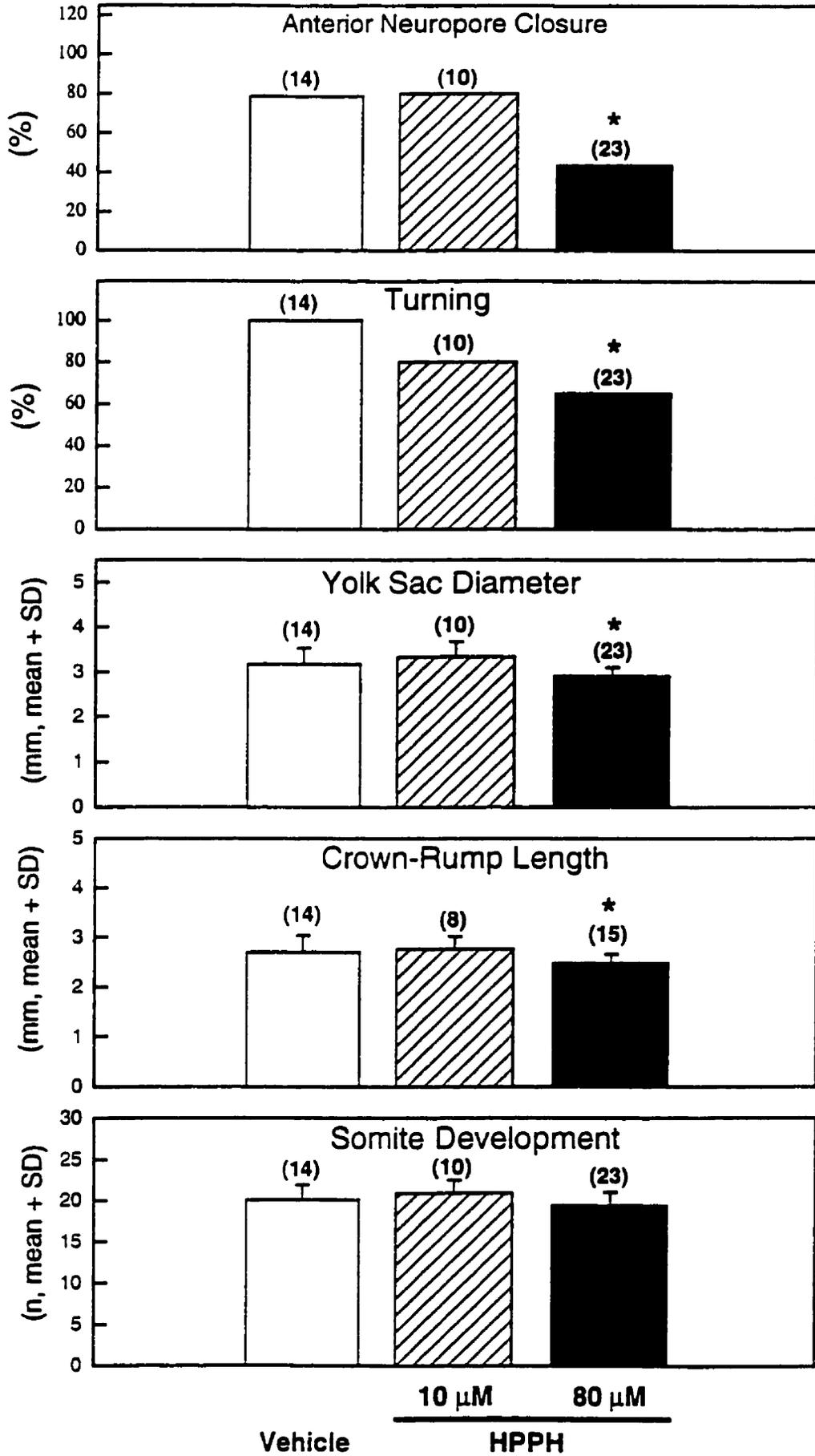
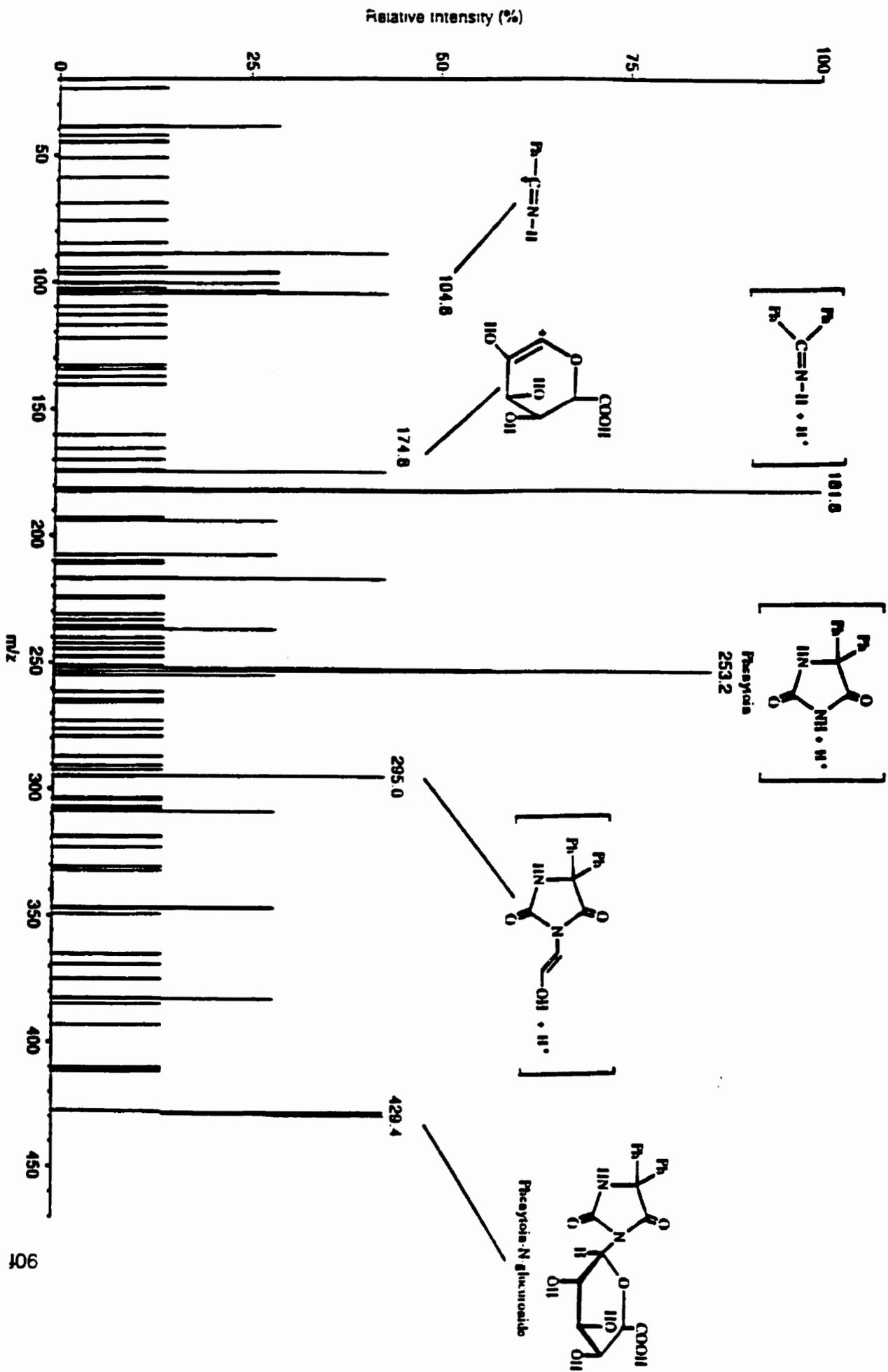


Figure 6. Collision activation spectra of the N3-glucuronide of phenytoin observed in the urine of Wistar and heterozygous +/- Gunn rats, including the structures assigned to each fragment. Abbreviations: m/z, mass/charge; Ph, phenyl.

Figure 6



In the urine of Wistar rats, as expected, a parent ion with a retention time of 1.42 min and an m/z value of 445 was evident, corresponding to the O-glucuronide of HPPH, the major para-hydroxylated metabolite of phenytoin (**fig. 1**). Importantly, this parent ion reflecting O-glucuronidation of HPPH was not observed in the urine of either +/j or j/j UGT-deficient Gunn rats.

2.3.5 DISCUSSION

Phenytoin is therapeutically and toxicologically important due to its antiepileptic efficacy and teratogenic potential, respectively. Although phenytoin is teratogenic in many animal species, including humans (Winn and Wells, 1995b), the danger to both the mother and fetus from uncontrolled seizures is considered to be greater than the possible teratogenic effects of phenytoin, and therapy generally is continued throughout pregnancy. UGTs are known to catalyze the glucuronidation and elimination of both phenytoin (Smith et al., 1977) and its major, para-hydroxylated metabolite, HPPH. This study demonstrated that both phenytoin and HPPH were equally effective initiators of micronucleus formation in rat skin fibroblasts. This genotoxic outcome may reflect teratologic initiation, as has been postulated for carcinogenic initiation, since HPPH also proved in mouse embryo culture to be equipotent to phenytoin in its embryotoxicity. Furthermore, UGT deficiencies resulted in decreased glucuronidation of phenytoin and HPPH, with a resultant enhancement in phenytoin- and HPPH-initiated DNA oxidation and micronucleus formation, suggesting that hereditary UGT-deficiencies may play an important role in teratological susceptibility.

There have been several hypotheses proposed for the mechanism of phenytoin-initiated teratogenesis (Hansen, 1991; Juchau et al., 1992; Winn and Wells, 1995b), including PHS- and/or lipoxygenase (LPO)-catalyzed bioactivation to a reactive intermediate (Winn and Wells, 1995b). PHS and LPOs produce prostaglandins, leukotrienes and related eicosanoids from polyunsaturated fatty acids such as arachidonic acid. In this synthetic pathway, xenobiotics such as phenytoin (Smith et al., 1991; Winn and Wells, 1995b) and HPPH (Nicol and Wells, unpublished results) can donate an electron, and thus be oxidized to a free radical intermediate (Winn and Wells, 1995b; Parman et al., 1996) (**fig. 1**).

This study in rat skin fibroblasts showed that HPPH can initiate DNA oxidation and genotoxicity, the latter reflected by enhanced micronucleus formation, and that these effects were enhanced over 3-fold in UGT-deficient cells. Thus, as has been postulated for phenytoin itself (Winn and Wells, 1995a,b), HPPH may contribute to the teratogenicity of phenytoin via the same

mechanism of peroxidase-catalyzed bioactivation and ROS-mediated oxidative damage to DNA and other targets (**fig. 1**). Similar results were seen in an *in vitro* horseradish peroxidase-H₂O₂ system, where both phenytoin and HPPH initiated the formation of 8-OH-2'-dG (Winn and Wells, 1995a). The results of the present study suggest that phenytoin-initiated DNA oxidation (Winn and Wells, 1995a) and hydroxyl radical formation (Kim and Wells, 1996b) in part may be mediated by HPPH bioactivation to a reactive free radical intermediate, which, similar to benzo[a]pyrene (Kim and Wells, 1995, 1996a), may constitute a molecular mechanism for both phenytoin- and HPPH-initiated micronucleus formation and, potentially, teratogenesis.

The studies in mouse embryo culture constitute the first direct evidence for an embryotoxic effect of HPPH, contrary to results from previous *in vivo* studies discussed below. HPPH was equipotent to phenytoin (Winn and Wells, 1995a) in this regard, initiating embryopathic effects at a concentration of 80 μM, which is within the therapeutic concentration range for phenytoin in maternal plasma. The spectrum of embryopathic effects for HPPH also was almost identical to that for phenytoin in embryo culture (Winn and Wells, 1995a), including decreases in anterior neuropore closure, turning, yolk sac diameter and crown-rump length. The only exception was no decrease in somite development, which for phenytoin is small but usually statistically significant (Winn and Wells, 1995a). At a lower HPPH concentration (10 μM), more likely to be encountered *in vivo*, where up to 93% (Chow and Fischer, 1982) of HPPH can be glucuronidated, HPPH exhibited no significant embryotoxicity in our embryo culture model, although the possibility of other embryopathic effects, such as neurotoxicity, from *in vivo* exposure to such lower concentrations of HPPH cannot be excluded. An embryopathic contribution from HPPH would be more likely in UGT-deficient mothers, in whom decreased glucuronidation of HPPH would lead to higher embryonic exposure to this potentially embryotoxic metabolite, as discussed below (**fig. 1**).

The potential teratologic contribution of HPPH during phenytoin therapy is particularly remarkable because equivalent genotoxicity was observed with only 10 μM HPPH, which is about one-tenth of the maternal therapeutic concentration for phenytoin (80 μM). The lower concentration of HPPH (10 μM) was genotoxic only in UGT-deficient cells, and not in UGT-normal

cells. UGTs were substantially genoprotective, suggesting that the reported apparent lack of *in vivo* HPPH teratogenicity (Harbison and Becker, 1974) and genotoxicity (Barcellona et al., 1987) was due to maternal glucuronidation preventing HPPH from reaching the embryo. If so, then pregnant women with certain hereditary UGT deficiencies may be at increased risk from the teratogenicity of phenytoin and related xenobiotics that are eliminated substantially via glucuronidation. Evidence from pregnant UGT-deficient Gunn rats, which show enhanced susceptibility to benzo[a]pyrene embryotoxicity (Wells et al., 1989a), suggests that UGT deficiencies are teratologically relevant. Human studies with acetaminophen *in vivo* (de Morais et al., 1992b) and with benzo[a]pyrene in an *in vitro* human lymphocyte model (Hu and Wells, 1993) indicate that human UGT deficiencies are relatively common, and result in decreased xenobiotic glucuronidation with enhanced bioactivation and cytotoxicity.

While this study presents the first evidence for HPPH-initiated DNA oxidation, micronucleus formation and embryotoxicity, phenytoin itself has been shown to initiate DNA oxidation in embryo culture (Winn and Wells, 1995a) during a gestational time when embryos have little or no demonstrable P450 for forming HPPH *in situ*. This study does present the first evidence for phenytoin-initiated micronucleus formation, which is consistent with its ability to irreversibly damage DNA via both oxidation and arylation (Winn and Wells, 1995a,b). The teratological relevance of DNA damage by phenytoin and HPPH is further supported by the enhanced teratogenicity of phenytoin in p53-deficient mice, which have compromised DNA repair (Laposa et al., 1996). A similarly enhanced teratological susceptibility of p53-deficient mice was observed for benzo[a]pyrene, another DNA-damaging teratogen and carcinogen (Nicol et al., 1995). An equivalent enhancement in micronucleus formation and embryotoxicity initiated by phenytoin and HPPH was not surprising, and a similar equivalence was reported using a rat embryo limb culture assay (Brown et al., 1986). However, the enhanced genotoxicity of phenytoin itself in UGT-deficient cells was unexpected, since we were not aware that phenytoin could be directly glucuronidated. While phenytoin potentially could be hydroxylated by P450s to HPPH, for which UGTs are expected to be protective, P450 activities in rat skin fibroblasts are negligible (Vienneau et al., 1995; Kim and Wells, 1995), and this is an unlikely explanation for

UGT protection against the observed *in vitro* genotoxicity of phenytoin. Also, phenytoin itself has been shown to oxidize DNA in embryo culture (Winn and Wells, 1995a), as discussed above. Thus, the only apparent mechanism for UGT-dependent protection against the genotoxicity of phenytoin itself is via direct glucuronidation of phenytoin. This hypothesis was evaluated by HPLC-MS/MS analysis of the urine from UGT-normal Wistar and UGT-deficient Gunn rats treated with a teratogenic dose of phenytoin. An N3-glucuronide conjugate of phenytoin was identified in UGT-normal rats, which we subsequently discovered had been reported previously in Wistar rats by Smith et al. (1977). More importantly, we found that the N3-glucuronide of phenytoin was not detected in *+/+* UGT-deficient Gunn rats, and the O-glucuronide of HPPH was not detected in either *+/+* or *+/+* UGT-deficient Gunn rats. These results provide the first evidence that UGT deficiencies lead to reduced *in vivo* glucuronidation of both phenytoin and its HPPH metabolite. Assuming a similar process in fibroblasts, as has been shown for benzo[a]pyrene (Vienneau et al., 1995), these results suggest that decreased glucuronidation resulted in enhanced DNA oxidation and genotoxicity initiated by both phenytoin and HPPH in UGT-deficient fibroblasts.

For both phenytoin and HPPH, maximal genotoxic susceptibility was observed in *+/+* UGT-deficient cells, with no further enhancement in *+/+* UGT-deficient cells. This suggests that these concentrations in UGT-deficient cells constitute the plateau of the concentration-response curve. Similar results were seen both *in vitro* (Vienneau et al., 1995; Kim and Wells, 1996a) and *in vivo* (de Moraes et al., 1992a; Hu and Wells, 1992, 1994), where *+/+* UGT-deficiencies increased acetaminophen bioactivation and toxicity, as well as benzo[a]pyrene and NNK-initiated micronucleus formation. These results suggest that hereditary UGT deficiencies may have considerable clinical relevance, since, unlike homozygous deficiencies, heterozygous deficiencies are relatively common.

In bacterial studies, mutagenicity initiated by both phenytoin and HPPH was shown to be dependent upon P450-catalyzed enzymatic bioactivation, requiring preincubation with a metabolic activating system (S9 liver fraction) (Sezzano et al., 1982). Phenytoin was mildly mutagenic in the TA 1538 strain of *Salmonella typhimurium* at 25 (38 μ M) and 250 μ g (381

μM)/2.6 ml (plate) upon preincubation with S9 from rats induced respectively with the P450 inducers 3-methylcholanthrene and Aroclor 1254. HPPH was more mutagenic than phenytoin after preincubation with similar S9 fractions, including S9 from rats induced with β -naphthoflavone at HPPH concentrations ranging from 25 (36 μM) to 250 (358 μM) $\mu\text{g}/2.6$ ml/plate. However, a conflicting study conducted under similar conditions found that phenytoin (25-1000 $\mu\text{g}/2.6$ ml/plate) (38-1524 μM) and HPPH (25-500 $\mu\text{g}/2.6$ ml/plate) (36-717 μM) were not mutagenic in all strains (TA97, TA98, TA100, TA1530, TA1537, TA1538) tested (Leonard et al., 1984). Similar contradictory results were reported for *in vivo* sister chromatid exchange in phenytoin-treated patients. Hadebank et al. (1982) found a significant increase in sister chromatid exchange in phenytoin monotherapy patients, while Hunke and Carpenter (1978) did not see a difference in patients with phenytoin serum concentrations ranging from 3.8 $\mu\text{g}/\text{ml}$ (15 μM) to 29.5 $\mu\text{g}/\text{ml}$ (117 μM). However, *in vitro* studies by Hunke and Carpenter (1978) found phenytoin concentrations ranging from 10 $\mu\text{g}/\text{ml}$ (40 μM) to 100 $\mu\text{g}/\text{ml}$ (396 μM) significantly increased sister chromatid exchange, suggesting phenytoin and/or its metabolite HPPH are mutagenic and genotoxic.

In our study, both phenytoin and HPPH at the equivalent of a human therapeutic concentration for phenytoin (80 μM) were equipotent in initiating micronuclei in skin fibroblasts cultured from $+/+$ UGT-normal Gunn rats versus DMSO-treated controls. *In vivo*, phenytoin at doses of 0.5 and 1.0 mg/kg, but not 6-20 mg/kg, initiated micronuclei in mouse bone marrow polychromatic erythrocytes (Montes de Oca-Luna et al., 1984). A somewhat contradictory *in vivo* study found that 100 mg/kg phenytoin initiated micronucleus formation only in fetal (day 13), but not in maternal, polychromatic erythrocytes (Barcellona et al., 1987). Furthermore, a molar equivalent dose (106 mg/kg) of HPPH did not initiate micronuclei in either fetal or maternal erythrocytes (Barcellona et al., 1987), substantiating an earlier study showing that HPPH administered *in vivo* at the molar equivalent of a teratogenic dose of phenytoin did not initiate teratogenesis in mice (Harbison and Becker, 1974). In contrast, our study found not only that HPPH can initiate micronucleus formation in $+/+$ UGT-normal rat skin fibroblasts, but also that micronucleus formation was increased in $+/j$ and j/j UGT-deficient fibroblasts treated with either 80 μM phenytoin or HPPH (**figs. 2 and 3**). During phenytoin therapy, approximately 60% of HPPH

normally would be glucuronidated (Browne and Chang, 1989), and in the mouse over 90% is glucuronidated (Chow and Fischer, 1982); however, even 10 μ M HPPH was as genotoxic as the 80 μ M concentration in UGT-deficient cells, and thus may contribute to genotoxicity, particularly in UGT-deficient people.

In summary, these results suggest that DNA oxidation may constitute a molecular mechanism for the initiation of micronuclei by both HPPH and phenytoin, as has been postulated for phenytoin teratogenicity (Winn and Wells, 1995a,b), and also may constitute a mechanism for HPPH embryotoxicity and other adverse effects. The results in mouse embryo culture provide the first direct evidence for HPPH-initiated embryotoxicity, the effectiveness of which was equivalent to that previously reported for phenytoin (Winn and Wells, 1995a). UGTs provided important protection against both phenytoin- and HPPH-initiated *in vitro* genotoxicity, and related *in vivo* studies with benzo[a]pyrene (Wells et al., 1989a) suggest that these *in vitro* results have teratologic relevance. The genotoxicity of both phenytoin and HPPH was as high in heterozygous +/j as in homozygous j/j UGT-deficient cells, suggesting that hereditary UGT deficiencies may have considerable relevance to clinical toxicologic susceptibility. However, although UGT-deficient animal models can reflect some human UGT deficiencies and their toxicologic consequences, further studies will be necessary to confirm the relevance of these results to teratologic susceptibility, particularly in humans.

2.4 STUDY #4: PHENYTOIN-INITIATED HYDROXYL RADICAL FORMATION: CHARACTERIZATION BY ENHANCED SALICYLATE HYDROXYLATION^{1,2}

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2. Published in Molecular Pharmacology 49(1):172-181, 1996.

2.4.1 ABSTRACT

Bioactivation of phenytoin and related teratogens by peroxidases such as prostaglandin H synthase (PHS) may initiate hydroxyl radical ($\bullet\text{OH}$) formation that is teratogenic. Salicylate is hydroxylated by $\bullet\text{OH}$ at the 3rd and 5th carbon atoms forming 2,3- and 2,5-dihydroxybenzoic acids (DHBA). *In vivo* salicylate metabolism produces only the 2,5-isomer, so 2,3-DHBA formation may reflect $\bullet\text{OH}$ production. This study validated the salicylate assay using the known $\bullet\text{OH}$ generator paraquat, and evaluated $\bullet\text{OH}$ production by phenytoin. Female CD-1 mice were treated with paraquat, 30 mg/kg ip, given 30 min after acetylsalicylic acid (ASA), 200 mg/kg ip. Blood was collected at 5, 15, 30 min, and 1 and 2 hr after paraquat, and plasma was analyzed for DHBA isomers and glucuronide conjugates by high-performance liquid chromatography with electrochemical detection. Paraquat increased 2,3-DHBA formation 19.2 fold, with substantial interindividual variability in the time of maximal formation ($p=0.0001$). The 2,3-DHBA glucuronide conjugates *in vivo* and in hepatic microsomal studies amounted respectively to about 11% and 0.43% of total 2,3-DHBA equivalents. To investigate putative $\bullet\text{OH}$ production initiated via PHS-catalyzed phenytoin bioactivation, ASA was given 30 min before phenytoin, 65 or 100 mg/kg ip, resulting in respective 7.6-fold ($p=0.02$) and 14.2-fold ($p=0.003$) increases in phenytoin-initiated maximal 2,3-DHBA formation. Maximal 2,3-DHBA formation was 2.1-fold higher when ASA was administered after rather than before the same dose (65 mg/kg) of phenytoin ($p=0.03$), indicating ASA inhibition of PHS-catalyzed phenytoin bioactivation. Urinary analysis was much less sensitive, and the 2,5-isomer reflected enzymatic rather than $\bullet\text{OH}$ -mediated hydroxylation. The paraquat studies demonstrate the importance of timing in accurately quantifying 2,3-DHBA formation, and suggest that glucuronidation does not interfere. The substantial, dose-dependent initiation of 2,3-DHBA formation by phenytoin, and its inhibition by ASA, provide the first *in vivo* evidence that PHS-dependent $\bullet\text{OH}$ formation could contribute to the molecular mechanism of phenytoin teratogenesis.

2.4.2 INTRODUCTION

The treatment of epilepsy generally involves the use of anticonvulsant drugs such as phenytoin (diphenylhydantoin; Dilantin®). Although phenytoin is teratogenic in many animal species (Wells, 1993) and humans (Hanson and Smith, 1975), treatment usually is continued throughout pregnancy to avoid the dangers to both mother and fetus from uncontrolled seizures.

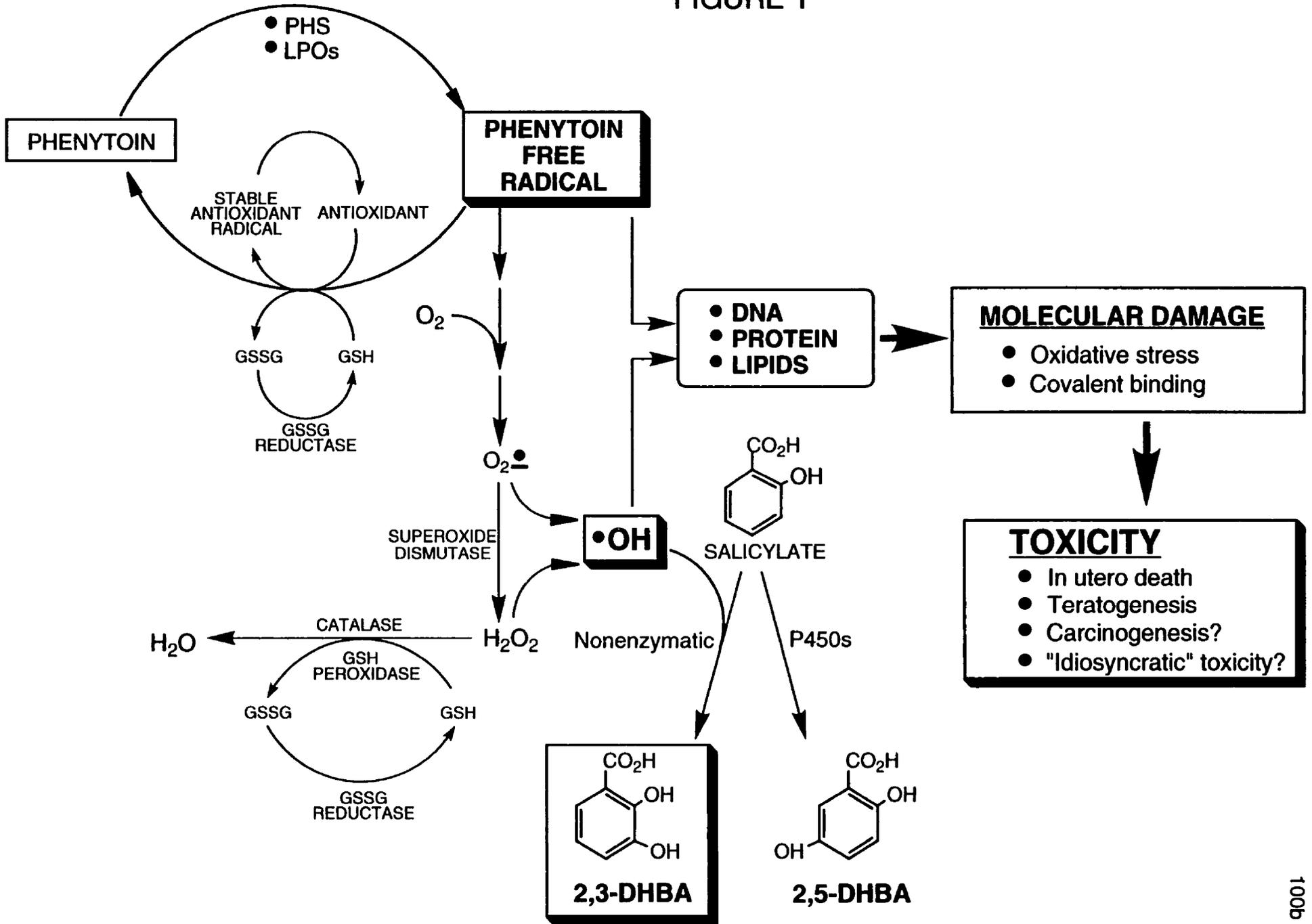
There are several postulated mechanisms by which phenytoin may initiate teratogenicity (Hansen, 1991). These include receptor-mediated effects of the parent phenytoin compound, reversibly binding to the glucocorticoid receptor or the bioactivation of phenytoin to an electrophilic arene oxide, catalyzed by the NADPH-dependent cytochromes P450 (P450). Although substantial evidence exists for a P450-catalyzed bioactivating pathway, discrepancies in this hypothesis may be explained by an alternative pathway involving peroxidase-catalyzed bioactivation of phenytoin (Wells, 1993).

Evidence from *in vivo* and *in vitro* studies suggests that peroxidases such as prostaglandin H synthase (PHS) and other enzymes such as lipoxygenases (LPOs) can bioactivate phenytoin and related xenobiotics to a potentially teratogenic, reactive free radical intermediate (Wells, 1993; Miranda et al., 1994; Yu and Wells, 1995; Parman et al., 1996) (**fig. 1**). The free radical, if not quickly detoxified by glutathione (GSH), can oxidize and/or covalently bind to essential macromolecules (DNA, proteins, lipids), and/or initiate the formation of reactive oxygen species (ROS), potentially initiating *in utero* death or teratogenesis. This mechanism could contribute to adverse reactions of phenytoin and related drugs in adults, including so-called idiosyncratic reactions (rash, fever, etc.) and reversible lymphoma, although this latter hypothesis has yet to be tested.

A potential molecular mechanism contributing to phenytoin-initiated teratogenesis may involve the production of ROS such as hydroxyl radicals ($\bullet\text{OH}$). Both *in vivo* (Liu and Wells, 1994, 1995a) and in embryo culture (Winn and Wells, 1995a), phenytoin can initiate oxidation of both maternal and embryonic DNA, protein and lipids. The antioxidative enzymes superoxide dismutase and catalase inhibit phenytoin-initiated DNA and protein oxidation, as well as

Figure 1. Phenytoin bioactivation and subsequent salicylate hydroxylation by hydroxyl free radicals ($\bullet\text{OH}$). Phenytoin may be bioactivated by peroxidases such as prostaglandin H synthase (PHS) and lipoxygenases (LPOs) to a putative phenytoin free radical. The phenytoin free radical may initiate the formation of reactive oxygen species such as superoxide anion ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and $\bullet\text{OH}$. Both the phenytoin free radical and ROS can initiate molecular damage to DNA, protein and lipids, potentially leading to teratogenesis. $\bullet\text{OH}$ also can hydroxylate salicylate forming both the 2,3- and 2,5-dihydroxybenzoic acids (DHBA), the former of which is unique to this reaction. Abbreviations: P450, cytochromes P450; GSH, glutathione; GSSG, oxidized glutathione.

FIGURE 1



teratogenicity in embryo culture (Winn and Wells, 1995a), corroborating the teratological relevance of phenytoin-initiated ROS production.

Aspirin (o-acetylsalicylic acid, ASA), with a half life of approximately 15 min, is hydrolyzed to salicylate (2-hydroxybenzoic acid) in plasma, liver, erythrocytes and synovial fluid (Flower et al., 1985). Salicylate is eliminated in the urine, either unchanged, conjugated with the endogenous amino acid glycine (aminoacetic acid) or with UDP-glucuronic acid (UDPGA), or is hydroxylated (oxidized) by P450s, primarily to 2,5-dihydroxybenzoic acid (2,5-DHBA), which may be conjugated with glycine, forming gentisuric acid (Levy et al., 1972; Grootveld and Halliwell, 1988). Depending on the dose administered, salicylate has a half-life of 2 to 30 hr (Flower et al., 1985). Dose-dependent elimination occurs due to saturation of the formation of salicyluric acid (o-hydroxyhippuric acid, glycine conjugate at the carboxyl group) and salicyl ether glucuronide (phenolic glucuronide, glucuronidation at hydroxyl group), two of the major salicylate metabolites in humans (Levy et al., 1972).

Salicylate can be hydroxylated to both 2,5-DHBA and the relatively unique 2,3-isomer (2,3-DHBA) by $\bullet\text{OH}$, the latter of which appears to be an effective assay for $\bullet\text{OH}$ formation (Halliwell et al., 1988). This reaction has been evaluated in many systems that produce $\bullet\text{OH}$, including hypoxanthine/xanthine oxidase (Floyd et al., 1984) and ischemia-reperfusion (Udassin et al., 1991). The hydroxylated products observed in these systems are 2,3-DHBA and 2,5-DHBA. However, since the latter isomer also is produced *in vivo* by P450s, quantitation of 2,5-DHBA likely results in an overestimation of actual $\bullet\text{OH}$ formation (Halliwell et al., 1991). Although 2,3-DHBA has been measured in healthy human volunteers, as well as in arthritic patients who had ingested aspirin (Grootveld and Halliwell, 1986, 1988), this isomer was found only in relatively low concentrations, and thus was suggested to be due to endogenous *in vivo* $\bullet\text{OH}$ production (Halliwell and Gutteridge, 1986). 2,3-DHBA is not produced by hepatic microsomal fractions obtained from rabbits and rats treated with P450 inducers (Ingelman-Sundberg et al., 1991), and no enzyme has been identified that can catalyze its formation (Halliwell et al., 1991). Thus, 2,3-DHBA formation appears to be an accurate measure of $\bullet\text{OH}$ production *in vitro*.

However, many if not most hydroxylated substrates are substantially conjugated with UDPGA *in vivo* (Dutton, 1980), and it is not clear to what extent this glucuronidation may complicate the interpretation of *in vivo* studies of salicylate hydroxylation. Furthermore, in *in vivo* studies, it is unlikely that a single time point will accurately reflect the maximal xenobiotic-initiated formation of •OH in different subjects.

Another consideration important to this study is that ASA can selectively and irreversibly inhibit the cyclooxygenase component, but not the hydroperoxidase component of PHS (Miyamoto et al., 1990). Previous *in vivo* and *in vitro* studies have demonstrated that pretreatment with ASA and other PHS inhibitors can reduce phenytoin teratogenicity, oxidation of molecular targets, and covalent binding to embryonic protein (Wells, 1993; Miranda et al., 1994; Liu and Wells, 1994, 1995a,b; Yu and Wells, 1995). These results suggest a role for PHS-catalyzed bioactivation of phenytoin to a reactive intermediate that initiates the formation of teratogenic ROS such as •OH.

This study in CD-1 mice was designed to validate the 3-hydroxylation of salicylate to 2,3-DHBA as an *in vivo* measure of •OH formation, and to use this assay to determine the potential *in vivo* contribution of PHS-dependent •OH formation to the molecular mechanism of phenytoin teratogenicity. Validation using paraquat, an herbicide known to initiate •OH formation (Yamazaki et al., 1990), included an assessment of the importance of interindividual variation in the time of peak DHBA formation in accurately estimating *in vivo* •OH formation. Potentially confounding effects of glucuronidation were addressed by the direct *in vivo* administration of 2,3-DHBA, and by *in vitro* glucuronidation studies using hepatic microsomes. The potential teratogenic contribution of •OH was characterized according to its dose- and time-dependent initiation by phenytoin. The putative role of PHS-catalyzed phenytoin bioactivation in •OH formation was determined by comparing 2,3-DHBA formation when ASA was administered after phenytoin, to that when ASA was given before, when it can more effectively

inhibit phenytoin bioactivation. The results provide insights into both the molecular mechanism of phenytoin teratogenesis and potential approaches in teratologic risk assessment.

2.4.3 MATERIALS AND METHODS

Animals

Virgin female CD-1 mice (Charles River Canada Ltd., St. Constant, Quebec) were housed in plastic cages with ground corn cob bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY, USA) and maintained in a temperature-controlled room with a 12 hr light-dark cycle. Food (Laboratory Rodent Chow 5001, PMI Feeds Inc., St. Louis, MO, USA) and tap water were provided *ad libitum*.

Chemicals

Phenytoin (diphenylhydantoin sodium), paraquat (1,1'-dimethyl-4,4'-bipyridylium), acetaminophen (N-acetyl-p-aminophenol, APAP), ASA, 2,3- and 2,5-DHBA, and β -glucuronidase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium bicarbonate and sodium hydroxide were purchased respectively from BDH Inc. (Toronto, Ontario) and Fischer Scientific Co. (Toronto, Ontario). All other reagents used were of analytical or HPLC grade.

2,3-DHBA Glucuronidation Studies

***In vitro* Glucuronidation:** Hepatic microsomes (final protein concentration, 1 mg/ml) from TCDD-induced mice were incubated with 2,3-DHBA (50 μ M), and UDPGA (10 mM) in 0.1 M phosphate buffered saline (PBS), pH 7.4, in glass borosilicate test tubes. Reactions were incubated at 37°C for either 30 min, 1 or 3 hr in a shaking water bath (final incubation volume, 300 μ l). Reactions were stopped by adding 75 μ l of 10% trichloroacetic acid (TCA). Samples were then centrifuged at 1000 x g for 20 min at 4°C (model TJ-6, Beckman, USA). The pellet was discarded and supernatants were extracted and analyzed for 2,3-DHBA and its glucuronides as described below.

***In Vivo* Glucuronidation:** CD-1 mice were injected ip with 2,3-DHBA (200 mg/kg), asphyxiated with CO₂ and blood was collected via cardiac puncture at either 15, 30 min or 1 hr.

Approximately 600-800 μ l of whole blood was obtained from each mouse and immediately microcentrifuged (Beckman Microfuge B, USA) at 18, 000 X g for 2 min. Plasma 100 μ l was removed and divided in half. Both aliquots were incubated for 20 hr at 37°C, with or without β -glucuronidase (32 μ g/ml). Peak disappearance by β -glucuronidase was used to confirm 2,3-DHBA glucuronide peaks. Incubations were similar to that described above except that β -glucuronidase was added instead of microsomes and UDPGA.

***In Vivo* •OH-Catalyzed Salicylate Hydroxylation in Murine Plasma**

All solutions were prepared immediately prior to use, employing an injection volume of 0.01 ml/g body weight and the route of administration was intraperitoneal (ip). Paraquat was dissolved in 0.9% sodium chloride with a final pH of 7.5, and administered in a dose of 30 mg/kg, which is the LD₅₀ for mice (Bus et al., 1976). Phenytoin was dissolved in 0.002 N sodium hydroxide with a final pH of 10.8-10.9, and administered in a teratogenic dose of 65 or 100 mg/kg (Wells, 1993). ASA was dissolved in a vehicle of 0.25 M sodium bicarbonate and injected in a dose of 200 mg/kg ip 30 min before paraquat or phenytoin, or 30 min after phenytoin. All treatment groups contained a minimum of 3 mice. Controls received an identical volume of drug vehicle, pH adjusted where appropriate, at times corresponding to ASA, paraquat or phenytoin administration.

All blood samples were taken via tail tip amputation and analyzed for plasma concentrations of 2,3-DHBA and 2,5-DHBA. In the paraquat study, blood was collected from the same mouse at 5, 15, 30 min, 1 and 2 hr. In the phenytoin study, blood was collected at 15, 30 min, 1, 2, 3 and 4 hr after ASA or phenytoin. Blood was collected using heparinized capillary tubes (Drummond Microcaps, Drummond Scientific Co., Broomall, Pa.) and immediately microcentrifuged (Beckman) at 18, 000 X g for 2 min to separate plasma from red blood cells. Plasma (40 μ l) was then transferred to a new microcentrifuge tube (Sarstedt, West Germany) and kept on ice until the end of the experiment. The plasma was then extracted as described below.

Detection and Quantitation

Plasma or supernatants (*in vitro* study) were mixed with 10 μ l of 0.1 mM N-acetyl-p-aminophenol (APAP, acetaminophen, internal standard), 25 μ l of 1 M hydrochloric acid (HCL), (Mallinckrodt, Inc. Paris Kentucky, USA) and 500 μ l of diethyl ether (AnalaR, BDH Inc. Tor. Ont. Can). The mixture was vortexed for 10 sec at room temperature and then microcentrifuged for 2 min to allow for separation of diethyl ether and aqueous layers. The top diethyl ether layer containing 2,3-DHBA, 2,5-DHBA and APAP was extracted and placed into a glass borosilicate test tube. A 500 μ l aliquot of diethyl ether was added to the remaining bottom layer, the mixture was processed as above and the diethyl ether layer was transferred to the same test tube. The addition and removal of diethyl ether was performed at least 4 times. The diethyl ether was evaporated under a stream of nitrogen. After evaporation the dried residue was reconstituted with 250 μ l of the mobile phase (97% sodium acetate/citric acid 0.03 M; 3% methanol).

High-performance liquid chromatography (HPLC) coupled with electrochemical detection (ESA model 5100A) was used to detect the ASA metabolites, including 2,3-DHBA and APAP, according to modified methods of Grootveld and Halliwell (1988). Briefly, an isocratic procedure was used, with a mobile phase consisting of 97% 0.03 M sodium acetate/citric acid and 3% methanol. The flow rate was set at 1.0 ml/min. Similar to Floyd et al. (1984), the electrochemical detector potential (oxidizing potential of +0.8 V) was optimized for ASA metabolites by creating a hydrodynamic voltammogram. All samples were measured against authentic standards of 2,3- and 2,5-DHBA.

Statistical Analysis

Statistical significance of differences between treatment groups in each study was determined by Students t-test or one factor analysis of variance (ANOVA) as appropriate using a standard, computerized statistical program (Statview, Abacus Conceptus, Inc.). DHBA data from paraquat studies were logarithmically normalized prior to statistical analysis. The level of significance was $p < 0.05$.

2.4.4 RESULTS

2,3-DHBA Glucuronidation Studies

β -Glucuronidase Hydrolysis and *In Vivo* Glucuronidation: HPLC chromatographic peaks for two glucuronide conjugates of 2,3-DHBA in plasma were characterized by HPLC peak disappearance upon incubation with β -glucuronidase, which almost completely eliminated both peaks (up to 99%) (**fig. 2 and 3**). The peak plasma concentration of both 2,3-DHBA and its two glucuronides occurred at 30 min, and was almost completely eliminated within 1 hr. Both glucuronides amounted to a maximum of only 11% of the total 2,3-DHBA plasma concentration (**fig. 3**).

***In vitro* Glucuronidation:** Further verification of 2,3-DHBA glucuronidation was established in a hepatic microsomal system incubated with the UGT cofactor, UDPGA, and 2,3-DHBA, for either 30 min, 1 or 3 hr. There was a significant time-dependent increase in 2,3-DHBA glucuronidation that was maximal at the final sampling time (3 hr) ($p=0.0001$) (**fig. 3**). However, 2,3-DHBA glucuronidation amounted to only 0.43% of the 2,3-DHBA concentration.

Paraquat-initiated \bullet OH Formation and Salicylate Hydroxylation in Murine Plasma

Paraquat-treated mice had substantially increased plasma 2,3-DHBA formation over the entire 2 hr sampling period ($p\leq 0.04$) (**fig. 4**). There was a remarkable interindividual variability in the time of maximal 2,3-DHBA elevation by paraquat, from 0.1 to 1.0 hr, with any single time substantially underestimating formation in some animals. When the maximal value was chosen for each animal, independent of time, there was over a 19.2-fold increase in the mean maximal 2,3-DHBA formation in the paraquat-treated group compared to vehicle controls ($p=0.0001$) (**fig. 4, insert**).

In contrast, formation of the 2,5-DHBA metabolite, which is catalyzed both by the P450s and \bullet OH, was decreased in paraquat-treated mice at early sampling times (0.1 and 0.25 hr) ($p<0.02$) (**fig. 7**). However, mean maximal 2,5-DHBA concentration in the paraquat-treated group

Figure 2. Analysis of plasma concentrations of 2,3-dihydroxybenzoic acid (DHBA) and its glucuronide conjugate by high-performance liquid chromatography (HPLC). HPLC peaks were identified with the use of authentic standards.

FIGURE 2

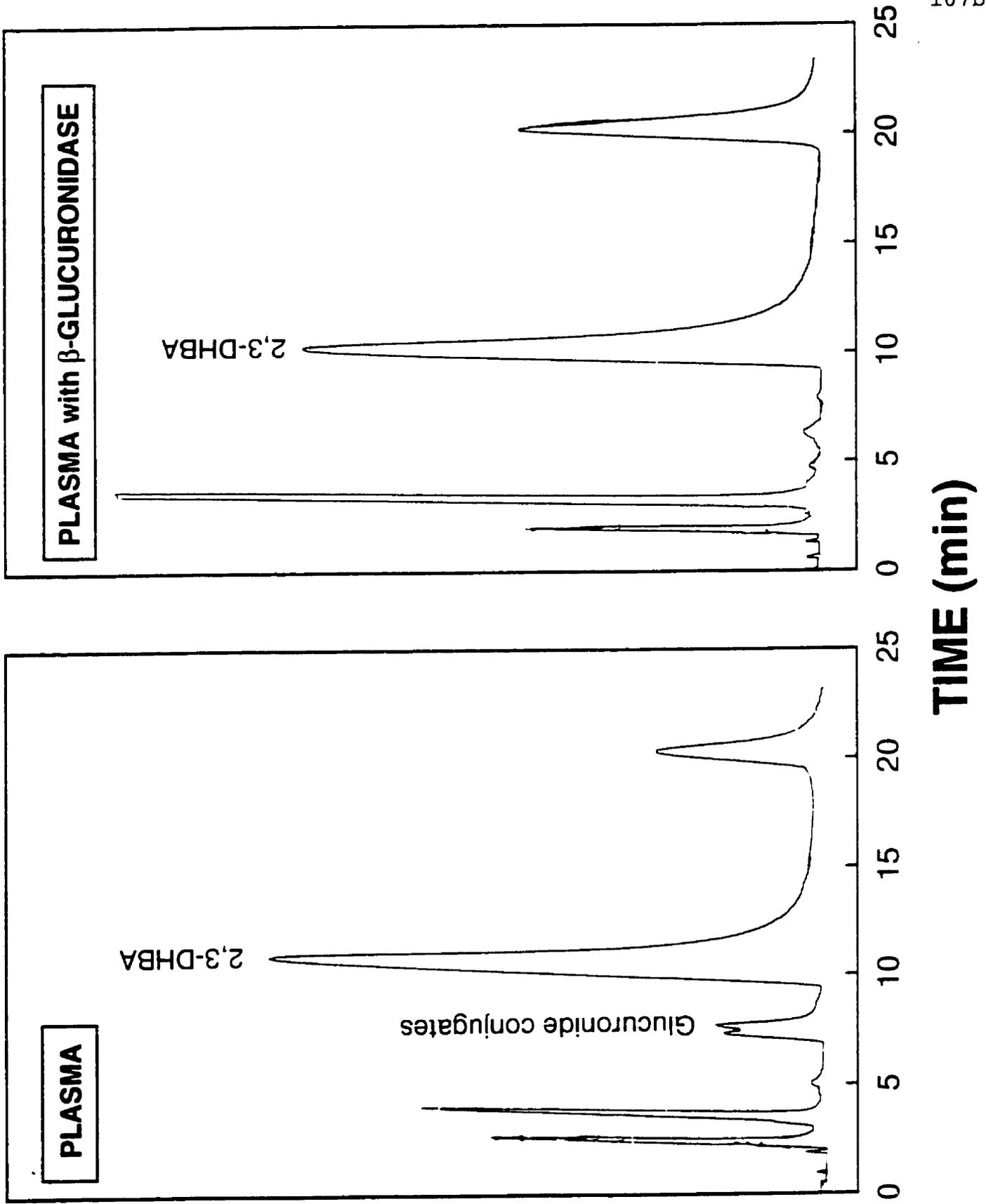
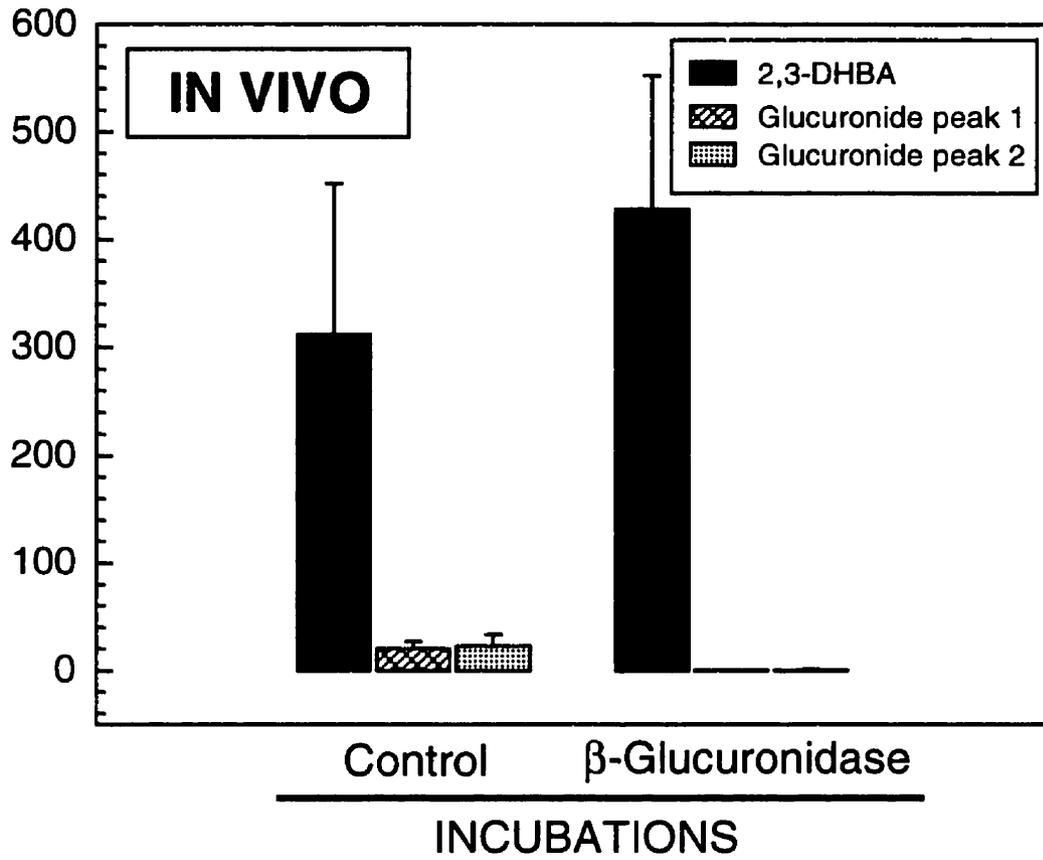


Figure 3. *In vivo* and *in vitro* characterization of 2,3-dihydroxybenzoic acid (DHBA) glucuronidation. The upper panel shows 2,3-DHBA glucuronidation in female CD-1 mice 30 min after injection with 2,3-DHBA (200 mg/kg ip). The lower panel figure shows *in vitro* 2,3-DHBA glucuronidation catalyzed by TCDD-induced hepatic microsomal UDP-glucuronosyltransferases (UGTs). 2,3-DHBA (50 μ M) and uridine diphosphate glucuronic acid (UDPGA, 10 mM) were incubated with hepatic microsomal protein (1 mg/ml) for 0.5, 1 and 3 hr at 37°C in a shaking water bath. Asterisks indicate a significant difference from other groups ($p < 0.05$).

FIGURE 3

CONCENTRATION

(μM , mean + SD)



2,3-DHBA GLUCURONIDE

(% of 2,3-DHBA, mean + SD)

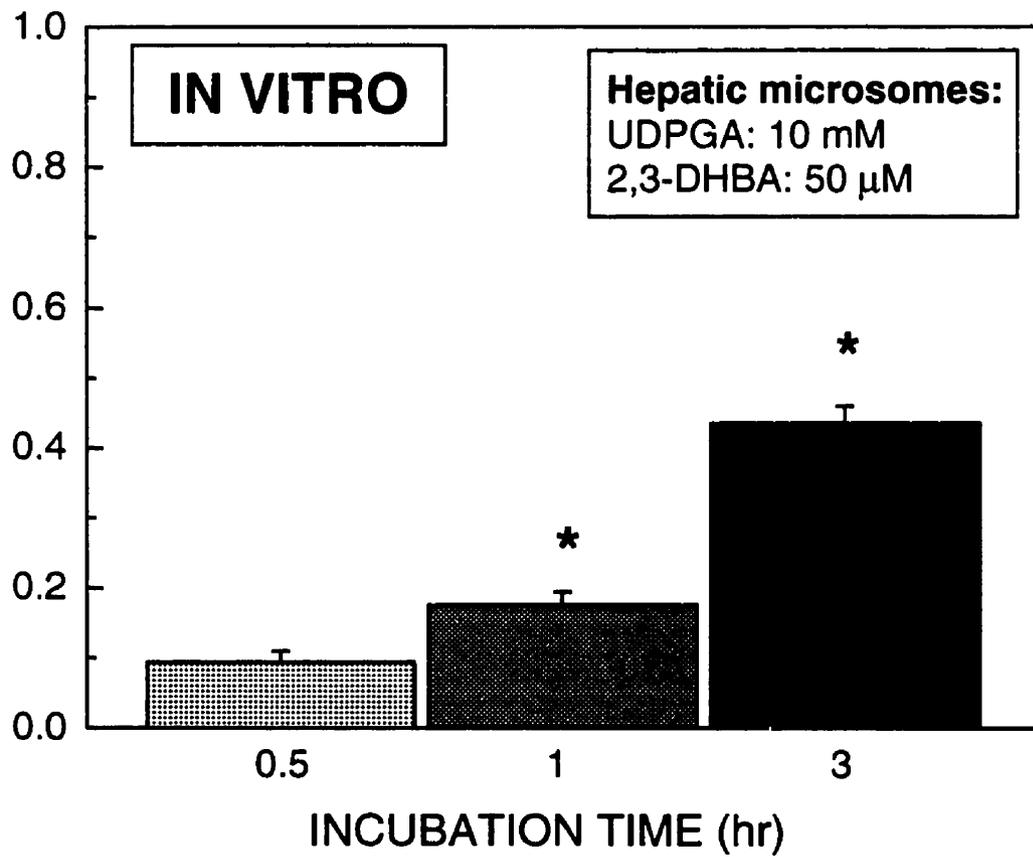


Figure 4. Paraquat (PQ)-initiated hydroxyl radical (\bullet OH) formation as measured via 2,3-dihydroxybenzoic acid (DHBA) production. Mice were injected with acetylsalicylic acid (ASA, 200 mg/kg ip) and 30 min later with paraquat. Blood was collected from each animal at 5, 15, 30 min, 1 and 2 hr after ASA. The thin lines indicate plasma 2,3-DHBA concentrations in individual mice, while the thick lines indicate the mean values at each time for the respective paraquat or control groups. In the control group, 3 of 6 mice did not have detectable concentrations of 2,3-DHBA at any time. Undetectable concentrations were assigned an arbitrary value of 0.001. The insert shows the mean maximal peak of 2,3-DHBA for individual mice, regardless of the time of occurrence. The number of animals is give in parentheses. Asterisks indicate a difference from respective controls ($p < 0.05$).

PLASMA 2,3-DIHYDROXYBENZOIC ACID (DHBA) (picomoles/ μ l plasma)

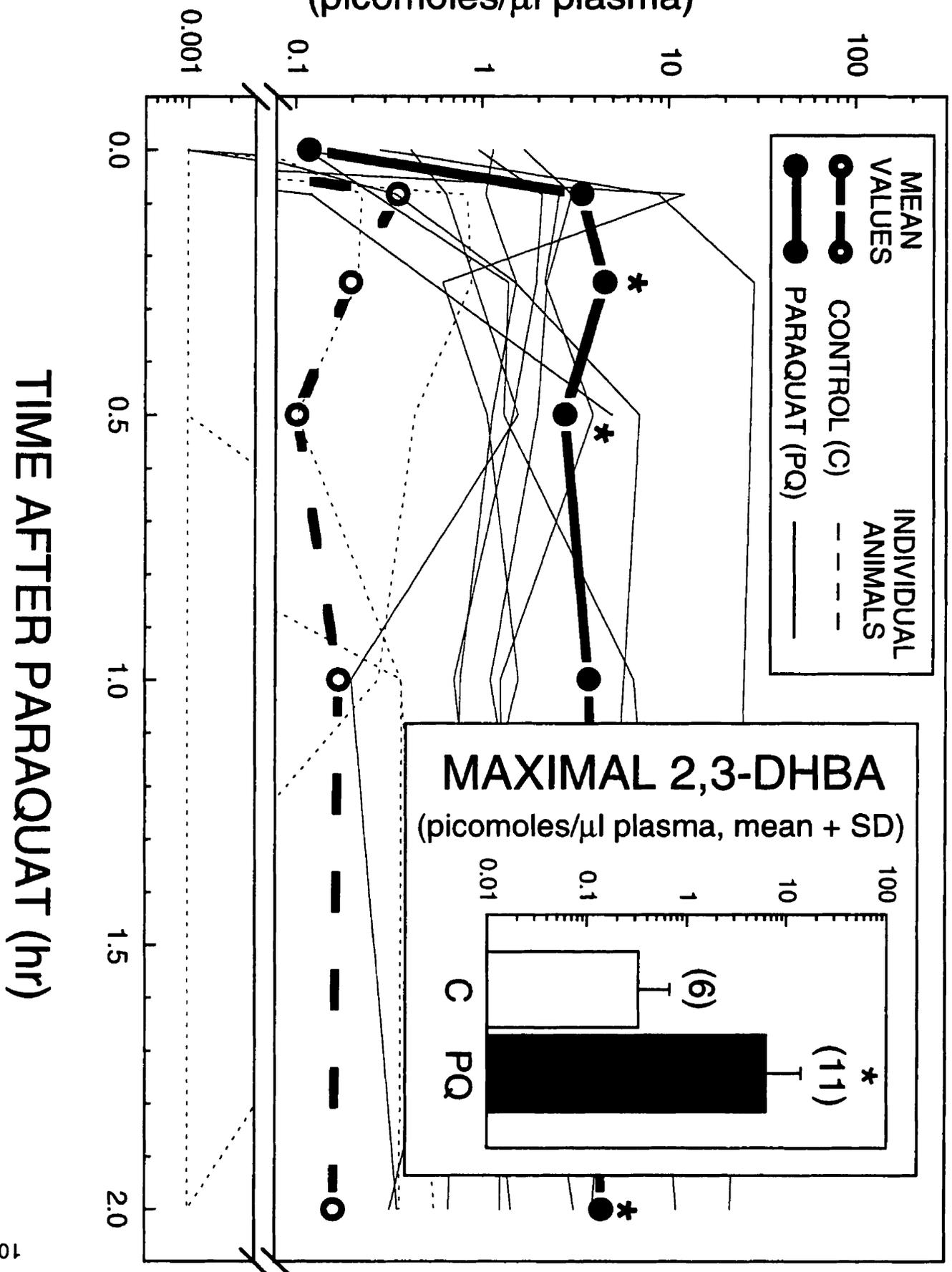


FIGURE 4

was not significantly decreased compared to vehicle controls (**fig. 7, insert**).

Phenytoin-initiated •OH Formation and Salicylate Hydroxylation in Murine Plasma

Phenytoin at both low and high doses after ASA pretreatment initiated a dose- and time-dependent increase in 2,3-DHBA formation above vehicle controls ($p < 0.05$) (**fig. 5**). The low and high phenytoin doses respectively increased mean maximal 2,3-DHBA formation by 7.6- ($p = 0.02$) and 14.2-fold ($p = 0.003$) above vehicle controls (**fig. 5**). The time of maximal 2,3-DHBA elevation by phenytoin varied from 0.25 to 1 hr. 2,3-DHBA concentrations initiated by the higher dose of phenytoin were significantly higher than with the lower dose at most time points ($p < 0.02$) (**fig. 5**), although the mean maximal concentrations were not significantly different (**fig. 5, insert**).

When the lower phenytoin dose was injected prior to ASA, there was a higher elevation in 2,3-DHBA formation over the 4 hr sampling period, compared to the group given phenytoin after ASA (**fig. 6**). The mean maximal 2,3-DHBA formation in the group given phenytoin before ASA was over 2-fold higher than the group given ASA before the same dose of phenytoin ($p = 0.03$) (**fig. 6, insert**).

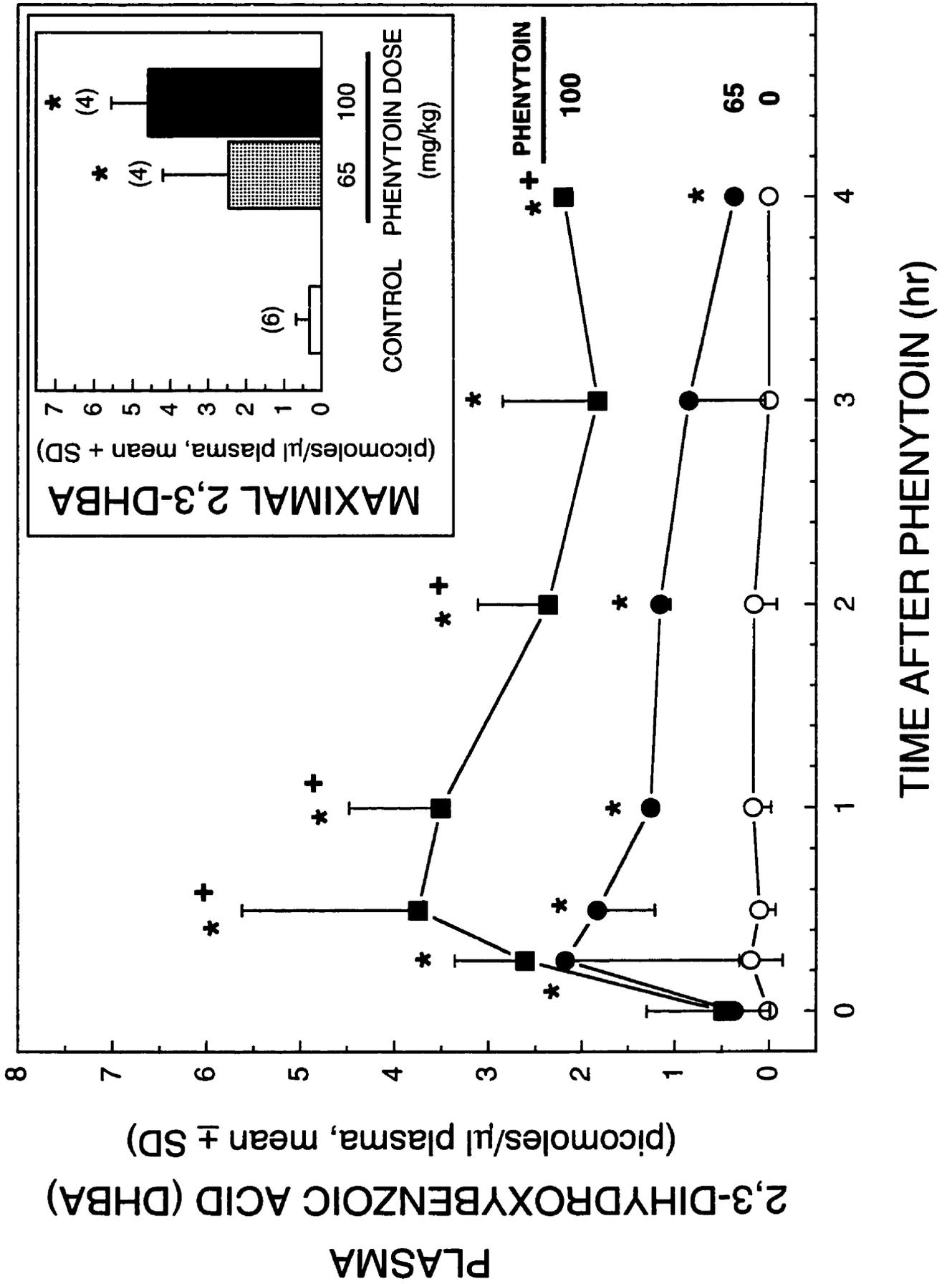
The 2,5-isomer was differentially affected by pretreatment with the low and high dose of phenytoin (**fig. 7**). Although not significant at individual time points, the lower dose of phenytoin appeared to decrease 2,5-DHBA formation over the entire sample period compared to vehicle controls, and there was a significant 49% decrease in mean maximal 2,5-DHBA concentration ($p = 0.04$) (**fig. 7, insert**). In contrast, the higher phenytoin dose significantly increased 2,5-DHBA formation at 30 min and 1 hr (**fig. 7**), although there was no increase in the mean maximal 2,5-DHBA concentration. The mean maximal 2,5-DHBA concentration initiated by the higher dose of phenytoin was 3-fold higher than observed with the lower dose ($p = 0.02$) (**fig. 7, insert**).

Urinary Dihydroxybenzoic Acid Formation

Mice pretreated with ASA and the lower phenytoin dose showed no increase in urinary 2,3-DHBA concentration over vehicle controls (**fig. 8**). However, pretreatment with the same low phenytoin dose given before rather than after ASA showed respective 3-fold ($p = 0.0001$) and

Figure 5. Phenytoin-initiated dose-dependent *in vivo* hydroxyl radical (\bullet OH) formation measured via 2,3-dihydroxybenzoic acid (DHBA) formation. Mice were injected with acetylsalicylic acid (ASA, 200 mg/kg ip) and 30 min later with phenytoin at a dose of either 65 or 100 mg/kg ip. The insert shows the mean maximal peak of 2,3-DHBA from mice treated with either the phenytoin vehicle or the low or high dose of phenytoin. The number of animals is given in parentheses. Asterisks indicate a difference from the time-matched vehicle control, and the plus symbols indicate a difference from the lower phenytoin dose ($p < 0.05$).

FIGURE 5



PLASMA
2,3-DIHYDROXYBENZOIC ACID (DHBA)
(picomoles/μl plasma, mean ± SD)

MAXIMAL 2,3-DHBA
(picomoles/μl plasma, mean + SD)

CONTROL PHENYTOIN DOSE
(mg/kg)

TIME AFTER PHENYTOIN (hr)

Figure 6. Comparison of phenytoin-initiated *in vivo* hydroxyl radical ($\bullet\text{OH}$) formation when acetylsalicylic acid (ASA) was administered before or after phenytoin. All animals received the same dose of phenytoin (65 mg/kg ip) and ASA (200 mg/kg ip). The insert shows the mean maximal peak of 2,3-dihydroxybenzoic acid (DHBA) from mice treated with either ASA followed by phenytoin, or phenytoin followed by ASA. The plus symbol indicates a difference from mice treated with ASA followed by phenytoin ($p < 0.05$).

FIGURE 6

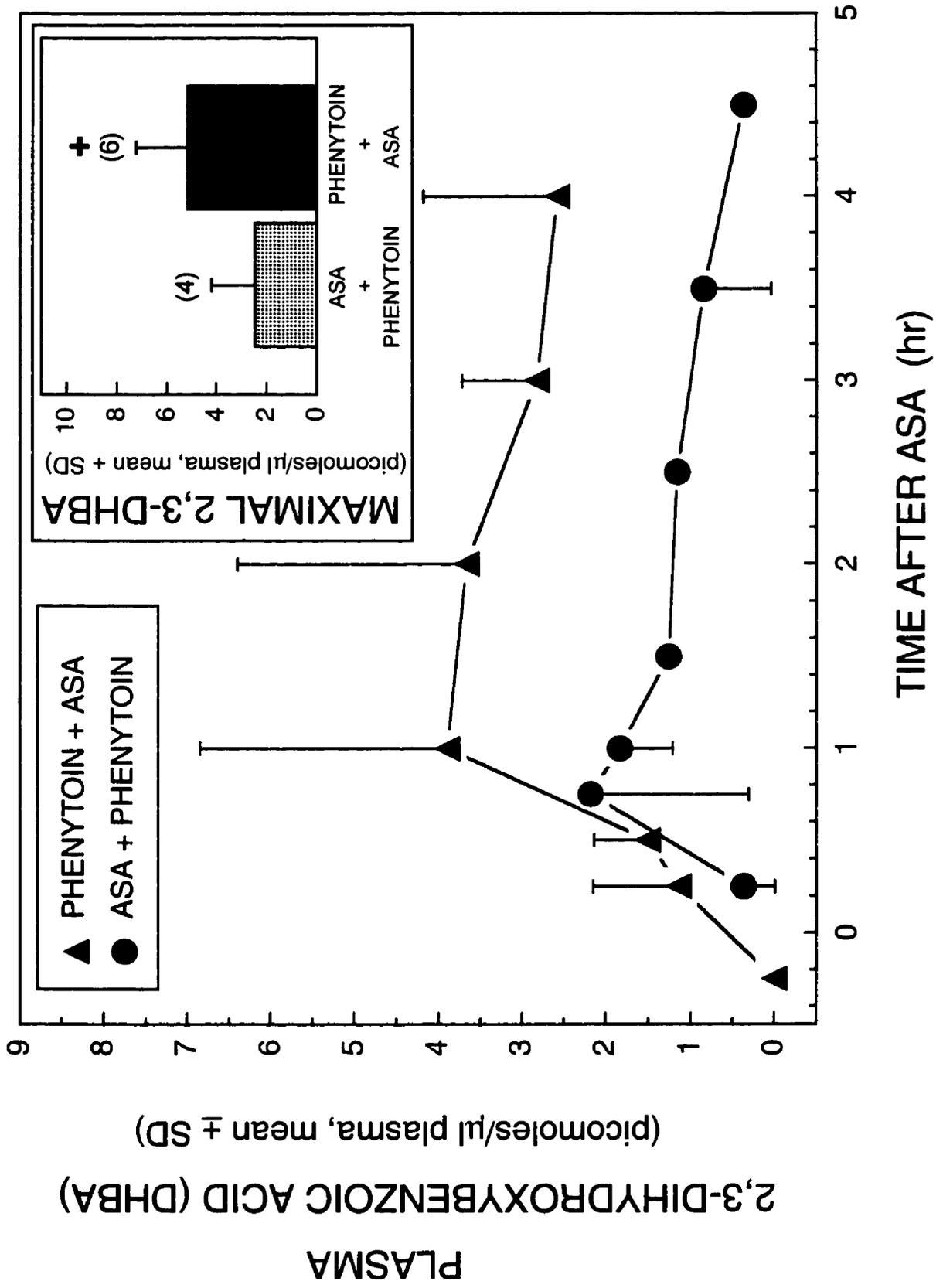


Figure 7. Formation of the 2,5-isomer of dihydroxybenzoic acid (2,5-DHBA) in mice treated with acetylsalicylic acid (ASA, 200 mg/kg ip) followed by paraquat (PQ, 30 mg/kg ip) or phenytoin (65 or 100 mg/kg ip). The upper panel shows plasma 2,5-DHBA formation from paraquat and vehicle control groups. The bottom panel shows plasma 2,5-DHBA formation from phenytoin (65 and 100 mg/kg) and vehicle control groups. The inserts show the mean maximal peak of plasma 2,5-DHBA. Asterisks indicate differences from vehicle control, and plus symbols indicate differences from mice treated with ASA plus the lower dose (65 mg/kg) of phenytoin ($p < 0.05$).

FIGURE 7

PLASMA 2,5-DIHYDROXYBENZOIC ACID (DHBA)

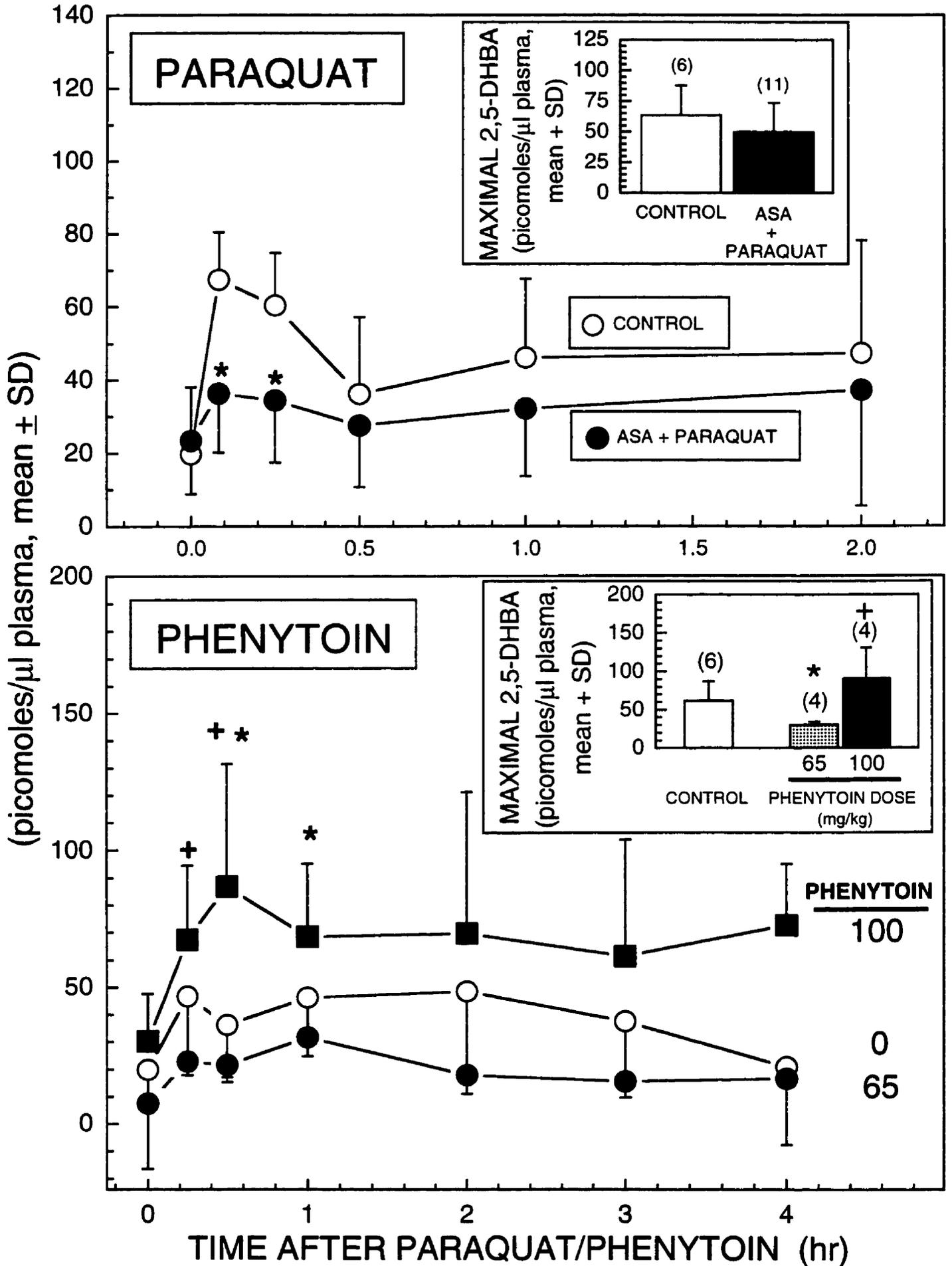
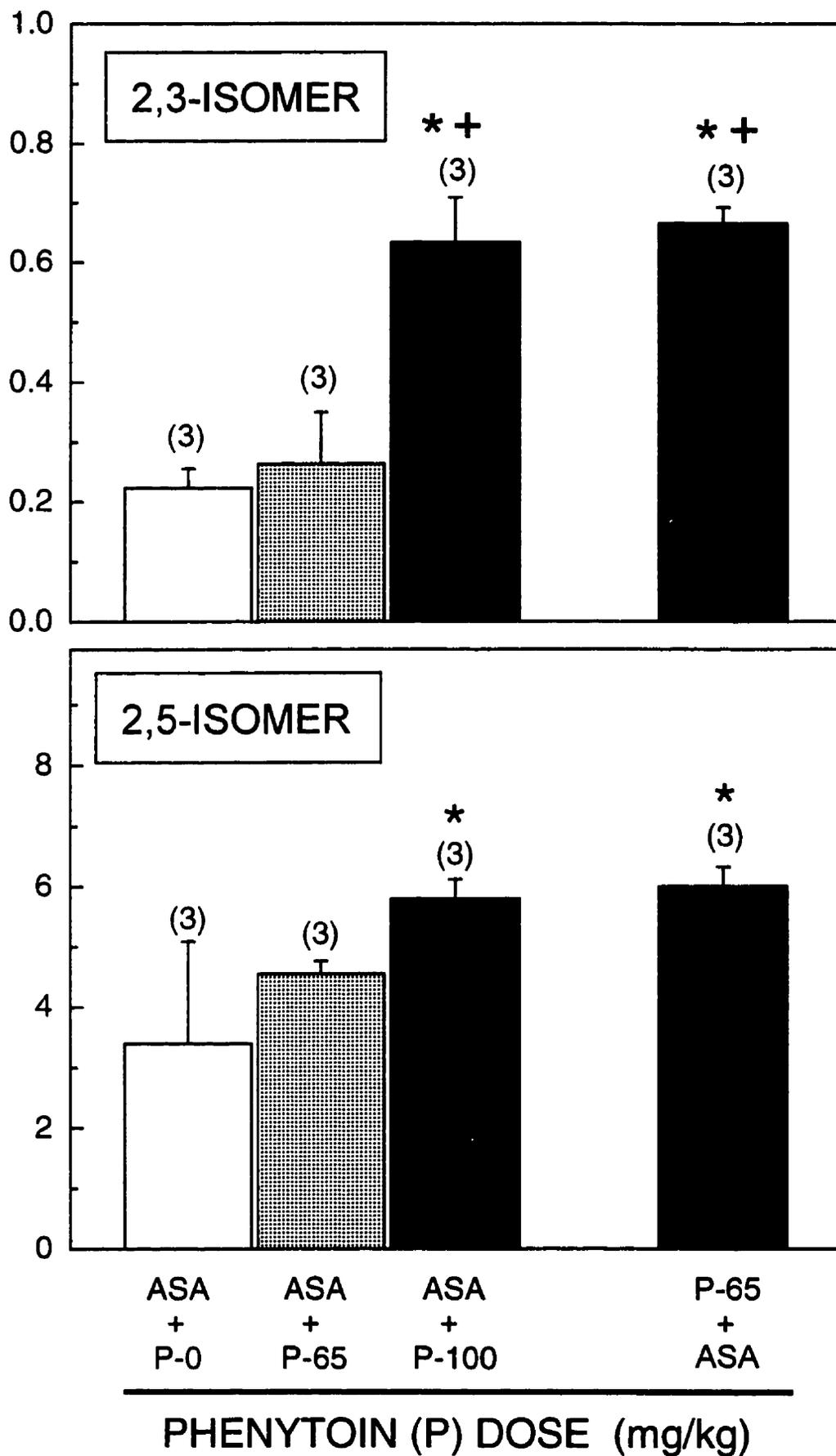


Figure 8. Urinary concentrations of 2,3- and 2,5-dihydroxybenzoic acids (DHBA). Mice were either treated with acetylsalicylic acid (ASA, 200 mg/kg ip) followed by phenytoin vehicle or phenytoin (P, 65 or 100 mg/kg ip), or were first treated with phenytoin (65 mg/kg ip) followed by ASA (200 mg/kg). Urine was collected for 4 hr after the last injection and analyzed. Asterisks indicate differences from vehicle control, and plus symbols indicate differences from mice treated with ASA followed by the lower dose (65 mg/kg) of phenytoin (ASA+P-65) ($p < 0.05$).

**URINARY
DIHYDROXYBENZOIC ACIDS**
 (nanomoles/ μ l urine, mean + SD)



1.8-fold ($p=0.05$) increases in 2,3- and 2,5-DHBA formation over vehicle controls, and 2,3-DHBA was increased 2.5-fold above the group given the same dose of phenytoin after ASA ($p=0.002$). Similar increases in both 2,3- and 2,5-DHBA occurred in the group pretreated with ASA followed by the high phenytoin dose ($p<0.05$) (**fig. 8**).

2.4.5 DISCUSSION

Recent attention has focused on free radical-mediated damage as a putative mechanism of various pathological conditions, including neurodegenerative diseases, carcinogenesis (Kehrer, 1993) and teratogenesis (Wells, 1993). Salicylate hydroxylation through the formation of 2,3-DHBA, as distinct from 2,5-DHBA, has been postulated to be an effective means of detecting both *in vivo* (Grootveld and Halliwell, 1986; 1988) and *in vitro* (Ingelman-Sundberg et al., 1991) •OH formation. In this study, we characterized salicylate hydroxylation as an *in vivo* probe for xenobiotic-initiated •OH formation using the known free radical initiator paraquat, and employed this approach in determining the *in vivo* production of •OH by phenytoin.

Initial *in vivo* and *in vitro* studies were conducted to determine the extent of 2,3-DHBA glucuronidation, which could cause a substantial underestimation of 2,3-DHBA formation. The conjugation of hydroxylated xenobiotics with UDPGA, catalyzed by UGTs, frequently is a major route of elimination (Dutton, 1980). Many xenobiotics such as acetaminophen (Prescott, 1980), benzo[a]pyrene (Nemoto, 1981) and particularly salicylate (Levy et al., 1972) are predominantly eliminated via glucuronidation, with glucuronide conjugates excreted in the urine and to a minor extent in the feces. Two glucuronide conjugates of 2,3-DHBA were detected *in vivo* and in *in vitro* hepatic microsomal studies (**figs. 2 and 3**). However, even after direct injection of 2,3-DHBA, only 11% was recovered as glucuronide conjugates *in vivo*. *In vitro* formation of glucuronide conjugates amounted to only 0.43% of total 2,3-DHBA. Thus, unlike most xenobiotics, 2,3-DHBA is not substantially glucuronidated in mice, which generally are similar to humans, and more active than rats, in their ability to glucuronidate xenobiotics such as the analgesic drug acetaminophen (Kalabis and Wells, 1990; de Morais et al., 1992a,b). Accordingly, the formation of glucuronide conjugates should not interfere critically with the *in vivo* estimation of •OH by 2,3-DHBA production.

Xenobiotic-initiated salicylate hydroxylation was investigated with the herbicide paraquat,

which can cause lipid peroxidation (Bus et al., 1976). Paraquat is metabolized by an NADPH-dependent cytochrome reductase which reduces paraquat to a free radical anion (Bus et al., 1976). *In vitro* studies with paraquat, using the spin trapping agent 5,5-dimethyl-1-pyrroline-1-oxide have shown that paraquat initiates superoxide anion and $\bullet\text{OH}$ formation (Yamazaki et al., 1990). A recent *in vitro* study using either a xanthine oxidase-hypoxanthine or rat hepatic microsomal system investigated paraquat-initiated salicylate hydroxylation (Tomita et al., 1994). This *in vitro* study showed a slight increase in 2,3-DHBA suggesting increased $\bullet\text{OH}$ formation. However the results were not significant.

Our study accordingly provides the first evidence for *in vivo* paraquat-initiated $\bullet\text{OH}$ formation measured by 2,3-DHBA formation. Mice treated with paraquat had substantially increased 2,3-DHBA formation over the entire 2 hr sampling period, indicating sustained paraquat-initiated $\bullet\text{OH}$ formation ($p \leq 0.04$) (**fig. 4**). Maximal 2,3-DHBA formation occurred at substantially different times and amounts in each individual mouse, which may indicate an important factor for the variable susceptibility seen in paraquat toxicity (Calderbank, 1968). The mean maximal 2,3-DHBA formation was over 19.2-fold higher in paraquat treated mice compared to vehicle controls ($p=0.0001$) (**fig. 4**). These results not only corroborate previous studies demonstrating that paraquat initiates $\bullet\text{OH}$ formation, but also show that the timing of $\bullet\text{OH}$ production *in vivo* varies considerably in individual mice, indicating the importance of multiple samplings from each mouse for accurate quantitation. Multiple sampling in human studies over time may prove to be critical in ascertaining the accurate peak production of $\bullet\text{OH}$ in any single individual. For example, a recent *in vivo* study measuring both plasma salicylate hydroxylation (2,3- and 2,5-DHBA) and oxidative stress (thiobarbituric acid reactant substances, TBARS) in healthy humans failed to show a positive correlation between 2,3-DHBA and TBARS (Coudray et al., 1995). However, this study took measurements at a single point. Given the temporal variability in $\bullet\text{OH}$ formation observed in our study, and the expectation that TBARS formation would follow a similarly variable but not necessarily congruent temporal pattern, studies based upon single sampling

times may prove prohibitively inaccurate.

Subsequent *in vivo* studies investigated PHS-dependent phenytoin bioactivation and $\bullet\text{OH}$ formation. PHS contains both a cyclooxygenase and hydroperoxidase component which respectively can oxidize arachidonic acid to prostaglandin G_2 , which is then reduced to prostaglandin H_2 . In the latter reaction, hydroperoxidases can use xenobiotics, such as phenytoin as a source of reducing equivalents, concomitantly cooxidizing the xenobiotic to a free radical (Marnett, 1990a).

The putative phenytoin free radical (Wells, 1993; Parman et al., 1996) potentially may initiate the production of ROS, catalyzed by transition metals such as iron (Fe) and copper (Cu). Free radical intermediates such as the paraquat radical are capable of reducing Fe^{3+} -complexes directly, which can further react with hydrogen peroxide (H_2O_2) forming $\bullet\text{OH}$ (Halliwell and Gutteridge, 1989). Free radicals in general also may initiate oxidation in the form of lipid peroxidation, which indirectly can lead to the formation of $\bullet\text{OH}$.

Catalytic iron may be available *in vivo* as iron loosely bound to membrane lipids, DNA and phosphate complexes such as ADP (Halliwell and Gutteridge, 1986; Gutteridge et al., 1990). In addition, ferritin is associated with lipid peroxidation, in the presence of ascorbate and H_2O_2 , possibly by increasing $\bullet\text{OH}$ formation (O'Connell et al., 1986). $\bullet\text{OH}$ production, in the presence of excess H_2O_2 , also is increased by methaemoglobin (Puppo and Halliwell, 1988) and hemosiderin (O'Connell et al., 1986); the latter being a weak promoter of $\bullet\text{OH}$ formation. Ascorbate may function in mobilizing iron from ferritin (O'Connell et al., 1986), and an excess of H_2O_2 may decompose haem, releasing iron (Puppo and Halliwell, 1988), both of which allow iron to participate in the generation of $\bullet\text{OH}$. On the other hand, transferrin and lactoferrin seem incapable of catalyzing the formation of $\bullet\text{OH}$ (Halliwell and Gutteridge, 1986; Aruoma and Halliwell, 1987).

Following ASA pretreatment, phenytoin in a dose-dependent fashion increased plasma

2,3-DHBA formation over the 4 hr sampling period (**fig. 5**), as well as the mean maximal 2,3-DHBA formation (**fig. 5, insert**) ($p < 0.05$). Phenytoin-initiated $\bullet\text{OH}$ formation in turn may initiate teratogenic oxidative stress. Previous studies show that phenytoin teratogenicity, oxidation of molecular targets (DNA, protein and lipids) and covalent binding to protein are reduced by the iron chelator deferoxamine, a variety of antioxidants and/or free radical spin trapping agents (Wells, 1993; Liu and Wells, 1994, 1995a,b). It is remarkable that the mean maximal plasma concentration of 2,3-DHBA initiated by the low dose of phenytoin (**fig. 5**) was almost half that initiated by an LD_{50} dose of paraquat (**fig. 3**). This dose of phenytoin is not associated with maternal lethality, but is highly teratogenic in mice (Yu and Wells, 1995). Thus, while plasma concentrations of 2,3-DHBA may reflect the overall potential for various xenobiotics to initiate $\bullet\text{OH}$ formation in a particular species, the nature and severity of toxicologic expression will vary considerably due to other factors, possibly including tissue-specific bioactivation, detoxification, antioxidative cytoprotection and molecular target repair and redundancy.

The potential toxicologic importance of PHS-catalyzed bioactivation was evident in the study comparing the same dose of phenytoin given before or after ASA. ASA, in addition to serving as a substrate for 2,3-DHBA formation, is also an inhibitor of PHS. Maximal 2,3-DHBA formation was 2.1-fold higher when the low dose of phenytoin was given before ASA, compared to the same phenytoin dose given after ASA, when PHS was inhibited (**fig. 5 and 6**). Indeed, 2,3-DHBA formation initiated by the low dose of phenytoin given before ASA was equivalent to that initiated by the high dose given after ASA (**figs. 5 and 6**). Interestingly, putative phenytoin-initiated $\bullet\text{OH}$ formation was not inhibited completely by ASA, suggesting that peroxidases other than PHS, such as LPOs, may contribute to phenytoin bioactivation. This hypothesis is consistent with the partial protection by ASA observed in *in vivo* studies of phenytoin teratogenicity (Wells, 1993), and the apparently more complete protection by eicosatetraynoic acid, a dual inhibitor of PHS and LPOs (Yu and Wells, 1995).

Earlier *in vivo* studies using ASA as an inhibitor of PHS and subsequent phenytoin bioactivation and toxicity, showed that ASA pretreatment, at a dose of 10 mg/kg (5% of dose in

this study), decreased phenytoin-induced cleft palates and phenytoin covalent binding to embryonic protein (Wells, 1993). This suggested PHS-dependent phenytoin bioactivation and teratogenesis, which was corroborated by *in vivo* and embryo culture studies in which phenytoin embryopathy was reduced by the dual PHS/LPO inhibitor eicosatetraynoic acid (Miranda et al., 1994; Yu and Wells, 1995). Similar embryoprotective effects of ASA were seen with the structurally related anticonvulsants trimethadione and dimethadione (Wells, 1993), and the sedative/hypnotic drug thalidomide (Arlen and Wells, 1996). Recent *in vivo* studies also have demonstrated that phenytoin-initiated lipid and protein oxidation and degradation were decreased by ASA pretreatment at a dose of 10 mg/kg (Liu and Wells, 1994).

Subsequent studies have demonstrated that phenytoin can initiate DNA oxidation, as measured by the formation of 8-hydroxy-2'-deoxyguanosine, *in vivo*, *in vitro* (Liu and Wells, 1995a) and in embryo culture (Winn and Wells, 1995a). *In vivo* phenytoin-initiated DNA oxidation was inhibited by ASA or the free radical spin trapping agent phenylbutylnitron, supporting respectively the importance of PHS-catalyzed bioactivation and free radical-mediated toxicity. In embryo culture, phenytoin-initiated DNA oxidation and embryopathy were completely inhibited by either superoxide dismutase or catalase, indicating a fundamental role for ROS in mediating phenytoin teratogenesis. DNA is a likely teratologically relevant molecular target, since transgenic knock-out mice deficient in the p53 tumor suppressor gene, which facilitates DNA repair, are more susceptible to the teratogenicity of phenytoin (Laposa and Wells, 1995) and another DNA-damaging teratogen, benzo[a]pyrene (Nicol et al., 1995).

Interestingly, unlike 2,3-DHBA, the 2,5-isomer was consistently decreased in paraquat-treated mice over the 2 hr sampling period, suggesting paraquat inhibition of P450-catalyzed salicylate hydroxylation ($p < 0.05$) (**fig. 7**). This inhibition may reflect direct paraquat inactivation of the P450 enzyme(s) responsible for salicylate hydroxylation, or may be due to intracellular depletion of the P450 cofactor NADPH. Similarly, when the lower dose of phenytoin was administered after ASA pretreatment, there were decreases in 2,5-DHBA both over the 4 hr sampling period, and in the mean maximal 2,5-DHBA production ($p < 0.05$) (**Fig. 7, insert**). Although the higher dose of phenytoin did increase 2,5-DHBA formation, likely reflecting $\bullet\text{O H}$

production, the decreased formation of this isomer with paraquat and the lower phenytoin dose show that the 2,5-isomer primarily reflects enzymatic hydroxylation and is not suitable for •OH estimation. This caution was first raised by Halliwell et al. (1991), and studies that have used 2,5-DHBA formation to quantify •OH production (Udassin et al., 1991) are difficult to interpret.

When urine was used to analyze salicylate metabolites, the results were considerably less sensitive than analysis of plasma samples, although they were corroborative. Unlike plasma analysis, the low dose of phenytoin given after ASA did not alter urinary 2,3- or 2,5-DHBA formation over a 4 hr collection period (**fig. 8**), demonstrating that urinary analysis is remarkably less sensitive in detecting *in vivo* •OH production. Otherwise, urinary and plasma analysis were corroborative, with the urinary 2,3- and 2,5-DHBA formation being increased both by the high dose of phenytoin, and by the low phenytoin dose when given before ASA. As with the plasma analysis, the 2,3-DHBA formation initiated by the low dose of phenytoin given before ASA, to avoid prior inhibition of PHS by ASA, was equivalent to that initiated by the high dose of phenytoin given after ASA, demonstrating the importance of PHS-catalyzed bioactivation of phenytoin in •OH formation. In general, however, *in vivo* studies depending upon only urinary 2,3-DHBA data may fail to detect •OH formation at lower levels.

Various end points (oxidation of molecular targets, amino acid hydroxylation) have been investigated in an attempt to measure *in vivo* •OH formation and oxidative stress. This is particularly difficult in humans where many of the techniques can be invasive or involve toxic probes. We demonstrate that salicylate hydroxylation measured in plasma and urine can be an effective tool not only in determining the molecular mechanism of xenobiotic-initiated toxicity, but also potentially in the monitoring and assessment of individual susceptibility. This technique may prove valuable in cases where drugs such as phenytoin, a known teratogen, must be taken throughout pregnancy in order to control seizures.

In summary, the paraquat studies demonstrated the importance of multiple sampling in accurately determining 2,3-DHBA formation, the relative insensitivity of urinary compared to

plasma analysis, the inappropriateness of the 2,5-isomer of DHBA in estimating •OH formation, and the low potential for confounding by 2,3-DHBA glucuronidation. In the phenytoin studies, our results provide the first direct *in vivo* evidence for PHS-dependent phenytoin bioactivation and subsequent •OH formation as a potential molecular mechanism in phenytoin embryotoxicity. This approach may prove useful in human teratological risk assessment.

2.5 STUDY #5: PHENYTOIN EMBRYOTOXICITY: PROTECTION BY UDP-GLUCURONOSYLTRANSFERASES (UGTS)^{1,2}

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2. To be submitted.

2.5.1 ABSTRACT

The teratogenic anticonvulsant drug phenytoin is eliminated primarily by UGT-catalyzed glucuronidation. Direct N-glucuronidation of phenytoin and O-glucuronidation of its major hydroxylated metabolite, 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH), are decreased or absent in UGT-deficient rats and both heterozygous (+/j) and homozygous (j/j) UGT-deficient fibroblasts are more susceptible to micronucleus formation initiated by phenytoin or HPPH (J. Pharmacol. Exp. Ther. 280: 200, 1997). This study tested the protective role of UGTs in phenytoin teratogenicity. Pregnant UGT-normal (+/+) and heterozygous (+/j) UGT-deficient Gunn and RHA rats were treated with phenytoin (1.5-150 mg/kg ip) on gestational days (GD) 11-14 (vaginal plug/smear=GD 1). Embryotoxicity was assessed on GD 21. Our results demonstrated that, at a dose as low as 15 mg/kg ($p=0.0004$), +/j UGT-deficient dams had a higher incidence of fetal resorptions and increased fetal postpartum lethality. (A teratogenic dose in other strains of UGT-normal rats is 100-150 mg/kg.) Malformations were not seen in either phenytoin-treated genotype. Phenytoin-initiated hydroxyl radical formation, as a potential mechanism of increased embryotoxicity, was assessed in UGT-deficient and UGT-normal male RHA rats. Both +/j and j/j rats had increased plasma concentrations of hydroxyl radical-mediated salicylate hydroxylation (i.e. 2,3-dihydroxybenzoic acid) versus +/+ UGT-normal controls ($p<0.05$). Lipid peroxidation was not increased in UGT-deficient animals. In *In vitro* studies using the human bilirubin UGT (i.e. UGT1A1), HPPH but not phenytoin was a substrate for human UGT1A1. This is the first evidence that pregnant UGT-deficient rats are more susceptible to phenytoin embryotoxicity, possibly due to increased reactive oxygen species production, and further suggests that pregnant epileptic women with some UGT deficiencies, including the prevalent bilirubin UGT deficiency, may be at increased risk for phenytoin teratogenicity.

2.5.2 INTRODUCTION

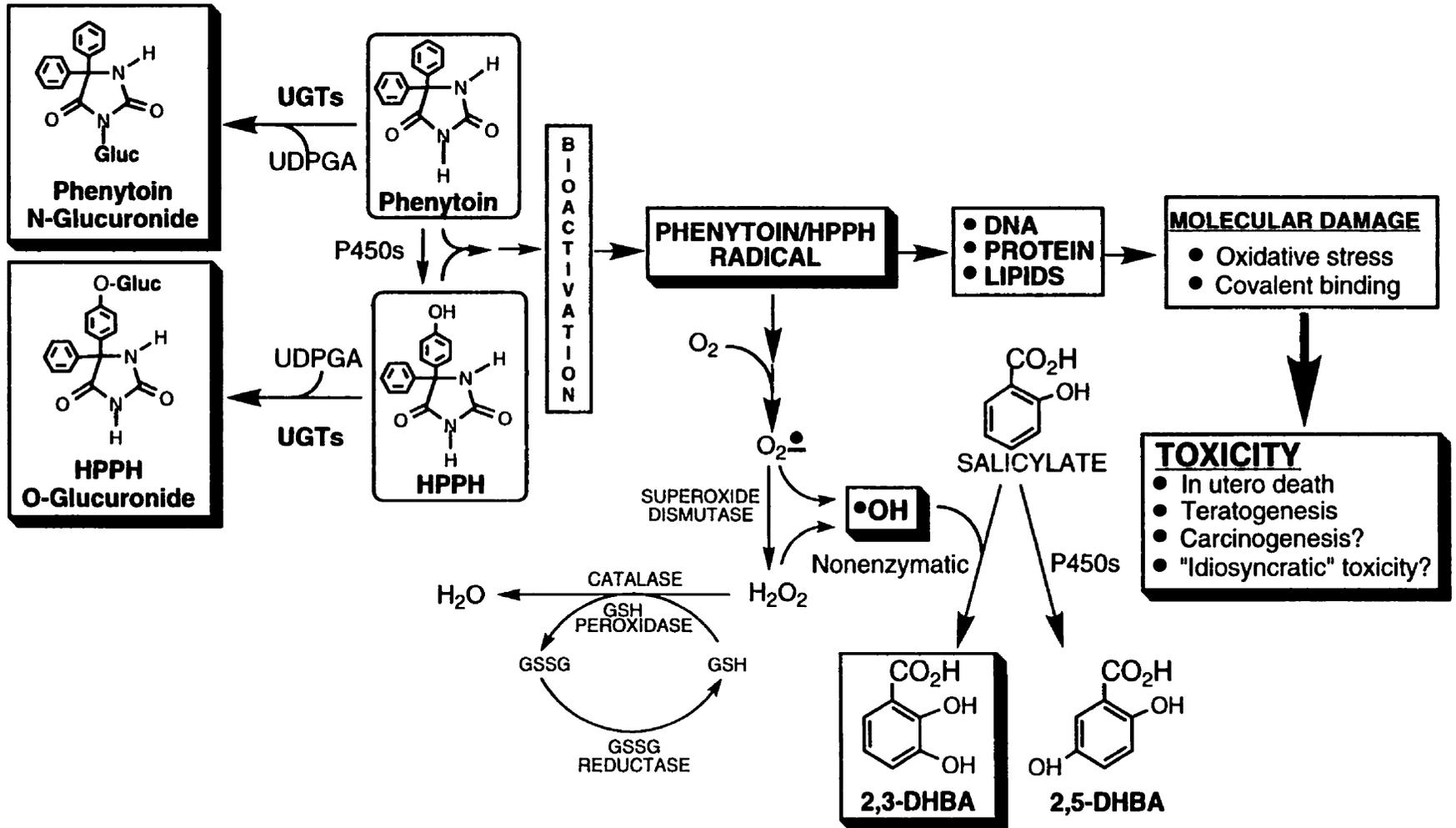
Phenytoin is a known animal and human teratogen (see Section 2.4) which may cause its toxicity through reactive oxygen species-initiated DNA damage (see Section 2.3 and 2.4) (**fig. 1**). As indicated above (see Section 2.2 and 2.3), NNK, B[a]P, phenytoin and its major hydroxylated metabolite HPPH are more genotoxic in skin fibroblasts cultured from UGT-deficient rats versus UGT-normal controls (see Section 2.3). Furthermore, this laboratory has previously demonstrated that UGT-deficiencies in both Gunn and RHA rats can increase the *in vivo* susceptibility to both acetaminophen and B[a]P-initiated toxicities. Therefore, the increased *in vitro* genotoxicity initiated by both phenytoin and HPPH in UGT-deficient cultured skin fibroblasts suggests that teratogenicity/embryotoxicity of phenytoin may be higher in UGT-deficient animals.

It appears that human UGT-deficiencies also can be a significant risk factor for increased toxicological susceptibility to xenobiotic toxicities (de Morais et al., 1992b; Hu and Wells, 1993, 1998). Recent evidence from our laboratory has shown that individuals with UGT deficiencies, including bilirubin UGT deficiencies (i.e. Gilbert's syndrome), may be at increased risk for either *in vivo* acetaminophen bioactivation or *in vitro* B[a]P-initiated toxicities. This evidence suggests that a decreased ability to glucuronidate either phenytoin and/or HPPH may significantly enhance the teratogenic potential in individuals treated with this anticonvulsant drug.

In the following study we investigated whether rats deficient in the UGT1 family of isozymes are at an increased risk for phenytoin-initiated reactive oxygen species production, lipid peroxidation and embryotoxicity/teratogenicity. Furthermore, we investigated whether the human bilirubin UGT isozyme (UGT1A1), which appears to be the most common human UGT deficiency (i.e. Gilbert's syndrome), can glucuronidate either phenytoin or HPPH. The objectives of these studies were to determine whether UGT deficiencies can be risk factors in phenytoin teratogenesis and whether the phenotypically apparent major human bilirubin UGT deficiency may be toxicologically important.

Figure 1. Postulated protective roles of UDP-glucuronosyltransferases (UGTs) in phenytoin and 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) bioactivation and toxicity. Hereditary UGT-deficiencies may allow greater bioactivation to free radical reactive intermediates that can initiate the formation of reactive oxygen species, such as hydroxyl radicals (HO•). These reactive intermediates and ROS can irreversibly damage DNA, protein and lipid via covalent binding and oxidation, potentially initiating teratogenesis and other toxicities. The contribution of molecular target oxidation to idiosyncratic drug reactions and reversible lymphoma initiated by phenytoin is speculative. HO• also can covalently bind to salicylate forming either the 2,3- or 2,5-dihydroxybenzoic acid isomer (DHBA), the latter of which can also be produced by salicylate hydroxylation catalyzed by cytochrome P450 (P450). Abbreviations: Gluc, glucuronide conjugate; GSH, glutathione; GSSG, oxidized glutathione; H₂O₂, hydrogen peroxide; O₂⁻, superoxide anion, UDPGA, uridine diphosphate glucuronic acid.

FIGURE 1



2.5.3 MATERIALS AND METHODS

Animals

Male and female HsdBlu:Gunn rats (Harlan Sprague Dawley Inc., Indianapolis, Indiana), RHA rats and Wistar rats (Charles River, St. Constant, Quebec), 180-200 g were housed in plastic cages. All animals were kept in a temperature-controlled room with a 12-hr light-dark cycle automatically maintained. Food (Laboratory Rodent Chow 5001, PMI Feeds Inc.; St. Louis, MO) and tap water were provided *ad libitum*. Animals were acclimatized for a minimum of one week. All animal studies were approved by the University Animal Care Committee in accordance with the standards of the Canadian Council on Animal Care.

Chemicals

Phenytoin (diphenylhydantoin sodium), acetaminophen (N-acetyl-p-aminophenol, APAP), ASA, 2,3- and 2,5-DHBA, UDPGA, [¹⁴C]-UDPGA and β -glucuronidase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium bicarbonate and sodium hydroxide were purchased respectively from BDH Inc. (Toronto, Ontario) and Fischer Scientific Co. (Toronto, Ontario). All other reagents used were of analytical or HPLC grade.

Phenytoin/HPPH-Initiated Lipid Peroxidation and Hydroxyl Radical Production.

UGT-normal Wistar and heterozygous (+/j) and homozygous (j/j) UGT-deficient RHA rats were injected ip with a teratogenic dose of phenytoin (150 mg/kg), or an equimolar dose of HPPH (191 mg/kg) and 30 min later with 200 mg/kg acetylsalicylic acid (ASA). Blood was collected via tail tip amputation at 15, 30 min, 1, 2, 3, and 4 hr and urine for the entire 4 hr, after ASA injection. Approximately 300 μ l of blood was collected using heparinized capillary tubes (Drummond Microcaps, Drummond Scientific Co., Broomall, Pa.) and immediately microcentrifuged (Beckman) at 18,000 x g for 2 min to separate plasma from red blood cells. Plasma (approximately 150 μ l) was then transferred to a new microcentrifuge tube (Sarstedt, West Germany) and kept on ice until all samples were collected. The plasma was then extracted and analyzed as described below.

Lipid Peroxidation. Lipid peroxidation was measured using a modified method of Esterbauer and Cheeseman, (1990). Plasma (50-100 μ l) or urine (100-200 μ l) from both phenytoin and HPPH-treated rats was incubated with a 1.6 M solution of perchloric acid, vortexed, stored at 4°C for 10 min and then centrifuged at 500 x g for 15 min. Supernatants were then mixed with an equal volume of 0.62% 2-thiobarbituric acid solution and boiled for 10 min. Samples were cooled to room temperature and then read at an absorbance of 532 nm for the presence of thiobarbituric acid reactive substances (TBARS). Concentrations of TBARS were calculated using a malondialdehyde standard curve. Since there were not enough plasma or urine samples, TBAR levels in fig. 6 are the combined data from both phenytoin and HPPH-treated rats.

2,3-Dihydroxybenzoic Acid (2,3-DHBA) Production. The plasma or urine (100 μ l) was mixed with 5 μ l of 0.1 mM N-acetyl-p-aminophenol (acetaminophen, internal standard), 65 μ l of 1 M hydrochloric acid (HCl), (Mallinckrodt, Inc. Paris Kentucky, USA) and 500 μ l of diethyl ether (AnalaR, BDH Inc., Toronto Canada). The mixture was vortexed for 10 sec at room temperature and then microcentrifuged for 2 min to allow for separation of diethyl ether layer, which contained salicylate, 2,3-DHBA, 2,5-DHBA and acetaminophen, from the aqueous layer. The diethyl ether layer was then removed and placed into a glass borosilicate test tube and a new 500 μ l aliquot of diethyl ether was added to the remaining bottom layer, the mixture was processed as above and the diethyl ether layer was transferred to the same test tube. The addition and removal of diethyl ether was performed at least 3 times. The diethyl ether was evaporated under a stream of nitrogen and the dried residue was reconstituted with 200 μ l of the mobile phase which consisted of 97% sodium acetate/citric acid 0.03 M and 3% methanol.

The reconstituted samples were then analyzed using high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ESA model 5100A) (Grootveld and Halliwell, 1988). Briefly, an isocratic procedure using a flow rate of 1 ml/min and a mobile phase consisting of 97% 0.03 M sodium acetate/citric acid and 3% methanol was used. The electrochemical detector potential (oxidizing potential of +0.8 V) was previously optimized for the ASA metabolites (salicylate, 2,3- and 2,5-DHBA) by creating a hydrodynamic voltammogram

(Kim and Wells, 1996b). All samples were measured against authentic standards of salicylate, 2,3- and 2,5-DHBA.

Bilirubin UGT (UGT1*1, UGT1A1) Substrate Specificity.

The glucuronidating capability of human bilirubin UGT (UGT1A1) for phenytoin, HPPH, acetaminophen and bilirubin was determined in microsomal protein (Gentest Corporation, Woburn, MA). The microsomal protein was quickly thawed at 30 °C for 30 min to repair ruptured vesicles (Burchell et al., 1989) and 500 µg was then incubated with 0.05 µg/µl alamethicin, a UGT activator, or its vehicle for 30 min at 20 °C. The radioactive (0.1 µCi) and non-radioactive (1.5 mM) UDPGA and substrates (phenytoin, 1mM; HPPH, 1 or 3 mM; APAP, 3mM; bilirubin, 0.5 mM) were added to activated microsomes and incubated for 1 hr at 37 °C in a final incubation volume of 200 µl. All concentrations indicate final concentrations in 200 µl. The incubations were terminated with 600 µl of ice-cold methanol, centrifuged and supernatants were then analyzed in one or all of the following ways. HPLC-UV (240 nm, C-18 column, flow rate=1ml/min) was used to detect phenytoin, HPPH or their glucuronides using a mobile phase of 15% acetonitrile, 1% acetic acid and 74% water. A mobile phase consisting of 3% methanol and 97% 0.2 N acetic acid at a flow rate of 0.8 ml/min and absorbance of 248 nm was used to detect acetaminophen. For bilirubin the HPLC conditions consisted of a C-18 column, a UV absorbance of 400 nm and flow rate of 1 ml/min using a 55% to 100% linear gradient of methanol in 0.1 M sodium acetate, pH 4 containing 5 mM 1-heptane sulfonic acid (Roy Chowdhury et al., 1981). Since all incubations had radiolabelled UDPGA, HPLC fractions also were collected every 1 min and radioactivity was quantitated using a liquid scintillation spectrometer. To further verify or detect phenytoin, HPPH, acetaminophen or bilirubin glucuronides, a modified thin layer chromatography (TLC) method of Bansal and Gessner (1980) was used. Briefly, the substrates and metabolites were separated on a 250 µm silica gel TLC plate, with a mobile phase consisting of n-butanol, acetone, glacial acetic acid, 30% ammonium and water at a ratio of 70:50:18:1.5:60.

Verification of glucuronides was conducted by incubating supernatants with β-glucuronidase (8000 units) and measuring peak disappearance. Supernatants were dried under

a stream of nitrogen, reconstituted with phosphate buffered saline (pH 7.0) and incubated with β -glucuronidase for 12 hr at 37°C. Incubations were stopped with ice-cold methanol (400 μ l), centrifuged and supernatants were analyzed via HPLC-UV, as above.

Phenytoin-initiated Embryotoxicity.

UGT-normal (+/+) and heterozygous UGT-deficient (+/j) Gunn and RHA rats were bred with either +/+ UGT-normal, or +/j or j/j UGT-deficient male rats. Pregnant +/+ and +/j Gunn or RHA rats were injected ip with phenytoin at doses of 1.5, 15, 75 and 150 mg/kg on gestational days (GD) 11-14 (vaginal plug/positive vaginal smear=GD 1). Rats were monitored for 24 hr after injection for overt toxicity. On GD 21 dams were killed and embryos were removed and then embryos were weighed, sexed, and examined for external physical anomalies. Fetuses delivered alive were monitored at room temperature for 30 min to determine postpartum lethality. Embryos were then fixed in Carnoy's solution for 2 days and fixed embryos were then examined for cleft palates. Internal anomalies were determined by embryo dissection. Hearts and kidneys were dissected to determine the presence of aortic/pulmonary stenosis and/or septal defects or hydronephrosis, respectively.

To determine the embryonic/fetal genotype, as well as the genotype of dissectable resorptions, the method of Huang et al. (1992) was used (NB: only adult rat +/j RNA was analyzed since the method was not working properly, for explanation see Results and Discussion). Briefly, total RNA was isolated using a QIAGEN RNeasy kit (Qiagen) and the UGT1 mRNA was converted to cDNA using a Superscript cDNA synthesis kit (Gibco). The first strand cDNA that was produced was then amplified using the polymerase chain reaction (PCR) and the PCR products were subsequently digested with the restriction enzyme *Bst* N1 (Stratagene). The primers used were 5' GTCACGTGACACAGTCAAAC 3' and 5' TTTGCTCCTGCCAGAGGTT 3'. The restriction fragments were then resolved on a 1% agarose gel, and genotypes were determined by fragment migration patterns.

Statistical Analysis

Statistical significance of differences between treatment groups in each study was determined by Students t-test or one factor analysis of variance (ANOVA) as appropriate using a standard, computerized statistical program (Statview, Abacus Conceptus, Inc.). The level of significance was $p < 0.05$.

2.5.4 RESULTS

Phenytoin and HPPH-initiated Hydroxyl Radical Formation

Phenytoin. Both the +/j and j/j UGT-deficient rats had an increase in phenytoin-initiated plasma 2,3-DHBA formation over time, reflecting an increase in hydroxyl radical formation ($p < 0.05$) (**fig. 2**). The mean maximal plasma 2,3-DHBA was increased 5.5-fold ($p < 0.05$) and 3.7-fold ($p = 0.09$) in +/j and j/j UGT-deficient rats, respectively (**fig. 2, inset**). Urinary 2,3-DHBA concentrations were increased in +/j and j/j UGT-deficient rats, with the latter also having a significant increase in concentrations of the 2,5-DHBA isomer ($p < 0.05$) (**fig. 3**). There were no significant differences in either plasma (**fig. 2**) or urinary (**fig. 3**) salicylate concentrations in rats of all three UGT phenotypes.

HPPH. The mean maximal 2,3-DHBA was increased 3.8-fold ($p = 0.08$) and 3.6-fold ($p < 0.05$) in +/j and j/j UGT-deficient rats (**fig. 4, inset**). Interestingly, the mean maximal 2,3-DHBA formation in UGT-deficient rats was similar to that initiated by phenytoin. Over time, a similar pattern of increased 2,3-DHBA was observed in both +/j and j/j UGT-deficient rats, but the differences from UGT-normals at each time point were not statistically significant (**fig. 4**). In contrast to that seen in phenytoin-treated rats (**fig. 2**), plasma 2,5-DHBA concentrations in UGT-normal Wistar rats were higher than in +/j UGT-deficient rats, but were not increased over j/j UGT-deficient animals ($p < 0.05$) (**fig. 4**). There were no differences in plasma and urinary salicylate concentrations in rats of all three UGT phenotypes (**figs. 4, 5**).

Phenytoin/HPPH-Initiated Lipid Peroxidation

Although plasma lipid peroxidation measured by the production of thiobarbituric acid reactive substances (TBARS) shows an upward trend over time, no clear increase could be demonstrated in either UGT-normal Wistar or +/j and j/j UGT-deficient rats (**fig. 6**). Due to the limitation of available sample, TBAR levels in plasma or urine are combined data from both phenytoin and HPPH-treated rats. Urinary TBARS were significantly increased in +/j UGT-deficient animals, with a similar trend seen in j/j UGT-deficient rats versus UGT-normal Wistar controls (**fig. 6**). The data in figure 6 for each UGT phenotype represents TBAR formation in the

Figure 2. Effect of UDP-glucuronosyltransferase (UGT) deficiencies on phenytoin-initiated plasma 2,3-dihydroxybenzoic acid (DHBA) formation, as a measure of hydroxyl radical (HO•) formation. Rats were injected ip with acetylsalicylic acid (ASA), 200 mg/kg, followed 30 min later by a teratogenic dose of phenytoin, 150 mg/kg, and blood was collected over a four hr period. Plasma was analyzed for 2,3-DHBA, as well as the ASA metabolites 2,5-DHBA and salicylate. Asterisks indicate a difference from UGT-normal Wistar controls ($p < 0.05$).

PLASMA CONCENTRATION

(picomoles/ μ l plasma, mean \pm SD)

FIGURE 2: PHENYTOIN

125b

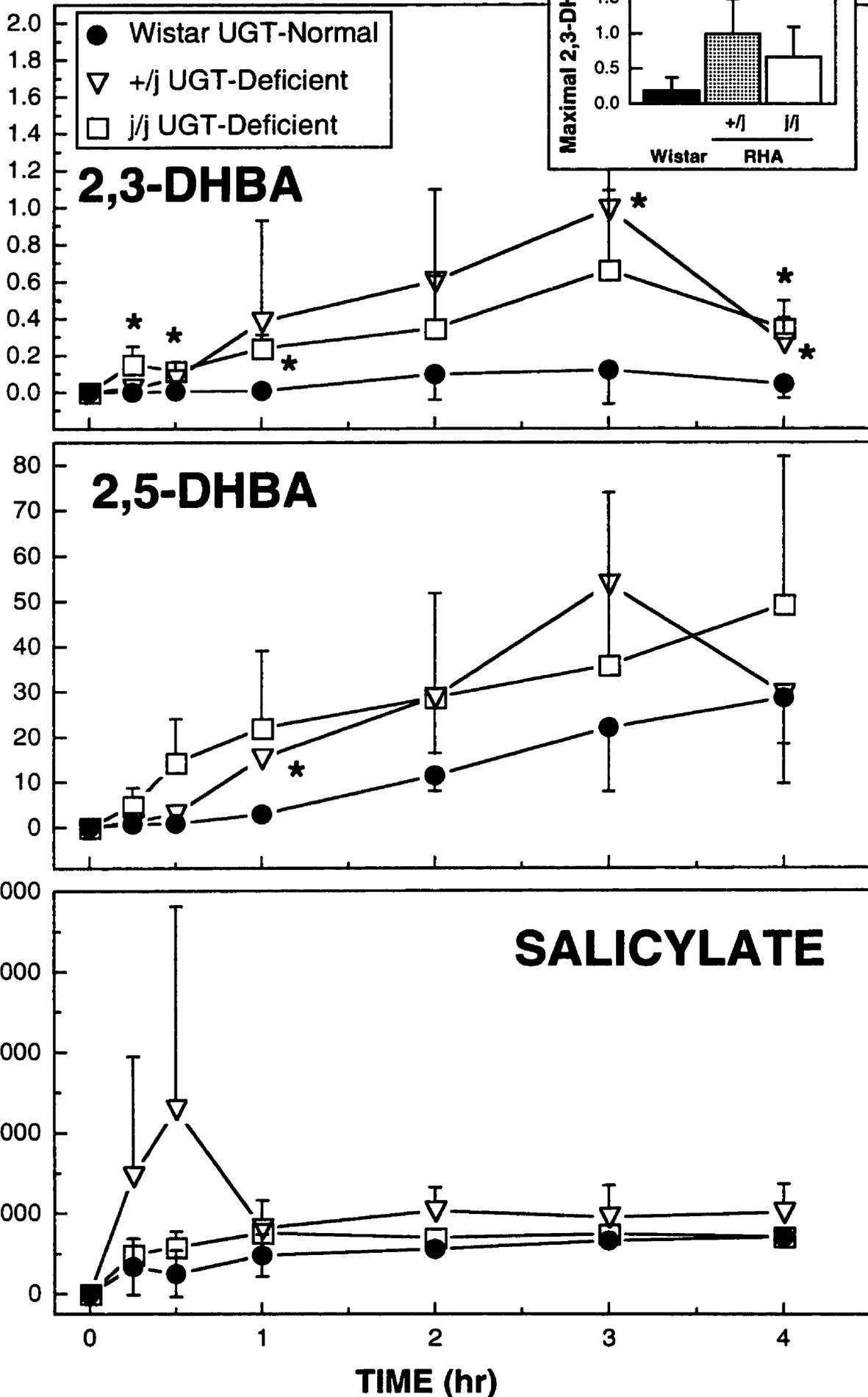


Figure 3. Effect of UDP-glucuronosyltransferase (UGT) deficiencies on phenytoin-initiated 2,3-dihydroxybenzoic acid (DHBA) formation, as a measure of hydroxyl radical (HO•) formation. Rats from fig. 2 were housed in metabolic cages and urine was collected over a four hr period and analyzed as per fig. 2. Asterisks indicate a difference from UGT-normal Wistar controls ($p < 0.05$).

FIGURE 3: PHENYTOIN

125d

URINARY CONCENTRATION

(picomoles/ μ l urine, mean+SD)

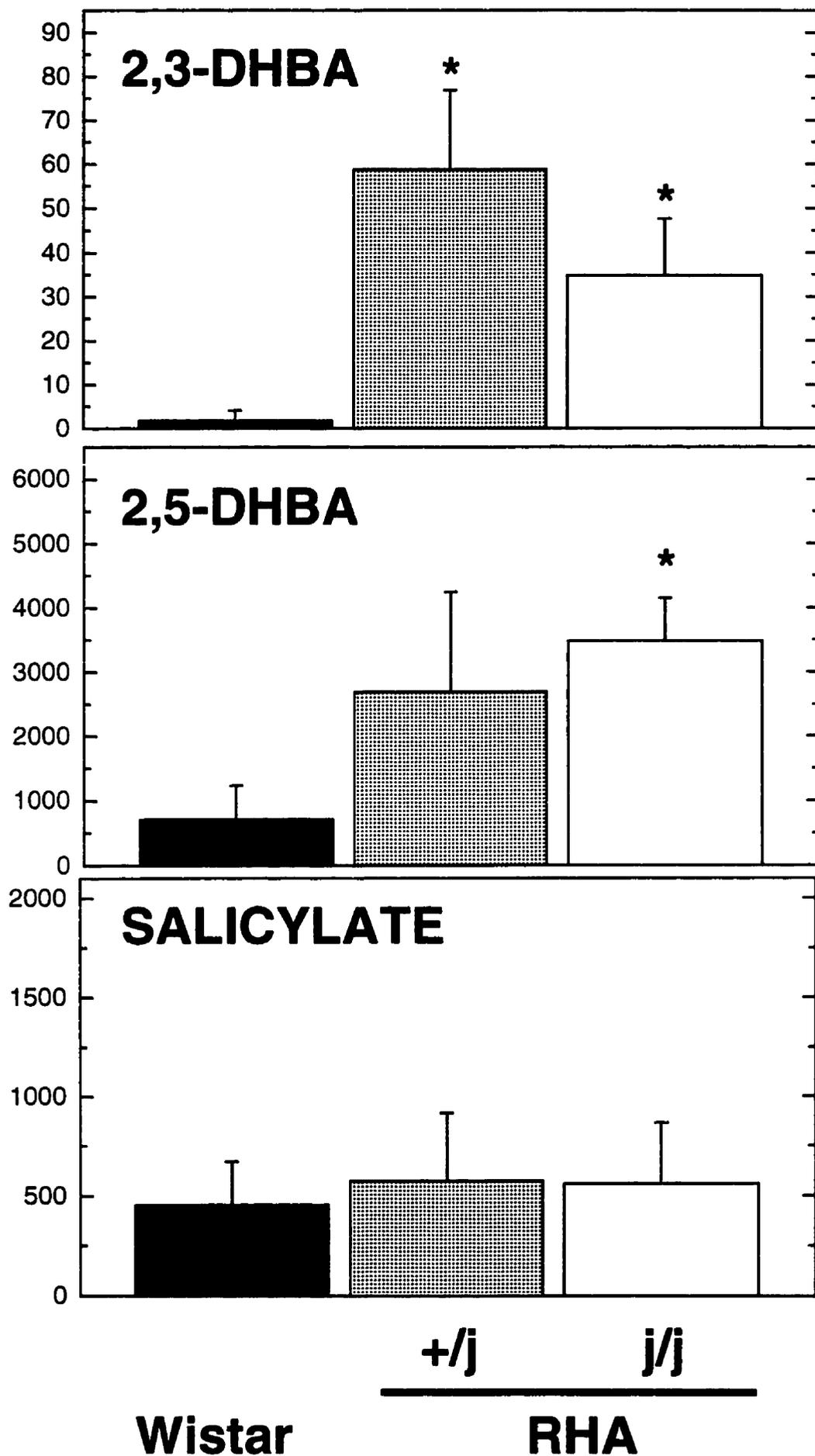


Figure 4. Effect of UDP-glucuronosyltransferase (UGT) deficiencies on 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH)-initiated plasma 2,3-dihydroxybenzoic acid (DHBA) formation, as a measure of hydroxyl radical (HO•) formation. Rats were injected ip with acetylsalicylic acid (ASA), 200 mg/kg, followed 30 min later by HPPH, 191 mg/kg (equimolar to 150 mg/kg phenytoin), and blood was collected over a four hr period. Plasma was analyzed for 2,3-DHBA, as well as the ASA metabolites 2,5-DHBA and salicylate. Plus symbols indicate a difference from +/j UGT-deficient rats ($p < 0.05$).

FIGURE 4: HPPH

PLASMA CONCENTRATION

(picomoles/ μ l plasma, mean \pm SD)

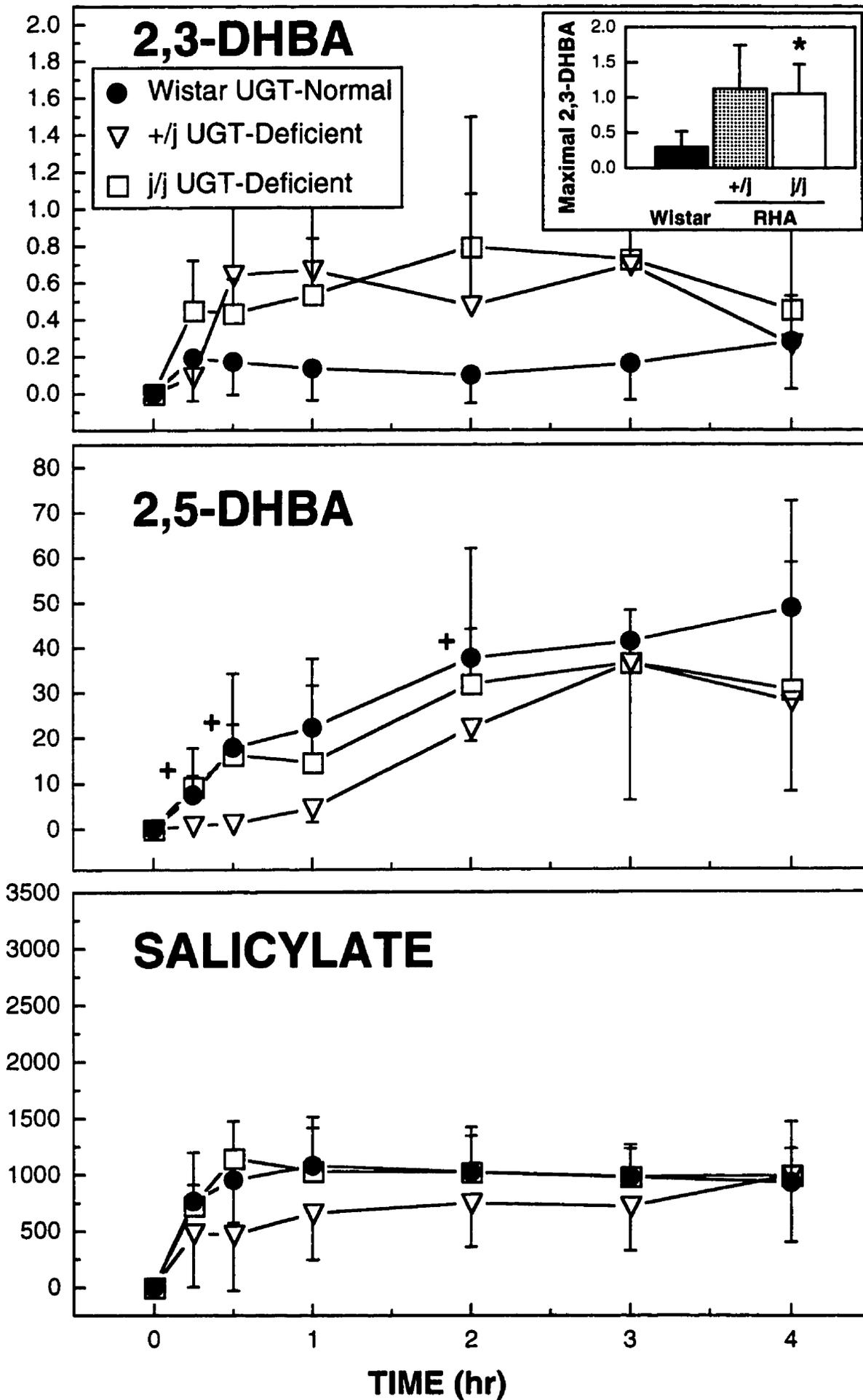


Figure 5. Effect of UDP-glucuronosyltransferase (UGT) deficiencies on 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH)-initiated 2,3-dihydroxybenzoic acid (DHBA) formation as a measure of hydroxyl radical (HO•) formation. Rats from fig. 4 were housed in metabolic cages and urine was collected over a four hr period and analyzed as per fig. 4.

FIGURE 5: HPPH

125h

URINARY CONCENTRATION

(picomoles/ μ l urine, mean+SD)

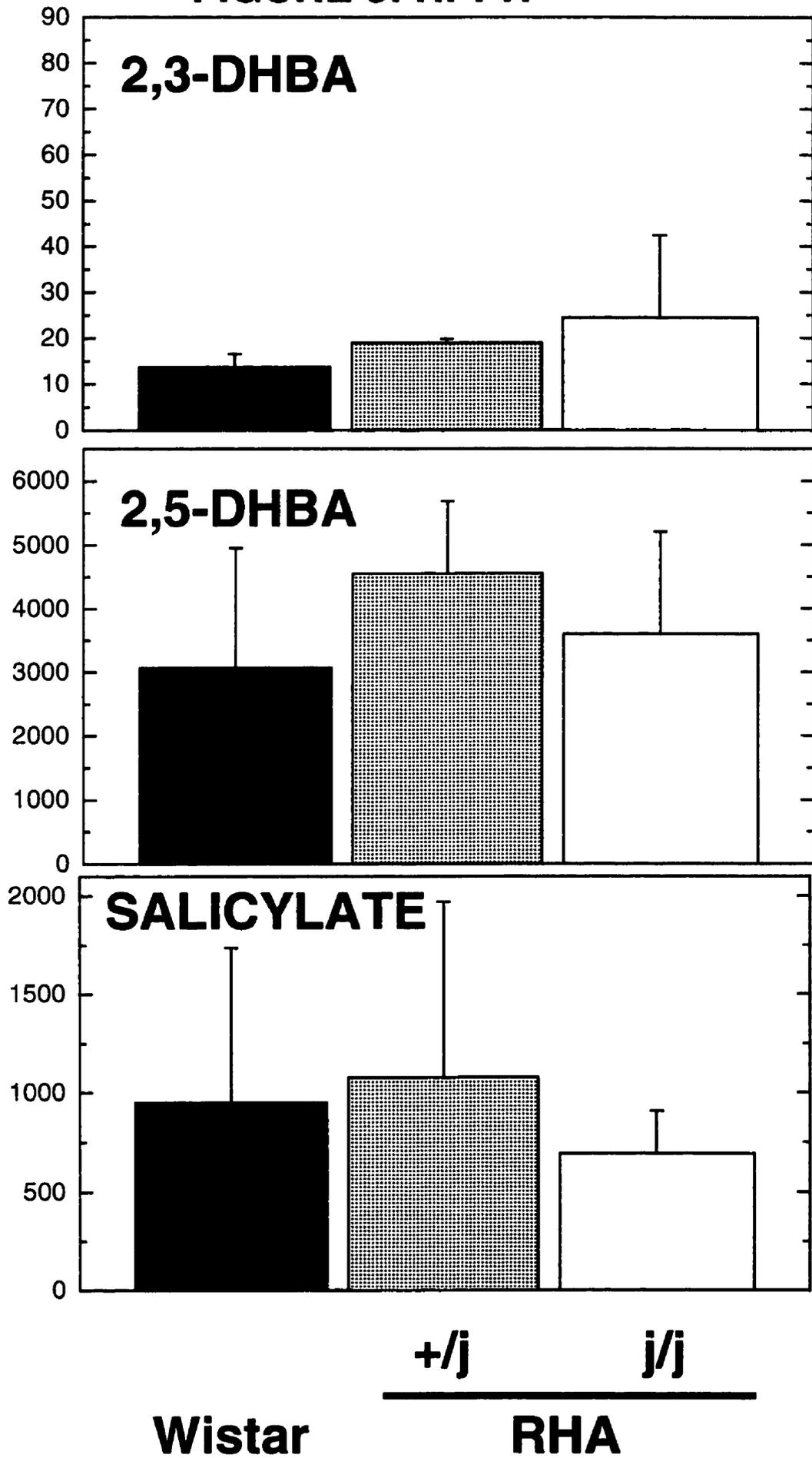
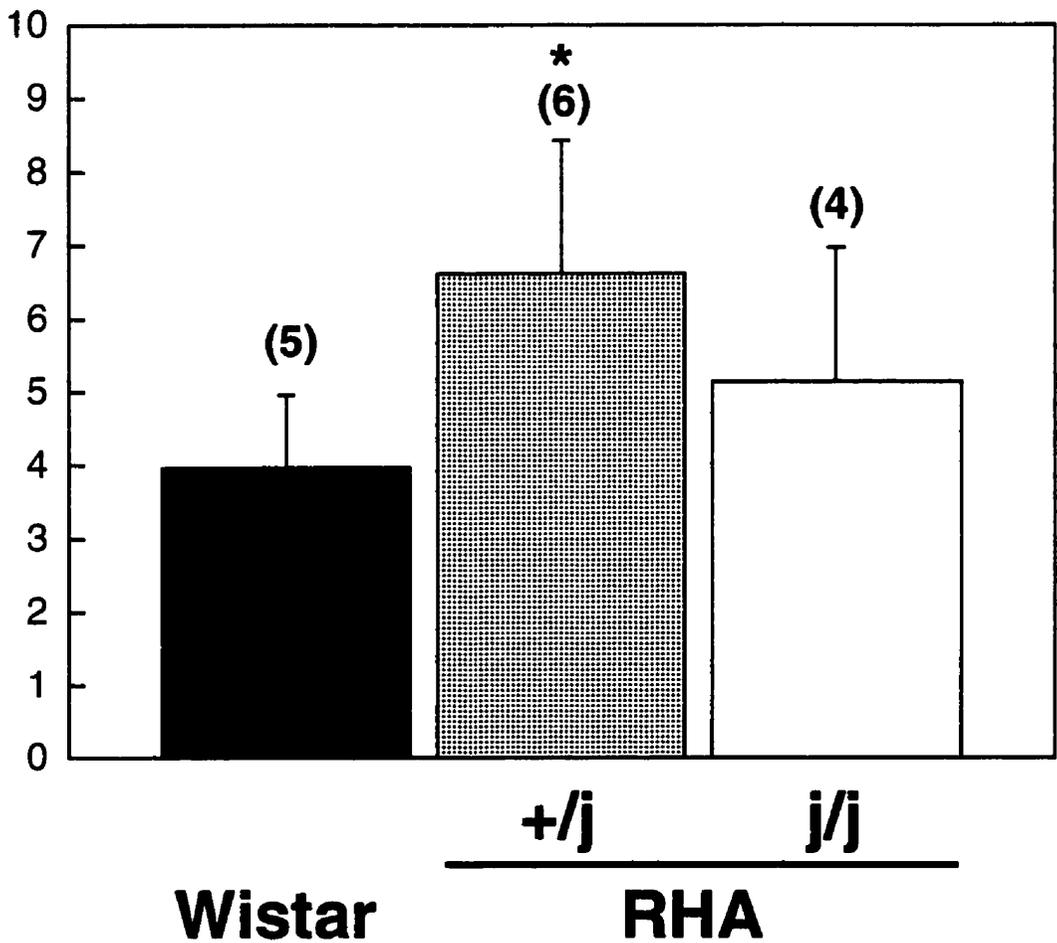
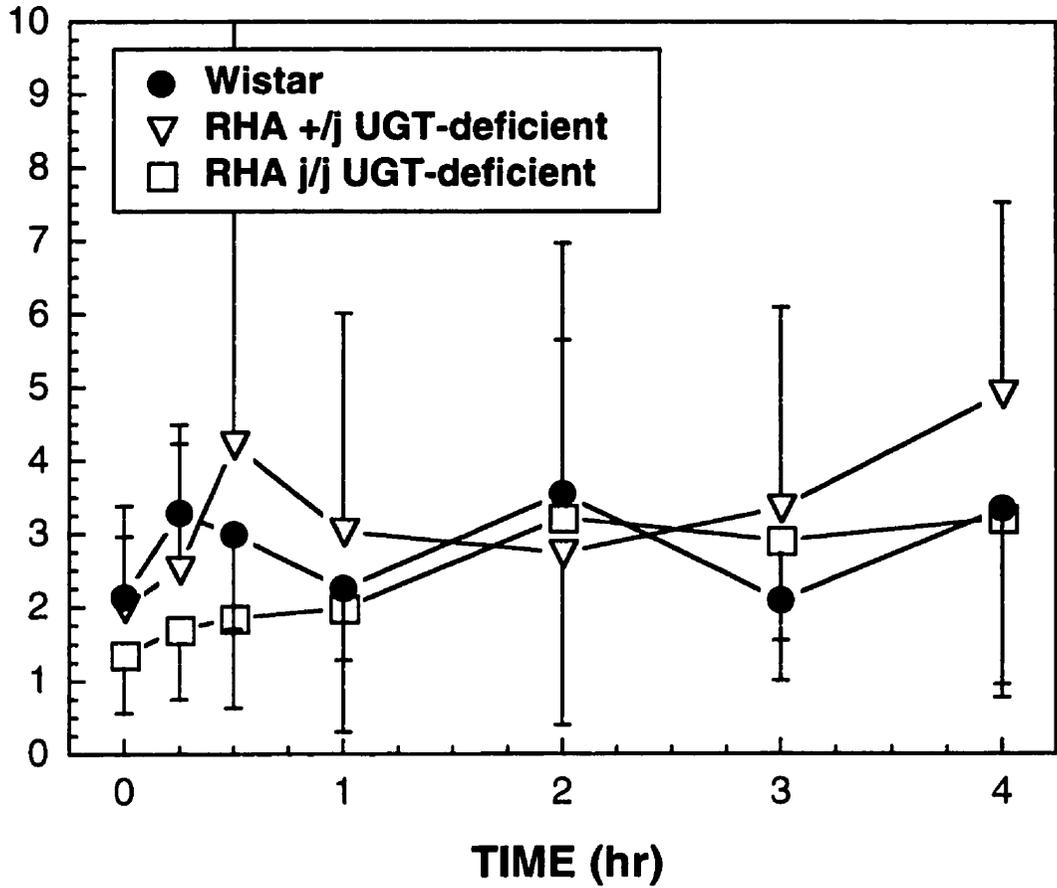


Figure 6. Effect of UDP-glucuronosyltransferase (UGT) deficiencies on phenytoin and 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH)-initiated lipid peroxidation, as measured by thiobarbituric acid reactive substance (TBARS) formation. Rats were injected with either 150 mg/kg phenytoin or an equimolar dose of 191 mg/kg HPPH and were housed in metabolic cages and blood and urine was collected over a four hr period and analyzed. The data for each UGT genotype includes plasma or urine from both phenytoin and HPPH-treated rats. The asterisk indicates a difference from UGT-normal Wistar controls ($p < 0.05$).

FIGURE 6: PHENYTOIN/HPPH

(TBARS, nmol/ μ l, mean \pm SD)

PLASMA
URINE



plasma or urine from both phenytoin and HPPH-treated rats.

Human Bilirubin UGT (UGT1A1) Substrate Specificity

The human UGT1A1 isozyme did not appear to glucuronidate either acetaminophen (**fig. 7**) or phenytoin. However, UGT1A1 did glucuronidate HPPH, and as expected, bilirubin was glucuronidated to both a mono- and diconjugated form (**figs. 7 and 8**). Although not statistically significant, HPPH (3 mM) glucuronidation was increased in alamethicin-activated microsomes ($p < 0.08$), which showed a similar activity at 1mM HPPH (**fig. 9**). HPPH glucuronides were confirmed by incubation with and without β -glucuronidase and observing HPLC peak disappearance (**fig. 10**).

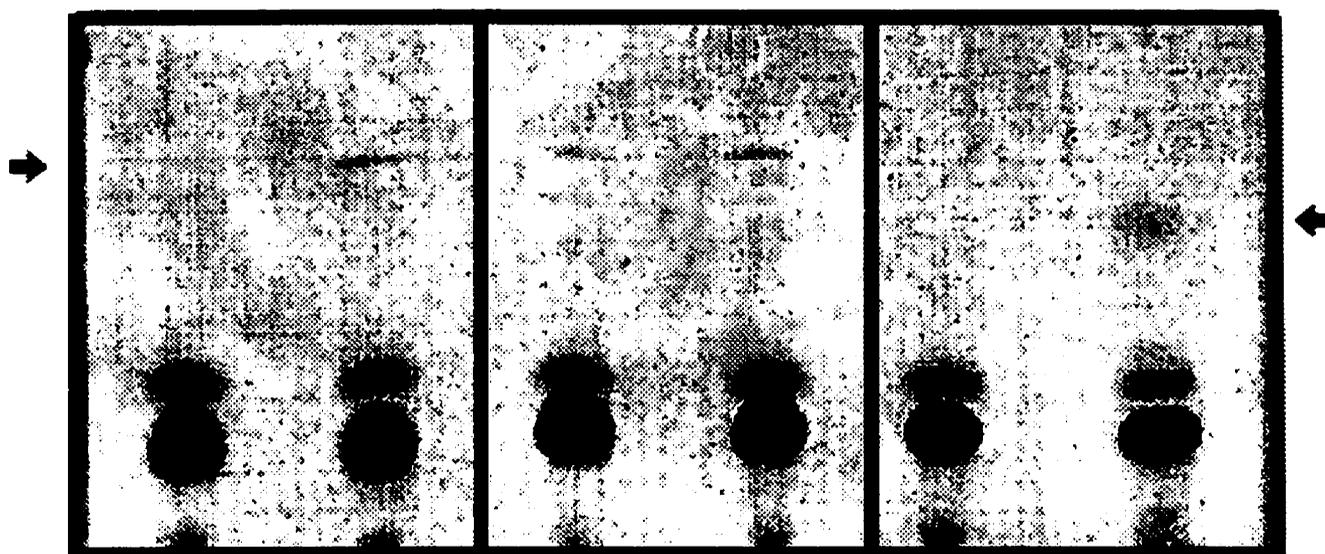
Phenytoin-initiated Embryotoxicity.

GUNN Rats. The resorption rate was increased in +/j UGT-deficient dams treated with a phenytoin dose of 15 mg/kg ($p = 0.0004$) versus similarly treated +/+ UGT-normal controls (**fig. 11**). However, although the 75 mg/kg dose appears to have caused an increase in the incidence of resorptions in +/j dams, it was not higher than the incidence in the 15 mg/kg treated study. Both 100 and 150 mg/kg phenytoin doses caused nearly 100% resorptions in both UGT phenotypes and thus studies with these doses were discontinued. Fetal weights were decreased in fetuses from +/j UGT-deficient dams treated with either 1.5 or 75 mg/kg ($p < 0.05$) (**fig. 11**). Postpartum lethality was increased in pups from +/j UGT-deficient dams treated with the 75 mg/kg dose ($p < 0.05$) (**fig. 12**). External and internal physical anomalies were not apparent in the fetuses from either UGT phenotype.

RHA Rats. Similar to the Gunn rat study, +/j UGT-deficient dams treated with 15 mg/kg phenytoin had an increased incidence of fetal resorptions versus +/+ UGT-normal controls ($p = 0.02$) (**fig. 13**). Interestingly, similar to the Gunn rat study, 75 mg/kg did not increase resorption rates over that of the 15 mg/kg group but appeared to be higher in +/j UGT-deficient dams. There is no apparent decrease in the fetal weights by phenytoin treatment in either phenotype. No external or internal physical anomalies were observed in any of the fetuses.

Figure 7. Autoradiogram of thin layer chromatographic plates. 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) (1 and 3 mM), acetaminophen (APAP) (3 mM) and bilirubin (0.5 mM) were incubated with UGT1A1, with and without the UGT activator alamethicin, as indicated. Arrows indicate glucuronides.

FIGURE 7

Human bilirubin UGT (UGT1A1)-catalyzed glucuronidation

DRUG/CHEMICAL :	NO DRUG	HPPH 1 mM	HPPH 3 mM	HPPH 3 mM	APAP 3 mM	BILIRUBIN 0.5 mM
ALAMETHICIN :	YES	YES	NO	YES	YES	YES

Figure 8. *In vitro* UGT1A1-catalyzed bilirubin mono- and diglucuronidation. Supernatants from an incubation with bilirubin and UGT1A1. UGT1A1 microsomes were incubated with bilirubin for 1 hr at 37°C and supernatants were analyzed by 1 min fraction collections and quantified by liquid scintillation spectrometry.

FIGURE 8

Human Microsomal UGT1A1-Catalyzed Bilirubin Glucuronidation

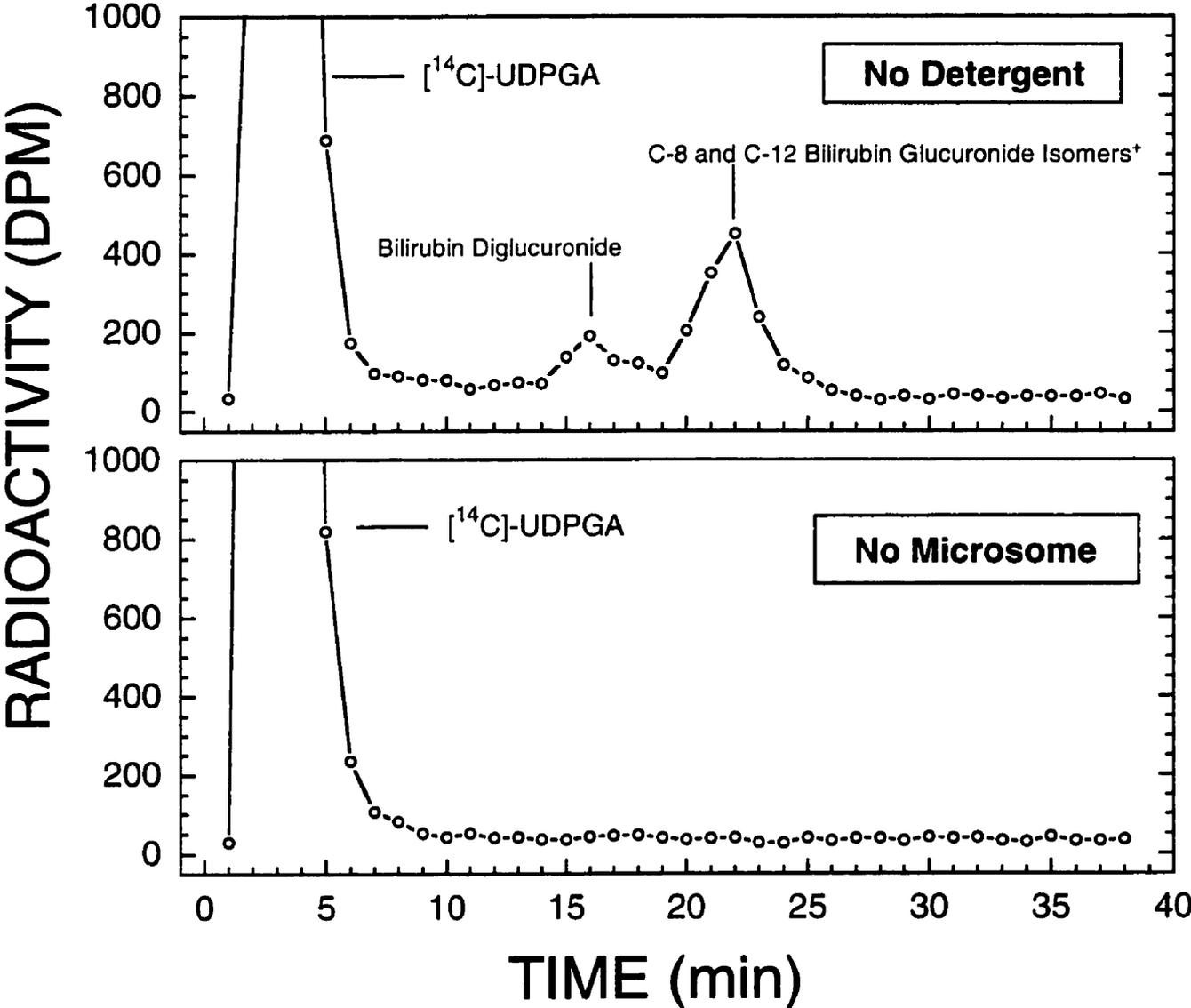


Figure 9. Effect of 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) concentration and alamethicin activation on UGT1A1-catalyzed glucuronidation of HPPH. UGT1A1 microsomes were incubated for 1 hr at 37°C and supernatants were analyzed by HPLC-UV.

FIGURE 9

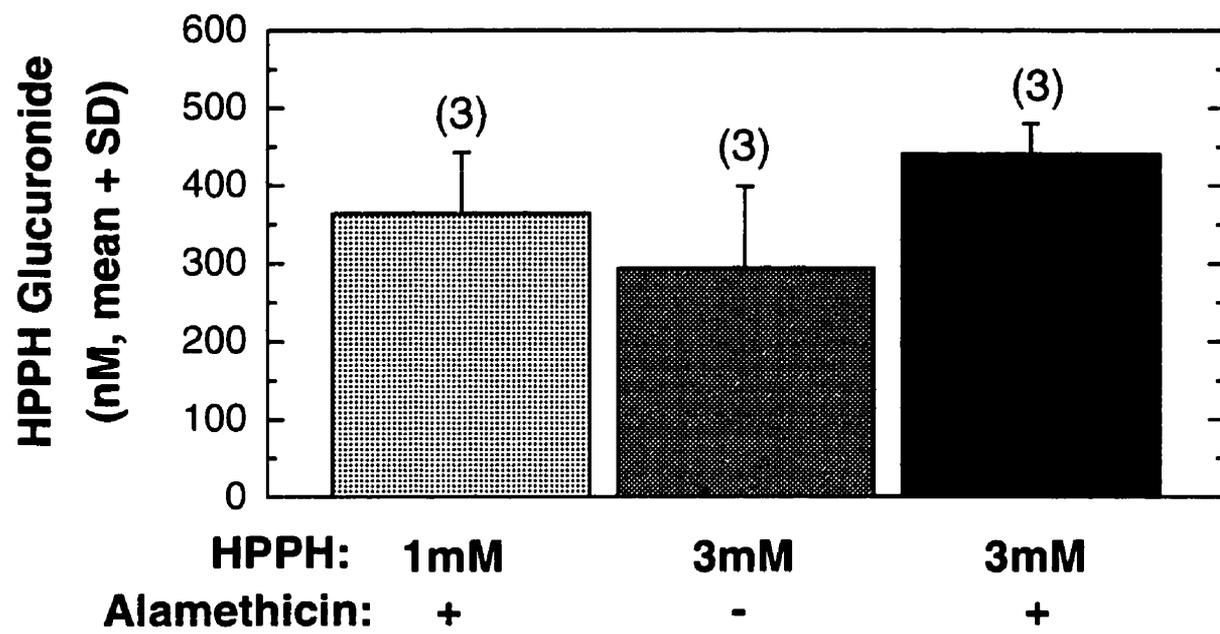


Figure 10. High-performance liquid chromatograms (HPLC)-UV of either; A) supernatants analyzed after UGT1A1 incubation with HPPH, B) reconstituted supernatants analyzed after incubation with the β -glucuronidase vehicle, and C) reconstituted supernatants analyzed after incubation with β -glucuronidase.

FIGURE 10

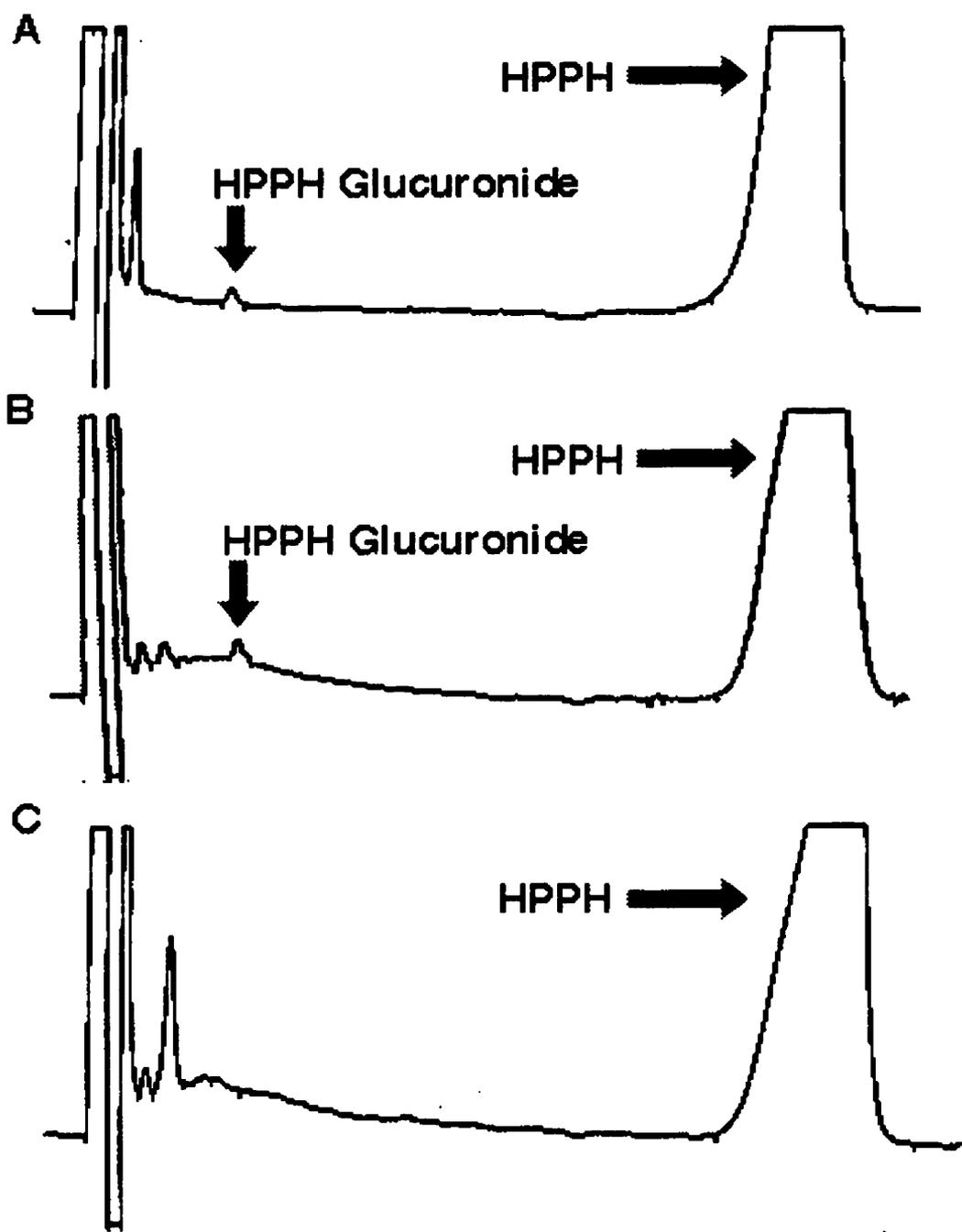


Figure 11. Phenytoin-initiated embryotoxicity in Gunn rats. Pregnant +/+ UGT-normal and +/- UGT-deficient Gunn rats were injected with phenytoin doses from 1.5 to 150 mg/kg, on gestation day (GD) 11 to 14. Dams were c-sectioned on GD21 and fetal resorptions and fetal body weight were determined. Plus symbol indicates a significant difference from UGT-normal ($p < 0.05$).

FIGURE 11
GUNN RATS

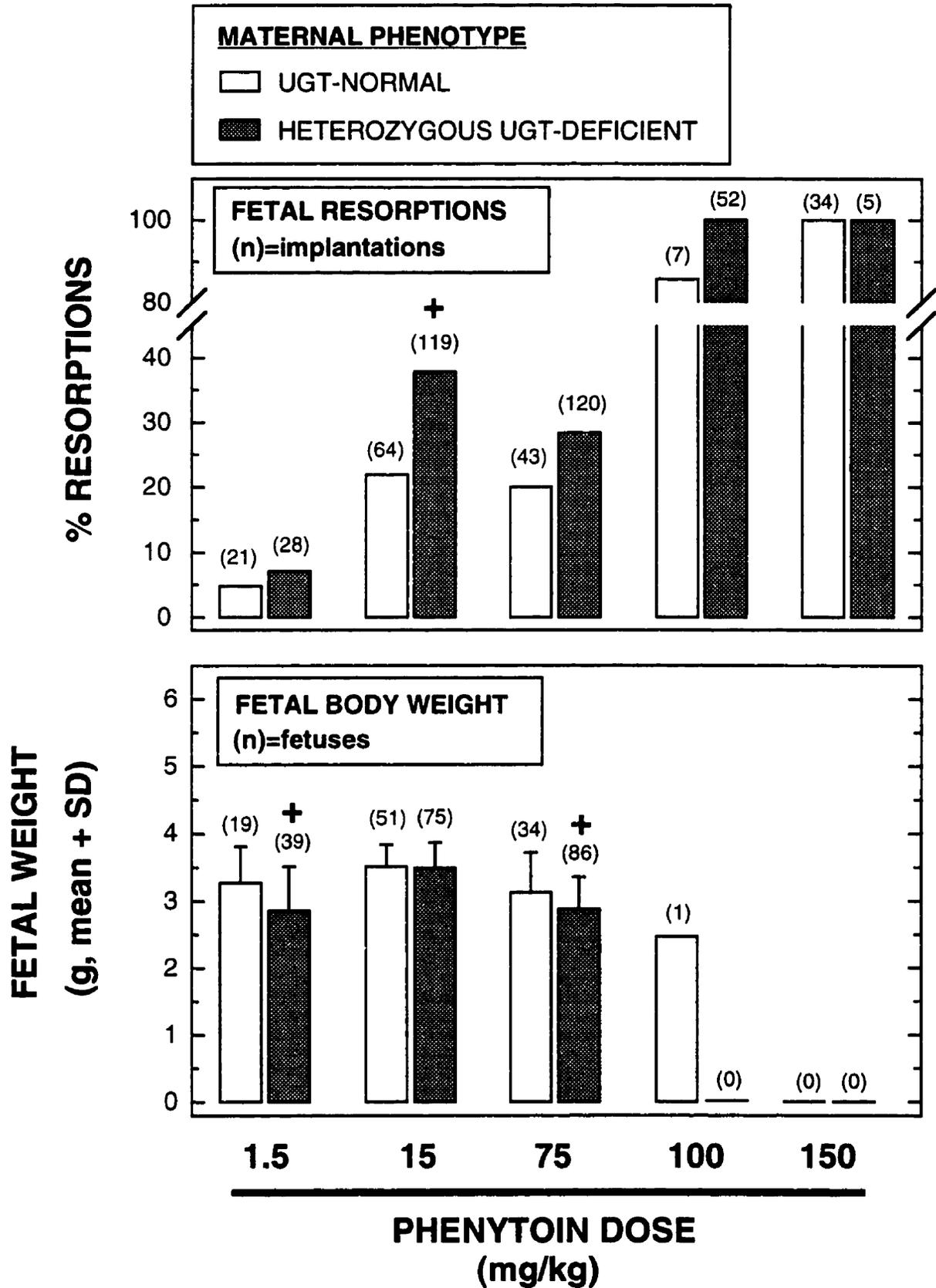


Figure 12. Postpartum lethality of new born pups from phenytoin-treated Gunn rats. Alive fetuses from fig. 11 were monitored for 30 min. to determine postpartum lethality.

FIGURE 12
GUNN RATS

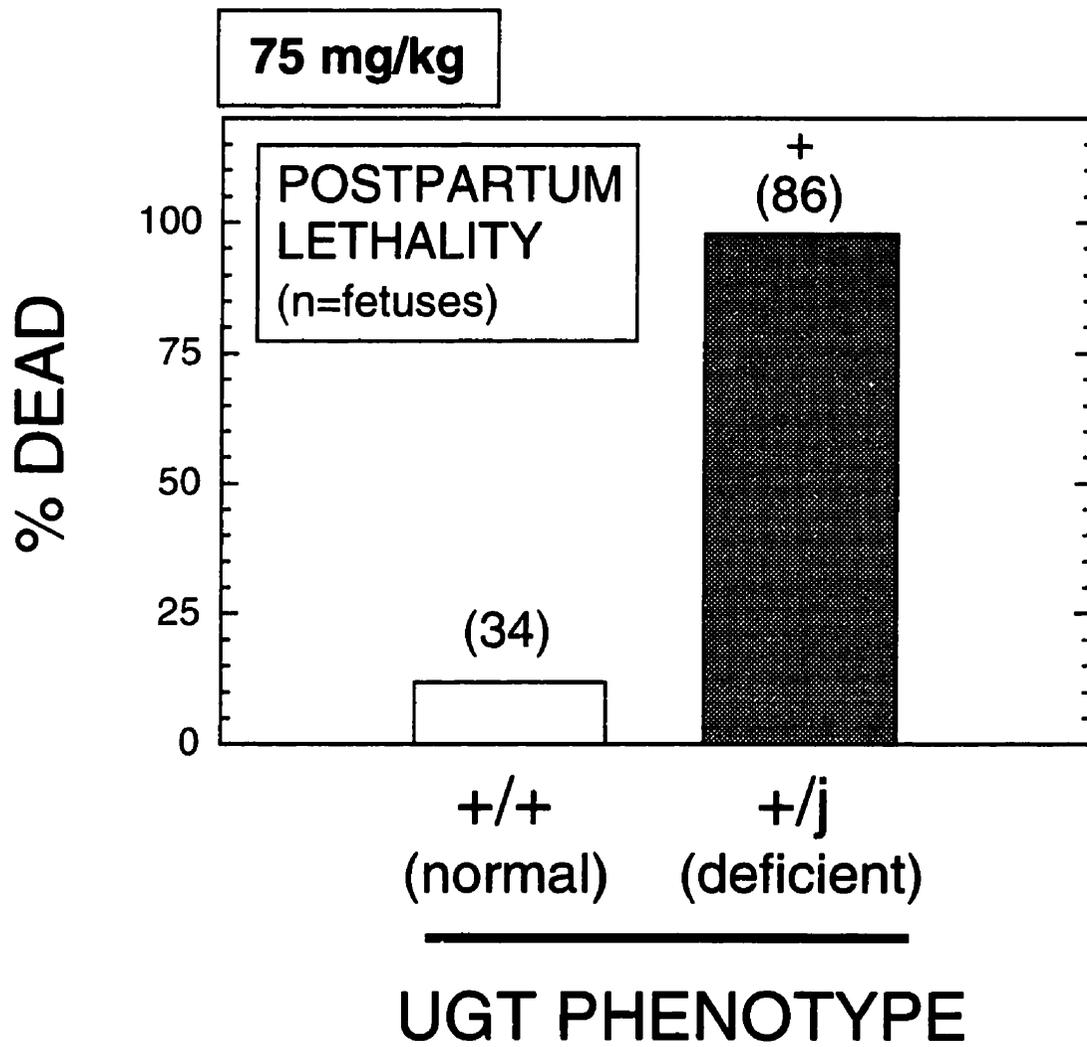
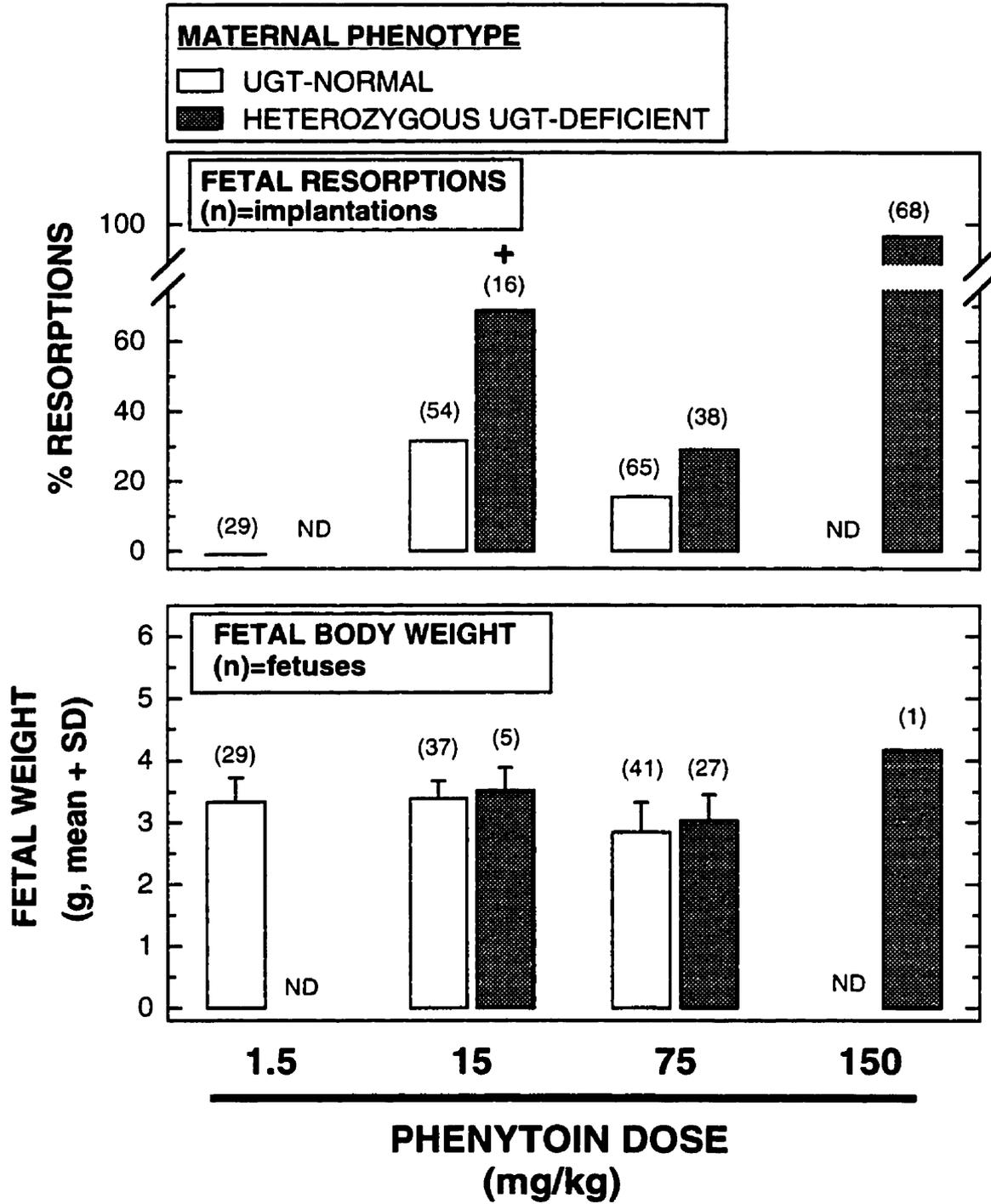


Figure 13. Phenytoin-initiated embryotoxicity in RHA rats. Pregnant $+/+$ UGT-normal and $+/-$ UGT-deficient RHA rats were injected with phenytoin doses from 1.5 to 150 mg/kg, on gestation day (GD) 11 to 14. Dams were c-sectioned on GD21 and fetal resorptions and fetal body weight were determined. Plus symbol indicates a significant difference from UGT-normal ($p < 0.05$).

FIGURE 13

RHA RATS

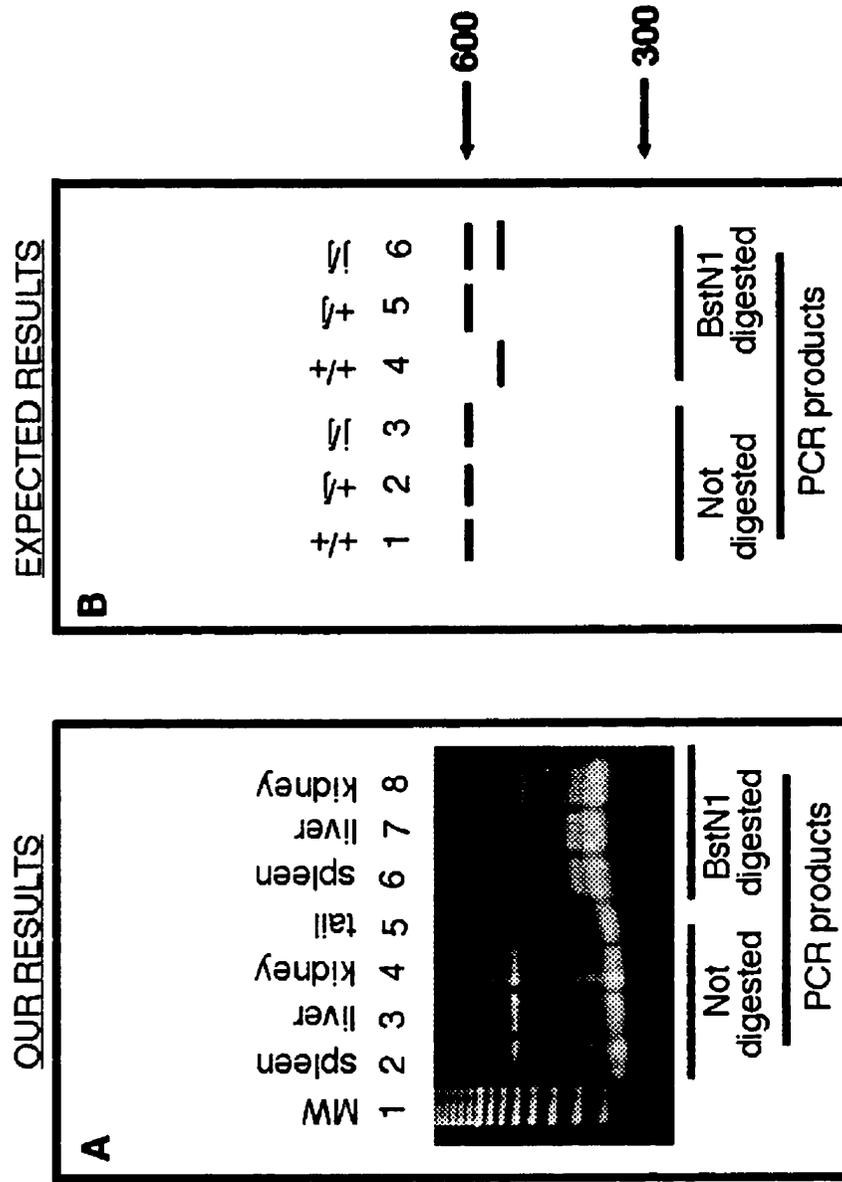


Genotyping. As shown in **figure 14a**, UGT1 cDNA created from mRNA, isolated from a heterozygous (+/j) UGT-deficient adult rat, was amplified using PCR to produce a band at around 600 bp. Upon *Bst*I digestion of this PCR product, the digested DNA did not show the expected characteristic restriction pattern (i.e a 500 and 600 bp band) that was published by Huang et al. (1992) (**fig. 14b**). As discussed previously a mutation in the *UGT1* gene complex is responsible for the loss of all UGT1 isozymes and this mutation also causes a loss of a *Bst*I restriction site. Therefore, as previously shown by Huang et al. *Bst*I digestion of PCR products from UGT-normal (+/+) and homozygous (j/j) UGT-deficient Gunn rats should have produced a single 500 (i.e complete digestion) and a single 600 bp band (i.e. no digestion), respectively. The heterozygous (+/j) UGT-deficient rats should have a double band pattern at 500 and 600 bp. **Figure 14b** diagrammatically demonstrates the expected restriction band pattern for +/+ UGT-normal, j/j and +/j UGT-deficient genotypes, as previously published by Huang et al. (1992).

Figure 14. PCR digestion patterns of UGT1 cDNA (mRNA). Panel A are genotyping results for a male heterozygous (+/j) Gunn rat. Lane 1, 100 bp molecular weight (MW) markers. Lanes 2 to 5 are non-digested PCR DNA and lanes 6 to 8 are BstNI-digested PCR DNA. PCR products were made from cDNA (mRNA) isolated from spleen (2 & 6), liver (3 & 7), kidney (4 & 8) and tail (5) of a heterozygous (+/j) UGT-deficient male Gunn rat.

Panel B diagrammatically demonstrates the expected genotyping results previously published by Huang et al. (1992). Lanes 1 to 3 are non-digested PCR DNA and Lanes 4 to 6 are BstNI-digested PCR DNA from homozygous (+/+) UGT-normal (lanes 1 and 4), homozygous (j/j) UGT-deficient (lanes 2 and 5) and heterozygous (+/j) UGT-deficient (lane 3 and 6) rat liver samples.

FIGURE 14



2.5.5 DISCUSSION

Phenytoin teratogenicity is a major concern in humans since the drug must often be taken throughout pregnancy to control potentially dangerous seizures. Phenytoin can be bioactivated to a free radical which can further initiate reactive oxygen species (**ROS**) production leading to macromolecular damage and toxicity (Kubow and Wells, 1989; Winn and Wells, 1995a,b; Kim and Wells, 1996b; Wells et al., 1997; Parman et al., 1996, 1998) (**fig. 1**). Previous reports have also shown that antioxidants/radical spin trap agents (vitamin E, caffeic acid, α -phenyl-*N*-t-butyl nitron, acetylsalicylic acid), and cytoprotective enzymes (superoxide dismutase, catalase) can reduce or completely abolish phenytoin-initiated embryotoxicity or teratogenicity (see review by Winn and Wells, 1995b; Wells et al., 1997).

In this study we demonstrate that phenytoin- or HPPH-treated heterozygous (+/j) and homozygous (j/j) UGT-deficient RHA rats have increased plasma and urinary concentrations of 2,3-DHBA, a measure of hydroxyl radical formation, over UGT-normal Wistar controls (**figs. 2-5**). In these same animals, plasma and urinary lipid peroxidation were measured and the latter was shown to be increased in +/j rats and a similar trend was seen in j/j rats (**fig. 6**). These results are in agreement with our earlier *in vitro* studies which showed that phenytoin and HPPH initiate more micronucleus formation and DNA oxidation in UGT-deficient cultured rat skin fibroblasts (see Section 2.4). This suggests that the increased genotoxicity seen in UGT-deficient cells may be, at least in part, due to increased phenytoin bioactivation and ROS production.

Perhaps more importantly, these *in vivo* studies demonstrating increased hydroxyl radical formation suggest a possible molecular mechanism for the increased embryotoxicity (i.e. resorption rates or in utero deaths) seen in both UGT-deficient Gunn and RHA rats compared to UGT-normal controls (**figs. 11-13**). These results demonstrate that maternal rat UGT genotype/phenotype is an important modulator of phenytoin embryotoxicity. What remains to be determined is whether phenytoin embryotoxicity is modulated by embryonic/fetal UGT genotype. The evidence to date suggests that, similar to most P450s, most UGT isozymes only begin to be significantly expressed later in gestation and more so after birth (Burchell and Coughtrie, 1989). The fetuses and embryonic/fetal resorptions from our study have been stored at -80°C to

evaluate, in the future, the UGT genotypes and therefore definitively determine the role of embryonic genotype, if any, on phenytoin embryotoxicity.

As discussed in the Results section the UGT genotyping assay did not result in the expected DNA restriction fragment pattern which was previously published by Huang et al. (1992). **Figure 14a** shows our results of the DNA restriction fragment pattern from a heterozygous (+/j) rat and **figure 14b** shows the expected DNA restriction pattern for +/+, +/j and j/j UGT genotypes published by Huang et al. (1992). Upon BstNI digestion, a band at about 500 bp in addition to the 600 bp band was expected, whereas we found no band at about 500 bp, and rather one at about 150 bp, and some lanes had no band at 600 bp. The reason for the aberrant DNA band pattern in our studies is unknown and does not allow us to genotype the rats. In order to verify that the proper DNA sequence (i.e. the proper UGT1 mRNA region containing the mutation) is being analyzed, the cDNA or PCR product should be sequenced in the future. Furthermore, although we have used the mRNA for our preliminary genotyping experiments, future work should use the rat genomic DNA for a number of reasons. Huang et al. (1992) also has published a genotyping assay based on genomic DNA which is simpler than for mRNA. The mRNA genotyping method requires mRNA isolation, cDNA production and the amplification of the cDNA. The DNA method would simply require DNA isolation and amplification, and also would avoid the added difficulty of RNA instability. More importantly, with regard to our phenytoin embryotoxicity studies the genotyping assay based on mRNA may prove to be inappropriate since the expression of most UGT isozymes (e.g. UGT1A1) predominantly occurs postnatally. Therefore genotyping embryos, fetuses and resorptions will require the use of genomic DNA.

Our *in vitro* studies demonstrating human bilirubin UGT1A1 (UGT1*1)-catalyzed glucuronidation of HPPH, suggest that this UGT isozyme may play an important role in phenytoin elimination through HPPH glucuronidation (**fig. 7-10**). This is particularly important since bilirubin UGT1A1 activity is deficient in a large percentage (>10%) of the population (i.e. Gilbert's syndrome) and therefore pregnant epileptic women with this particular UGT isozyme deficiency may be at an increased risk for phenytoin teratogenicity. However, in light of the fact that the

human UGT2B15 isozyme also can glucuronidate HPPH (UGT2B7 cannot glucuronidate HPPH) (see table 3 of Section 1.2.2.4), further work clearly must be conducted in order to demonstrate an association or possible causal link between glucuronidation and teratological susceptibility to phenytoin or other related xenobiotics.

Our *in vitro* studies also show that acetaminophen (**APAP**), at least at the concentration we used, is not a substrate for the human bilirubin UGT1A1 (UGT1*1) (**fig. 7**), which is in agreement with previous data (Senafi et al., 1994) (see Section 1, table 2b). As discussed above (see Section 1.2.4), Gilbert's syndrome is believed to be the result of a promoter region defect located upstream of the bilirubin *UGT1A1* gene, and therefore only the UGT1A1 isozyme is thought to be deficient (Monaghan et al., 1996). If this is true, one would not expect Gilbert's syndrome patients to be deficient in APAP glucuronidation. However, studies from our laboratory are particularly interesting since it was shown that Gilbert's syndrome patients, who were clinically diagnosed based upon bilirubin glucuronidation, were also deficient in their ability to glucuronidate APAP, which also resulted in increased APAP bioactivation (de Morais et al., 1992). This would suggest that, at least for some individuals in the de Morais et al. study, the molecular mechanism(s) leading to the clinical diagnosis of Gilbert's syndrome, must have been the result of defects that not only caused a deficiency in bilirubin UGT1A1 activity (i.e decreased bilirubin glucuronidation), but also a deficiency in the UGT isozyme(s) that glucuronidate APAP. A possible explanation may be a mutation in the common 3' exon region, that encodes for all UGT1 isozymes, leading to a mild UGT1 deficiencies resulting in a deficiency in both bilirubin and APAP glucuronidation. Therefore, this may indicate that genotyping individuals for the homozygous UGT1A1 promoter region defect, thought to be responsible for most if not all Gilbert's syndrome individuals, may in fact be an underestimation of the prevalence of this syndrome, and suggests that mild deficiencies in bilirubin glucuronidation may occur concomitantly with other UGT1 or even UGT2 deficiencies. Similarly a recent report has found that individuals have both the bilirubin UGT deficiency and a deficiency in the enzyme glucose-6-phosphate dehydrogenase, which can also be responsible, via an alternative mechanism, for jaundice (Kaplan et al., 1997).

SECTION 3: SUMMARY, CONCLUSION AND FUTURE STUDIES

3.1 SUMMARY AND CONCLUSION

UGTs are an important family of isozymes for the elimination of numerous endobiotics and xenobiotics. We have studied the protective role of UGTs by investigating how UGT deficiencies can increase the competing xenobiotic bioactivation pathway, which is responsible for most, if not all, of the toxicities we examine. Although other mechanisms, including receptor-mediated events, can contribute to xenobiotic toxicity, we have shown in our studies (see Section 2.1, 2.2) and in previous studies (Winn and Wells, 1995b), that the inhibition of enzyme (P450 and peroxidase)-catalyzed xenobiotic bioactivation, or an increase in cell cytoprotective mechanisms (adding exogenous catalase or superoxide dismutase) can significantly or completely abolish toxicity, such as DNA damage (oxidation, micronucleus formation) and embryotoxicity. This thesis has presented data that continue to support previous evidence suggesting that UGT deficiencies may predispose individuals to increased xenobiotic bioactivation and toxicity, such as teratogenesis and possibly carcinogenesis.

Previous studies demonstrated that B[a]P-initiated micronucleus formation was increased in UGT deficient fibroblasts (Vienneau et al., 1996). However, that study never addressed the molecular mechanisms and lesions involved in the process of micronucleus formation, which up until now, has been suggested to be indicative of carcinogenic initiation. We have demonstrated (see Section 2.1), through the use of chemical inducers and inhibitors, that B[a]P-initiated micronucleus formation can result from either P450- or peroxidase [e.g. prostaglandin H synthase (PHS)]-catalyzed B[a]P bioactivation to a reactive intermediate that can further initiate reactive oxygen species production. We further show that the oxidation of DNA and possibly protein may be responsible for, or at least associated with, the process of micronucleus formation. Therefore our *in vitro* studies suggest that the processes involved in carcinogenic initiation also are responsible for the formation of micronuclei, indicating that micronucleus formation reflects the potential for carcinogenic initiation. One unexpected discovery in the above set of studies was the remarkable finding that TCDD induces not only CYP1A1 but also PHS protein. Subsequent studies have shown that this PHS protein may be PHS-2, and that induction may involve the

dioxin-responsive element (**DRE**) (Kraemer et al., 1992, 1996). Our findings are toxicologically important since they suggest that previous studies using TCDD as a CYP1A1 inducer and xenobiotic bioactivator may need to be reinterpreted in light of the fact that PHS also can bioactivate various xenobiotics to toxic reactive intermediates (see Section 1.4). However, it remains unknown whether TCDD induction of PHS occurs *in vivo*, or whether this induction is an *in vitro* phenomenon.

In further studies, we used the micronucleus assay to demonstrate the importance of UGT-catalyzed glucuronidation in regard to the *in vitro* genotoxicity initiated by both the tobacco specific carcinogen, NNK (see Section 2.2), and the teratogenic anticonvulsant drug phenytoin and its major metabolite HPPH (see Section 2.3). We have shown that these xenobiotics can increase DNA oxidation and micronucleus formation in UGT-deficient rat skin fibroblasts versus UGT-normal cells, and perhaps more importantly, we have demonstrated that a heterozygous UGT deficiency can increase toxicological susceptibility to xenobiotics to a similar extent as that seen in homozygous UGT-deficient cells. This is particularly important since heterozygous UGT-deficient animals are phenotypically normal (i.e. normal plasma bilirubin levels), which would suggest that, under normal non-stressful conditions, a heterozygous UGT deficiency may be a somewhat benign disorder. In fact, in the human population, many individuals are often unaware that they have Gilbert's syndrome until they are either genotyped or phenotyped after fasting (Monaghan et al., 1996). However, our studies demonstrate that xenobiotic exposure, above that which can be compensated for by heterozygous UGT-deficient fibroblasts, may predispose the cells to toxicities, to a similar degree as that seen in homozygous UGT-deficient fibroblasts. This phenomenon of heterozygous susceptibility is not restricted to our *in vitro* studies, but also is demonstrated *in vivo*, where phenytoin can initiate a similarly significant increase in hydroxyl radical formation in both UGT-deficient genotypes over that seen in UGT-normal controls (see Section 2.5). Furthermore, increased heterozygous susceptibility, similar to that for homozygous genotypes, has been seen previously in studies investigating the toxicological importance of enzyme deficiencies such as the cytoprotective enzyme glucose-6-phosphate dehydrogenase (Nicol and Wells, 1996, 1997, 1998), and the p53 tumor suppressor gene (Nicol et al., 1995).

The susceptibility of heterozygous UGT-deficient animals to xenobiotic-initiated toxicity may prove to be particularly important in humans, since heterozygous deficiencies are likely to be more prevalent in the human population. Unlike Gilbert's or Crigler-Najjar syndromes which appear to be recessive disorders occurring only when a homozygous deficiency is present, heterozygous individuals potentially susceptible to xenobiotic toxicity may be much more prevalent in the human population. For instance, Gilbert's syndrome is thought to be due or related to a homozygous variant of the UGT1A1 exon promoter area [i.e. extra TA element, (TA)₇TAA] that appears to occur in the Scottish (10-13%) and Canadian Inuit (17-19%) populations at a relatively high rate, whereas the heterozygous genotype is significantly more prevalent (45-51%) (Monaghan et al., 1996; 1997) (see Sections 1.2.4 and 4.1). Therefore these individuals, and possibly others with a heterozygous mutation in the coding or non-coding regions of *UGT1* or *UGT2* genes, may be susceptible to xenobiotic exposure, even though they are otherwise phenotypically normal.

Our *in vitro* results with phenytoin and its HPPH metabolite suggest that HPPH-initiated toxicity may be potentially important *in vivo* (see Section 2.3). HPPH was equipotent to phenytoin in its ability to initiate micronucleus formation in all three UGT genotypes, suggesting that the teratogenicity of phenytoin may be partially mediated by its metabolite, HPPH. As discussed in section 2.3, although maternally administered HPPH was shown to be non-teratogenic *in vivo* (Harbison and Becker, 1974), we have shown HPPH to be embryotoxic, and other studies have shown HPPH to be mutagenic *in vitro* (Sezzano et al., 1982). Studies in our laboratory have shown that HPPH, similar to phenytoin, can be enzymatically bioactivated to a toxic free radical intermediate (Parman et al., 1998). Our *in vivo* studies (see Section 2.5) confirmed these findings, demonstrating that the *in vivo* production of hydroxyl radical is not only increased in UGT-deficient animals, but also equally initiated by phenytoin or HPPH. Therefore, our studies suggest that the lack of HPPH teratogenicity seen previously (Harbison and Becker, 1974) may have been due to maternal elimination of HPPH via glucuronidation prior to reaching the embryo. This suggests that in the case of UGT deficiencies, individuals treated with phenytoin and deficient in the UGT isozymes responsible for HPPH glucuronidation, may be at

an increased risk for phenytoin teratogenicity.

We have begun to examine the potential contribution of bilirubin UGT1A1 in the glucuronidation of phenytoin and HPPH and have discovered that HPPH, but not phenytoin, is a substrate for the bilirubin UGT1A1 isozyme (see Section 2.5). As discussed above, this may have important toxicological implications, since bilirubin UGT1A1 is frequently deficient in the general population. Therefore this research suggests a risk factor for the anticonvulsant drug phenytoin and its metabolite HPPH, since pregnant epileptic women must continue phenytoin treatment over the entire pregnancy (see Future Human Studies below).

Although *in vitro* and *in vivo* animal studies may be somewhat limited in their human applicability, we feel that the body of evidence, which does include previous human studies (de Morais et al., 1992b; Hu and Wells, 1993, 1998), suggests that UGT deficiencies, whether heterozygous or homozygous in nature, may significantly predispose individuals to xenobiotic-initiated toxicities. UGTs are particularly important since the superfamily of UGT isozymes glucuronidate most xenobiotics, and glucuronidation is a major route of elimination for most of these compounds. In conclusion, our studies have shown that diverse toxicities, including embryotoxicity and possibly carcinogenesis, can be substantially modulated by UGT-catalyzed elimination of potentially toxic xenobiotics, and that hereditary UGT deficiencies may have considerable toxicological relevance.

3.2 FUTURE STUDIES

3.2.1 ANIMAL STUDIES

The fortuitous discovery of the Gunn and RHA rat have been extremely helpful for the study of UGTs as mediators of xenobiotic susceptibility. However, these animal models pose some concern with regard to the level and activity of other toxicologically important enzymes, including P450s, which may be different from other rat strains. When Gunn rats were compared to Wistar rats, the former strain was shown to have a relatively lower level of intracellular heme (Celier and Crestiel, 1991), and although CYP1A1/1A2, as expected, were induced by the polycyclic aromatic hydrocarbon, 3-methylcholanthrene, and therefore possibly by TCDD as well, there also was a suppression of CYP2C11 (Celier and Cresteil, 1989, 1991). Although these studies suggest that experiments investigating xenobiotic-initiated toxicity in Gunn versus Wistar rats in the presence of TCDD must be carefully interpreted since the P450 isozyme responsible for xenobiotic bioactivation may be altered in one strain versus the other, subsequent studies have shown that the suppression of CYP2C11 is not specific to the Gunn rat strain but appears to occur in other rat strains such as the Fischer 344 strain (Safa et al., 1997). Nevertheless, strain difference in our studies (Gunn versus Wistar) should not be a concern since CYP2C11 is not responsible for the bioactivation of most of the xenobiotics that we have investigated, and although CYP2C11 can form B[a]P depurinating adducts (Todorovic et al., 1991), this would only be a concern if we had performed induction studies in the Gunn vs Wistar rat strain. The B[a]P-initiated micronucleus studies with TCDD induction were all performed in different Gunn UGT genotypes and thus CYP2C11 activity would be expected to be similar in all treatment groups (see Section 2.1). Additionally, previous studies conducted in our laboratory have shown that CYP1A1, CYP2B activity, total P450 content and the activity of the cytoprotective enzyme glutathione S-transferase were the same in Wistar rats, as compared to +/j and j/j UGT-deficient Gunn rats (Hu and Wells, 1992). This suggests that the toxicological susceptibility seen in UGT-deficient Gunn rats versus Wistar rats was most likely a function of a decrease in UGT-catalyzed xenobiotic glucuronidation.

To avoid the problem of strain differences in drug metabolizing enzymes, in many of our studies, we used the different UGT genotypes (+/+, +/j, j/j) Gunn or RHA rats, the latter of which also are congeneric. This theoretically should avoid strain differences since the Gunn and definitely the RHA rat will only be different in UGT1 enzyme activity. However, caution again must be used since the resulting phenotype in the congeneric animals may affect the interpretation of results. For example, Kapitulnik and Gonzalez (1993) investigated the mRNA and protein levels of CYP1A1 in Gunn rat litter mates and demonstrated that pups from the same litter that were homozygous for the UGT1 deficiency (i.e. jaundiced) had substantially higher levels of CYP1A1 than heterozygous, non-jaundiced litter mates. This study shows that although the genetic makeup of the rats were similar, except for the *UGT1* locus, the phenotype (i.e. increased bilirubin levels) was thought to be responsible for a dramatic increase in CYP1A1 levels. Recent studies have suggested that bilirubin may in fact be an endogenous ligand for the Ah receptor and therefore the CYP1A1 induction seen, may have been due to an Ah receptor-mediated effect (Sinal and Bend, 1997; Denison et al., 1998). However, the role of bilirubin as an inducer of CYP1A1 via the Ah receptor remains questionable, since Kapitulnik and Gonzalez (1993) saw an increase in CYP1A1 in the jaundiced Gunn pups only up to the age of 1 month. If elevated bilirubin concentrations were solely responsible for the increased level of CYP1A1, we would not expect to see a decrease in CYP1A1 levels in adults, similar to that seen in non-jaundiced Gunn rats, since the homozygous UGT-deficient Gunn rats remain jaundiced throughout their lifetime.

Recent advances in molecular biology have allowed investigators to create genetically engineered mice that have been manipulated to selectively "knockout" a specific gene of interest. This same technology can be used to create mice that are selectively knocked out for a particular UGT1 or UGT2 isozyme. Recently, Nebert and Duffy (1997) have discussed their approach at creating a *UGT1a6* knockout mouse. The advantages of creating Ugt knockout mice, versus the continued use of Gunn or RHA rats, would be both practically and scientifically important. From a practical standpoint for toxicological and teratological research, mice would be cheaper, easier to handle, require less xenobiotic exposure per gram of body weight (i.e. more sensitive species), and would be easier to breed than rats. From a scientific perspective, one could create Ugt1

knockout mice that would not necessarily be bilirubin Ugt1A1-deficient and thus, as discussed above, avoid the possible problems associated with a jaundiced animal. Ugt2 knockout mice also could be constructed to study the toxicity associated with exposure to xenobiotics that are predominantly glucuronidated by Ugt2 isozymes. These studies regarding the importance of UGT2 catalyzed xenobiotic glucuronidation could not be conducted since the Gunn and RHA rats have normal UGT2 activities. Therefore once the UGT isozyme(s) responsible for the glucuronidation of the xenobiotic of interest have been determined, knockout mice for these Ugts could be created to investigate, unequivocally, their toxicological protective role against xenobiotic-initiated toxicities. Ugt isozyme knockout mice may be advantageous for teratology studies since the homozygous Gunn and RHA rats are unable to breed. Therefore teratological studies, should be conducted in Ugt knockout mice since the homozygous genotype may be able to breed and thus may prove to be a more revealing study than that being conducted in UGT-normal versus heterozygous (+/j) UGT-deficient Gunn and RHA rats (see Section 2.5).

Much of the work from this laboratory has suggested that UGTs are critical modulators against xenobiotic (e.g. B[a]P and NNK) toxicity, which includes potential carcinogenic initiating events (i.e. DNA oxidation and covalent binding). Future work in this field should build on this past knowledge of data and therefore study the importance of UGT deficiencies in either B[a]P or NNK-initiated carcinogenesis. Since all of the previous work has been conducted in the Gunn or RHA rat, it may be the most appropriate animal model to use. However, if toxicological studies in Ugt isozyme knockout mice support the hypothesis that UGTs are an important modulator of toxicity, then one may consider in the future to investigate xenobiotic-initiated carcinogenesis in the knockout mice.

3.2.2 HUMAN STUDIES

The toxicological importance of bilirubin UGT deficiency and the associated jaundice has been the major focus of most investigators in this field. Previous work from our laboratory has shown that humans with at least the bilirubin UGT1A1 deficiency (i.e. Gilbert's syndrome) have an increase in either *in vivo* acetaminophen bioactivation or *in vitro* B[a]P cytotoxicity (de Morais

et al., 1992b; Hu and Wells, 1993, 1998). These studies have also demonstrated that, in so called “normal” individuals (i.e. do not have Gilbert’s syndrome), other underlying UGT deficiencies exist that can compromise the glucuronidation of acetaminophen or B[a]P and increase bioactivation and/or toxicity. Therefore these so called, “normal” individuals appear to have deficiencies in other UGT isozymes (i.e. not bilirubin UGT1A1) responsible for the glucuronidation of B[a]P-hydroxylated metabolites or acetaminophen, and thus may represent an unrecognized toxicologically susceptible group of individuals that are not phenotypically obvious.

This work suggests that the characterization of toxicologically important human UGT isozymes must be carried out. We (see Section 2.5) and other laboratories (see Section 1.2.2.4) have begun to investigate UGT isozyme-substrate specificity of individual human and animal UGTs. Continued work from this lab should be conducted to determine the UGT isozymes responsible for the glucuronidation of phenytoin, HPPH, B[a]P and NNK, and/or their metabolites. As discussed in the Introduction, the production of single UGT isozyme-transfected cells now makes this task easier. Information regarding the UGT isozyme-substrate specificity for these toxicologically important substrates will allow future investigators to study the role of these particular UGT isozyme deficiencies in relation to diseases such as smoking (B[a]P, NNK)-induced lung cancer or phenytoin-induced birth defects. In fact, since we have shown that HPPH (the major phenytoin metabolite) is glucuronidated by bilirubin UGT1A1, we can quickly genotype (see Section 4.1) epileptic phenytoin-treated mothers and their children for the variant of the bilirubin UGT1A1 promoter area seen in Gilbert’s syndrome patients. This study may demonstrate that the extra TA element seen in 10-13% of the population may be associated with a predisposition to phenytoin/HPPH-initiated teratogenesis.

SECTION 4: APPENDICES

4.1 HUMAN UGT1A1 (UGT1*1) PROMOTER (TATAA) DEFECT GENOTYPING

4.1.1 INTRODUCTION

We have shown that human bilirubin UGT1A1 (UGT1*1) glucuronidates HPPH, the major p-hydroxylated metabolite of phenytoin, and further show that UGT1 deficiencies can enhance phenytoin and HPPH-initiated micronucleus formation and embryotoxicity (see Sections 2.3 and 2.5). Therefore, since UGT1A1 deficiencies (i.e. Gilbert's syndrome) are quite prevalent in the human population (>10%) we were interested in the teratologic potential of embryonic/fetal phenytoin exposure in pregnant epileptic mothers. We began to investigate the teratologic potential of UGT1A1 deficiency by first genotyping normal human control individuals for the variation in the TATAA promoter region, which is thought to be responsible for Gilbert's syndrome (Monaghan et al., 1996).

As discussed above (see Section 1.2.4) Monaghan et al. (1996) demonstrated that individuals with Gilbert's syndrome were homozygous for an extra TA element in the promoter sequence, located directly upstream of the bilirubin *UGT* (*UGT1A1*, *UGT1*1*) exon. The genotype designations are 6/6, 6/7 or 7/7 for individuals that have; six TA repeats on both alleles, a six and a seven TA repeat on each allele, or have seven TA repeats on both alleles. The genotyping method involves amplifying the promoter sequence and resolving the 98 bp or 100 bp product on a polyacrylamide sequencing gel. The method was kindly given to us by Professor Brian Burchell (University of Dundee, Dundee, Scotland) and involves the following steps (Please note that this method is patent protected):

4.1.2 METHODS

- Step#1: Synthesize the following primers:
 HP3GM3: 5' GTCACGTGACACAGTCAAAC 3'
 HP3GM4: 5' TTTGCTCCTGCCAGAGGTT 3'
- Step #2: Extract DNA from whole blood using a Qiagen blood kit.
- Step #3: End-label the forward primer (HP3GM3). (NB: 1X = amounts for one 20 µl PCR reaction)
- | | |
|--|-----------------------|
| •Primer HP3GM3 @ 10 pmol/µl | 4X
1 µl |
| • [γ- ³² P]-ATP (10 µCi) (NEN Dupont) | 1 µl |

- | | | |
|-----------|---|--|
| | • 10X T4 Polynucleotide kinase buffer (Promega) | 0.5 μ l |
| | • T4 Polynucleotide kinase @ 10 U/ μ l (Promega) | 0.5 μ l |
| | • ddH ₂ O | 2 μ l |
| | <u>Total</u> | <u>5μl (2 pmol/μl)</u> |
| Step #4: | Prepare PCR reaction master mixture. | |
| | | <u>1X (i.e. for one reaction)</u> |
| | • dNTPs each @ 2mM | 2 μ l |
| | • 10X Promega MgCl ₂ -free buffer | 2 μ l |
| | • MgCl ₂ @ 25 mM | 1.2 μ l |
| | • Primer HP3GM4 @ 10 pmol/ μ l | 0.5 μ l |
| | • Primer HP3GM3 @ 10 pmol/ μ l | 0.25 μ l |
| | • Primer HP3GM3 @ 2 pmol/ μ l (radiolabelled) | 1.25 μ l |
| | • ddH ₂ O | 12.32 μ l |
| | • Taq Polymerase @ 5 U/ μ l | <u>0.08 μl</u> |
| | <u>Total</u> | <u>19.6 μl</u> |
| Step #5: | Take 19.6 μ l of the reaction master mix and add 0.1 to 0.2 μ g of DNA in a 0.4 μ l volume. | |
| Step#6: | Amplify DNA using the following conditions. 5 min at 95°C followed by 30 cycles of 95°C 30 sec, 58°C 40 sec and 72°C 40 sec. The PCR products will either be 98 bp [(TA) ₆ TAA] or 100 bp [(TA) ₇ TAA] depending on the genotype of the individual. | |
| Step #7: | Prepare samples for electrophoresis.
Mix 5 μ l of PCR product with 5 μ l of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and heat sample at 95°C for 1 min and immediately place on ice for at least 5 min prior to loading. | |
| Step #8: | Prepare a standard sequencing gel [6% denaturing polyacrylamide gel (19 acrylamide: 1 bis acrylamide)] | |
| Step #9: | Prerun gel for 30 min (do not exceed 50°C). Load 2-4 μ l of PCR/dye mixture and run at 110 Watts for 1 hr and 40 min. | |
| Step #10: | Autoradiography: Cover gel with cling-film and expose gel to autoradiographic film, between intensifying screens at -70°C for 15 min and then develop film. By examining the migration pattern of the PCR products, one can determine the genotype of the individual. The 6/6 genotype should have a single band at 98 bp, while the 7/7 genotype should have a single band at 100 bp, and the 6/7 genotype should have both bands. | |

4.1.3 RESULTS AND DISCUSSION

We have genotyped normal individuals for the presence of the extra TA element by first amplifying the promoter area using the above primers. In order to verify that we are amplifying the correct region of the *UGT1A1* gene (cDNA), we first sequenced the PCR product to determine if it contains the promoter sequence of interest (Biotechnology Services, University of Toronto).

The sequence of the TATAA promoter region is listed for four control individuals (see below). None of the samples contained the extra TA element and therefore were homozygous for the six TA repeat promoter sequence and thus did not have Gilbert's syndrome. No pregnant epileptic women taking phenytoin have been genotyped, but future studies should investigate the relationship between phenytoin exposure, birth defects and the TATAA promoter sequence of the *UGT1A1* gene. As discussed above, since HPPH is glucuronidated by human UGT1A1 and since HPPH is more genotoxic in UGT-deficient cells, this UGT1A1 isozyme may prove to be an important toxicological determinant of human phenytoin teratogenicity.

CONTROL SAMPLE TATAA PROMOTER SEQUENCES:

Sample (CN): 5'- TGCCATATATATATATATAAGTAGGAGAGGGCGAACCTCTKGCAAGGAG-3'

Sample (LW): 5'-CCATATATATATATATAAGTAGTAGAGRGCGARCATCTCGNAC-3'

Sample (PK): 5'-GCCATATATATATATATAAGTGTGAGAGRGGGAGACATCTCGGCGGCGACMCG-3'

Sample (TP): 5'-TGCCATATATATATATATAAGTAGGAGAGGGCGAACCTCTGG SAGGNNNNNNN-3'

Note: The sequences above contain a number of IUPAC ambiguous codes (italicized) as listed below. Note that no ambiguities exist within the critical TATAA sequence.

IUPAC Ambiguous codes:

B	not A
D	not C
H	not G
K	G or T
M	A or C
N	any base
R	A or G
S	C or G
V	not T
W	A or T
Y	C or T

4.2 INVESTIGATION OF THE TOBACCO-SPECIFIC CARCINOGEN 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) FOR *IN VIVO* AND *IN VITRO* MURINE EMBRYOPATHY AND EMBRYONIC *ras* MUTATIONS^{1,2}

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1. A preliminary report was presented at the 35th annual meeting of the Society of Toxicology [Fundam. Appl. Toxicol. 30 (Suppl. No. 1, Part 2: 198, 1996)]. This research was supported by a grant from the Medical Research Council of Canada.
2. Published in Journal of Pharmacology and Experimental Therapeutics (in press).
3. Perry Kim aided in the first draft of the manuscript and conducted the original teratology studies investigating NNK-initiated teratogenicity at the three different gestational times (see fig. 2). All other work was conducted by Louise Winn.

4.2.1 ABSTRACT

The teratological potential of the carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is unknown. *In vivo*, NNK (100 mg/kg ip) was administered to pregnant CD-1 mice during organogenesis, with or without pretreatment with the P450 inducer phenobarbital (60 mg/kg ip). With NNK alone, 3 of 374 fetuses had open eye and one had a cleft palate, which were not observed in 160 controls. With phenobarbital plus NNK, 2 fetuses had a cleft palate, 2 had exencephaly and 1 had a kinky tail, while phenobarbital controls showed no anomalies ($p < 0.05$). NNK-initiated fetal postpartum lethality was enhanced by phenobarbital pretreatment. There were no fetal skeletal anomalies or alterations in resorptions or fetal body weight in any group. In embryo culture, gestational day 9.5 embryos exposed to 10 μM NNK had decreases in yolk sac diameter, crown-rump length and somite development ($p < 0.05$), and 100 μM NNK decreased anterior neuropore closure and crown-rump length ($p < 0.05$). Embryos exposed to 100 μM NNK were assessed for *K-ras* codon 12 mutations and none were detected. This is the first evidence of NNK teratogenicity and embryotoxicity, the molecular mechanism of which appears to differ from that for its carcinogenicity.

4.2.2 INTRODUCTION

Fetal effects of exposure to cigarette smoke include decreases in human fetal birth weight, intrauterine growth retardation, premature delivery, perinatal mortality, spontaneous abortion and fetal malformations including cleft palate (Khoury et al., 1987; Khoury et al., 1989). Exposure of children to cigarette smoke is also associated with increased rates of sudden infant death syndrome, respiratory illness, asthma and middle ear effusion (Law and Hackshaw, 1996; Am. Acad. Ped., 1997).

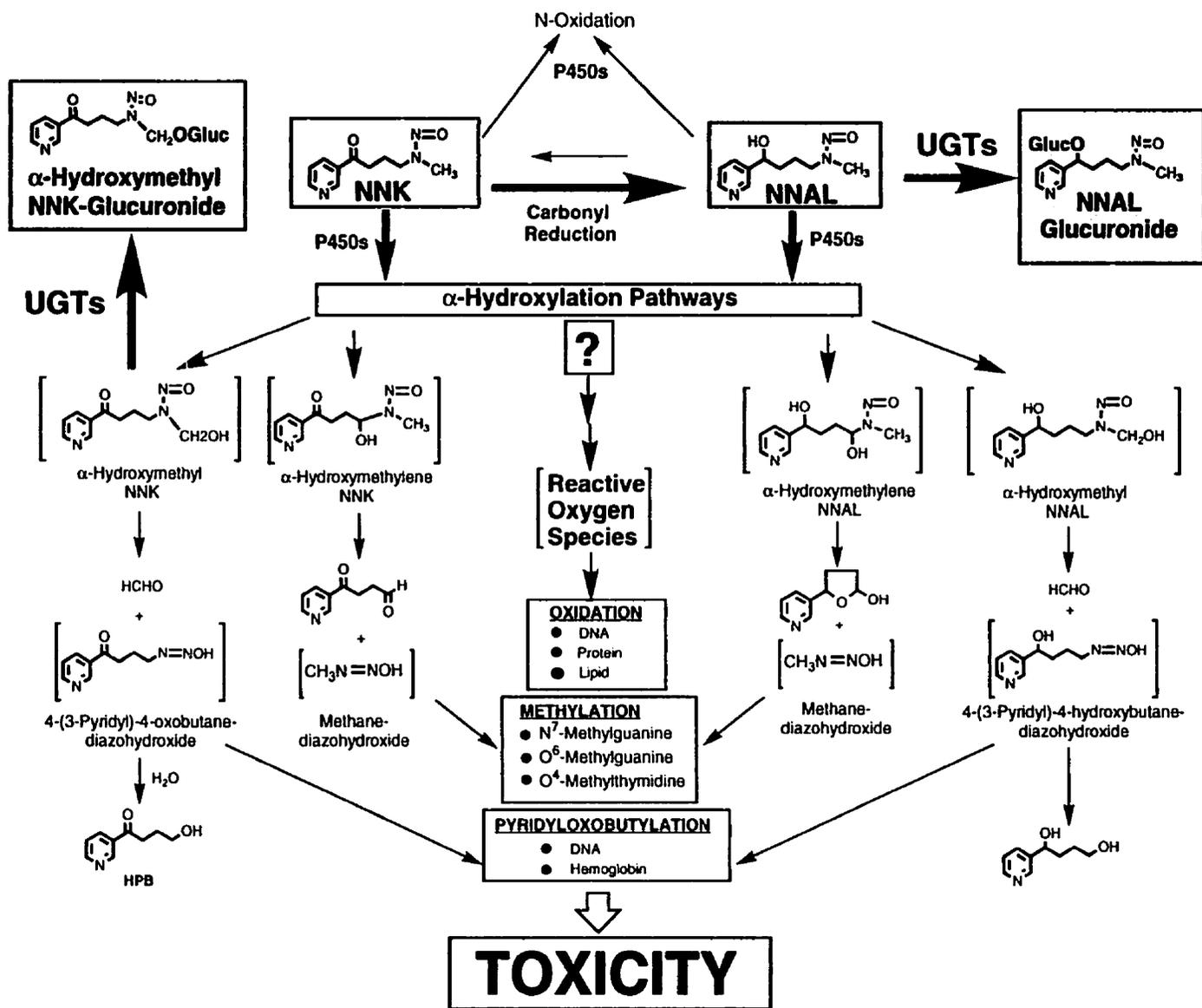
At least 43 of the 3800 chemicals found in cigarette smoke, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are carcinogenic in experimental animals (Hecht, 1996). NNK is the most potent carcinogen of at least seven identified tobacco-specific nitrosamines, and is present at high levels in commercial tobacco products (Hoffmann and Hecht, 1985).

Although NNK-initiated carcinogenesis is well documented, it is still unknown if *in utero* exposure to NNK is teratologically important. Studies have shown that NNK can cross the mouse and hamster placenta, and can initiate various tumors in fetuses born to NNK-treated dams (Anderson et al., 1989; Correa et al., 1990). In humans, there is an increase in DNA-carcinogen adducts in the placenta from women who smoked during their pregnancy compared to nonsmokers (Everson et al., 1988). Furthermore, maternal administration of radiolabelled NNK in mice results in covalent binding to gestational day 18 fetal tissues (Castonguay et al., 1984), and NNK initiates both micronucleus formation in fetal hamster liver (Alaoui-Jamali et al., 1989), and DNA oxidation in fetal mouse tissues (Sipowicz et al., 1997), indicating placental transfer and bioactivation of NNK by maternal and/or fetal tissues.

NNK, a derivative of the N-nitrosation of nicotine, can undergo reversible carbonyl reduction leading to the formation of two enantiomers of the N-nitroso alcohol, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (**fig. 1**). P450-catalyzed α -hydroxylation of either NNK or NNAL at either the α -methyl or the α -methylene carbons can lead to the formation of reactive intermediates capable of methylating DNA (O^6 -methyldeoxyguanine, 7-

Figure 1. Postulated metabolic pathway mediating the toxicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK is converted to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). Both NNK and NNAL can be α -hydroxylated by various cytochromes P450 (P450s) at either the α -methyl or the α -methylene carbons, producing reactive intermediates that can methylate or pyridyloxobutylate DNA. NNK can also initiate DNA oxidation, possibly via the formation of ROS, but the bioactivating enzymes have not been characterized.

FIGURE 1



methyldeoxyguanine, O⁴-methyldeoxythymidine) or pyridyloxobutylating DNA, respectively (Hecht, 1996).

Although NNK can form an electrophilic reactive intermediate, recent studies have shown that NNK also may initiate the formation of reactive oxygen species (ROS). Radical “scavengers” reduced the amount of NNK-initiated DNA single strand breaks in cultured human lung cells, suggesting that at least part of the genotoxicity of NNK was ROS-mediated (Weitberg and Corvese, 1993). Similarly, Xu et al. (1992) showed that multiple dosing of NNK increased DNA oxidation in mouse lung, which was reduced with concomitant administration of green tea that contains an antioxidant (polyphenol, (-)-epigallocatechin gallate). Similarly, in rat skin fibroblasts, NNK-initiated micronucleus formation was inhibited by the antioxidative enzyme superoxide dismutase (Kim and Wells, 1996a).

The results of the above studies support the hypothesis that, in addition to the toxicity initiated by electrophiles, alternative ROS-initiated DNA damage may contribute to NNK toxicity. It is unclear at this point whether P450s and/or other enzymes, such as peroxidases, bioactivate NNK to reactive intermediates capable of producing ROS. Peroxidases, such as prostaglandin H synthase (PHS) are known to bioactivate various xenobiotics to free radical intermediates, leading to ROS formation (Marnett, 1990a,b).

Ras proteins are involved in signal transduction controlling cell growth and differentiation, and are expressed at relatively high levels throughout all stages of development where a high degree of cellular differentiation is occurring (organogenesis) (Slamon and Cline, 1984; Barbacid, 1987). Ras oncogenes have acquired specific point mutations that code for Ras proteins which are constitutively active, and they are the most prevalent oncogenes detected in human cancers (Barbacid, 1987; Bos, 1989). Mutations in codon 12 of the K-*ras* gene have been detected in NNK-initiated lung tumors in mice (Belinsky et al., 1989; Ronai et al., 1993). Since embryogenesis, like tumorigenesis, is a process whereby cells proliferate extensively, but in a tightly controlled, tissue-specific fashion, oncogenes such as ras may play a role in ROS-mediated chemical teratogenesis.

The objective of this study was to determine the teratologic and embryotoxic

consequences of NNK exposure in CD-1 mice, using *in vivo* and embryo culture approaches to assess both maternal and embryonic contributions. The teratologic role of P450-catalyzed biotransformation was evaluated *in vivo* by maternal pretreatment with the P450 inducer phenobarbital. PCR and PIREMA techniques were used to evaluate whether mutations in embryonic ras may mediate the teratogenicity of NNK.

4.2.3 METHODS

Animals

Virgin female CD-1 mice (Charles River Canada Inc., St. Constant, Quebec) weighing 20-25 g were housed in plastic cages with ground corn cob bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY). Animals were kept in a temperature-controlled room with a 12 hr light-dark cycle automatically maintained. Food (Purina Rodent Chow, Ren's Feed and Supply, Oakville, Ontario) and tap water were provided ad libitum. One male mouse was housed with three females overnight between 1700 and 0900 hr. Pregnancy was ascertained the next morning by the presence of a vaginal plug, and this time was designated as gestational day (GD) 1.

Chemicals

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was purchased from Chemsyn Science Laboratories (Lenexa, Kansas). Phenobarbital was purchased from British Drug Houses (BDH) (Toronto, Ontario). Hanks' balanced salt solution (HBSS), Waymouth's MB 752/1, fetal bovine serum (FBS), sodium bicarbonate solution, HEPES, L-glutamine and penicillin-streptomycin came from Gibco BRL (Toronto, Ontario). Male rat serum (MRS) was obtained from retired CD-1 male breeder rats (Charles River) as described elsewhere (Winn and Wells, 1995a). Amplitaq[®], MgCl₂ and the PCR buffer were obtained from Perkin Elmer Canada Ltd. *Bst*N1 was purchased from New England BioLabs (Mississauga, Ontario, Canada). Primers were synthesized and obtained from the Hospital for Sick Children Biotechnology Service Centre (Toronto, Ontario, Canada). All other reagents used were of analytical grade.

***In vivo* study**

NNK Dosing. NNK, dissolved in 0.9% saline, was administered to pregnant CD-1 dams as a single ip dose of 100 mg/kg in an injection volume of 0.01ml/g on two consecutive days, either GDs 10 and 11, 11 and 12, or 12 and 13, to determine the period of maximal susceptibility. In the P450 induction studies, phenobarbital dissolved in saline (60 mg/kg, i.p) was administered

on GDs 8, 9, and 10 followed by treatment with NNK (100 mg/kg, ip) on GDs 11 and 12.

Since an optimal teratogenic dose and dosing regimen for NNK is not known, the dose for these experiments is based upon the most relevant studies performed with NNK. Anderson et al. (1989) evaluated the transplacental carcinogenesis of NNK in mice and found that the LD₅₀ with respect to survival to weaning of NNK was 150 mg/kg. They also found that NNK at a dose of 100 mg/kg caused a significant increase in both lung and liver tumors in the offspring of treated dams. This dose also had a relatively high incidence of survival to weaning (80%) and was therefore the dose chosen in our study. Additionally, this dose was compared on a molar basis to the teratogenic dose of benzo[a]pyrene (B[a]P) which is a model teratogen and carcinogen (Shum et al., 1979). B[a]P causes teratogenic effects at doses in the range of 192-1152 μ mol/kg (50-300 mg/kg) (Shum et al 1979) and the molar dose of NNK used in our studies is within that range (481 μ mol/kg). The dosing period was chosen such that the NNK doses would cover most of the period of organogenesis.

Teratological assessment. Dams were observed for at least 1 hr after NNK administration to assess any overt signs of toxicity. On gestational day 19, one day prior to spontaneous delivery, dams were sacrificed by cervical dislocation. Following laparotomy, the uterus was exteriorized and the number and location of fetuses and resorptions were noted. Fetuses were then weighed and monitored under a heat lamp for 2 hr to detect postpartum deaths and then fixed in Carnoy's solution. At least two days after fixing fetuses were examined for cleft palates, ectopic kidneys and hydrocephalus.

Skeletal anomalies were assessed by the method of Staples and Schnell (1964). Briefly, embryos were removed from the Carnoy's fixative, eviscerated and then placed in 95% ethanol for 48 hr. Embryos were then removed and placed into a 1% solution of potassium hydroxide (KOH) for 12 to 24 hr to digest the embryonic tissue. Digested embryos were then placed into fresh 1% KOH solution and with 6 drops of Alizarin Red stain (5 ml saturated Alizarin Red in 50% acetic acid, 10 ml glycerine, 60 ml 1% aqueous chloral hydrate). Embryos were then kept in the Alizarin/KOH solution for about 12 hr or until the skeleton was adequately stained. The stained

fetal skeletons were then placed in increasing concentrations of propylene glycol for a minimum of 4 hr each (30%, 50%, 70%, 95%) and finally stored in 100% propylene glycol with one crystal of thymol until skeletal anomalies could be assessed. Skeletal anomalies looked for included: malformed sternebrae, delayed ossification of distal phalanges and the supraoccipital bone, and polydactyly.

Embryo culture study

Pregnant CD-1 dams were sacrificed on GD 9.5 by cervical dislocation and embryos were explanted according to the method of New (1978). Explanted embryos were kept at 37°C in a holding bottle which contained pre-gassed (5% CO₂ in air, Cannox Canada) "holding medium" (50 ml Waymouth's MB 752/1, 14 mM NaHCO₃, 2.5 mM HEPES, 1.0 mM L-glutamine and 17 ml male rat serum) until all embryos from all dams were explanted. Embryos at a similar stage of development (4-6 somite pairs) were pooled and cultured in 25-cm² sterile cell culture flasks (Corning Glasswork Inc., Corning, NY, USA) which contained 10 ml of CO₂ saturated embryo culture medium [50 ml holding-medium, penicillin (50 units/ml) and streptomycin (50 mg/ml)]. Flasks were incubated at 37°C (Forma Scientific, Toronto, Ontario) on a platform rocker (Bellco Biotechnology, Vineland, NJ).

Embryotoxicity. Embryos were exposed to NNK (10 and 100 µM) or the DMSO vehicle for 24 hr. After the culture period, embryonic morphological and developmental parameters were observed using a dissecting microscope (Carl Zeiss, FRG) as described elsewhere (Winn and Wells, 1995a). Developmental parameters included dorsal-ventral flexure (turning), anterior neuropore closure and somite development. Morphological assessment included yolk sac diameter (mm) and crown-rump length (mm).

K-ras 12 mutational analyses. Embryonic DNA was isolated from CD-1 embryos, cultured in the presence or absence of NNK (100 µM) for 24 hr as described above, using a

QIAamp tissue kit (Qiagen, CA). Embryonic DNA was then analyzed for K-*ras* codon 12 mutations using the polymerase chain reaction-primer introduced restriction with enrichment for mutant alleles (PCR-PIREMA) method described by Mills et al. (1995). This is a highly sensitive assay which detects mutant alleles present at the level of 0.1%. Embryonic DNA was first amplified using PCR with fully matched primers flanking exon 1 of the K-*ras* gene (Model GeneAmp PCR System 9600, Perkin Elmer). The primers used were as follows: 5'-ACT GAG TAT AAA CTT GTG GTG GTT GGA GCT-3' (sense) and 5'-CGG CGT TAC CTC TAT CGT AGG GTC-3' (antisense). The PCR reaction included: 1 μ M of each primer, 10 μ M of each nucleotide and 1.2 mM MgCl₂ in a 50 μ l reaction volume, cycled 25 times at 94°C for 1 min, 55°C for 2 min and 74°C for 3 min. A 5 μ l aliquot of this PCR product was then amplified in a second PCR step using a 5'-mismatched sense primer (5'-ACT GAG TAT AAA CTT GTG GTG GTT GGA CCT-3') which introduces a *Bst*I restriction site into normal alleles. This PCR reaction contained 1 μ M of each primer (5'-mismatched sense and 5'-matched antisense), 4 μ M of each nucleotide and 0.6 mM MgCl₂ in a 50 μ l reaction volume and was cycled 25 times at 94°C for 1 min, 40°C for 2 min and 74°C for 3 min. A 2.5 μ l aliquot of this PCR step was then digested overnight with *Bst*I in a final volume of 10 μ l. The second PCR step was then repeated on a 5 μ l aliquot of the digestion product followed by overnight digestion with *Bst*I. A final PCR reaction was then carried out using a 5 μ l aliquot of the digestion product, the 5'-mismatch primer, 120 μ M of each nucleotide and 1.25 mM MgCl₂ in a 50 μ l reaction volume and was cycled 40 times at 94°C for 1 min, 55°C for 2 min and 74°C for 3 min. After overnight digestion with *Bst*I the PCR products were electrophoresed on a 2 % agarose gel and stained with ethidium bromide.

Statistical analysis

Statistical significance between treatment groups in each study was determined using a standard, computerized statistical program (Statview, Abacus Concepts, Inc.) Groups were compared using a one factor analysis of variance (ANOVA). Binomial data were examined using the Chi-square test. The minimum level of significance used throughout was $p < 0.05$.

4.2.4 RESULTS

In vivo study

To our knowledge, there are no previous studies that have investigated the teratological effects of NNK, therefore we examined fetuses for external and internal morphological anomalies, including skeletal defects. All pregnant CD-1 dams treated with 100 mg/kg of NNK with or without phenobarbital induction survived to GD 19 and showed no obvious signs of toxicity, nor did the body weights of pregnant dams differ between the groups.

When NNK alone was administered on GDs 10 and 11, 11 and 12, or 12 and 13, there was no significant difference from controls in mean fetal weight, postpartum lethality, fetal resorptions or gross fetal anomalies (**fig. 2**). However, there were three fetuses from NNK-treated dams with an open eye and one with a cleft palate, while no anomalies were observed in control groups treated with either saline or phenobarbital alone (**table 1**). There were no fetal skeletal anomalies, ectopic kidneys or hydrocephaly in any of the NNK-treated fetuses or in the saline or phenobarbital controls.

Pretreatment of dams with the P450 inducer phenobarbital caused a significant increase in NNK-initiated postpartum lethality, but did not effect mean fetal weight or fetal resorptions ($p < 0.05$) (**fig. 3**). In addition to two fetuses with a cleft palate, two fetuses with exencephaly and one with a kinky tail occurred in dams treated with both phenobarbital and NNK (**table 1**). While the incidence of these anomalies was not statistically different from the phenobarbital alone control group, it was different from the combined saline and phenobarbital alone controls ($p < 0.05$). The two fetuses with exencephaly were born dead but fully formed. Exencephaly and kinky tail were not observed when NNK was administered alone, nor in the controls treated with either saline or phenobarbital alone.

Embryo culture study

The low NNK concentration (10 μ M) significantly reduced yolk sac diameter, crown rump length and somite development, but had no effect on anterior neuropore closure or turning

Figure 2. *In vivo* teratogenicity of NNK in CD-1 mice. NNK (100 mg/kg ip) was given to pregnant dams on two consecutive gestational days (GDs) at three different times during organogenesis: either GDs 10 and 11, 11 and 12, or 12 and 13. The number of fetuses or implantations is given in parentheses. *, Difference from saline control group ($p < 0.05$).

FIGURE 2

EMBRYOPATHY (% + SE)

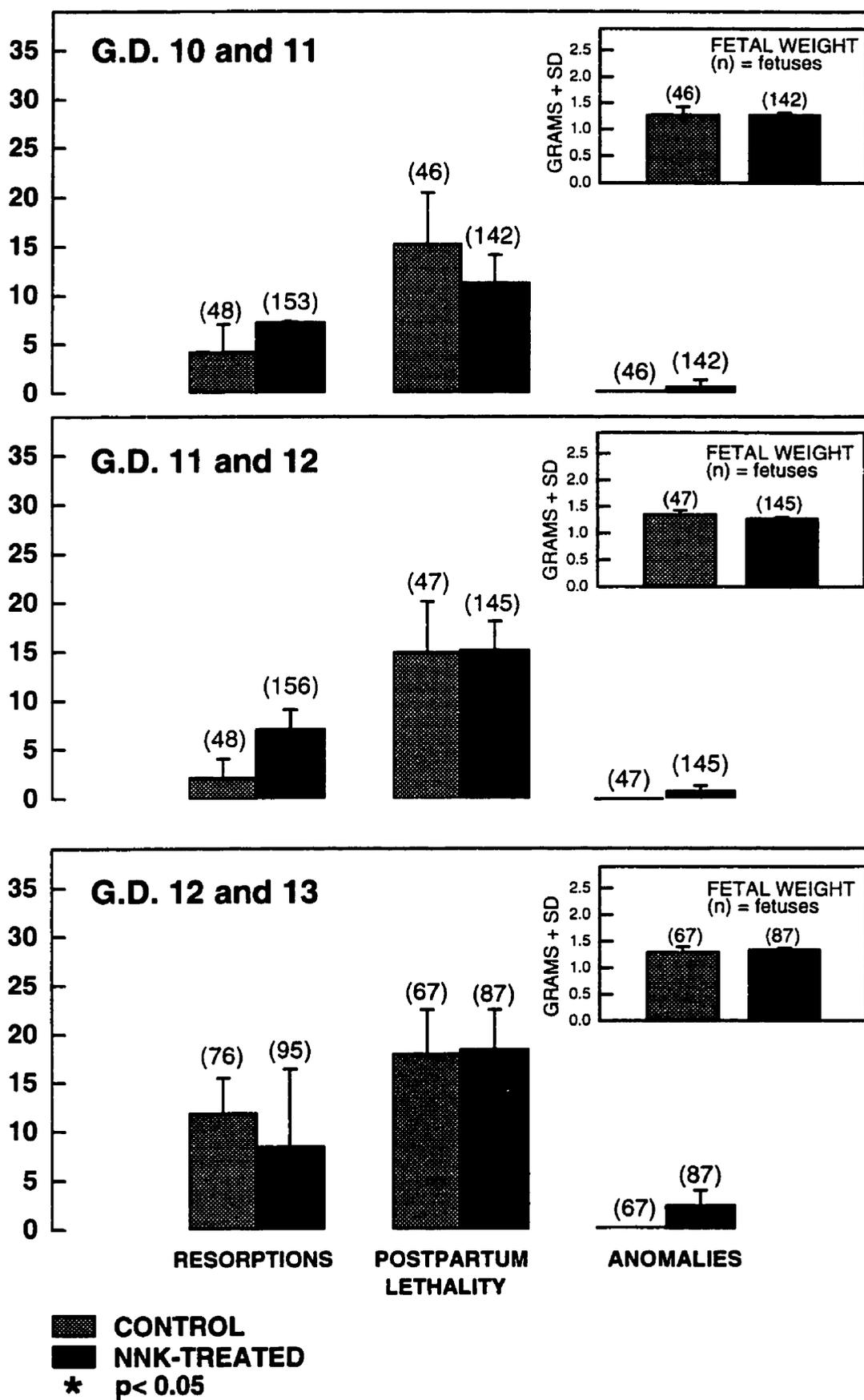


Figure 3. *In vivo* effect of phenobarbital pretreatment on NNK teratogenicity in CD-1 mice. Pregnant dams were pretreated with phenobarbital (60 mg/kg ip) on GDs 8, 9 and 10, followed by treatment with NNK (100 mg/kg ip) on GDs 11 and 12. The number of fetuses or implantations is given in parentheses. *, Difference from phenobarbital alone control group ($p < 0.05$). †, Difference from NNK alone group ($p < 0.05$). +, Difference from combined saline and phenobarbital alone controls ($p < 0.05$).

FIGURE 3

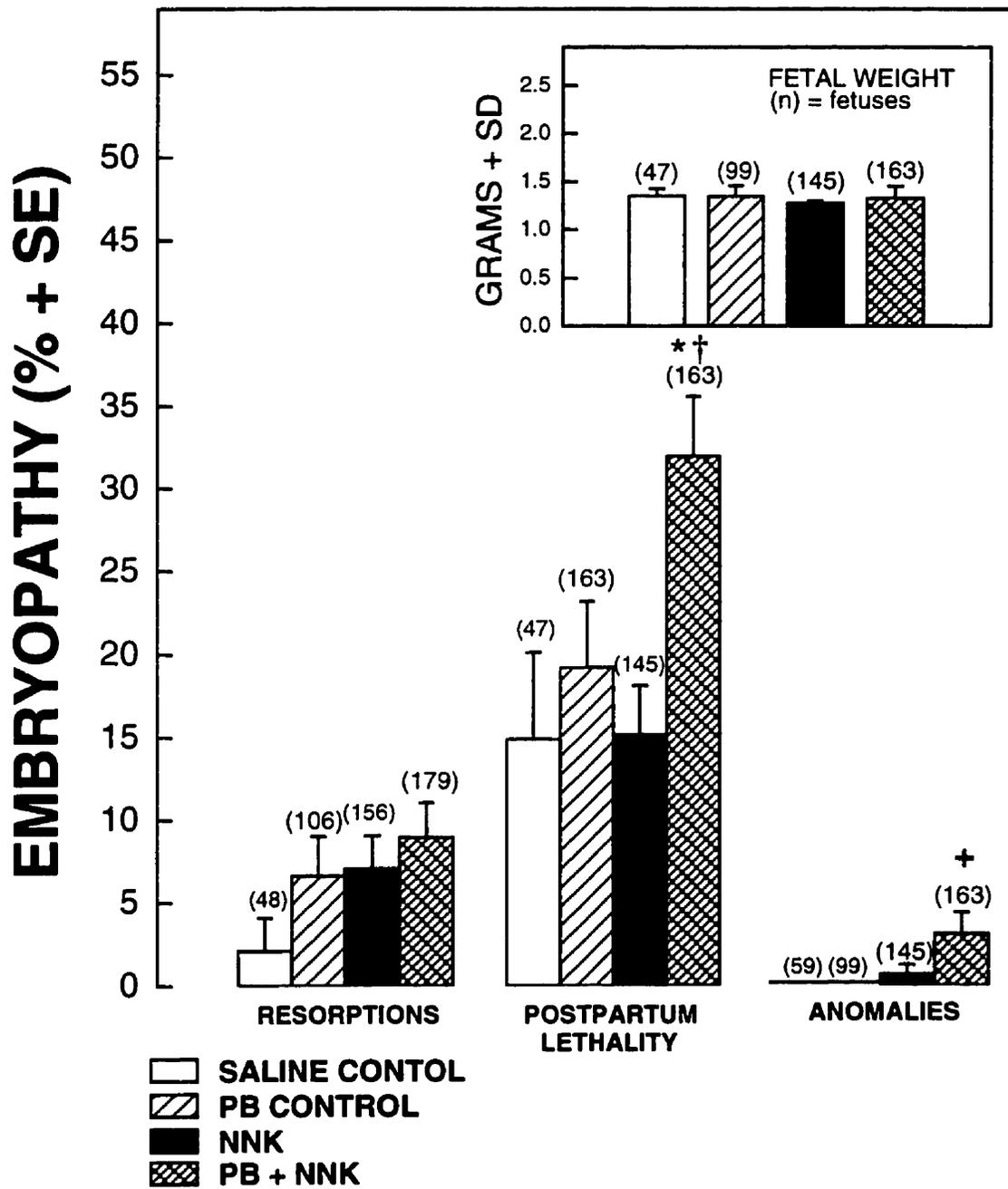


TABLE 1. Fetal structural anomalies in pregnant CD-1 mice administered 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), with or without pretreatment using the cytochromes P450 inducer phenobarbital^a.

TREATMENT	(n)	ANOMALIES ^b	(n)
Saline Vehicle	160	none	
NNK (GDs 10 and 11)	142	open eyelid	1
NNK (GDs 11 and 12)	145	open eyelid	1
NNK (GDs 12 and 13)	87	open eyelid	1
		cleft palate	1
Phenobarbital	99	none	
Phenobarbital + NNK (GDs 11, 12)	163	exencephaly ^c	2
		kinky tail ^c	1
		cleft palate	2

(n) = number of fetuses.

a. NNK (100 mg/kg ip) was administered on two consecutive gestational days (GDs) at three different times during organogenesis. Pretreatment with phenobarbital (60 mg/kg ip) was administered on GDs 8, 9 and 10.

b. There were no skeletal anomalies, ectopic kidneys or hydrocephaly in any groups.

c. Anomalies seen in fully formed fetuses that were born dead.

($p < 0.05$) (**fig. 4**). The higher NNK concentration (100 μM) significantly decreased anterior neuropore closure and crown-rump length but did not decrease turning, yolk sac diameter or somite development ($p < 0.05$) (**fig.4**).

K-ras mutational analysis

DNA from embryos cultured with NNK (100 μM) analyzed for *K-ras* 12 mutations using the PCR-PIREMA method did not show any mutations (**fig. 5**). This technique is based on the introduction of a new restriction site into normal alleles, therefore a nondigested band at 118 bp should indicate the presence of a mutation, whereas a digested band at 89 bp would indicate that there was no mutation present. The positive control sample with the known mutation produced one strong nondigested band (118 bp), whereas the normal tumor samples, the embryonic DMSO and the embryonic NNK samples all produced two bands (118 and 89) with similar intensity, indicating that NNK did not cause a *K-ras* codon 12 mutation. The lack of complete digestion in normals is due to the high misincorporation rate of the taq polymerase (Mills et al., 1995).

Figure 4. *In vitro* embryotoxicity of NNK in embryo culture. Day 9.5 embryos were incubated for 24 hr at 37°C in the presence of NNK (10 µM or 100 µM) or its DMSO vehicle. The number of embryos is given in parentheses. *, Difference from DMSO control embryos (p<0.05).

NNK EMBRYOTOXICITY IN CD-1 MURINE EMBRYO CULTURE

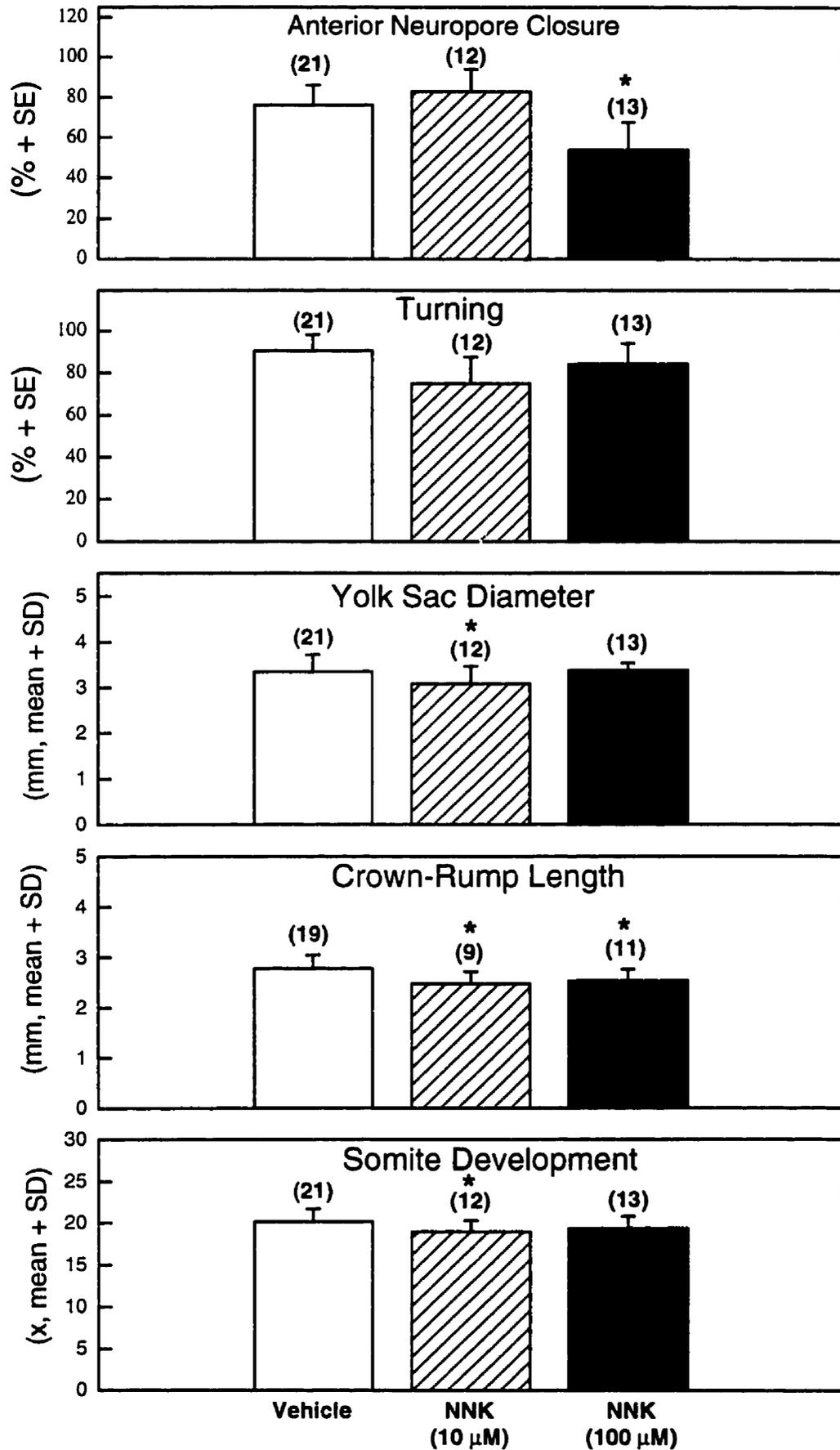
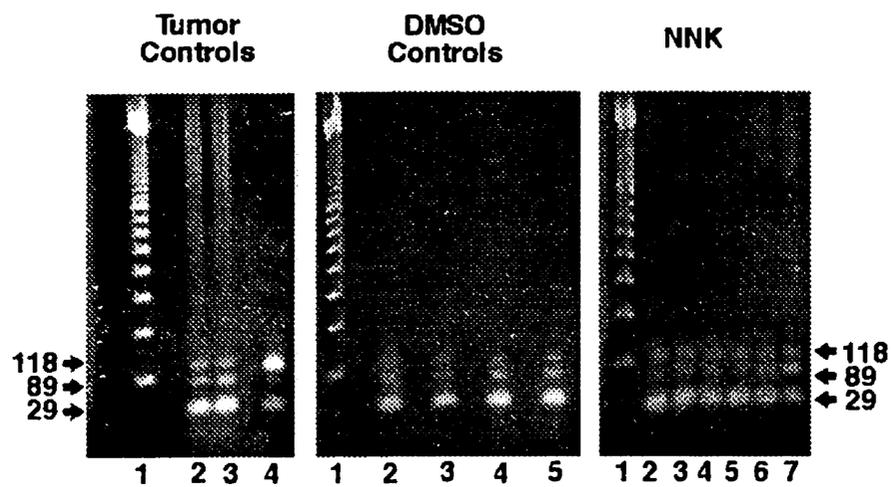


Figure 5. Mutational analysis of codon 12 of the *K-ras* gene using the PCR-PIREMA technique. Tumor controls include DNA isolated from murine lung with a normal sequence (lanes 2 and 3, negative controls) and a known TGT mutation (lane 4, positive control). DNA also was isolated from CD-1 embryos exposed to DMSO vehicle (controls) (4 lanes) and NNK (100 μ M) (6 lanes). Lane 1 in each case shows the 100 base-pair ladder.

FIGURE 5



4.2.5 DISCUSSION

Although previous studies have shown that NNK can initiate transplacental carcinogenesis (Anderson et al., 1989; Correa et al., 1990), no studies evaluating the potential teratogenic or embryotoxic effects of NNK have been conducted. We evaluated the effect of maternal administration of NNK during embryonic organogenesis to determine whether NNK was teratogenic. In transplacental carcinogenesis studies, NNK was administered to pregnant dams late in gestation, after organogenesis, when there is substantially higher activity of most embryonic P450s, the enzyme superfamily which, at least in adults, is thought to be primarily responsible for NNK bioactivation (Hecht, 1996) (**fig. 1**).

The results from our embryo culture studies provide the first evidence that NNK is embryotoxic, and suggests that the embryo itself can bioactivate NNK to a reactive intermediate. Although NNK toxicity in adult mice is known to be mediated by P450-catalyzed bioactivation to electrophilic reactive intermediates capable of methylating or pyridyloxobutylating DNA (**fig. 1**) (Hecht, 1996), the enzymes involved in embryonic bioactivation of NNK remain unknown. Expression of most P450s in rodent embryonic tissue during organogenesis is thought to be low to negligible (Juchau et al., 1992; Raucy and Carpenter, 1993), and whether or not this activity is involved in embryonic bioactivation remains to be determined. However, there are some P450 isozymes, including CYP1B1, which are expressed at high levels in rodent embryonic tissues (Savas et al., 1994; Walker et al., 1995), although it is not known whether NNK or its metabolites are substrates for these P450 isozymes.

Peroxidase-catalyzed bioactivation of NNK leading to the formation of reactive oxygen species (ROS) also may be involved in the embryonic bioactivation and embryotoxicity of NNK. NNK-initiated micronucleus formation in rat skin fibroblasts is blocked by the dual P450/peroxidase inhibitor 1-aminobenzotriazole, and by the dual PHS/LPO inhibitor eicosatetraynoic acid (Kim and Wells, 1996a). Given the low P450 activity in such cultured cells, the protection against NNK genotoxicity afforded by these peroxidase inhibitors suggests an important role for peroxidases such as PHS in NNK bioactivation. It has recently been

postulated that NNK can initiate the formation of ROS (Xu et al., 1992; Weitberg and Corvese, 1993; Kim and Wells, 1996a), and it has been shown that the antioxidative enzyme superoxide dismutase can prevent NNK-initiated micronucleus formation (Kim and Wells, 1996a). ROS can damage essential cellular macromolecules including DNA, which may be a critical determinant in chemically-initiated teratogenesis since mice deficient in DNA repair are more susceptible to the teratogenicity of both benzo[a]pyrene and phenytoin (Nicol et al., 1995; Laposa and Wells, 1995).

Several studies have shown that NNK-initiated lung tumors in mice contain *K-ras* 12 mutations (Belinsky et al., 1989; Ronai et al., 1993), which are thought to occur from the direct methylation of DNA resulting in a G to A transition mutation. In humans, 16% of lung tumors and 24% of adenocarcinomas have been shown to have mutated *K-ras* genes (Rodenhuis and Slebos, 1992). We did not see any mutations in the *K-ras* 12 codon in any of the NNK-treated embryos, which suggests that the mechanism of NNK-initiated embryotoxicity may be different from that for NNK-initiated carcinogenesis.

Although our embryo culture studies showed that NNK can initiate embryotoxicity, our *in vivo* results using NNK alone suggest that NNK is not a potent teratogen, at least with respect to structural defects. NNK can produce tumors in mice and rats at doses as low as 5 mg/kg (Hecht et al., 1988; Prokopczyk et al., 1991), and can initiate transplacental carcinogenesis in pregnant mice and hamsters at doses as low as 1 mg/kg when administered late in gestation (Anderson et al., 1989; Correa et al., 1990). In our studies, NNK alone at a dose of 100 mg/kg was not significantly teratogenic, although it did cause one cleft palate and three open eye defects, neither of which were observed in any controls. In our experience, open eye and cleft palate are rare in the CD-1 mouse, therefore we suspect that given a larger control group, these anomalies likely would prove to be statistically associated with NNK, which is consistent with the two additional fetuses with cleft palate in the group treated with both phenobarbital and NNK, and the statistically significant association in humans of cleft palate with smoking (Khoury et al., 1989). The observation that NNK was embryotoxic in embryo culture, but not significantly teratogenic *in vivo*, suggests that *in vivo*, maternal elimination of NNK and its metabolites via glucuronidation

may protect the fetus from exposure to high levels of NNK and its metabolite. This may be particularly relevant in humans, where it is known that 2-12% of the population have deficiencies in UGTs (Monaghan et al., 1996) and, unlike in rodents, the production and subsequent glucuronidation of NNAL (carbonyl reduced form of NNK) is extensive (Morse et al., 1990; Carmella et al., 1993) (**fig. 1**). In rat skin fibroblasts, NNK-initiated micronucleus formation is enhanced in UGT-deficient cells (Kim and Wells, 1996a).

Since our *in vivo* study found that NNK alone was not significantly teratogenic, we evaluated whether or not pretreatment with phenobarbital could enhance the teratogenicity of NNK. Many of the other compounds present in tobacco smoke, or exposure to other drugs/xenobiotics such as alcohol, which often is concomitant, could potentially modulate enzymes implicated in NNK bioactivation and/or detoxification in the placenta (Manchester and Jacoby, 1982), or possibly the embryo. In mice, CYP2B1 and CYP2B2 have been shown to catalyze the α -hydroxylation of NNK, and are the two major P450 isozymes inducible by phenobarbital pretreatment (Thomas et al., 1983; Hecht, 1996).

We found that pretreatment of dams with phenobarbital caused a significant increase in NNK-initiated postpartum lethality, although mean fetal weight or fetal resorptions were unaffected (**fig. 3**). An increase in postpartum lethality may be potentially relevant in humans given that exposure to tobacco smoke has been associated with an increased incidence of sudden infant death syndrome.

When compared to the combined controls treated with phenobarbital alone and with saline, phenobarbital pretreatment also enhanced the incidence of fetal anomalies initiated by NNK, including cleft palate, exencephaly and kinky tail. This not only is consistent with the interpretation above that NNK alone is teratogenic, but also suggests that embryonic P450-catalyzed bioactivation may contribute to the teratologic mechanism. An additional possibility includes enhanced maternal P450-catalyzed formation of a stable metabolite that can cross the placenta and undergo embryonic bioactivation. Since the apparent increase in anomalies in the group treated with both phenobarbital and NNK was not statistically different when compared only to the phenobarbital alone controls, it is possible that the association was fortuitous;

however, for the reasons given above in the discussion of studies with NNK alone, we believe that this teratologic enhancement is biologically significant.

It is important to note that the dose of NNK used in our *in vivo* study was very high (100 mg/kg), even considering that pregnant women would be exposing their fetus for many months to irreversible macromolecular damage initiated by tobacco-related xenobiotics. If a pregnant woman smoked 20 nonfiltered average cigarettes (425 ng of NNK/cigarette) (Adams et al., 1987) a day for 9 months and weighed in the range of 50-70 kg, she would be exposing herself and her fetus to about 0.04 mg/kg of NNK, which is over 3 orders of magnitude lower than the dose used. Even considering that, to provide an equivalent plasma concentration, the dose for a mouse may need to be roughly ten times that for a human, the dose in our study was about 250 times higher. However, while our results appear to represent the extreme teratologic potential for NNK, there can be any number of complicating factors when attempting to extrapolate these results in embryo culture and *in vivo* in mice to humans.

In summary, although NNK is a highly potent animal carcinogen, our results indicate that, compared to dual carcinogen/teratogens like benzo[a]pyrene, NNK is a relatively weak structural teratogen in our mouse models, although this effect was enhanced *in vivo* by pretreatment with the P450 inducer phenobarbital. This suggests that human fetal anomalies initiated by cigarette smoking may be caused by teratogenic tobacco constituents more potent than NNK, and possibly by NNK with concomitant exposure to drugs and environmental chemicals that enhance NNK teratogenicity. However, complicating factors such as potential synergistic effects of other constituents of tobacco smoke, and substantial differences in rodent and human embryonic bioactivating activities, preclude direct extrapolation of these results. NNK did not cause mutations in codon 12 of the *K-ras* gene, suggesting teratologic mechanisms different from that postulated for carcinogenesis. For a comprehensive understanding of the teratological potential of NNK, further studies are warranted to determine the functional fetal consequences of NNK exposure during the latter half of pregnancy, when brain development is predominant, and the activities of P450 isozymes are increasing.

SECTION 5: REFERENCES

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