

**REGULATION OF THE MEVALONATE PATHWAY BY DIETARY FAT
AND CHOLESTEROL IN MAMMARY CANCER DEVELOPMENT**

by

Ahmed El-Sohemy

**A thesis submitted in conformity with the requirements for the
degree of Doctor of Philosophy
Graduate Department of Nutritional Sciences
University of Toronto**

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ABSTRACT

Dietary lipids have been implicated in breast cancer development but their precise role and molecular mechanisms of action are poorly understood. The objective of this investigation was to examine in rodent models a mechanism by which dietary polyunsaturated fatty acids (PUFAs) and cholesterol could regulate mammary tumorigenesis. The hypothesis is that mevalonate synthesis mediates the effects of dietary PUFAs and cholesterol on mammary tumorigenesis. 3-Hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase is the rate limiting enzyme in cholesterol biosynthesis that catalyzes the formation of mevalonate which is essential for cell proliferation. Dietary cholesterol was found to increase serum cholesterol and inhibit the development of rat mammary tumors while oxidized cholesterol abrogated both of these effects. Dietary cholesterol decreased mevalonate synthesis in the mammary glands but not in mammary tumors. Mevalonate is also a precursor of isoprenoids that are required for the function of growth regulatory proteins such as H-Ras. Cholesterol inhibited the development of mammary tumors initiated by either 7,12-dimethylbenz[a]anthracene or N-methyl-N-nitrosourea, carcinogens that give rise to tumors with different frequencies of H-ras mutations. Thus, inhibition of mevalonate

synthesis may be a mechanism by which cholesterol inhibits tumorigenesis, but the effects are independent of *H-ras* mutations. The objective of the second part of this work was to determine whether a decrease in mevalonate synthesis could explain the known inhibitory effects of n-3 PUFAs on experimental mammary tumorigenesis. Compared to n-6 PUFAs, n-3 PUFAs decreased mevalonate synthesis in rat mammary glands while increasing the levels of a protein and mRNA transcript with some homology to the low-density lipoprotein receptor (LDL-R). n-3 PUFAs decreased mammary gland HMG-CoA reductase activity and protein levels in wild-type but not LDL-R-deficient mice, suggesting that the LDL-R is required for n-3 PUFAs to decrease mevalonate synthesis in the mammary gland. The changes in HMG-CoA reductase activity observed in these experiments are in agreement with changes in mammary tumorigenesis suggesting that this biochemical pathway may mediate the effects of dietary cholesterol and fat on cancer development. Understanding how lipids regulate breast cancer development may lead to mechanism-based strategies to prevent this disease.

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LIST OF ABBREVIATIONS

ACAT	Acylcholesterolacyltransferase
ALA	α -Linolenic acid
CM	Chylomicron
DHA	Docosahexaenoic acid
DMBA	7,12-Dimethylbenz[a]anthracene
EPA	Eicosapentaenoic acid
FFA	Free fatty acids
FPTase	Farnesyl protein transferase
HDL	High density lipoprotein
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
IDL	Intermediate density lipoprotein
LA	Linoleic acid
LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
LPL	Lipoprotein lipase
MNU	N-Methyl-N-nitrosourea
MUFA	Monounsaturated fatty acid
OA	Oleic Acid
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
SRE	Sterol response element
SREBP	Sterol response element binding protein
TG	Triglyceride
VLDL	Very low density lipoprotein

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Breast cancer is the most commonly diagnosed neoplasm among women in Canada and accounts for 30% of all new cancer cases (NCIC 1998). The 1998 Canadian Cancer Statistics predict over 19,000 new cases of breast cancer and an estimated 5,000 deaths annually (NCIC 1998). Incidence has been increasing steadily over the years but mortality has not. This may be due to a combination of better screening methods and more effective treatments. Although the causes of breast cancer are not known, there are several well known risk factors. Individuals with inherited mutations in genes such as *BRCA-1* or *BRCA-2* have a high risk of breast cancer (Miki et al. 1994; Wooster et al. 1995), but these account for only a small percentage of total cases (~5%) (Easton 1997). Factors that increase life-time exposure to hormones such as early age of menarche and/or late age of menopause are associated with increased risk of breast cancer (Henderson et al. 1982). The high incidence of breast cancer in Canada is not seen among all populations. International breast cancer rates vary by as much as five-fold with industrialized nations such as Canada, US, and Denmark having much higher rates than developing countries such as China, Ecuador, and many African nations (IARC 1987). Individuals that migrate from countries with low incidence to countries with high incidence acquire the higher rate of their adopted country (Haenszel 1982), but this is usually not observed until the second generation (Ziegler et al. 1993). These observations suggest that environmental factors may be important determinants of risk. Of the environmental factors, diet has been the most thoroughly investigated (Doll 1996).

In 1981, Doll and Peto estimated that as much as 50% of breast cancer cases in North America can be prevented by dietary changes (Doll and Peto 1981). The World Cancer Research Fund in association with the American Institute for Cancer Research, recently published a 670-page report entitled, "Food, Nutrition and the Prevention of Cancer: A Global Perspective." (AICR 1997). It was estimated that approximately 30-40% of all cancers may be prevented by dietary and life-style changes. Diet is strongly believed to influence the development of breast cancer but the precise role of different dietary factors remains uncertain. Dietary fat has been the most extensively studied nutrient implicated in breast cancer development and its effects appear to be due to both the type and amount of fatty acids in the diet. Specifically, some classes of fatty acids increase risk while others decrease risk. Current evidence suggests that dietary cholesterol may not play a significant role in breast cancer. However, some studies suggest that cholesterol oxidation products may be important contributing factors. Several mechanisms have been proposed to explain how fat modulates breast carcinogenesis, yet none have been proven conclusively. A number of experimental approaches can be applied to study the association between fat and breast cancer. Molecular biology provides a powerful tool to investigate the mechanisms of action and has shed light on our understanding of basic cellular processes that are regulated by lipids. Identification of the molecular and biochemical pathways that mediate the effects of fat will allow more definitive recommendations to be made to reduce the prevalence of this disease.

The objective of this work was to investigate the role of dietary fat and cholesterol in experimental breast carcinogenesis and to examine a potential mechanism of action.

1.2 MODELS USED TO STUDY DIET AND BREAST CANCER

1.2.1 Epidemiological Studies

Epidemiology plays an important role in identifying and describing potential nutritional factors that are involved in the development of breast cancer. Ecological studies make correlations between putative risk factors and breast cancer incidences or mortality rates among large geographic areas. An example would be the international correlation studies that examine the incidences and mortality rates between countries (IARC 1987). Ecological studies, however, are generally regarded as the weakest in epidemiological investigations because many correlations may be spurious (Oliveria et al. 1997). Case-control studies offer the advantage of being conducted over a relatively short period of time but, the major limitation is that current intake may not reflect past intake which is probably a more relevant determinant of risk. Cohort studies are much more costly and time consuming but can provide more convincing evidence of associations. The strongest epidemiological evidence for a link between diet and breast cancer comes from intervention studies (Henderson 1992), yet very few have been conducted. In addition to the tremendous cost and time required, there are usually inconsistent findings from other types of studies leaving insufficient evidence to warrant an intervention trial. The development of early

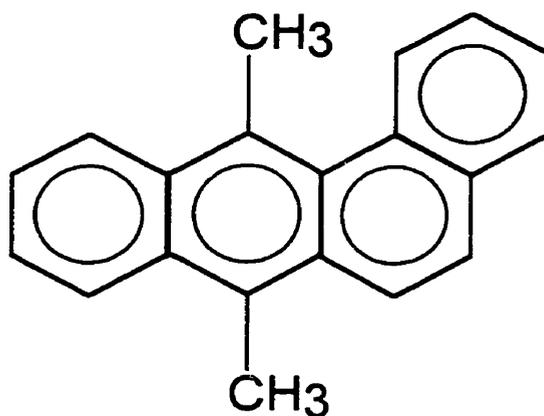
biomarkers of disease would greatly facilitate the undertaking of such studies. Mammographic breast densities have recently been used as a marker of breast cancer to examine the effects of diet in a two year intervention trial (Boyd et al. 1997). Meta-analyses are being used more often to compare the results of similar studies. In as much as meta-analyses are touted as superior analytical tools, design flaws in the studies selected will still be carried through and become incorporated into the final analysis. Molecular epidemiology is a relatively new field of study that is growing rapidly (Ambrosone and Kadlubar 1997). This approach associates a disease with a genetic or biological marker such as mutations, DNA-adducts, genetic polymorphisms, receptor-status etc. These types of studies are likely to provide important new links between nutritional and genetic risk factors.

There are several limitations to human studies designed to evaluate the role of diet in breast cancer development. These include insufficient differences between the diets in the populations studied to allow detection of associations, large measurement errors associated with dietary questionnaires, and many confounding dietary variables (Bingham et al. 1994). There is also a lack of appropriate standardized methods of dietary assessment and lack of suitable biomarkers of exposure. These methodological issues have made it difficult to establish unequivocally the role of diet in breast cancer.

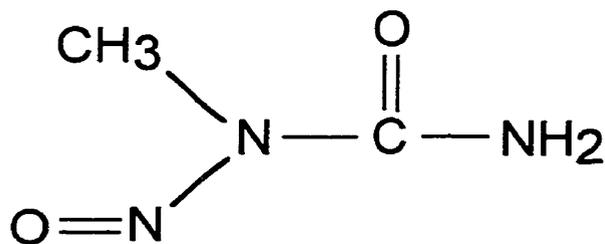
1.2.2 Animal Models

In order to assess the biological plausibility of epidemiological associations and to study the mechanisms of action of dietary factors in cancer development, a great deal of emphasis is placed on experiments using rodents.

The most widely used model of human breast cancer development is the rat, using either 7,12-dimethylbenz[a]anthracene (DMBA) or N-methyl-N-nitrosourea (MNU) as carcinogens (Figure 1-1) (Rogers and Lee 1988). Rat strains differ in their susceptibilities to these carcinogens. For example, the Sprague-Dawley is highly susceptible while the Fisher strain is of intermediate susceptibility (Chan et al. 1977). The MNU model is simpler mechanistically than the DMBA model because MNU is a direct acting carcinogen that does not require the enzymes of drug metabolism for its activation (McCormick et al. 1981; Tamulski et al. 1976). Furthermore, MNU has a short half-life (1h) in the animal following injection so that cancer initiation is rapid. MNU is water soluble and is usually administered ip or iv, whereas DMBA is a lipid soluble compound that is administered ig. Single doses of either of these agents (50 mg/kg MNU or 100 mg/kg DMBA) given to 50-55 day old virgin female rats produces essentially 100% breast adenocarcinoma incidence with a short latency (~12 weeks) in susceptible strains (Russo and Russo 1996; Thompson et al. 1992). At this age, the animals are maturing sexually and readily develop cancer due to active organogenesis and high proliferation rates in the glandular epithelium (Russo and Russo 1994). Induction of mammary tumors by either MNU or DMBA occurs in a dose-dependent fashion with relatively low toxicity (Thompson and Adlakha 1991; Thompson et al. 1992). Recently, Thompson *et al* (1995) showed that 21 day old rats given MNU develop mammary tumors within 4 weeks and by 8 weeks virtually all animals develop tumors. The growth characteristics of these tumors and their response to dietary factors, however, has not been adequately investigated.



7,12-dimethylbenz[a]anthracene (DMBA)



N-methyl-N-nitrosourea (MNU)

Figure 1-1. Chemical structure of 7,12-dimethylbenz[a]anthracene (DMBA) and N-methyl-N-nitrosourea (MNU).

Although pathologists have identified many different morphological types of breast cancer in humans, rat mammary cancers are much less morphologically heterogeneous and are often classified as either adenocarcinomas or papillary carcinomas (Komitowski et al. 1982). Administration of MNU or DMBA primarily gives rise to adenocarcinomas (Welsch 1985). Despite the similarities between MNU- and DMBA-induced tumors and their response to dietary factors, there are notable differences at the genetic level. The *H-ras* oncogene, activated by a G → A transition mutation in codon 12 is found in ~85% of MNU-induced rat mammary tumors (Sukumar et al. 1983; Zarbl et al. 1985). In contrast, DMBA-induced tumors contain few *H-ras* mutations (~20%), and those are usually A → T transversion mutations in codon 61 (Zarbl et al. 1985). Human breast tumors do not commonly harbor *ras* mutations (Thor et al. 1986), however, both human and rat mammary tumors overexpress the Ras protein product p21 (Bos 1989; DeBortoli et al. 1985). In spite of this difference at the genetic level between rat and human tumors, MNU- and DMBA-induced malignant rat mammary tumors share a very similar pathogenesis and have many features in common with the development of intraductal and infiltrating ductal carcinomas in humans (Russo et al. 1990).

The mouse model has also been used extensively to study mammary carcinogenesis and responds like the rat to the effects of dietary factors (Abraham et al. 1984; Cameron et al. 1989; Craig-Schmidt et al. 1993). Six weekly injections of DMBA or a single injection of MNU yields a high tumor incidence in susceptible strains with a latency of approximately 16 weeks (Guzman et al. 1992; Medina 1974). BALB/c is the most commonly used strain because of its susceptibility to develop mammary

tumors. Unlike rats, mice receiving MNU require transplantation of pituitary isografts to sustain adequate levels of prolactin for tumor development (Guzman et al. 1992), whereas those receiving DMBA do not require hormone treatment (Medina et al. 1983). Mice harbouring the mouse mammary tumor virus develop tumors spontaneously but, tumor latency is prolonged (Kamano et al. 1989). Transgenic mice that overexpress various oncogenes develop mammary tumors spontaneously and can be used to study the effects of dietary factors (Amundadottir et al. 1996). The development of knockout mice using homologous recombination to delete certain genes provides a unique opportunity to isolate and study a specific molecular mechanism. This advantage will lead to increasing use of the mouse model since knockout rats are not yet available.

The transplanted tumor model is used to study dietary effects on tumor growth and development (Rose et al. 1997). Human breast cancer cells are injected into the mammary fat pads of athymic mice and the growth of these cells into palpable tumors is assessed. This model offers the advantage of evaluating the effects of diet on human cells but, does not adequately assess mechanisms of prevention since the cancer cells have already been established.

1.2.3 Cell Culture Systems

In vitro models are not commonly used to study the effects of dietary factors on breast carcinogenesis. The need for digestion and metabolism of nutrients makes it difficult to investigate the effects of whole foods in cell culture, however, the effects of individual fatty acids, vitamins and phytochemicals on the growth of breast cancer

cells can be investigated. Since this model can be manipulated quite easily, mechanistic studies can be carried out that can not otherwise be done *in vivo*. The MCF-7 (hormone-dependent) and MDA-MB-231 (hormone-independent) breast cancer cell lines are commonly used along with the MCF-10A non-transformed mammary epithelial cell line to study mechanisms that may mediate the effects of dietary factors on breast cancer.

1.3 LIPID METABOLISM

In order to elucidate the mechanisms by which dietary lipids regulate cancer development, there is a need to understand how they are metabolized, transported, localized to subcellular compartments and regulate gene expression. Differences in lipid metabolism exist between various species including humans and rodents (Spady and Dietschy 1983). However, there are also considerable genetic differences among humans (Clifton and Abbey 1997), and between different strains of rats and mice (Paigen et al. 1985). Although such genetic differences should be recognized, there are many common features of the pathways involved in regulating lipid metabolism and these will be discussed here.

1.3.1 Fatty Acid Metabolism

Dietary lipids consist mostly of triglycerides (TGs) but also contain monoglycerides, diglycerides, free fatty acids (FFA), phospholipids, free cholesterol and cholesterol esters. TGs consist of three fatty acids esterified to a glycerol

backbone and are predominantly digested by lipases in the small intestine. The resultant 2-monoglycerides and FFA are emulsified by bile acids and incorporated into mixed micelles that are taken up by enterocytes (Spector 1984). FFA are re-esterified to the glycerol moiety of the 2-monoglyceride to reproduce TGs that are packaged with other lipids into chylomicrons (CM). These lipoproteins are transported through the lymphatics and enter the circulation at the thoracic duct (Spector 1984). TGs in CM are extensively hydrolyzed by lipoprotein lipases (LPL) in the endothelium of blood vessels in extrahepatic tissues resulting in more dense CM remnants containing apolipoprotein (apo) B-48, apoC and apoE. Once the apoC is removed, the remnants are rapidly cleared by the liver (Cooper 1997). Lipids are then repackaged by the liver into very low-density lipoprotein (VLDL) particles which become the major carriers of fat in the blood. VLDL carry the full length form of apoB (B-100) and can be internalized by the VLDL receptor which is abundant in muscle, adipose and heart but not the liver (Takahashi et al. 1992). Approximately 5% of circulating fat is FFA while the rest is found as TGs transported in lipoproteins (Spector 1984). Further hydrolysis of TGs from VLDL produces more dense intermediate-density lipoproteins (IDL) that are finally converted into even more dense low-density lipoproteins (LDL). The increasing density of lipoproteins results from the loss of TGs that are less dense than water. LDL are internalized by the LDL receptor (LDL-R) through receptor mediated endocytosis, a discovery that earned Goldstein and Brown the 1985 Noble Prize in Physiology and Medicine (Brown and Goldstein 1986). Uptake of the various lipoproteins occurs in the liver as well as extrahepatic tissues. A number of receptors have been identified that recognize different apo moieties on lipoproteins (Brown et al.

1997). In addition to these receptors, there are also 'receptor-independent' pathways of uptake (Spady et al. 1985), and possibly other receptors that have not yet been identified.

FFA liberated from lipoproteins by the action of LPL are transported in the blood bound to albumin or fatty acid binding proteins (FABPs) (Spector 1984). These carrier molecules dock to the outer plasma membrane and the fatty acids are transported to the interior of the cell by fatty acid transfer protein (Hirsch et al. 1998). Once inside, FFA are shuttled by a carrier protein called Z-protein that functions similarly to FABPs in the circulation. FFA can be incorporated into the lipid bilayer of plasma membranes, serve as precursors to other molecules (e.g. eicosanoids), undergo β -oxidation for energy, re-esterify for storage or be transported to the nucleus to regulate gene expression (Sessler and Ntambi 1998).

Fatty acids can be classified as either saturated (SFAs), monounsaturated (MUFAs) or polyunsaturated (PUFAs), depending on the number of double bonds (Mayes 1996). Oleic acid (OA, 18:1n-9) is the most common MUFA and can be obtained in the diet or synthesized *de novo*. PUFAs can be further classified as either n-3 or n-6 PUFAs depending on the position of the first double bond from the terminal methyl carbon (i.e. at the third or sixth carbon respectively). This difference between n-3 and n-6 PUFAs leads to marked differences in their nutritional, biochemical and biological functions (Ackman and Cunnane 1992). Linoleic acid (LA, 18:2n-6) and α -linolenic acid (ALA, 18:3n-3) are essential fatty acids that can not be synthesized by the body. Plant oils such as corn and safflower are a rich source of LA while flaxseed (also called linseed) oil is a rich source of ALA (Thompson 1995). Marine fish oils are

one of the richest sources of long chain n-3 PUFAs in the diet, the most abundant being eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Sargent 1997). LA and ALA can undergo elongation and desaturation to produce long chain fatty acids. ALA is a precursor of EPA and DHA whereas LA is a precursor of arachidonic acid (AA, 20:4n-6) which can also be found in the diet. These long chain fatty acids can be converted into bioactive lipids such as the eicosanoids (prostaglandins (PG), thromboxanes, leukotrienes) that mediate diverse biological functions (Goetzl et al. 1995).

In humans, essential fatty acid deficiency is rare. Although the exact requirements for adults is not well defined, approximately 2% of calories as LA and 0.5% of calories as ALA are sufficient to prevent symptoms of deficiency (Holman et al. 1982). Similarly, only estimates are available for requirements in rodents which are 12 g per kg diet LA and 2 g per kg diet ALA (Reeves et al. 1993). This is approximately equivalent to 90 mg LA and 15 mg ALA per day for a growing female rat. The exact level, however, depends on age, sex, strain and developmental status.

1.3.2 Cholesterol Metabolism

Cholesterol ($C_{27}H_{46}O$, MW 386) has been one of the most extensively studied molecules in science. Thirteen Nobel Prizes have been awarded to scientists that devoted most of their careers to studying some aspect of cholesterol (Brown and Goldstein 1986). Since the body is capable of synthesizing cholesterol, it is not considered an essential nutrient although it is required to sustain mammalian life.

Despite daily intakes of over 400 mg of cholesterol in a typical Western diet, the body still synthesizes 800 mg or more per day (Quintao and Sperotto 1987). When dietary cholesterol is increased or decreased, endogenous synthesis decreases or increases, respectively (Jones et al. 1996). Exogenous cholesterol enters the circulation along with other dietary lipids in the form of CM (see above). The liver is the major organ regulating serum cholesterol and is the major site of cholesterol biosynthesis (Dietschy et al. 1993). Cholesterol entering the liver *via* CM remnants is esterified predominantly to OA by acylcholesterol-acyltransferase (ACAT) before it is repackaged into VLDL. As TGs from VLDL are hydrolyzed by LPL, the lipoproteins become relatively enriched with cholesteryl esters. Ultimately, LDL become the major carriers of cholesterol in the blood.

When uptake of cholesterol is enhanced and intracellular cholesterol levels rise, *de novo* synthesis of cholesterol is reduced to prevent excessive accumulation of cholesterol within the cell (Brown and Goldstein 1986). Thus, individual cells meet their cholesterol requirements by uptake of cholesterol-rich lipoproteins or through *de novo* biosynthesis (Brown and Goldstein 1986; Goldstein and Brown 1984). Figure 1-2 shows the uptake of LDL through the LDL-R and a LDL-R-independent pathway.

Cholesterol that is liberated from dying cells or membranes undergoing turnover is cleared by the 'Reverse Cholesterol Transport' pathway (Fielding and Fielding 1995). Cholesterol is esterified onto high density lipoprotein (HDL) by lecithin cholesterol acyltransferase (LCAT) in the plasma and transported to the liver or other tissues that require high levels of cholesterol such as the adrenal glands. Once at the cell surface, HDL particles transfer their cholesterol through a recently discovered

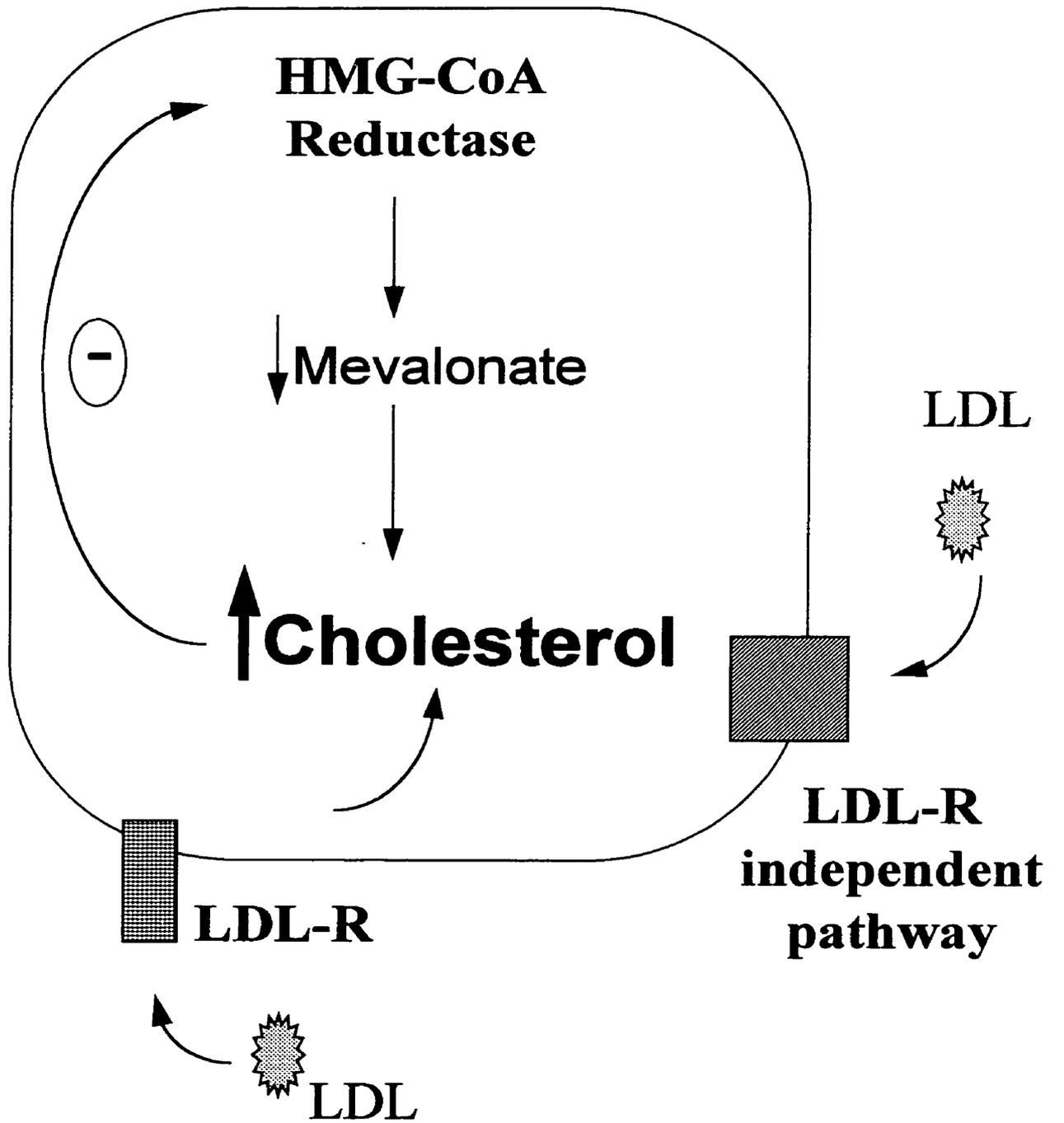


Figure 1-2. Cellular uptake of LDL by LDL-R and LDL-R-independent pathways.

HDL receptor (Acton et al. 1996). Unlike the LDL-R that completely internalizes LDL, the HDL receptor is a 'docking' receptor that allows HDL to attach to the cell surface, deliver its cholesterol, dissociate and continue to circulate in the blood with the potential of gathering more cholesterol (Steinberg 1996). Alternatively, cholesterol from HDL can be transferred to LDL or VLDL by cholesterol ester transfer protein (Tall 1998).

In the 1940s, Konrad Bloch showed that all 27 carbon atoms of cholesterol are derived from acetate, earning him the Noble Prize in Physiology and Medicine in 1964. The first step is the synthesis of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) from acetyl-CoA and acetoacetyl-CoA, a reaction catalyzed by HMG-CoA synthase (Figure 1-3) (Bloch 1965). HMG-CoA reductase (EC 1.1.1.34) catalyzes the 'rate limiting step' by converting HMG-CoA to the six carbon (C6) mevalonate. Hence, this pathway is sometimes called the mevalonate pathway. Mevalonate is decarboxylated to yield isopentenyl pyrophosphate (PPi) which is used to synthesize geranyl-PPi (C10) and farnesyl-PPi (C15). Two of the C15 molecules condense to form squalene (C30), a reaction catalyzed by squalene synthase which is the first committed step in the synthesis of cholesterol. Squalene cyclizes in the presence of molecular oxygen to form lanosterol (C30) which is finally converted to cholesterol (C27) by the loss of three methyl groups. Depending on the cell type, cholesterol may then be used for the synthesis of steroid hormones, bile acids or vitamin D. Recently, cholesterol was also found to be essential for the modification of the hedgehog signaling protein which is involved in embryogenesis (Porter et al. 1996).

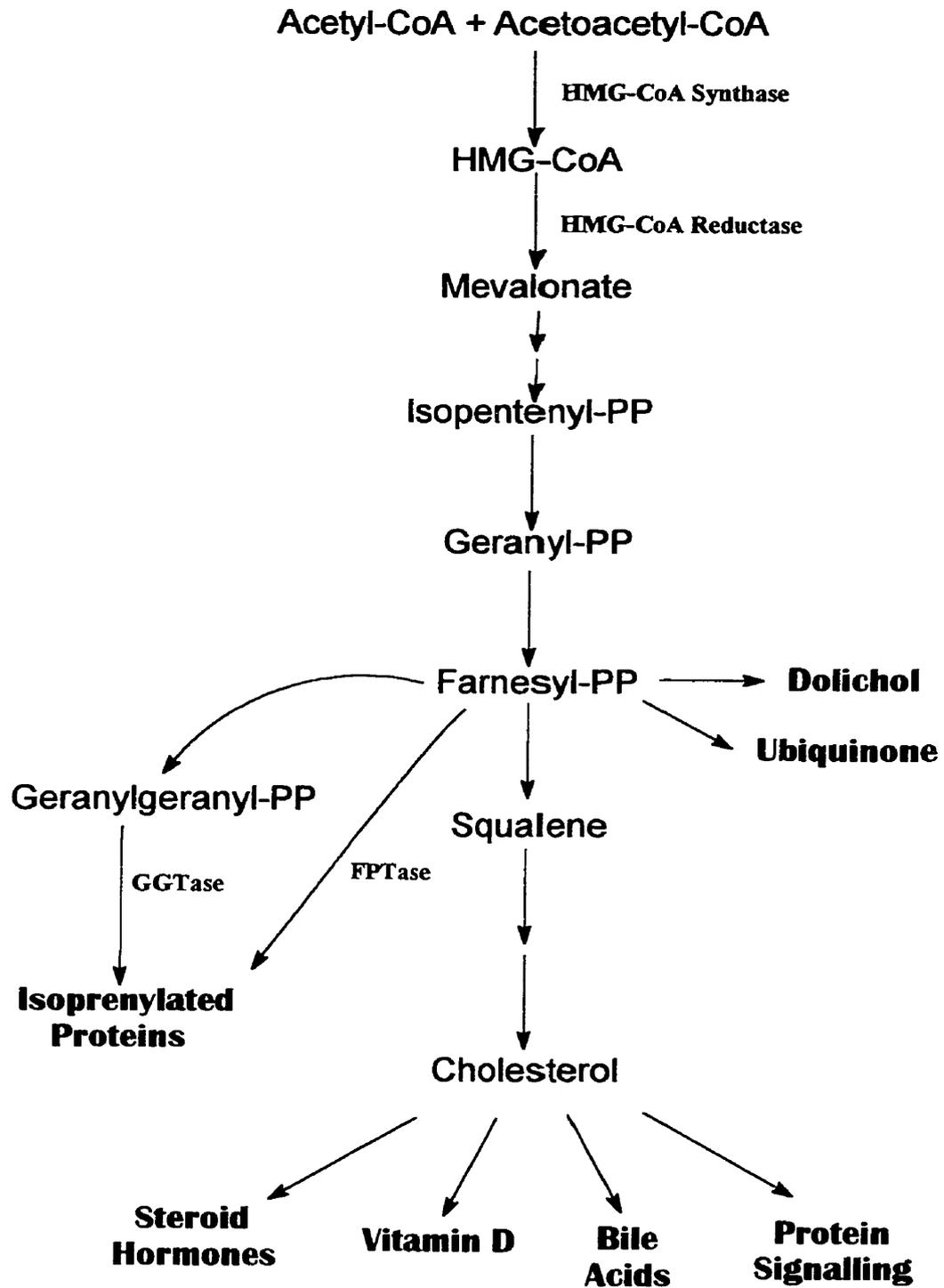


Figure 1-3. Cholesterol biosynthesis (or mevalonate) pathway.

Rodents are not the preferred model to study cholesterol metabolism because they are thought to be resistant to changes in serum cholesterol and thus respond differently than humans. While this may be true for some strains, both rats and mice display wide variabilities in responsiveness to dietary cholesterol (Paigen et al. 1985; Van Zutphen and Den Bieeman 1981). Placed on the same hypercholesterolemic diet, some strains show a marked increase in serum cholesterol (responders), while other strains show no such response (non-responders) (Paigen et al. 1985; Van Zutphen and Den Bieeman 1981). This variation is also observed in the human population (Katan et al. 1986; McCombs et al. 1994). Indeed, some individuals respond to dietary cholesterol by reducing serum levels, indicating a highly effective feedback system (Ginsberg et al. 1994).

Perhaps the most important difference in cholesterol metabolism between humans and rodents is that LDL are the major carriers of blood cholesterol in humans, whereas HDL are the major carriers in rodents. Infusion of radio-labeled LDL, however, is very rapidly cleared in rats (Spady et al. 1985), suggesting that they may have a greater requirement for cholesterol. This notion is supported by the observation that cholesterol synthesis is much greater in rodents than in humans (Spady and Dietschy 1983). In spite of this difference, studies using rodents have provided useful information on lipid metabolism in humans and are still commonly used to study mechanisms of diseases that can not be conducted using humans (Breslow 1996). Indeed, almost every major lipid disorder described in humans has also been demonstrated in rodents (Breslow 1996).

1.3.3 Fatty Acid Regulation of Cholesterol Metabolism

One of the common biological effects of fatty acids is to alter serum lipoproteins and cholesterol metabolism. The different effects of different fatty acids are mainly due to fatty acid chain length and degree of unsaturation. A review of studies in humans on the effects of fat on serum lipids indicates that SFA increase serum cholesterol, PUFAs lower serum cholesterol, and MUFAs have no significant effect on serum cholesterol (Hegsted et al. 1993). These changes in total cholesterol correlate with changes in LDL-cholesterol (Hegsted et al. 1993). The effects of PUFAs also depend on whether they are n-3 or n-6. Most studies on the effects of n-3 and n-6 PUFAs have compared the effects of fish oils (rich source of n-3) to plant oils (rich source of n-6). n-3 PUFAs consistently lower VLDL by inhibiting its formation in the liver (Nestel et al. 1987). The effects on other lipoproteins, however, are less consistent (Harris 1996). Supplementing a Western diet with fish oils does not lower LDL-cholesterol (Harris 1996), although replacing dietary saturated fats with fish oils leads to a significant reduction in total- and LDL-cholesterol levels (Harris et al. 1983).

1.3.4 Regulation of HMG-CoA reductase

Since HMG-CoA reductase catalyzes the rate limiting step in the cholesterol biosynthetic pathway, there has been considerable interest in the mechanisms that regulate its activity and expression. HMG-CoA reductase was initially thought to be a 53 kDa protein (Ness et al. 1979). Although this was found to be a proteolytic fragment of the full length 97 kDa protein, the fragment still retains full catalytic activity (Liscum et al. 1983). The size of HMG-CoA reductase was confirmed by the nucleotide

sequence of a ~4.8 kb mRNA showing that the protein has 887 amino acids and a molecular weight of 97 kDa (Chin et al. 1984). HMG-CoA reductase has also been observed to form dimers through disulfide linkages and can be detected as a 200 kDa protein by SDS-PAGE (Ness et al. 1985). HMG-CoA reductase is localized to the endoplasmic reticulum with the N-terminus bound to the membrane and the C-terminus, containing the catalytic site, projecting into the cytoplasm (Roitelman and Shechter 1986).

In cultured cells, depletion of LDL increases HMG-CoA reductase whereas an increase in LDL decreases HMG-CoA reductase activity by as much as 90% (Larsson 1996). Only the addition of mevalonate completely abolishes reductase activity (Goldstein and Brown 1990). The precise mechanisms by which increased levels of intracellular cholesterol signal the down-regulation of HMG-CoA reductase to prevent accumulation of cholesterol within the cell are not clear (Liscum and Underwood 1995). Down-regulation may be due to an oxygenated metabolite of cholesterol that is produced endogenously (Axelson and Larsson 1995). There is evidence that malignant cells lack feedback regulation by exogenous cholesterol (Siperstein and Fagan 1964), although, this has been demonstrated only in hepatomas and leukemias (Siperstein 1995). Some tumors, however, do exhibit a functional feedback mechanism (Cavenee et al. 1978).

Transcriptional control is one of the mechanisms by which cholesterol down-regulates HMG-CoA reductase (Goldstein and Brown 1984). Sterol response elements (SRE) and their binding proteins (SREBP) are believed to mediate transcriptional regulation (Brown and Goldstein 1998). Most of these studies, however, have been

carried out using cultured cells. Work by Ness and colleagues indicates that the transcription of HMG-CoA reductase is not affected in rats fed cholesterol (Ness et al. 1991). However, when rats are fed lovastatin, a competitive inhibitor of HMG-CoA reductase and inducer of hepatic *HMG-CoA reductase* gene expression, cholesterol does suppress the mRNA levels (Lopez et al. 1997). The authors concluded that competitive inhibitors of HMG-CoA reductase 'unmask' transcriptional control of cholesterol *in vivo*, but cholesterol feeding alone does not alter hepatic mRNA levels. Since the liver is the major organ regulating serum cholesterol and is a major site of cholesterologenesis (Dietschy et al. 1993), most of the *in vivo* experiments have examined changes in this organ. Much less is known about the regulation of HMG-CoA reductase in extrahepatic tissues.

HMG-CoA reductase is regulated through a multivalent feedback mechanism that is controlled by both sterol and non-sterol metabolites (Nakanishi et al. 1988; Ness et al. 1994a). Although cholesterol is the major end-product in the mevalonate pathway, mevalonate is also used for the synthesis of dolichol, ubiquinone, isopentenyladenine and various isoprenoids (Goldstein and Brown 1990). A decrease in enzyme activity may occur by phosphorylation, increased enzyme degradation, decreased translation and decreased transcription (Edwards et al. 1983). Several factors have been reported to alter the expression and/or activity of HMG-CoA reductase, for example; insulin (Ness et al. 1994b), dietary isoprenoids (Elson 1995), lycopene (Fuhrman et al. 1997), β -carotene (Moreno et al. 1995), psyllium (Turley et al. 1991), fatty acids (Choi et al. 1989), and retinoic acid (Dimitroulakos and Yeger

1996). Interestingly, the effects of these compounds on HMG-CoA reductase correlate well with their effects on cell proliferation or tumorigenesis.

1.4 CHOLESTEROGENESIS, MEVALONATE AND TUMORIGENESIS

Since cancer cells exhibit high rates of cholesterol synthesis, it has been postulated that limiting the availability of cholesterol may inhibit cancer development (Chen et al. 1978; Siperstein 1995). Almost twenty years ago, researchers observed that inhibitors of HMG-CoA reductase decrease DNA synthesis and inhibit cell proliferation (Huneeus et al. 1979). These drugs, collectively known as 'statins', were first developed for the treatment of hypercholesterolemia (Endo et al. 1976). Inhibition of proliferation by statins is completely reversed by mevalonate but is only partially reversed by cholesterol (Habenicht et al. 1980; Huneeus et al. 1979). Partial restoration by cholesterol appears to occur *via* a 'sparing' effect on mevalonate, thereby allowing more of it to be used for cell proliferation instead of sterol synthesis. Therefore, mevalonate synthesis not cholesterol synthesis *per se*, is relevant to proliferation and carcinogenesis. In 1991, two different groups reported that the growth inhibitory effects of statins occurs in a cell-cycle dependent manner (Jakobisiak et al. 1991; Keyomarsi et al. 1991). Cells deprived of mevalonate become growth arrested in the G₁-phase and may undergo apoptosis (programmed cell death) if insufficient mevalonate is available (Reedquist et al. 1995).

Although the availability of mevalonate, not cholesterol, determines the proliferative potential of a cell (Siperstein 1984), cholesterol synthesis correlates well

with mevalonate synthesis and may give an approximate measure of it. The rate of cholesterol biosynthesis in mammary tumors was found to be 6-fold greater than in normal mammary glands (Rao et al. 1988), and HMG-CoA reductase gene expression is elevated in a variety of tumors (Engstrom and Schofield 1987). Competitive inhibitors of HMG-CoA reductase inhibit the development of rat and mouse mammary tumors *in vivo* (Alonso et al. 1998; Inano et al. 1997) as well as the proliferation of human breast cancer cells *in vitro* (Larsson 1994; Wejde et al. 1992; Wejde et al. 1993). Thus, inhibiting the mevalonate pathway may be an effective approach for treating and/or preventing breast cancer (Rao 1995). Indeed, it has been suggested that the chemopreventive effects of some of the constituents found in fruits and vegetables are due to their ability to inhibit mevalonate synthesis (Elson 1995; Elson and Yu 1994). For example, d-limonene, found in citrus fruits, has been shown to inhibit HMG-CoA reductase and rat mammary tumorigenesis (Elegbede et al. 1984), and is currently under investigation as an anti-cancer agent (McNamee 1993). The precise mevalonate-derived metabolite required for cell-cycle progression, however, has not yet been identified.

1.4.1 Ras Partitioning

The *ras* gene family (*H-*, *K-* and *N-ras*) of proto-oncogenes encode a group of heterotrimeric G-proteins that are involved in signal transduction and regulate cell proliferation (Barbacid 1987). By convention, *ras* refers to the gene while Ras refers to the 21 kDa protein product (also referred to as p21^{ras}). In addition to serving an important function in normal cell growth and proliferation, overexpression and/or

mutated forms of *ras* are associated with malignant transformation and have been found in a variety of animal and human tumors (Bos 1989; Lowy and Willumsen 1993). Ras proteins exist in an active (GTP-bound) and inactive (GDP-bound) state (Lowy and Willumsen 1993). To become functional, they must undergo a series of post-translational modifications to increase their hydrophobicity thereby allowing them to become localized to the plasma membrane and trigger signal transduction pathways (Figure 1-4) (Gelb 1997). Schmidt *et al* (1984) observed that an isoprenoid derived from mevalonate is incorporated post-translationally into cellular proteins with molecular weights of 13-58 kDa. Ras proteins were discovered to belong to this class of proteins that are isoprenylated (Casey *et al.* 1989; Hancock *et al.* 1989; Schafer *et al.* 1989). The first step, catalyzed by farnesyl protein transferase (FPTase), is the addition of a farnesyl moiety to a cysteine that is four amino acids from the C-terminus of the protein (Casey *et al.* 1989; Newman and Magee 1993). The protein must then undergo proteolytic removal of the three terminal amino acids, carboxymethylation of the now terminal farnesyl-cysteine residue and palmitoylation of another cysteine (Der and Cox 1991). Some *ras* mutations give rise to proteins that lack GTPase activity resulting in constitutively active proteins that permanently bind GTP. One of the proteins that controls the Ras mitogenic activity is GTPase-activating protein (GAP) which promotes GTP hydrolysis and keeps Ras in the inactive GDP-bound state (Lowy and Willumsen 1993). Mutant Ras proteins that fail to interact with GAP, remain in the GTP-bound state and send unchecked mitogenic signals to the nucleus for proliferation. Since Ras is overexpressed in human breast cancer (Bos 1989; DeBortoli *et al.* 1985), suppression of Ras farnesylation by inhibitors of FPTase has

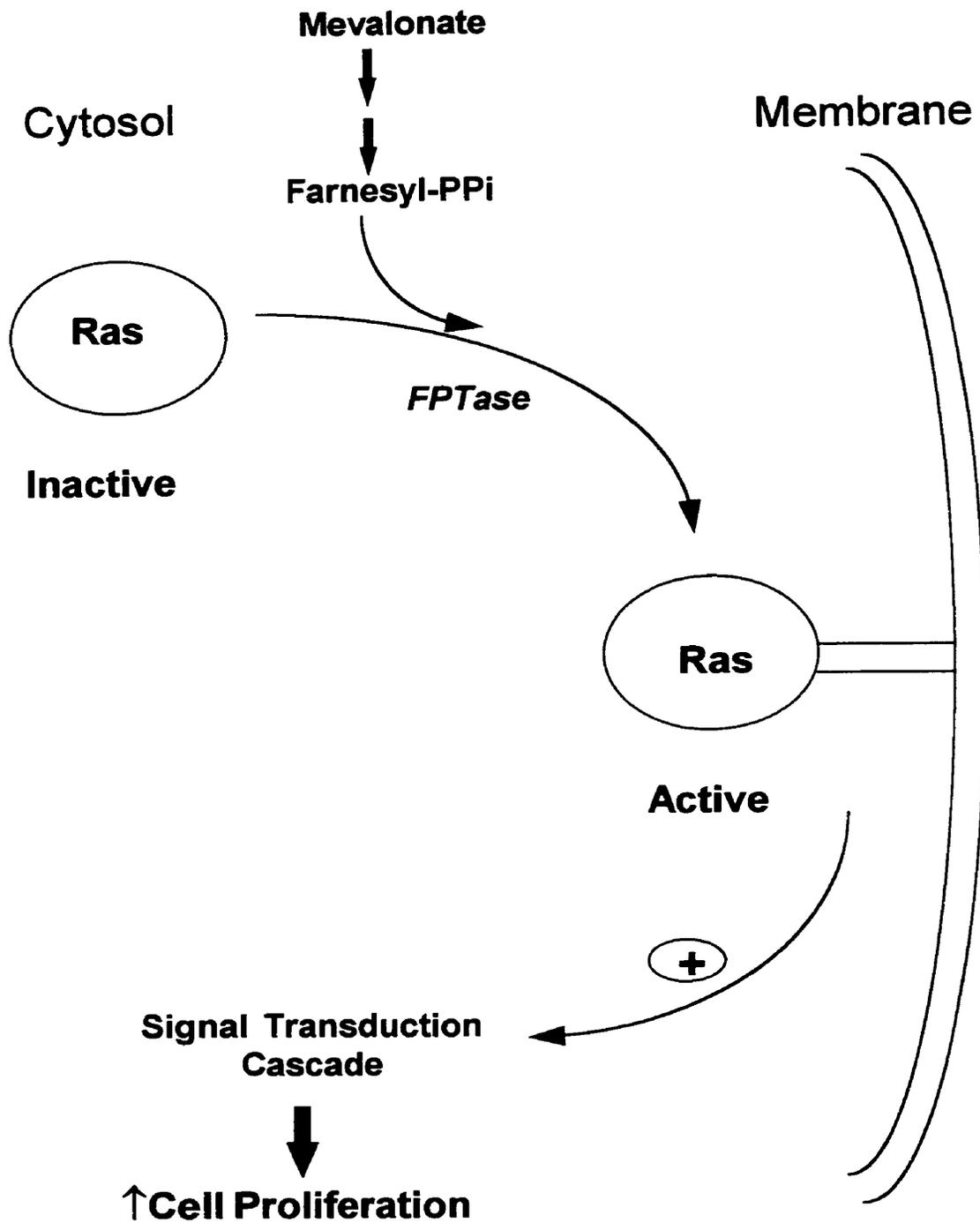


Figure 1-4. Ras partitioning and activation.

recently been proposed for the treatment of the disease (Kelloff et al. 1997). Indeed, competitive inhibitors of FPTase have been shown to inhibit the development of mammary tumors in mice (Kohl et al. 1995). It has also been postulated that the reduction in Ras membrane localization is one of the possible molecular mechanisms by which HMG-CoA reductase inhibitors exert their anticancer effects (Goldstein and Brown 1990). Specific inhibitors of either HMG-CoA reductase or FPTase lead to an accumulation of cytosolic Ras (inactive), reduction of membrane-bound Ras (active) and decreased tumor cell growth (Cox and Der 1992; Kohl et al. 1993). However, De Clue *et al* (1990) showed that inhibition of cell proliferation by HMG-CoA reductase inhibitors is independent of Ras function. This was demonstrated using a transformed cell line that requires myristate instead of farnesyl to become membrane-bound and functional. This important finding indicates that a decrease in mevalonate synthesis may inhibit tumorigenesis independently of changes in Ras partitioning. Although Ras may still be a suitable target for chemoprevention, other mevalonate-derived products involved in cell proliferation have not yet been identified (Larsson 1996).

1.5 FAT AND BREAST CANCER

The association between dietary fat and breast cancer remains one of the most contentious issues in nutritional sciences. Since dietary fat is a complex mixture of diverse lipophilic compounds, it is not possible to make generalizations regarding the effects of 'fat' on metabolic pathways or disease processes. The high caloric density of fat also makes it difficult to control the intake of energy while keeping other nutrients

constant in studies that compare different levels of fat. In this regard, it may not be clear whether any effects are due to fat *per se*, or to differences in energy intake between the experimental diets. Nevertheless, several lines of evidence do suggest that different classes of fatty acids may play an important role in cancer development or prevention.

1.5.1 Human Studies

The results of epidemiological studies relating dietary fat and breast cancer have not been consistent. International correlation studies have provided some of the most compelling evidence for an association between fat and breast cancer. The correlations between per capita fat consumption and breast cancer incidence are striking and are as high as 0.8 (Carroll et al. 1986). Case-control studies have also shown a positive association between fat and breast cancer. A meta-analysis of 12 case-control studies by Howe *et al* (1990) shows a significant positive correlation between overall fat consumption and risk of breast cancer. Many studies that examine the association between fat and breast cancer do not adequately correct for energy intake. Therefore, it is difficult to conclude whether any observed effects are due to the calories or to some other aspect of fat. This distinction is critical since energy restriction has a major inhibitory effect on breast carcinogenesis (Kritchevsky et al. 1984).

Most prospective cohort studies have failed to observe an effect of dietary fat (Hunter et al. 1996; van den Brandt et al. 1993; Willett et al. 1987). It has been argued that a greater difference in fat consumption than those observed in these studies (20%

versus 40% of energy) may be required to observe a significant difference (Boyd et al. 1993; Carroll 1992). Hunter *et al* (1996) pooled the raw data from several large cohort studies and examined the effect of fat as a percentage of energy intake in over 330,000 women with almost 5,000 cases of breast cancer. Subjects were divided into quintiles of fat consumption ranging from 15% of calories to over 45% of calories from fat. The results show no differences between any of the quintiles of fat intake. However, the food frequency questionnaire method of dietary assessment used in this analysis has been criticized for not being suitable to detect differences within the population studied (Prentice 1996; Wynder et al. 1997). Since many of the earlier studies have compared total fat with breast cancer risk, the inconsistent findings may be due, at least in part, to the different types of fatty acids in the diet.

Not all studies examining the effects of fat on breast cancer have examined the role of different types of fatty acids. Recently, a prospective study has shown that MUFAs are associated with a reduced risk of breast cancer while PUFAs are associated with an increased risk (Wolk et al. 1998). Although no distinction was made between n-3 and n-6 PUFAs, the majority of PUFAs consumed are n-6 (i.e. LA) (Okuyama et al. 1997). Thus, a general effect of PUFAs are interpreted as an effect of n-6 PUFAs. A few studies, however, have distinguished between n-3 and n-6 PUFAs. Using data from the MRFIT study, Dolecek and Granditis (1991) showed that the n-3/n-6 PUFA ratio is negatively correlated with overall cancer mortality. Marine fish oils are a rich source of dietary long chain n-3 PUFAs (Sargent 1997). Kaizer *et al* (1989) compared breast cancer incidence and mortality rates with approximate fish consumption in different countries and found an inverse relationship between percent

calories from fish and breast cancer rates. They hypothesized that the n-3 PUFAs contained in fish are protective against breast cancer. Two other ecological studies also showed that dietary fat derived from fish correlates negatively and vegetable fat positively with breast cancer mortality (Hursting et al. 1990; Sasaki et al. 1993). The WCRF/AICR panel made the conclusions shown in Table 1-1 regarding the role of different types of fat.

A more direct approach to evaluate the fat-breast cancer association is to use biomarkers of fat intake. The fatty acid profile of adipose tissue has been used as an estimate of the proportion of different types of fat consumed and the values correlate well with long term intake (London et al. 1991). Zhu *et al* (1995) examined fatty acid intake and composition of breast adipose tissue in post-menopausal breast cancer patients and patients with benign breast disease. Dietary intake of EPA and DHA was significantly lower in breast cancer patients than in patients with benign breast disease. Accordingly, the percentage of DHA in the breast adipose tissue was significantly lower in breast cancer patients than in patients with benign breast disease. The variability in the levels of EPA was large and no significant differences were found.

Table 1-1.

Summary of WCRF/AICR panel conclusions regarding the role of different types of fatty acids on breast cancer risk.

Type of fat	Remarks
Total	"Diets high in total fat <i>possibly increase</i> the risk of breast cancer."
SFA	"Diets high in saturated fat <i>possibly increase</i> the risk of breast cancer."
MUFA	"Diets high in monounsaturated fat per se <i>possibly have no relationship</i> with the risk of breast cancer, independent of that of total fat."
*PUFA	"Diets high in polyunsaturated or vegetable fats <i>possibly have no relationship</i> with the risk of breast cancer, independent of any contribution to total fat intake."
n-3 PUFA	"The evidence regarding n-3 fatty acids and breast cancer is too sparse; <i>no judgment</i> is possible."

*Although not stated explicitly, this presumably refers to n-6 PUFAs.

(Source: (AICR 1997))

In a more recent study, levels of n-3 or n-6 PUFAs in adipose tissue showed no consistent association by themselves with breast cancer risk (Simonsen et al. 1998). However, the ratio of long-chain n-3 PUFAs to total n-6 PUFAs showed a significant inverse correlation. Although the current evidence from human studies regarding either n-3 or n-6 PUFAs is not strong, the n-3/n-6 ratio in the diet may be a more significant factor and the studies that have made this measure are so far consistent (Dolecek and Granditis 1991; Simonsen et al. 1998). Modest increases in n-3 PUFAs and decreases in n-6 PUFAs can significantly alter the n-3/n-6 ratio that some have postulated is an important variable in understanding the association between fat and several chronic diseases including breast cancer (Okuyama et al. 1997). As discussed in a previous section (1.2.1), there are several limitations to epidemiological studies that can not be resolved using current methodologies. More convincing evidence for the role of fat in breast cancer development has come from studies using laboratory animals.

1.5.2 Animal studies

Over the past fifty years, numerous studies using rodents have investigated the effects of dietary fat on mammary carcinogenesis and these have been the subject of several recent reviews (Fay et al. 1997; Freedman et al. 1990; Ip 1997; Welsch 1995). In the early 1940's, Tannenbaum first showed that a high-fat diet increases the incidence of spontaneous mammary tumors in mice while energy restricted diets have the opposite effect (Tannenbaum 1942; Tannenbaum 1945). In 1967, Gammal *et al* used the DMBA-induced mammary tumor model to demonstrate the promoting effects

of a high fat diet over a much shorter period of time than the spontaneous tumor model used by Tannenbaum (Gammal et al. 1967). Since then, numerous studies have examined the role of fat in mammary tumorigenesis with emphasis on the level of fat (Freedman et al. 1990), types of fatty acids (Fay et al. 1997) and mechanisms of action (Welsch 1987). From these studies it appears that fat exerts its effect primarily on the post-initiation stage of carcinogenesis and the magnitude of the effect depends to a great extent on the type and amount of fat in the diet (Welsch 1992). Since energy restriction results in a marked inhibition of tumor development (Kritchevsky et al. 1984; Tannenbaum 1945), there has been some concern that the promoting effect of fat is merely a result of its high energy content (Carroll 1986). However, a meta-analysis of over 100 animal studies concluded that the promoting effect of fat is independent of its energy content (Freedman et al. 1990). Although several mechanisms have been proposed to explain the effects of fat, no single mechanism has emerged as conclusive.

One of the difficulties in evaluating the role of different types of fatty acids in mammary tumorigenesis is establishing the appropriate control diet since a minimum amount of essential fatty acids is required for normal growth and development (Reeves et al. 1993). Furthermore, Ip *et al* (1985) showed that approximately 4% LA is required in the diet for optimal tumor growth. Since there is no true reference diet, studies have compared the effects of one type of fat to another at various levels in the diet and the effects are, therefore, relative. Vegetable oils rich in n-6 PUFAs such as corn, safflower or sunflower oil have a greater tumor promoting effect than fats or oils rich in SFA such as coconut oil, butter or lard (Carroll and Hopkins 1979). This difference is

largely due to the LA content of vegetable oils which increases tumor yield up to a certain level, above which SFA promote as much as n-6 PUFAs (Hopkins and Carroll 1979). Olive or palm oil are rich in MUFA and do not exert a significant effect on mammary tumor development (Carroll and Khor 1971; Lasekan et al. 1990).

n-3 PUFAs derived mainly from fish oils appear to inhibit mammary tumor development. Jurkowski and Cave (1985) showed in MNU-initiated rats that as the percentage of fish oil in the diet increased from 0.5%-20%, tumor latency progressively lengthened and tumor incidence and tumor burden decreased. Using the DMBA model, Braden and Carroll (1986) examined the effects of feeding either menhaden (a marine fish) oil or corn oil to rats at levels of either 3, 10 or 20% in the diet. As the level of fat in the diet increased, the difference in tumor yield diverged with more tumors in the corn oil group and fewer tumors in the menhaden oil group. In mice initiated with DMBA, Cameron *et al* (1989) and Craig-Schmidt *et al* (1993) have shown that a high corn oil diet leads to a greater tumor incidence than a high fish oil diet. These studies on the inhibitory effects of fish oils may be criticized for using diets with inadequate amounts of LA that are necessary for tumor growth. Yet, even with diets containing sufficient amounts of LA, several investigators have reported the inhibitory effects of fish oils (Abou-El-Ela et al. 1989; Cave and Jurkowski 1987; Cohen et al. 1993; Ip et al. 1986; Karmali et al. 1987). Cave and Jurkowski (1987) compared the effects of various blends of corn and menhaden oils, and found that a diet containing 15% menhaden oil + 5% corn oil had a tumor latency period that was considerably longer than the 20% corn oil group. Ip *et al* (1986) also showed that rats consuming a diet containing 12% menhaden oil + 8% corn oil had a reduced tumor burden

compared to those fed 20% corn oil. In a transplanted tumor model, an 8% fish oil + 15.5% corn oil diet caused a 40% inhibition of tumor volume compared to a 23.5% corn oil diet (Karmali et al. 1987). Therefore, the protective effects of fish oils are probably due to the presence of inhibitory n-3 PUFAs as opposed to the absence of enhancing n-6 PUFAs.

A recent meta-analysis on the effects of different types of fat in experimental mammary tumorigenesis found a small but not statistically significant protective effect of n-3 PUFAs (Fay et al. 1997). The lack of effect appears to be due to the selection criteria used which excluded several supportive studies that were well designed. Nevertheless, the authors concluded that shifting the proportion of fat towards a higher n-3/n-6 PUFA ratio probably decreases tumor burden. Not all studies, however, support the hypothesis that n-3 PUFAs derived from fish oils are protective (reviewed by Ip 1997). One of the reasons is that there is no clear dose-response effect in several studies that show a protective effect of fish oils (Braden and Carroll 1986; Cohen et al. 1993; Ip et al. 1986). Once the LA requirements have been met, there appears to be an optimal n-3/n-6 PUFA ratio that is required to maximize tumor inhibition and this is hypothesized to be a mixture of approximately equal parts of menhaden or other fish oil with corn or safflower oil (Ip 1997). Although this is equivalent to an n-3/n-6 ratio of ~0.4, the precise ratio has not been determined. In support of this notion, Cohen *et al* (1993) treated rats with MNU, fed them various blends of corn and menhaden oils in a 23% fat diet, and found the fewest tumors in the group fed an equal amount of the two oils. However, another study found that 15% fish oil + 5% corn oil gave the same incidence of DMBA-induced mammary tumors as 10%

fish oil + 10% corn oil (Bunce and Abou-El-Ela 1990). Inconsistencies among studies may be due to differences in the levels of lipid peroxidation or fatty acid content of the fish or vegetable oils used.

The inhibitory effects of n-3 PUFAs have also been evaluated using other oils derived from plants which are mostly rich in ALA. Inhibitory effects have been reported for fats rich in ALA such as flaxseed, primrose and perilla oils (Abou-El-Ela et al. 1987; Hirose et al. 1990; Kamano et al. 1989; Nakayama et al. 1993; Thompson 1995), but the effects do not appear to be as strong as those of fish oils (Bunce and Abou-El-Ela 1990). Thus, the long chain n-3 PUFAs (e.g. EPA and DHA) found mostly in fish oils appear to be more potent inhibitors of mammary tumorigenesis than their precursor ALA. In spite of this, few animal studies have examined the effects of feeding individual long chain n-3 PUFAs on mammary tumorigenesis. Recently, Noguchi and co-workers showed that low doses (0.5 ml twice per week) of purified EPA and DHA significantly inhibited the promoting effect of a high-fat diet on DMBA-induced mammary tumors in rats (Noguchi et al. 1997). The role of other fatty acids in mammary tumorigenesis has also been examined but the results are not striking and the effects are relative to other fats that have been described above (Welsch 1992). Of interest, however, is the finding that conjugated linoleic acid (CLA) found naturally in dairy products, inhibits mammary tumor development when fed at <1% in the diet (Ip et al. 1991). The presence of dietary fatty acids that are potent inhibitors of mammary tumorigenesis may explain some of the inconsistencies among the human studies.

1.5.3 Cell Culture Studies

The effects of individual fatty acids on the growth of cultured mammary epithelial cells have been investigated. Since cancer cells already proliferate rapidly, few studies have examined whether n-6 PUFAs increase the proliferation of these cells even further. Rose and Connolly (1990) observed that LA stimulates the proliferation of MDA-MB-231 human breast cancer cells. LA has also been shown to stimulate the proliferation of non-transformed mouse and human mammary epithelial cells in culture (Balakrishnan et al. 1989; Bandyopadhyay et al. 1987).

Several studies have examined the growth inhibitory effects of n-3 PUFAs on normal and neoplastic human mammary epithelial cell lines. Grammatikos *et al* (1994) showed that EPA or DHA inhibit the growth of the estrogen positive MCF-7 human breast cancer cell line by as much as 50% in a concentration-dependent manner (6 - 30 μ M). The growth of the non-cancerous human mammary epithelial cell line MCF-10A was not inhibited by EPA or DHA at concentrations below 24 μ M, but there was significant inhibition at 30 μ M. ALA and AA inhibited proliferation much less extensively than EPA or DHA, while LA had no effect at concentrations up to 30 μ M (Grammatikos et al. 1994). Chajès *et al* (1995) showed that EPA and DHA at 65 and 60 μ M respectively, produce a significant inhibition of the growth of the two hormone-independent human breast tumor cell lines MDA-MB-231 and HBL-100. Indeed, presence of 60 μ M DHA during the incubation period reduced growth of both lines by almost 95%. In that study (Chajès et al. 1995), DHA inhibited growth of MCF-7 cells but EPA did not for reasons that are unclear. In a third study by Noguchi *et al* (1995),

EPA and DHA produced concentration-dependent (0 - 24 μ M) inhibition of the growth of MDA-MB-231 cells. At the highest concentration, EPA and DHA produced approximately 50% and 60% inhibition respectively. Finally, Rose and Connolly (1990) showed a concentration-dependent (0 - 8 μ M) inhibition of growth of MDA-MB-231 cells by both EPA and DHA. At the highest concentration, DHA produced about a 66% inhibition while EPA produced about 30% inhibition. Differences in the magnitude of the effects between these studies may be due to a variety of reasons. Highly unsaturated fatty acids are known to oxidize very readily in air leading to the formation of toxic peroxidation products. Indeed, this is one of the mechanisms that has been proposed to explain the anti-cancer effects of n-3 PUFAs (Begin et al. 1986). Therefore, the extent to which fatty acid peroxidation occurred in these different studies may explain some of the differences. The purity of the reagents and the presence of fatty acids in the serum may also explain some of the inconsistencies.

Most of the research on the role of fat in breast cancer has focused on the fatty acid content and relatively fewer studies have examined the role of cholesterol.

1.6 CHOLESTEROL AND BREAST CANCER

1.6.1 Human Studies

Although cholesterol has long been implicated in the development of cardiovascular disease (Brown and Goldstein 1992), descriptive and analytical epidemiological studies have shown no associations between dietary cholesterol and breast cancer (McMichael et al. 1984). However, the strong correlations between the

intake of cholesterol and animal fat and protein make it difficult to determine the independent effects of cholesterol (Mendola et al. 1995). Studies relating the intake of cholesterol and breast cancer have recently been reviewed (AICR 1997). Of the five prospective and seven case-control studies, only one case-control study showed a significant increase in breast cancer risk with increasing intake of cholesterol (Rohan et al. 1988). Among the remaining studies, the odds ratios ranged from 0.5-1.3. A pooled analysis by Hunter *et al* (1996) also found no significant association between dietary cholesterol and risk of breast cancer. The WCRF/AICR review panel concluded that, "Diets high in cholesterol probably have no relationship with the risk of breast cancer" (AICR 1997).

Serum cholesterol levels have been used as a surrogate measure of dietary intake, as well as an independent risk factor for cancer risk (McMichael et al. 1984). Some studies have shown that elevated serum cholesterol is a risk factor for breast cancer and is associated with decreased survival in breast cancer patients (Cowan et al. 1990). Other studies report an inverse association between serum cholesterol and overall cancer mortality (Vatten and Foss 1990). There are a number of difficulties, however, in interpreting associations between serum cholesterol and human cancer. As mentioned earlier, placed on the same hypercholesterolemic diet, some individuals show a marked increase in serum cholesterol (responders), while others show no such response (non-responders), or even a decrease in serum levels (Beynen et al. 1987). Thus, serum levels are a poor indicator of dietary cholesterol. Furthermore, the well established effects of other dietary components such as fat and fiber on serum cholesterol may be further confounding (Hegsted et al. 1993). The results may also be

obscured by the presence of a tumor which has been shown to lower markedly serum cholesterol levels (Vitols et al. 1985).

1.6.2 Animal Studies

In 1966, Szepeswol observed that feeding mice a diet high in lard and cholesterol greatly increases the number of spontaneous mammary tumors in mice (Szepeswol 1966). However, cholesterol or lard alone had no effect. Cohen and Chan (1982) used the MNU-induced mammary tumor model to study the effects of high- (20%) and low- (5%) fat (lard) diets with and without 2% cholesterol in Fisher rats. Regardless of the level of fat in the diet, cholesterol had no effect on tumor development. In other studies on mammary carcinogenesis, Klurfeld and Kritchevsky (1981) fed diets containing 1.5% cholesterol plus 0.5% bile salts to Sprague-Dawley rats initiated with DMBA. They observed a small increase in the mammary tumor yield per rat compared with controls, but tumor incidence and size were not changed. Although the effect of cholesterol in their experiment was not distinguished from that of the bile salts, they concluded that diet-induced hypercholesterolemia significantly enhances the development of mammary tumors. Nakayama *et al* (1993) also showed a small, but not statistically significant, increase in mammary tumor incidence in Sprague-Dawley rats initiated with DMBA and fed 0.2% cholesterol. Although Sprague-Dawley rats fed cholesterol should have increased levels in their serum, the animals in that study did not for reasons that are unclear.

Cholesterol is known to readily oxidize in air giving rise to a variety of oxidation products that can inhibit the absorption of cholesterol (Smith 1981). Some cholesterol

oxidation products have also been shown to be mutagenic and carcinogenic (Morin et al. 1991; Peng and Morin 1992). Although oxidized cholesterol has been suggested to play a role in breast cancer development (Petrakis et al. 1981; Wrensch et al. 1989), its effects on experimental mammary tumorigenesis have not been adequately investigated. Only one study that was conducted recently examined the effects of cholesterol oxidation products on mammary tumorigenesis (El-Bayoumy et al. 1996). Cholesterol epoxides had no effect on initiation and the effects on promotion were not investigated. Genetic differences in cholesterol absorption and metabolism between rat strains and the extent of cholesterol oxidation could explain any differences among studies relating cholesterol to mammary tumorigenesis (Klurfeld and Kritchevsky 1981; Smith 1981; Van Zutphen and Den Bienman 1981).

1.7 PROPOSED MECHANISMS

Despite several mechanisms being proposed to explain how dietary lipids modulate mammary carcinogenesis, no mechanisms have emerged as conclusive. Several biochemical pathways are modulated by dietary fat or cholesterol but it is not clear whether any of these alterations are causally related to cancer development. Since the role of cholesterol and its oxidation products in experimental mammary tumorigenesis are not well established, this section will only focus on proposed mechanisms involving the effects of fatty acids. It should be noted, however, that cholesterol may alter membrane fluidity and potentially change the function of cell

surface receptors (Liscum and Underwood 1995). Such changes in membrane fluidity have been used to explain some of the biological effects of cholesterol.

1.7.1 Prostaglandins

A widely investigated mechanism relating both the promoting effects of n-6 PUFAs and the inhibitory effects of n-3 PUFAs is prostaglandin (PG) biosynthesis. PGs are a class of eicosanoids that are produced from AA through the cyclooxygenase (COX) pathway. They are involved in diverse cellular functions such as inflammation, proliferation, differentiation and cellular adhesion (Smith et al. 1996). Support for this pathway derives from several lines of evidence. Mammary tumors synthesize greater levels of PGs than normal mammary glands (Karmali et al. 1989), and inhibitors of PG synthesis such as indomethacin inhibit mammary tumorigenesis (McCormick et al. 1985). To determine whether a decrease in PG synthesis could abrogate the tumor promoting effects of a high-fat diet, Carter *et al* (1983) gave indomethacin to rats fed either a high- or low-fat diet. Indomethacin had little effect on tumor incidence in rats fed the low-fat diet but abolished the promoting effects of the high fat diet. Whether this effect is mediated through the production of specific PGs *per se* or *via* other pathways that are affected by indomethacin is not clear. In a follow-up study, Carter *et al* (1989) found that the cyclooxygenase inhibitors carprofen and indomethacin similarly reduce PGE₂ levels in the serum and the mammary epithelium, but only indomethacin inhibited DMBA-induced mammary tumorigenesis suggesting that a reduction in PGE₂ synthesis is not sufficient to inhibit mammary tumor development. Thus, promotion by high fat and inhibition by indomethacin may occur

through different pathways and changes in PG synthesis do not necessarily correlate with tumor burden. Karmali *et al* (1989) compared the effects of n-3 and n-6 PUFAs on DMBA-induced mammary tumorigenesis and PG synthesis. The group fed a high n-3 PUFA diet had the fewest tumors and the lowest levels of PGE₂ in normal mammary glands and tumors.

Two isoforms of COX have been identified, a constitutive (COX-1) and inducible (COX-2) isoform (Smith *et al.* 1996). COX-1 maintains normal physiological functions whereas COX-2 is involved in inflammation and is overexpressed in a variety of tumors (Vane 1994). Indeed, specific inhibitors of COX-2 are being developed as novel chemotherapeutic agents (DeWitt and Smith 1995). Badawi *et al* (1998) recently showed that COX-2 mRNA levels are expressed in the mammary glands of rats fed n-6 PUFAs but are undetectable in those fed n-3 PUFAs. Although the chemopreventive effects of selective inhibitors of COX-2 are believed to be due to a decrease in PG synthesis (Vane 1994), these agents also inhibit tumorigenesis in the absence of changes in PG synthesis and in cancer cells that do not express COX-1 or COX-2 (Hanif *et al.* 1996). Thus, changes in the expression of COX-2 may not be causally related to tumorigenesis.

1.7.2 Hormones

The well established role of hormones in breast cancer development has led researchers to examine whether changes in various hormones could explain the effects of fat on mammary tumorigenesis. One of the uncertainties with this mechanism is that the promoting effect of a high-fat diet has been shown to occur in the absence

of any changes in the levels of estrogen or prolactin (Ip and Ip 1981; Ip et al. 1980). Furthermore, the promoting effects of fat can still be demonstrated in ovariectomized rats (Rogers 1997). Finally, n-3 PUFAs inhibit and n-6 PUFAs promote the proliferation of hormone-independent breast cancer cell lines (Chajès et al. 1995; Rose and Connolly 1990), suggesting that hormones are unlikely to play a major role in mediating the effects of fat.

Insulin is another hormone that may be involved in breast cancer development (Bruning et al. 1992). Insulin resistance is believed to be a risk factor for breast cancer and has been postulated to mediate the effects of fat on the development of the disease (Stoll 1998). It is too early, however, to determine whether insulin resistance mediates the effect of fat on breast cancer.

1.7.3 Oxidation Products

In cultured human cell lines, n-3 PUFAs have a marked cytotoxic effect on tumor cells but virtually no effect on normal cell lines (Begin et al. 1986). This toxic effect is independent of the cytostatic effects of n-3 PUFAs and appears to involve the formation of lipid peroxides which are produced readily by highly unsaturated fatty acids such as EPA and DHA. It is not clear whether this 'selective' toxicity is due to a specific feature of malignant cells, or whether it is an effect on highly proliferative cells. Interestingly, however, LA stimulates the proliferation of normal and malignant human mammary epithelial cells while increasing intracellular lipid peroxide concentrations (Cunningham et al. 1997). Therefore, the formation of lipid peroxides seems to occur with fatty acids that inhibit as well as with fatty acids that promote, suggesting that lipid

peroxide levels do not correlate with tumorigenesis. Others have also shown that the effects of anti-oxidants (Horvath and Ip 1983) or a high-fat diet (Lane et al. 1985), on tumorigenesis do not correlate with changes in lipid peroxidation. Therefore, this mechanism is unlikely to be a significant mediator of the effects of fat on mammary tumorigenesis.

1.7.4 Other Mechanisms

Other mechanisms explaining the promoting effects of n-6 PUFAs and/or the inhibiting effects of n-3 PUFAs have been proposed. These include alterations in the immune system (Vitale and Broitman 1981), changes in fatty acid processing (Grammatikos et al. 1994), alterations in cell membrane structure and function (Burns and Spector 1990), modulation of gene expression (Telang et al. 1988), changes in polyamine synthesis (Abou-El-Ela et al. 1989), differences in caloric intake (Welsch et al. 1990), differential expression of the *H-ras* oncogene (Hu et al. 1995; Lu et al. 1995) and modification of estrogen metabolism (Osborne et al. 1988). As with the other mechanisms discussed, none of these studies have demonstrated conclusively that they mediate the effects of fat on mammary carcinogenesis. Furthermore, most of the studies examining the mechanism of fat show correlations and do not demonstrate a causal link. Since cancer is a multistage and multifactorial disease, it is possible that no single mechanism could explain how lipids modulate breast carcinogenesis. Nevertheless, establishing the functional significance of one or more mechanisms will allow more definitive recommendations to be made for the prevention of breast cancer.

1.8 HYPOTHESIS AND ORGANIZATION OF THESIS

The overall objective of this research is to investigate a molecular mechanism by which dietary lipids may modulate mammary carcinogenesis. The hypothesis is that mevalonate synthesis mediates the effects of dietary polyunsaturated fatty acids and cholesterol on mammary tumorigenesis. Mevalonate is a precursor of cholesterol but also plays an important role, independent of cholesterol, in regulating DNA synthesis and cell proliferation. Furthermore, the role of mevalonate in cell proliferation may or may not involve the post-translational modification of Ras, a growth regulatory protein involved in tumorigenesis.

This thesis is in two parts. Since the role of dietary cholesterol and oxidized cholesterol in mammary tumorigenesis remains unclear, the first part involves experiments using carcinogens to evaluate the effects of cholesterol on tumorigenesis. Chapter 2 focuses on the effects of dietary cholesterol and its oxidation products on MNU-induced rat mammary tumor development. In view of the results from that chapter, the objective of Chapter 3 was to compare the effects of dietary cholesterol on the development of MNU- and DMBA-induced rat mammary tumors to determine whether *H-ras* mutations mediate the effects of cholesterol.

The objective of the second part of this thesis is to examine a potential mechanism of action of n-3 and n-6 PUFAs on mammary tumorigenesis. Relative to each other, there is considerable evidence that n-6 PUFAs (ie. LA) promote while n-3 PUFAs inhibit mammary tumorigenesis. Therefore, a tumorigenesis study was not conducted. Instead, normal rodent mammary tissues were used since the

preneoplastic cells that ultimately develop into neoplasms are more similar to normal than malignant cells. Normal cells are the most appropriate cell type to study mechanisms of prevention since preneoplastic cells are difficult to identify and isolate. Chapter 4 describes the effects of n-3 and n-6 PUFAs on the mevalonate pathway. Chapter 5 contains detailed mechanistic studies including possible effects mediated through the LDL-R. Finally, in Chapter 6, mice with a targeted disruption of the *LDL-R* gene were used to determine whether this receptor mediates the effects of n-3 PUFAs on HMG-CoA reductase, as established in the previous two chapters using rats (4 & 5).

CHAPTER TWO

EFFECTS OF DIETARY CHOLESTEROL AND OXIDIZED CHOLESTEROL ON RAT MAMMARY TUMORIGENESIS

(Modified from: A. El-Soehy, W.R. Bruce and M.C. Archer. *Carcinogenesis*, 17:159-
162, 1996)

2.1 ABSTRACT

The purpose of this study was to determine the effects of dietary cholesterol and oxidized cholesterol on MNU-induced mammary tumor development. Sprague-Dawley rats were administered 50 mg/kg MNU at 50 days of age and fed either a control AIN-76 diet or the control diet supplemented with 0.3% cholesterol or 0.3% oxidized cholesterol for up to 26 weeks. The oxidized cholesterol was prepared by heating cholesterol at 110°C for 48 hours. Gas chromatographic analysis of the oxidized cholesterol revealed a 2% yield of cholesterol oxidation products in addition to a large amount of unchanged cholesterol (>96%). Tumor incidence in the cholesterol group (67%) was significantly lower than the control group (96%) $p < 0.05$, but the oxidized cholesterol group (79%) was not different from the control or cholesterol groups. The average number of tumors per animal was lower in the cholesterol group (1.5) than in the control (2.8) or oxidized cholesterol groups (2.3), $p < 0.005$. Total serum cholesterol was greater in the cholesterol (6.79 ± 0.85 mmol/L) and the oxidized cholesterol groups (6.16 ± 0.65 mmol/L) than in the controls (3.39 ± 0.16 mmol/L), $p < 0.05$, although there were no differences between the cholesterol and the oxidized cholesterol groups. These results show that dietary cholesterol inhibits mammary tumor development in this model. Elevated serum cholesterol may down-regulate HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis, in preneoplastic and/or tumor cells, thereby inhibiting cell proliferation.

2.2 INTRODUCTION

Cholesterol is known to oxidize readily in air giving rise to a variety of products (Smith 1981), many of which have been detected in foods such as processed meats and dairy products (Sander et al. 1989). Some products of cholesterol oxidation are known to be cytotoxic, mutagenic, immunosuppressive and carcinogenic (Morin et al. 1991; Peng and Morin 1992; Peng et al. 1979; Sevanian and Peterson 1986). Petrakis *et al* (1981) detected cholesterol α - and β - epoxides in nipple aspirate fluid of women with benign breast disease. In a subsequent study (Gruenke et al. 1987), the levels of cholesterol β -epoxide were found to be higher in nulliparous women than in those who were lactating, parous or had previously breast fed. It was postulated that the lower risk of breast cancer in women who have had a full term pregnancy may be related to the lower exposure of their ductal epithelial cells to cholesterol epoxide. Furthermore, Wrensch *et al* (1989) found higher than normal levels of cholesterol β -epoxide in breast fluid aspirates of women with benign breast disease, with or without atypical hyperplasia of the epithelium. They postulated that this may be a factor in the increased incidence of breast cancer associated with hyperplasia.

In spite of this circumstantial evidence suggesting that cholesterol oxidation may be involved in breast cancer development, its role in experimental mammary carcinogenesis has not been explored. Thus, the objective of this study was to determine whether cholesterol oxidation products promote rat mammary tumorigenesis.

2.3 MATERIALS AND METHODS

Animals

Pathogen-free female Sprague-Dawley rats (43 days old) purchased from Charles River Laboratories (St. Constant, Quebec, Canada), were housed at $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$ at 50% humidity with a 12 hour light-dark cycle. They were acclimatized on the standard AIN-76 control diet for seven days before the start of the experiment with food and water provided *ad libitum*.

Diets

Cholesterol (5-[6]-cholesten-3-ol) was purchased from Sigma Chemical Co. (St. Louis, MO) and stored in a dark sealed container at -20°C to prevent oxidation. To prepare oxidized cholesterol, cholesterol was spread evenly to a thickness of about 5 mm on the surface of a glass tray, and heated at 110°C for 48 hours in an electric oven as previously described (Kendall et al. 1992). The control AIN-76 diet consisted of 50% sucrose, 20% casein, 15% starch, 5% alphacel, 5% corn oil, 3.5% mineral mix, 1% AIN-76 vitamin mix, 0.3% dl-methionine and 0.2% choline. To the control diet was added 0.3% cholesterol or 0.3% oxidized cholesterol at the expense of sucrose, levels that had previously been shown to enhance colonic preneoplasia (Kendall et al. 1992). The same batches of cholesterol and oxidized cholesterol were used throughout the experiment. The diets were prepared weekly and stored in dark plastic bags at -20°C . Fresh diet was added to the cages three times per week.

Experimental Protocol

At 50 days of age, the animals received an ip injection of 50 mg/kg MNU (Sigma Chemical Co., St. Louis, MO) dissolved in 0.05% acetic acid in normal saline and used within 30 minutes of preparation. Three days after carcinogen administration, the rats were randomized into the three dietary groups (24/group). They were weighed bi-weekly and palpable lesions were recorded weekly. Moribund animals, those with tumors larger than 15 mm, or those remaining after 26 weeks, were anaesthetized, blood samples taken by cardiac puncture and then sacrificed by cervical dislocation. Tumors were excised, fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histopathological examination (Dr. Alan Medline, Department of Pathology, Northwestern Hospital, North York). Serum prepared from the blood samples was stored at -70°C prior to analysis for total-, LDL- and HDL- cholesterol (Vita Tech Laboratories, Toronto, Canada).

Gas Chromatography

Analyses of cholesterol and oxidized cholesterol were performed on a Varian 3400 gas chromatograph with a flame ionization detector. Cholesterol, 5 α -cholestane, 7-ketocholesterol, 3 β ,5 α ,6 β -cholestanetriol, 7 β -hydroxycholesterol and 5 α ,6 α -epoxide, purchased from Sigma Chemical Co. (St. Louis, MO), were analyzed as their trimethylsilyl ether derivatives on a fused silica capillary SE-30 column (0.25 mm x 30 m, Altech, Guelph, Canada) with a liquid phase thickness of 0.25 μ m. 5 α -Cholestane was used as an internal standard. Samples were injected at a column temperature of 270°C that was raised to 300°C at a rate of 10°C/min. The injector and detector

temperatures were 275 and 320°C respectively. Nitrogen was used as the carrier gas with a flow rate of 0.4 ml/min. All samples and standards were analyzed in triplicate.

Statistical Analyses

Tumor incidence data were analyzed by Chi-Square test of independence. The average number of tumors per animal and serum cholesterol values were compared using analysis of variance followed by Student's t-test and Duncan's *post hoc*.

2.4 RESULTS

To study the effects of cholesterol and oxidized cholesterol on the promotion of mammary carcinogenesis, female Sprague-Dawley rats were injected with MNU and randomized them to the control diet or to diets supplemented with 0.3% cholesterol or 0.3% oxidized cholesterol. Gas chromatographic analysis showed that the purity of the cholesterol was ~98%, while the oxidized cholesterol contained about 2% of oxidation products and 96% unchanged cholesterol. The major oxidation products were 3 β ,5 α ,6 β -cholestanetriol, 7 β -hydroxycholesterol, 7-ketocholesterol and 5 α ,6 α -epoxide, as assessed by comparison of retention times to those of authentic standards. There were also a few minor unidentified peaks.

The final body weights of the animals in the three groups did not differ. The cumulative tumor incidences for the three groups of animals is shown in Figure 2-1. All tumors were shown to be adenocarcinomas by histopathological examination. The final tumor incidence in the cholesterol-fed group was significantly lower than the control (67% vs. 96%, $p < 0.05$). The tumor incidence in the animals fed oxidized cholesterol (79%) did not differ from the control or cholesterol groups. The average number of tumors per animal is shown in Figure 2-2. As was the case for tumor incidence, animals in the cholesterol group had fewer tumors (1.5) than in the control (2.8) or the oxidized cholesterol groups (2.3), $p < 0.005$. Two animals in the cholesterol group had tumors that regressed then subsequently reappeared. The number of tumors per tumor bearing rat did not differ significantly between groups; the means (ranges) were: control, 2.9 (1-8), cholesterol, 2.3 (1-8) and oxidized cholesterol, 2.9 (1-10).

Table 2-1 shows the serum cholesterol values for the three dietary groups. Total serum cholesterol was significantly greater in both the cholesterol and the oxidized cholesterol groups than the control group, $p < 0.01$. LDL-cholesterol levels in the cholesterol and oxidized cholesterol groups were significantly greater than in the control group, $p < 0.05$. However, both LDL-cholesterol and total cholesterol did not differ between the groups fed cholesterol and the oxidized cholesterol. HDL-cholesterol was lower in the oxidized cholesterol group than in the control and cholesterol groups.

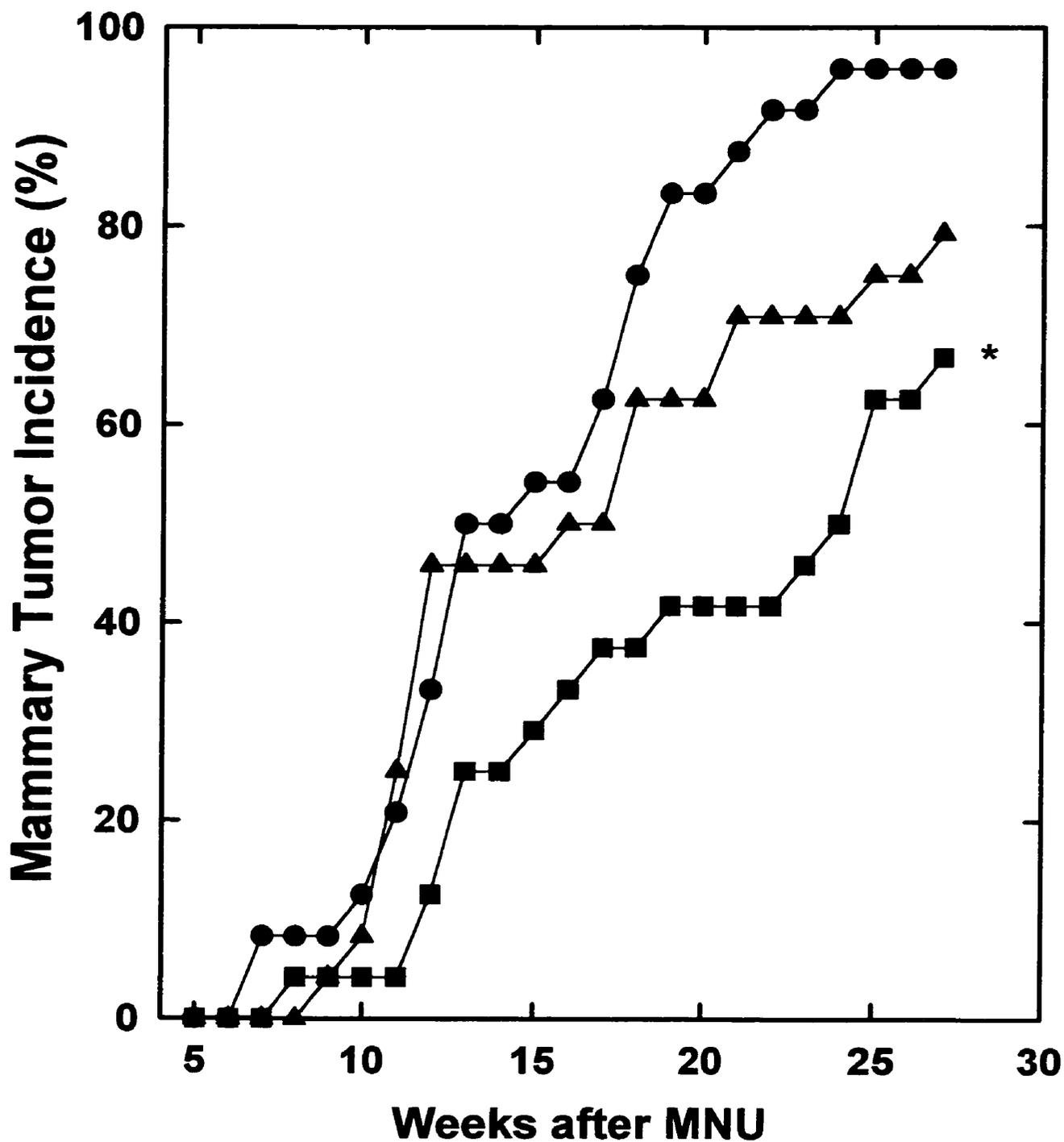


Figure 2-1. Mammary adenocarcinoma incidence in Sprague-Dawley rats initiated with MNU then fed a control diet (●), 0.3% oxidized cholesterol (▲), or 0.3% cholesterol (■). *Significant difference in the final tumor incidence from the control group ($P < 0.05$) ($n=24$).

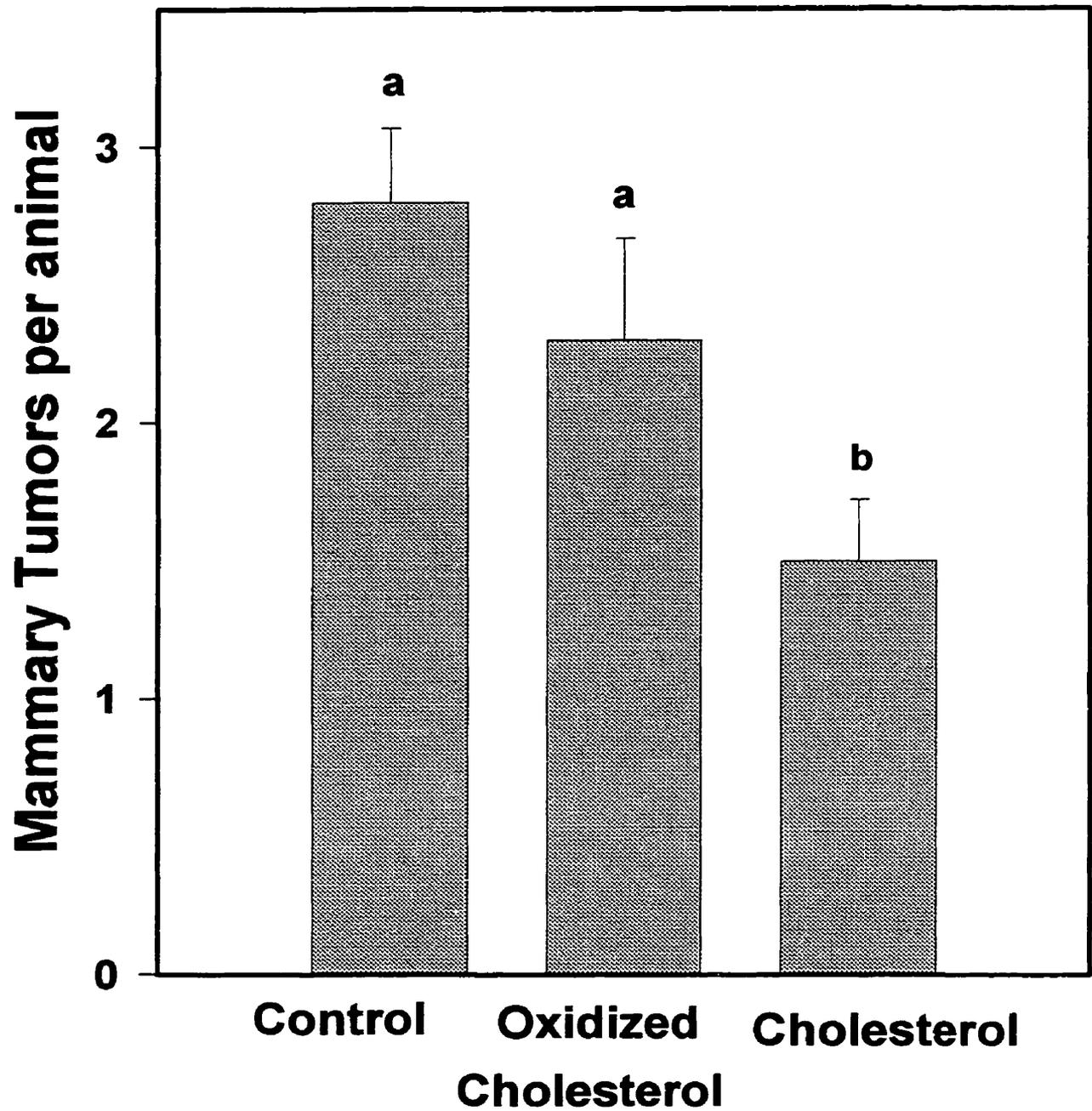


Figure 2-2. Average number of tumors per animal in groups fed control, 0.3% cholesterol or 0.3% oxidized cholesterol diets (n=24). Groups not sharing the same letter are significantly different ($P < 0.05$).

Table 2-1. Effect of dietary cholesterol or oxidized cholesterol on serum lipids.

Group	Total Cholesterol (mmol/L)	LDL Cholesterol (mmol/L)	HDL Cholesterol (mmol/L)
Control	3.39 ± 0.16	1.42 ± 0.10	1.76 ± 0.23
Cholesterol	6.79 ± 0.85 ^a	4.79 ± 0.98 ^b	1.30 ± 0.13
Oxidized Cholesterol	6.16 ± 0.65 ^a	4.14 ± 0.88 ^b	1.04 ± 0.10 ^b

Values are mean ± SEM (n=8).

^aSignificantly different from controls, $P < 0.01$.

^bSignificantly different from controls, $P < 0.05$.

2.5 DISCUSSION

In these experiments that were designed to examine the effects of oxidized cholesterol on the promotion of MNU-induced rat mammary tumors, it was first shown that oxidation of cholesterol by the method of Kendall *et al* (1992) gives a product that contains only about 2% of oxidation products, the remainder being unchanged cholesterol. It was, therefore, necessary to monitor the effects of unoxidized cholesterol in the diets of an additional group of animals. Unexpectedly, this group that was fed a diet containing 0.3% cholesterol, had a significantly lower incidence of tumors and a smaller number of tumors per animal than animals fed the control diet containing no cholesterol.

In previous studies, Klurfeld and Kritchevsky (1981) fed cholesterol plus bile salts to Sprague-Dawley rats initiated with DMBA. They observed a small increase in the mammary tumor yield per rat compared to controls, but tumor incidence and size were not changed. However, the effect of cholesterol in these experiments was not distinguished from that of the bile salts. Nakayama *et al* (1993) also showed a small, but not statistically significant, increase in mammary tumor incidence in Sprague-Dawley rats initiated with DMBA and fed 0.2% cholesterol in a diet similar to that used in the present experiment. It is not clear why these results differ from ours, but differences have been shown in the endocrine responsiveness of DMBA- and MNU-induced tumors (Welsch 1985), and in their patterns of oncogene activation (Waldmann *et al.* 1993; Zarbl *et al.* 1985).

Cohen and Chan (1982) found that cholesterol at a level of 2% in the diet of Fisher rats had no effect on MNU-induced mammary tumor development, regardless of the amount of fat in the diet. Their diets, however, were higher in saturated fat and lower in unsaturated fat than in the present study. Since animals fed saturated fat have a lower tumor incidence than those fed unsaturated fat (Fay et al. 1997), a protective effect of cholesterol may have been masked. Furthermore, Fisher rats are of intermediate sensitivity towards chemically induced mammary tumors, having a maximum tumor incidence of about 50% (Chan et al. 1977). We used the highly sensitive Sprague-Dawley strain that has a maximum tumor incidence of close to 100%, which may have enabled us to detect inhibitory effects more easily. Fisher rats also differ from Sprague-Dawley rats in their response to dietary cholesterol. Placed on the same hypercholesterolemic diet, some rat strains have been demonstrated to show a marked increase in serum cholesterol (responders), while other strains show no such response (non-responders) (Van Zutphen and Den Bienman 1981). Although serum cholesterol measurements were not made by Cohen and Chan (1982), Fisher rats appear to be non-responders (Klurfeld and Kritchevsky 1981). Sprague-Dawley rats used in the present study, however, are responders (Van Zutphen and Den Bienman 1981), a result confirmed by the measurements of the effect of dietary cholesterol on serum levels (Table 2-1).

The elevated serum cholesterol levels in the animals fed cholesterol, may be associated with the inhibition of tumorigenesis that was observed. This cholesterol can enter cells *via* the LDL-R and act as a negative feedback inhibitor of endogenous cholesterol biosynthesis by reducing the levels of HMG-CoA reductase (Goldstein and

Brown 1990). Thus, in our model, exogenous cholesterol may inhibit *de novo* cholesterol synthesis by decreasing mevalonate synthesis in preneoplastic mammary epithelial cells, thereby inhibiting their proliferation and subsequent development into neoplasms. In support of this notion, we have recently shown that dietary cholesterol inhibits the development of preneoplastic lesions in the colons of mice (El-Soehy et al. 1996b). Others have also shown that dietary cholesterol inhibits the development of MNU-induced colon tumors (Cohen et al. 1982) and UV-light induced skin tumors (Black et al. 1979). In addition to effects on preneoplastic cells, the growth of mammary adenocarcinoma cells may also be inhibited by cholesterol.

The rats fed oxidized cholesterol had a somewhat lower incidence of tumors than the controls, and a somewhat higher incidence than those fed cholesterol, although the effects were not statistically significant. It is possible that one or more of the cholesterol oxidation products are tumor promoters that abrogate the inhibitory effect of cholesterol to some extent. However, a recent study by El-Bayoumy *et al* (1996) shows that cholesterol epoxides do not induce mammary tumors in rats. Whatever the mechanism, our results suggest that it is important in studies of the biological effects of cholesterol, to minimize oxidation. Indeed, cholesterol stored at room temperature may contain significant levels of oxidation products (Peng et al. 1991).

In summary, it has been shown that dietary cholesterol inhibits the promotion of MNU-induced mammary tumors in Sprague-Dawley rats. The elevated cholesterol in the serum of these animals may mediate this inhibition. It was also shown that cholesterol heated to produce about 2% of oxidation products does not inhibit tumorigenesis.

CHAPTER THREE

EFFECTS OF DIETARY CHOLESTEROL ON MNU- AND DMBA- INDUCED RAT MAMMARY TUMORIGENESIS

3.1 ABSTRACT

The purpose of this study was to determine whether a decrease in mevalonate synthesis mediates the inhibitory effect of dietary cholesterol on mammary tumor development and whether the inhibition is dependent on the frequency of *H-ras* mutations. Female Sprague-Dawley rats (30/group) were given a single dose of either MNU (50 mg/kg ip) or DMBA (100 mg/kg ig), carcinogens that produce tumors with either a high (MNU) or low (DMBA) frequency of *H-ras* mutations. Rats were fed either a control AIN-93G diet or the control diet supplemented with 0.3% cholesterol for up to 19 weeks. Although tumors appeared more rapidly in animals given DMBA, dietary cholesterol significantly delayed the average time to tumor appearance in both the MNU group (10 versus 14 weeks, $P < 0.02$) as well as the DMBA group (7 versus 9 weeks, $P < 0.01$). HMG-CoA reductase activity was higher in mammary tumors than in normal mammary glands. However, cholesterol decreased HMG-CoA reductase in the mammary glands and not in tumors. These findings suggest that cholesterol may inhibit mammary tumorigenesis by decreasing the rate of mevalonate synthesis in preneoplastic mammary epithelial cells. This inhibition does not appear to be related to the frequency of *H-ras* mutations.

3.2 INTRODUCTION

In the previous chapter it was shown that dietary cholesterol inhibits the development of MNU-induced mammary tumors in Sprague-Dawley rats and hypothesized that elevated serum cholesterol decreases mevalonate synthesis by

down-regulating HMG-CoA reductase. Mevalonate is also a precursor of farnesyl-PPi that is required for the post-translational modification of Ras to increase its hydrophobicity thereby allowing it to anchor to the plasma membrane and activate signal transduction pathways (Casey et al. 1989; Hancock et al. 1989). Since competitive inhibitors of HMG-CoA reductase cause a build-up of cytosolic Ras (inactive) and a decrease in membrane-bound (active) Ras (Jakobisiak et al. 1991; Sinensky et al. 1990), it has been postulated that the reduction in Ras membrane localization is one of the possible mechanisms by which these drugs exert their anticancer effects (Goldstein and Brown 1990). Moreover, inhibitors of Ras farnesylation inhibit colony formation of cells transformed with the *ras* oncogene but not those transformed with other oncogenes (Kohl et al. 1993). Studies have consistently shown that MNU-induced tumors have a high frequency (~85%) of H-*ras* mutations whereas DMBA-induced tumors have a low frequency of H-*ras* mutations (~20%) (Kito et al. 1996; Sukumar et al. 1983; Waldmann et al. 1993; Zarbl et al. 1985). Tumors harbouring these mutations produce H-Ras proteins that are constitutively active.

The inhibitory effects of dietary cholesterol on rat mammary tumorigenesis that was reported in Chapter 2 has not been demonstrated by others (Cohen and Chan 1982; Klurfeld and Kritchevsky 1981; Nakayama et al. 1993). This may be due to genetic differences in cholesterol metabolism between the rat strains selected, the purity and level of cholesterol, the composition of the basal diets or the carcinogen used. One of the studies used the same strain and similar experimental design as that used in Chapter 2, but instead of MNU used DMBA as the carcinogen (Nakayama et

al. 1993). On the basis of this result and the results from the previous chapter, it was hypothesized that cholesterol selectively inhibits the development of tumors harbouring H-*ras* mutations. The objective of this experiment was to determine whether the inhibitory effect of dietary cholesterol on mammary tumor development is dependent on the frequency of H-*ras* mutations and whether a decrease in mevalonate synthesis may be a mechanism by which cholesterol inhibits tumor development.

3.3 MATERIALS AND METHODS

Animals and Diet

Pathogen-free female Sprague-Dawley rats (43 days old) purchased from Charles River Laboratories (St. Constant, Quebec, Canada), were housed at $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and 50% humidity with a 12 hour light-dark cycle. They were acclimatized for one week on the control AIN-93G diet (Reeves et al. 1993) with food and water provided *ad libitum*. At 50 days of age, one-half of the animals were given 50 mg/kg MNU ip and the other half given 100 mg/kg DMBA ig, doses that give similar latency periods and tumor incidences (Thompson et al. 1992).

Experimental Protocol

After carcinogen treatment, the animals were randomized into four groups (30/group) and fed either a control AIN-93G diet or the control diet supplemented with 0.3% cholesterol, at the expense of cornstarch, for 19 weeks (Dyets, Bethlehem, PA). An additional group (n=18) of animals received no carcinogen and were used to measure

HMG-CoA reductase activity in normal tissues. These animals were fed either the control or 0.3% cholesterol diet for one week. Animals were weighed and palpated for mammary lesions weekly. Moribund animals, those with tumors larger than 15 mm, or those remaining after 19 weeks, were anaesthetized, blood samples taken by cardiac puncture and then sacrificed by cervical dislocation. Tissues were dissected, immediately frozen in liquid nitrogen and stored at -70°C .

Preparation of Microsomes

All tissue preparations were performed over ice or at 4°C . Tissues were homogenized in buffer A (20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 70 mM KCl, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 50 μM leupeptin) and centrifuged for 10 min at 500xg. Supernatants were centrifuged for 15 min at 9000xg followed by a final spin for 1 h at 100,000xg. Microsomes were assayed for HMG-CoA reductase activity or were solubilized in Laemmli buffer containing 8 M urea for immunoblotting. The concentration of total microsomal protein was determined using the Bio-Rad protein dye binding assay according to the manufacturer's protocol, with bovine serum albumin as a standard.

HMG-CoA reductase Enzyme Activity

The activity of HMG-CoA reductase was determined using the radiochemical assay described by Ness *et al* (1987), and expressed as picomoles of mevalonolactone formed per minute and per milligram of microsomal protein. Briefly, 50-100 μg of microsomal protein were preincubated at 37°C for 5 min in buffer B (100 mM

phosphate (pH 7.4), 70 mM KCl, 10 mM DTT, 5 mM EDTA, 5 mM EGTA and 50 μ M leupeptin), followed by a further 5 min with an NADPH regenerating system (1U glucose-6-phosphate dehydrogenase, 20 mM glucose-6-phosphate, 2 mM NADP). The assay was initiated by the addition of 14 C-HMG-CoA (20000 dpm/nmol) at a final concentration of 80 μ M and volume of 75 μ l, and terminated after 30 min by the addition of 5 μ l HCl containing 3 H-mevalonolactone as a recovery standard. After a 1 h incubation at 37°C, to allow complete lactonization of mevalonate, samples were centrifuged for 1 min at 3,000xg to remove the denatured protein, 40 μ l of supernatant applied to a silica gel G plate (Analtech, Newark, DE) and mevalonolactone separated by thin layer chromatography using a toluene:acetone (1:1) solvent system. The region corresponding to mevalonolactone (R_f 0.7) was scraped into scintillation fluid and radioactivity measured using a β -counter with a dual-labeling program.

Serum Cholesterol

Blood was taken from anaesthetized animals by cardiac puncture using a 21 gauge needle and 5cc syringe. Serum was prepared by centrifuging blood samples at 2500xg for 10 min and stored at -70°C prior to analysis for total cholesterol using a colorimetric assay as described by the manufacturer (Boehringer Mannheim, Laval, Quebec).

Statistical Analyses

Time to tumor appearance was determined using the Mantel-Haenzel procedure (Mantel 1966). Tumor incidence data were analyzed by Chi-Square test of

independence. The average number of tumors per animal and serum cholesterol values were determined using a one-way ANOVA followed by the Student's t-test with Bonferroni *post hoc* test for multiple comparisons (GraphPad Prism™, San Diego, CA).

3.4 RESULTS

To determine whether the anti-tumorigenic effects of cholesterol are dependent on the frequency of H-*ras* mutations in the preneoplastic cells that develop into tumors, the effects of dietary cholesterol on the development of MNU- and DMBA-induced mammary tumors were compared in Sprague-Dawley rats. Doses that have previously been shown to yield similar tumor incidences and latencies were chosen (Thompson et al. 1992). For reasons that are unclear, however, in the present experiment DMBA produced somewhat more tumors that appeared more rapidly than with MNU (Figures 3-1 and 3-2). The composition of the diets is shown in Table 3-1.

At the end of the 19 week experiment, cholesterol feeding resulted in higher serum cholesterol levels in rats treated with either MNU (6.02 ± 1.44 vs 2.85 ± 1.00 mmol/L, $P < 0.01$) or DMBA (6.04 ± 1.37 vs 2.02 ± 0.37 mmol/L, $P < 0.01$), compared to animals fed the control diet. As expected, cholesterol significantly delayed the average time to tumor appearance in the MNU group (10 vs 14 weeks, $P < 0.02$) (Figure 3-1). In rats treated with DMBA, cholesterol also delayed the average time to tumor appearance (7 vs 9 weeks, $P < 0.01$) (Figure 3-2). In rats treated with DMBA, the final tumor incidence in the control group (100%) did not differ from the cholesterol group (93.3%), whereas in rats treated with MNU, tumor incidence was greater in the control

(96.6%) than in the cholesterol (66.6%) group ($P < 0.05$). Cholesterol reduced the average number of tumors per rat in the MNU group but not in the DMBA group (Figure 3-3).

It was next determined whether a decrease in mevalonate synthesis in mammary glands or tumors could explain the inhibitory effects of cholesterol on tumorigenesis. Dietary cholesterol significantly decreased HMG-CoA reductase enzyme activity in the normal mammary gland but had no effect in mammary tumors (Figure 3-4). HMG-CoA reductase activity was greater in mammary tumors induced by MNU or DMBA than in normal mammary glands, regardless of whether or not cholesterol was present in the diet.

Table 3-1. Composition of experimental diets.

<i>Ingredient</i>	Experimental Diet*	
	<i>Control</i>	<i>0.3% Cholesterol</i>
Casein	20	20
Cornstarch	39.7486	39.4486
Dyetrose	13.2	13.2
Sucrose	10	10
Cellulose	5	5
Soybean oil	7	7
t-BHQ	0.0014	0.0014
Salt Mix	3.5	3.5
Vitamin Mix	1	1
L-Cystine	0.3	0.3
Choline Bitartrate	0.25	0.25
Cholesterol	-	0.3

*Diets based on the AIN-93G formulation (Reeves et al. 1993).

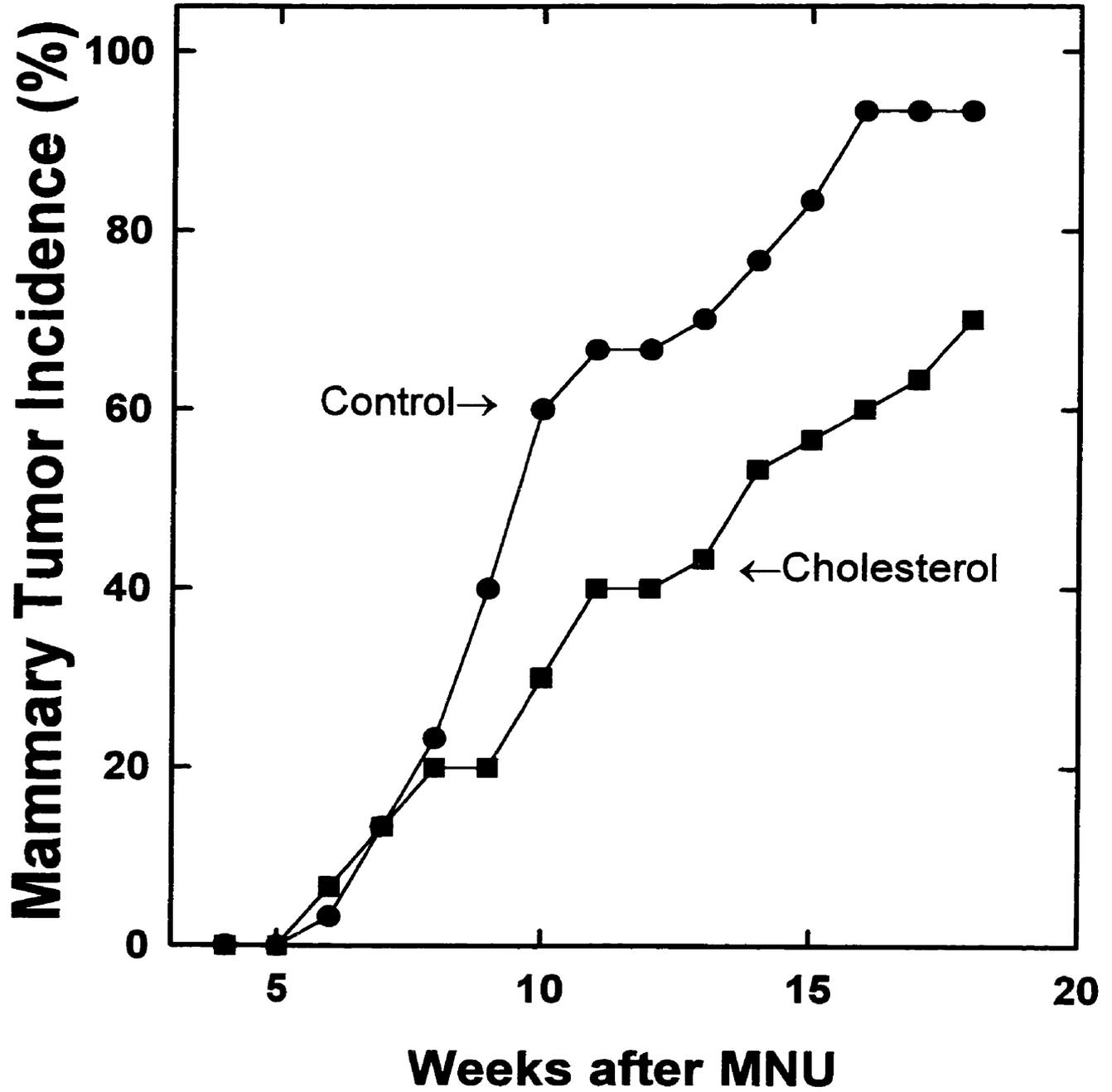


Figure 3-1. Cumulative mammary tumor incidence in rats initiated with MNU.

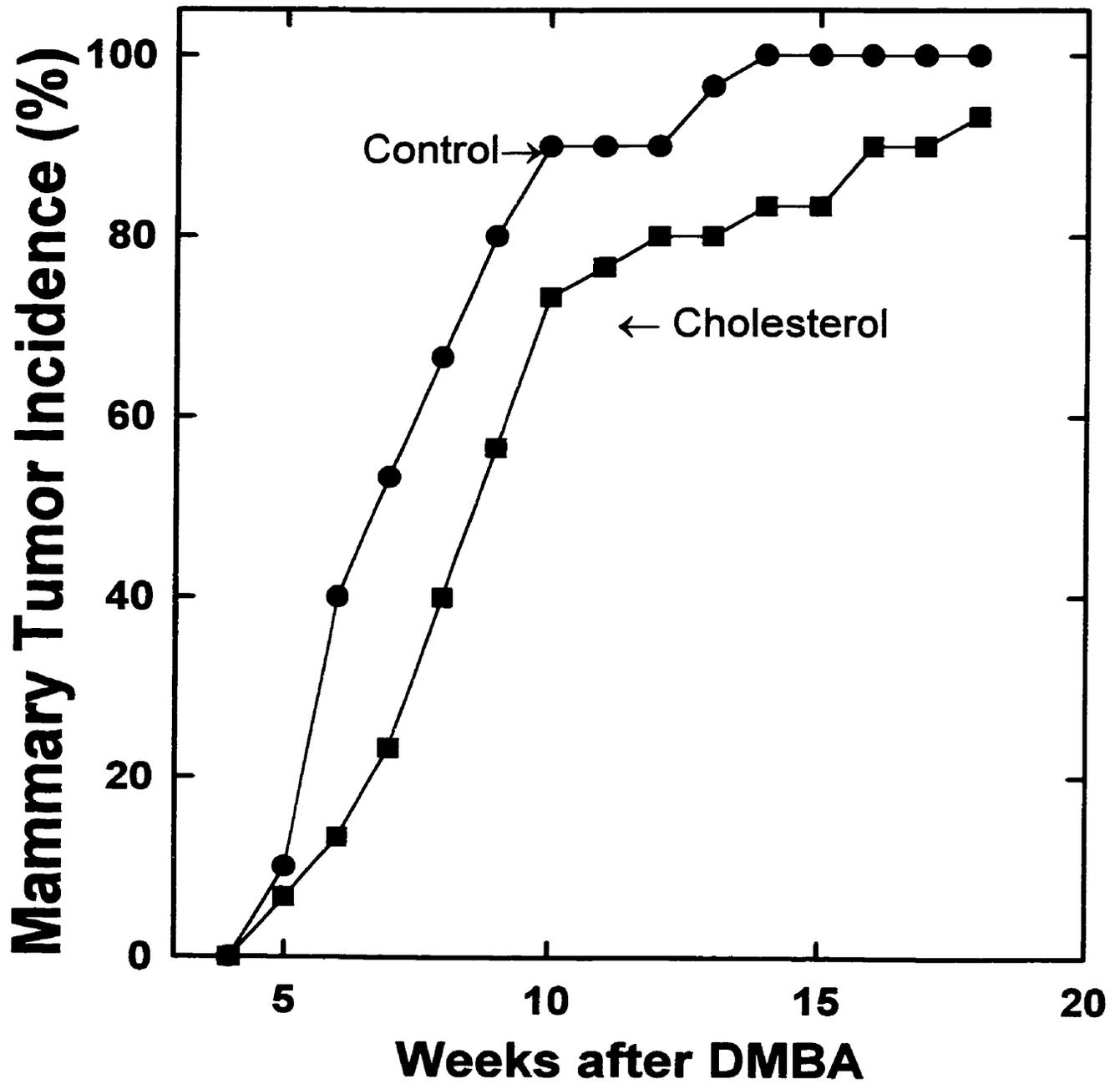


Figure 3-2. Cumulative mammary tumor incidence in rats initiated with DMBA.

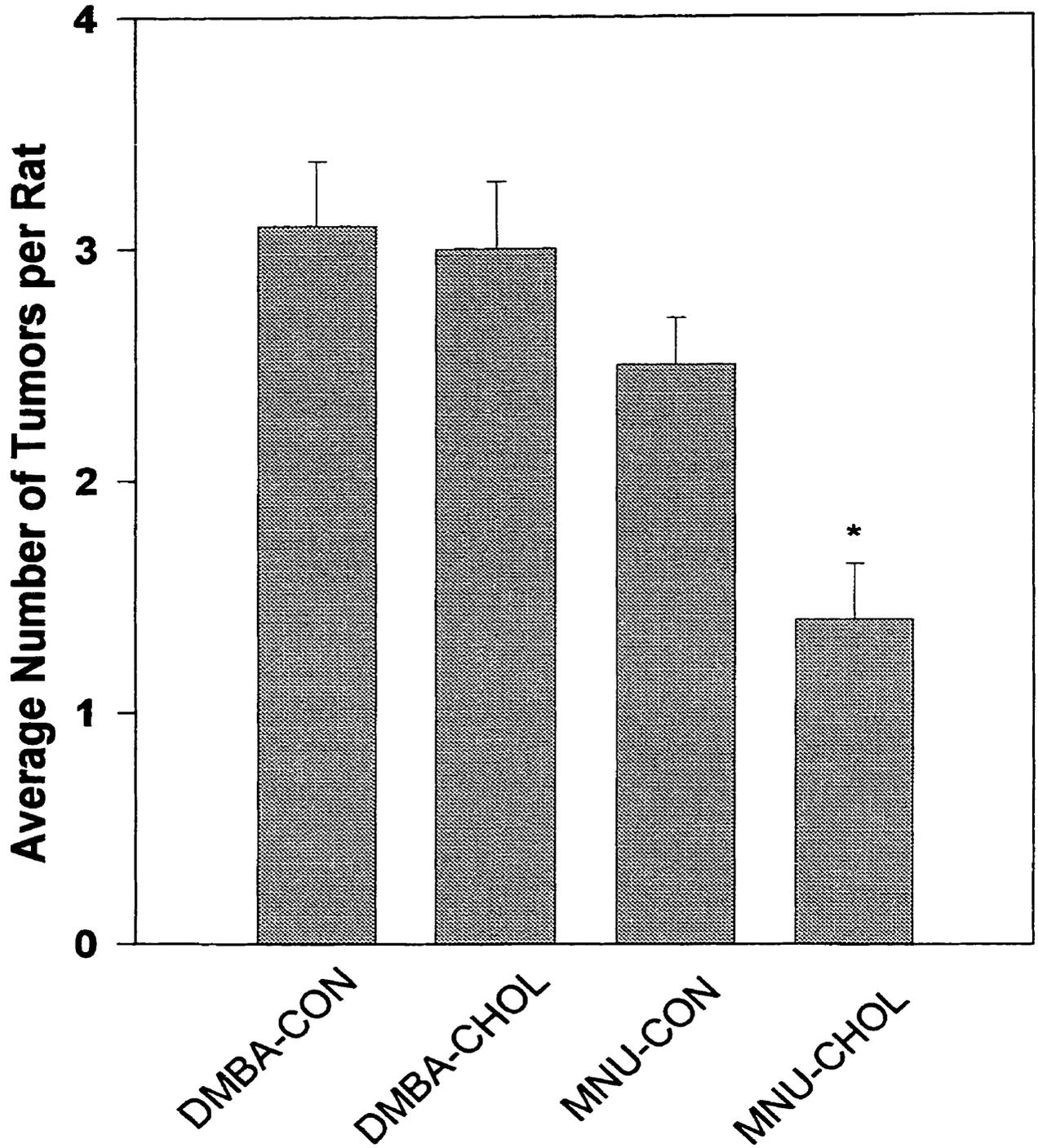


Figure 3-3. Average number of mammary tumors per rat in animals fed a control (CON) or 0.3% cholesterol (CHOL) diet. (*) Indicates statistically significant difference compared to other groups ($P < 0.05$).

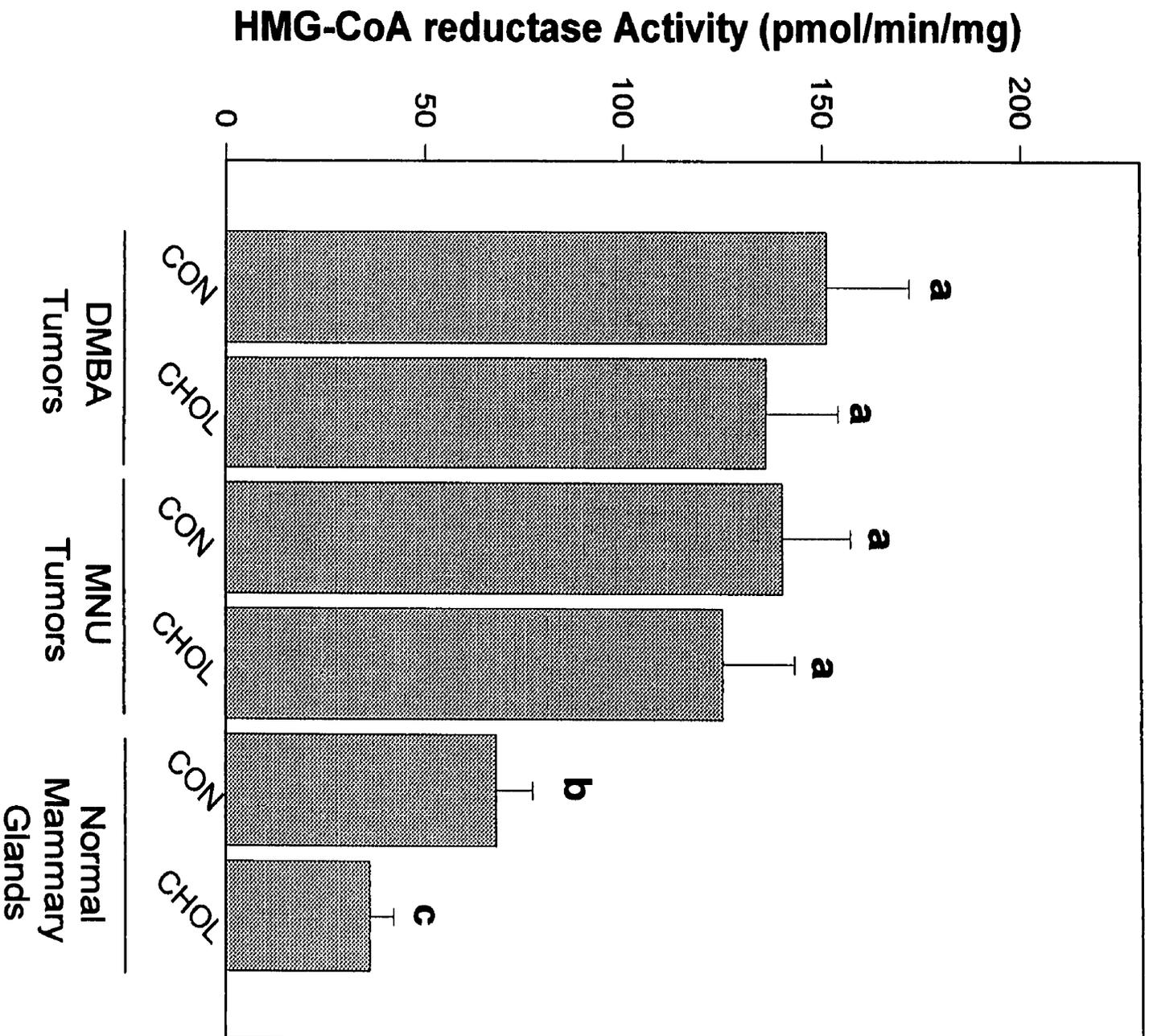


Figure 3-4. HMG-CoA reductase enzyme activity in tumors induced by either DMBA or MNU and normal mammary glands from rats fed a control (CON) or 0.3% cholesterol (CHOL) diet. Different letters indicate significant differences between groups ($P < 0.05$).

3.5 DISCUSSION

This study was designed to determine whether a decrease in mevalonate synthesis may mediate the inhibitory effect of dietary cholesterol on mammary tumor development and whether the inhibition of tumor development is dependent on the frequency of preneoplastic cells that develop into tumors harbouring *H-ras* mutations. Farnesyl-PPi derived from mevalonate is required for the post-translational modification of H-Ras proteins. Farnesylation of H-Ras enables it to become membrane-bound and functional. H-Ras proteins are much more commonly mutated in tumors induced by MNU than those induced by DMBA (Kito et al. 1996; Sukumar et al. 1983; Waldmann et al. 1993; Zarbl et al. 1985). The results of the present study show that cholesterol inhibits the development of mammary tumors induced by either of these carcinogens, suggesting that the effects on cancer development may be independent of mutated *H-ras*.

HMG-CoA reductase activity was reduced by cholesterol in normal mammary glands suggesting that a decrease in mevalonate synthesis may be a mechanism by which cholesterol inhibits tumorigenesis. The higher activity of HMG-CoA reductase that was observed in mammary tumors compared to normal mammary glands is consistent with previous reports showing elevated rates of cholesterol synthesis in mammary tumors versus normal mammary glands (Rao et al. 1988). Indeed, HMG-CoA reductase is overexpressed in a variety of tumors (Engstrom and Schofield 1987), suggesting that an increased rate of mevalonate synthesis may be necessary to maintain the high proliferation rates of cancer cells. Failure of cholesterol to down-regulate HMG-CoA reductase in mammary tumors indicates that tumor cells may lack

a feedback regulatory mechanism. This observation has been demonstrated by others using hepatomas and leukemias (Siperstein 1995; Siperstein and Fagan 1964), and may represent an adaptive mechanism by tumor cells to prevent a decrease in the mevalonate pool.

The results of the present study show that cholesterol significantly inhibits the development of mammary tumors induced by either MNU (Figure 3-1) or DMBA (Figure 3-2). The average number of tumors per animal and final tumor incidences were also reduced by cholesterol in rats treated with MNU but not those treated with DMBA. This result may be due to the greater yield and more rapid appearance of tumors caused by the DMBA. Although the inhibition of tumorigenesis by cholesterol may be mediated by the mevalonate pathway, this effect does not appear to be dependent on the frequency of tumors harbouring *H-ras* mutations. In support of this notion, there is evidence that the antiproliferative effects of selective inhibitors of HMG-CoA reductase are independent of Ras function (De Clue et al. 1990).

It is not clear why a previous study using the same strain and a similar experimental design did not observe a decrease in DMBA-induced tumorigenesis by dietary cholesterol (Nakayama et al. 1993). The cholesterol used in that study may have been extensively oxidized since serum levels were not elevated in the Sprague-Dawley rats that were used (Nakayama et al. 1993). Oxidized cholesterol is known to inhibit the absorption of cholesterol (Smith 1981) and as shown in the previous chapter, mammary tumorigenesis is not significantly inhibited by cholesterol that has been oxidized (El-Sohemy et al. 1996a).

Although cholesterol has long been implicated in the development of cardiovascular disease (Brown and Goldstein 1992), its role in human cancer development has not been thoroughly investigated. A review of the results from epidemiological studies examining a possible relationship between dietary cholesterol and breast cancer concluded that cholesterol probably has no relationship with the risk of breast cancer (AICR 1997). Since the intake of animal fat and protein, which may increase risk, correlate with the intake of cholesterol, it is unlikely that a protective effect of cholesterol can be detected in epidemiological studies (AICR 1997; Mendola et al. 1995). Furthermore, the oxidation state of cholesterol is an important confounding variable that is not controlled for in most studies. Although it is clearly inappropriate to recommend cholesterol to reduce the prevalence of breast cancer, the results of the present study underscore the importance of the mevalonate pathway in mammary tumorigenesis and suggest that other means of reducing mevalonate synthesis may be an effective approach to preventing and/or treating this disease.

In summary, a decrease in mevalonate synthesis by dietary cholesterol may mediate the inhibitory effects of cholesterol on tumorigenesis, but this effect is independent of the frequency of H-ras mutations.

CHAPTER FOUR

REGULATION OF HMG-CoA REDUCTASE BY DIETARY n-3 AND n-6 PUFAs

(Modified from: A. El-Soehy and M.C. Archer. *Cancer Res.*, **57**: 3685-3687, 1997)

4.1 ABSTRACT

Dietary n-6 PUFAs enhance rat mammary tumor development while n-3 PUFAs inhibit, yet the mechanisms are unclear. The objective of this study was to determine whether a difference in mevalonate synthesis could be a mechanism by which n-3 and n-6 PUFAs modulate mammary carcinogenesis. Female Sprague-Dawley rats were fed diets containing either Menhaden (n-3) or Safflower (n-6) oil in a 7% fat diet for one week. In comparison to the n-6 diet, the n-3 diet significantly reduced the activity and immunodetectable levels of HMG-CoA reductase in mammary glands thereby suppressing the formation of mevalonate. Serum cholesterol was lower in the n-3 group than in the n-6 group (1.91 ± 0.18 versus 2.61 ± 0.37 mmol/L, $P < 0.01$). Extrahepatic tissues meet most of their cholesterol requirements from circulating cholesterol and the internalized cholesterol down regulates HMG-CoA reductase. Thus, the concomitant decrease in serum cholesterol and mammary gland HMG-CoA reductase levels suggests that changes in circulating cholesterol levels do not solely determine the activity of extrahepatic reductase. In conclusion, the mevalonate pathway may be a mechanism through which different types of dietary PUFAs modulate breast cancer development.

4.2 INTRODUCTION

Several potential mechanisms for the effects of dietary fat on mammary tumorigenesis have been proposed and reviewed extensively, yet the molecular mechanism still remains unclear (Welsch 1992; Welsch 1995). In the previous two

chapters, cholesterol was shown to inhibit rat mammary tumorigenesis and it was hypothesized that this may be mediated by a decrease in mevalonate synthesis. Since dietary recommendations to increase cholesterol can not be made, we wondered whether the mevalonate pathway could mediate the effects of other dietary factors. Although n-3 PUFAs inhibit HMG-CoA reductase activity in the liver (Choi et al. 1989), their effects on extrahepatic tissues were not known.

The objective of the present study was to determine whether the known effects of dietary n-3 and n-6 PUFAs on rat mammary tumorigenesis could occur *via* changes in mevalonate synthesis in the mammary gland.

4.3 MATERIALS AND METHODS

Materials

All reagents for electrophoresis and immunodetection were purchased from Bio-Rad Laboratories (Richmond, CA). The rabbit polyclonal anti-HMG-CoA reductase antibody was a gift from Dr. D.G. Hardie (Clarke and Hardie 1990), and the phosphoprotein phosphatase was a gift from Dr. J.H. Shand (Shand and West 1991). 3-Hydroxy-3-methyl[3-¹⁴C]glutaryl-CoA and [5-³H]mevalonolactone were from Du Pont-New England Nuclear (Mississauga, Ontario, Canada), and all other reagents and chemicals were obtained from Sigma (St. Louis, MO).

Animals and Diets.

Female Sprague-Dawley (SD) rats were purchased from Charles River Laboratories (St. Constant, Quebec, Canada) at 43 days of age and housed at 23°C and 50% humidity with a 12-hour light/dark cycle. Animals were maintained on a standard AIN-93G control diet (Reeves et al. 1993) (Dyets, Bethlehem, PA) for one week. They were then randomized into two groups (n=7) and fed for one week diets in which the 7% Soybean oil in the AIN-93G diet was replaced by 1% Soybean oil (to ensure adequate amounts of essential fatty acids) plus either 6% Safflower oil or 6% Menhaden oil (Reeves et al. 1993). The fatty acid composition of these diets is shown in Table 4-1, and was determined using the method of Ulberth and Henninger (1992). The n-6 PUFA content of the diets is predominantly due to LA. At the end of the experiment, rats were anaesthetized, blood samples taken by cardiac puncture and animals sacrificed by cervical dislocation. Serum was prepared from blood samples and stored at -70°C prior to analysis for total cholesterol using a kit from Boehringer Mannheim (Laval, Quebec, Canada).

Preparation of Microsomes

Liver and mammary gland microsomes were prepared as described in Chapter 3.

HMG-CoA reductase Enzyme Activity

The activity of HMG-CoA reductase was determined using a radiochemical assay as described in Chapter 3.

Immunoblotting

Immunoblotting was carried out essentially as described by the manufacturers for the reagents (Bio-Rad) and detection kit (Amersham). Microsomal proteins solubilized in Laemmli buffer containing 8 M urea were heated for 5 min at 95°C, electrophoresed over 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes. After incubating overnight at 4°C with anti-HMG-CoA reductase (1:2000) (Clarke and Hardie 1990), membranes were probed with HRP-conjugated anti-rabbit antibody (1:5000) and signals detected by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL). The 97 kDa HMG-CoA reductase band was identified by comparison with the migration of pre-stained molecular weight markers. Total proteins were stained with Coomassie blue to ensure equal loading.

4.4 RESULTS

After one week of feeding AIN-93G diets containing either Menhaden or Safflower oil, food intake and body weights did not differ between the two groups. As anticipated, the n-3/n-6 PUFA ratio was 100-fold greater in the Menhaden oil diet than in the Safflower oil diet (Table 4-1). Serum cholesterol levels were significantly lower in the n-3 group (1.91 ± 0.18 mmol/L) than in the n-6 group (2.61 ± 0.37 mmol/L), $P < 0.01$ (Figure 4-1). Levels of immunodetectable HMG-CoA reductase protein were lower in the mammary glands and livers of animals fed the Menhaden oil diet compared to those fed the Safflower oil diet, as shown in the representative Western blots in Figure 4-2.

Table 4-1. Fatty acid content of experimental diets.

<i>Fatty acid</i>	Group	
	<i>n-3 Diet</i>	<i>n-6 Diet</i>
14:0	6.65	0.35
16:0	10.43	5.97
16:1n-7	7.71	-
18:0	2.27	2.25
18:1n-9	8.01	9.93
18:1n-7	2.25	0.58
18:2n-6	7.57	47.80
18:3n-3	1.32	0.96
18:4n-3	1.04	-
20:5n-3	8.67	-
22:5n-3	1.29	-
22:6n-3	2.70	-
Other	10.10	2.16
Total	70.00	70.00
<i>n-3:n-6 ratio</i>	2.00	0.02

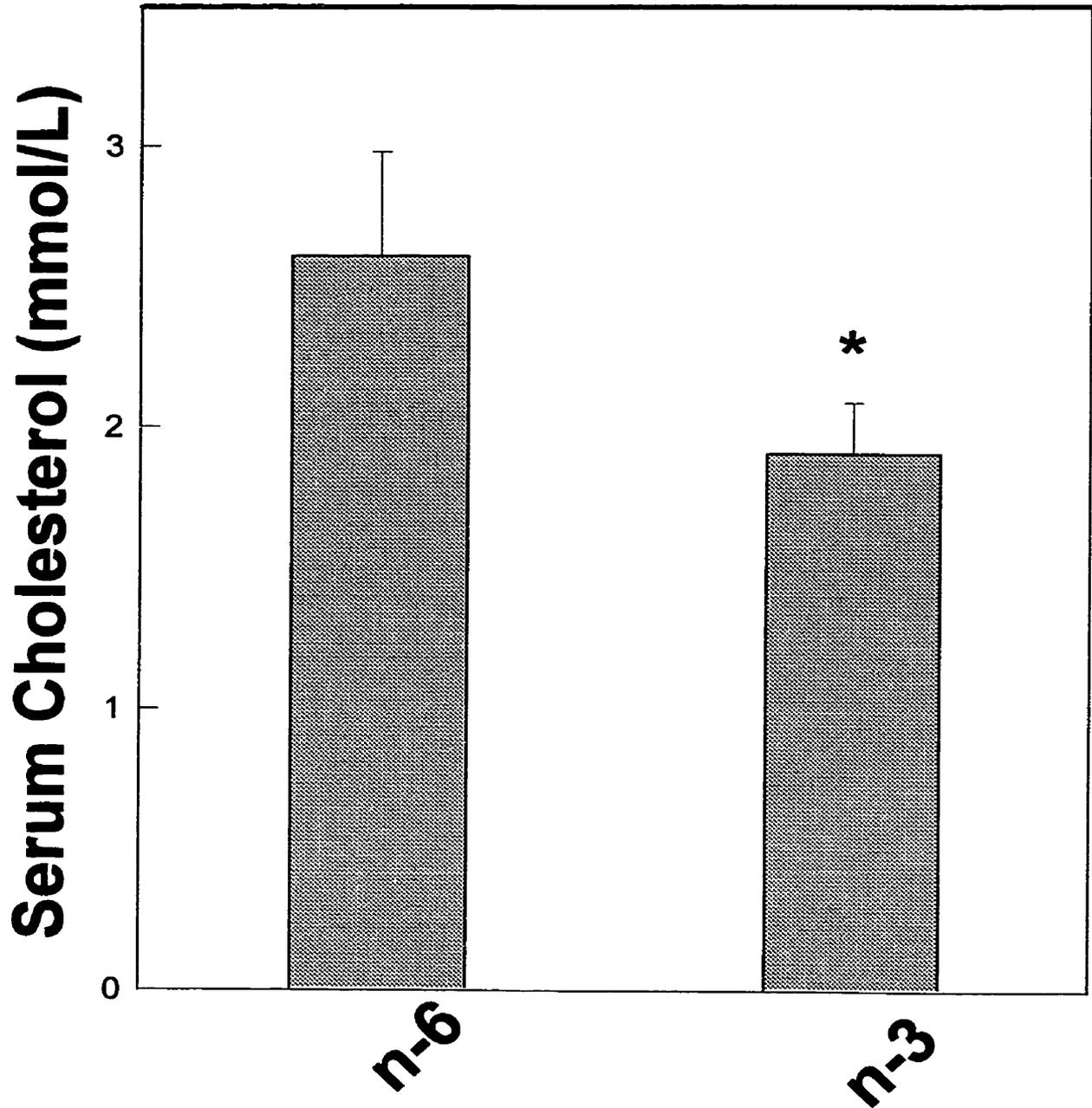


Figure 4.1. Total serum cholesterol from rats fed n-6 or n-3 PUFAs. (*) Indicates significantly different from n-6 group ($P < 0.01$).

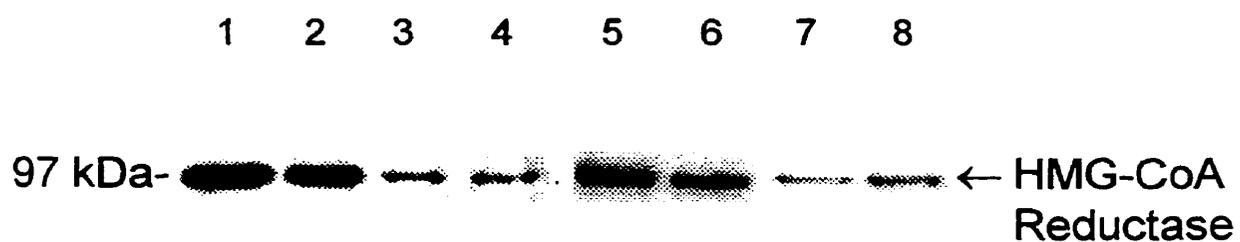


Figure 4.2. Representative immunoblot of HMG-CoA reductase from mammary glands (lanes 1-4) and livers (lanes 5-8) of rats fed either n-6 (lanes 1,2,5 and 6) or n-3 (lanes 3,4,7 and 8) PUFAs.

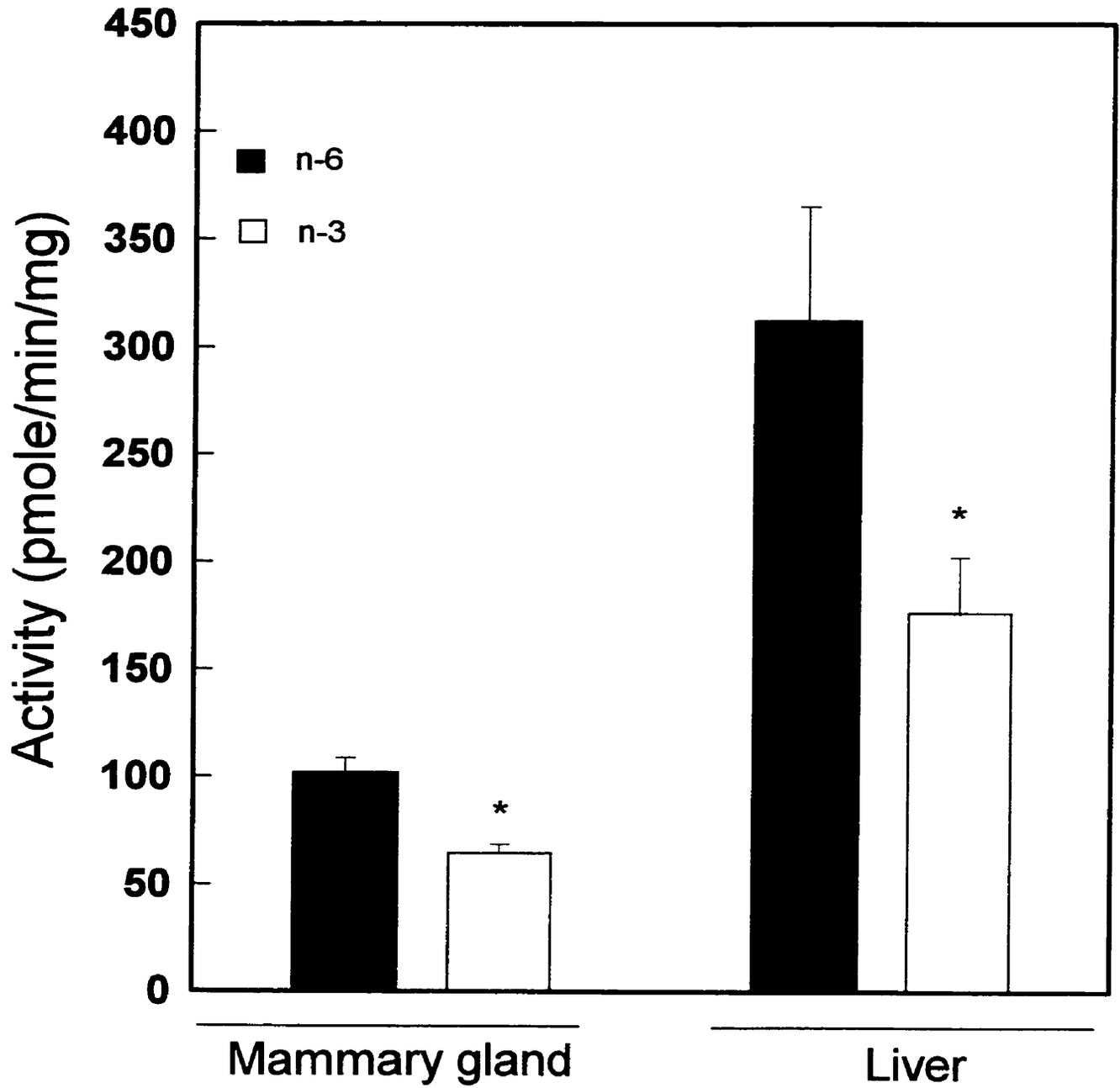


Figure 4.3. HMG-CoA reductase enzyme activity from liver and mammary glands of rats fed either n-6 or n-3 PUFAs. (*) Indicates significantly different from n-6 group ($P < 0.05$).

The activity of HMG-CoA reductase in the mammary glands and livers is shown in Figure 4-3. In rats fed the n-3 PUFA diet, the activity was significantly lower than those fed the n-6 PUFA diet in both the mammary glands (64.6 ± 4.1 versus 101.7 ± 7.1 pmol/min/mg, $P < 0.005$) and livers (176.1 ± 26.2 versus 312.0 ± 53.3 pmol/min/mg, $P < 0.05$).

4.5 DISCUSSION

In the present study, a mechanism was investigated by which dietary fats rich in either n-3 or n-6 PUFAs could modulate mammary carcinogenesis. The results demonstrate that HMG-CoA reductase activity and levels are significantly lower in mammary glands of rats fed an n-3 PUFA diet than in those fed an n-6 PUFA diet. Inhibitors of HMG-CoA reductase are known to inhibit tumorigenesis both *in vitro* and *in vivo* (Bennis et al. 1993; Larsson 1994; Narisawa et al. 1994; Soma et al. 1995), as well as the proliferation of normal and neoplastic mammary epithelial cells (Larsson 1994). The findings of the present study, therefore, suggest that regulation of mevalonate synthesis in preneoplastic mammary epithelial cells by dietary fats may explain, at least in part, their modulating effects on mammary carcinogenesis and mammary tumor growth.

Rao *et al* (1988) have suggested that stimulation of *de novo* cholesterologenesis in the mammary gland may explain the promoting effects of a high fat corn oil diet, and studies in other tissues and cells have also suggested an important role for cholesterol biosynthesis in tumor development (Chen et al. 1978). However, mevalonate, not

cholesterol *per se*, is required by mammalian cells for entry into the S-phase of the cell cycle (Fairbanks et al. 1984; Habenicht et al. 1980; Huneeus et al. 1979; Siperstein 1995), a notion that supports the findings of the present study. Nevertheless, cholesterol biosynthesis may be an approximate measure of mevalonate synthesis. Mevalonate is also a precursor of farnesyl pyrophosphate that is required for the processing, membrane localization and subsequent activation of Ras (Der and Cox 1991). Inhibition of Ras processing may inhibit malignant transformation (Kohl et al. 1993). Singh *et al* (1997) have recently shown that dietary Menhaden oil inhibits colon tumorigenesis and Ras membrane localization. Our observations support their hypothesis that this inhibition occurs at the level of HMG-CoA reductase.

As reported by others (Harris 1997), in the present study Menhaden oil led to lower levels of serum cholesterol than Safflower oil. This difference may be attributed to changes in hepatic HMG-CoA reductase since the liver is the major site of lipoprotein synthesis and largely determines the levels of circulating cholesterol (Anderson and Dietschy 1977). In Chapters 2 & 3 it was shown that raising serum cholesterol by dietary cholesterol inhibits mammary carcinogenesis. Others have shown that lowering serum cholesterol by cholestyramine increases the incidence of mammary tumors (Gabriel et al. 1987). The authors of these two reports speculated that the increase or decrease in serum cholesterol leads to a decrease or increase, respectively, in mammary gland cholesterologenesis, since extrahepatic tissues meet most of their cholesterol requirements by internalizing circulating lipoproteins, the carriers of cholesterol in the blood (Brown and Goldstein 1986). The internalized cholesterol suppresses *de novo* cholesterologenesis by down regulating HMG-CoA

reductase. In the present study, however, the decrease in serum cholesterol by Menhaden oil was associated with a concomitant decrease in HMG-CoA reductase in the mammary glands, suggesting that levels of circulating cholesterol are not solely responsible for the activity of the extrahepatic enzyme. Thus, changes in serum cholesterol alone are unlikely to predict tumor development. Rather, the effects of such changes on extrahepatic mevalonate synthesis may correlate better with tumorigenesis. Indeed, dietary cholesterol raises serum cholesterol while dietary Menhaden oil lowers serum cholesterol, yet both of these dietary factors inhibit HMG-CoA reductase and both inhibit mammary tumorigenesis (Cohen et al. 1993; El-Sohemy et al. 1996a).

In summary, mevalonate synthesis is inhibited in the mammary glands of rats fed an n-3 PUFA diet compared to an n-6 PUFA diet suggesting that changes in mevalonate synthesis may account for the inhibitory effects of n-3 PUFAs and/or the promoting effects of n-6 PUFAs on mammary carcinogenesis.

CHAPTER FIVE

EFFECTS OF n-3 AND n-6 PUFAs ON HMG-CoA REDUCTASE AND LDL-RECEPTOR GENE EXPRESSION

5.1 ABSTRACT

In Chapter 4 it was shown that HMG-CoA reductase activity and protein levels are decreased in mammary glands of rats fed n-3 PUFAs and it was hypothesized that this may be a mechanism by which these fatty acids inhibit carcinogenesis. The objective of this study was to determine whether up-regulation of the LDL-R mediates the decrease in HMG-CoA reductase by n-3 PUFAs and whether this may be due to changes in gene expression. Female Sprague-Dawley rats were fed 7% fat diets that were rich in either n-3 (Menhaden oil) or n-6 (Safflower oil) PUFAs for one week. Whole mammary glands were used for all analyses since, for technical reasons, the isolated cell fractions had undetectable levels of HMG-CoA reductase. A semi-quantitative RT-PCR method was used to compare the levels of gene expression. Although HMG-CoA reductase mRNA levels were higher in the liver than in the mammary gland, no differences were detected between the dietary groups in either tissue. n-3 PUFAs did not alter LDL-R protein or mRNA levels in either the liver or mammary gland. However, the expression of an unidentified transcript (~2 Kb) and an unidentified membrane-bound protein (~300 kDa) that appear to be homologous to the LDL-R, were markedly elevated only in the mammary glands by n-3 PUFAs. Thus, n-3 PUFAs may decrease HMG-CoA reductase by increasing the activity of a receptor similar to the LDL-R. Down-regulation of the reductase appears to occur at the post-transcriptional level.

5.2 INTRODUCTION

In the previous chapter, it was shown that n-3 PUFAs decrease the levels of HMG-CoA reductase protein and enzyme activity compared to n-6 PUFAs, and it was hypothesized that this may be a mechanism by which n-3 PUFAs inhibit mammary tumorigenesis. HMG-CoA reductase is regulated through a multivalent feedback mechanism that is controlled, in part, by intracellular cholesterol levels (Goldstein and Brown 1990). Cells meet their cholesterol requirements through *de novo* synthesis and by uptake of cholesterol-rich lipoproteins such as LDL (Goldstein and Brown 1984). The liver is the major site of cholesterologenesis and is the major organ regulating serum cholesterol (Dietschy et al. 1993). Internalization of LDL is mediated through the LDL-R as well as a LDL-R-independent pathway (Spady et al. 1985). When uptake of LDL is enhanced and intracellular cholesterol levels rise, HMG-CoA reductase is down-regulated to prevent excessive accumulation of cholesterol (Goldstein and Brown 1984). As a result, the synthesis of mevalonate that is also required for cell replication is reduced.

The purpose of this study was to determine whether the LDL-R mediates the down-regulation of HMG-CoA reductase by n-3 PUFAs, and whether any changes in these proteins are due to changes in gene expression.

5.3 MATERIALS AND METHODS

Materials

Reagents for electrophoresis and immunodetection were purchased from Bio-Rad Laboratories (Richmond, CA) and all other reagents and chemicals were obtained from Sigma (St. Louis, MO). The anti-LDL receptor antibody was a generous gift from Dr. A.D. Cooper (Stanford University, Palo Alto, CA). Primers used for PCR were purchased from ACGT (Toronto, Ontario, Canada).

Animals and Diet

Female Sprague-Dawley (SD) rats were purchased from Charles River Laboratories (St. Constant, Quebec, Canada) at 43 days of age and housed at 23°C and 50% humidity with a 12-hour light/dark cycle. Animals were fed *ad libitum* a standard AIN-93G control diet (Reeves et al. 1993) (Dyets, Bethlehem, PA) for one week. They were then randomized into two groups (n=12) and fed for one week diets in which the 7% Soybean oil in the AIN-93G diet was replaced by 1% Soybean oil (to ensure adequate amounts of essential fatty acids) plus either 6% Safflower oil or 6% Menhaden oil (Reeves et al. 1993). These are the same diets that were used in Chapter 4. At the end of the experiment, rats were anaesthetized then sacrificed by cervical dislocation. Mammary glands from one-half of the animals were quick frozen in liquid nitrogen while glands from the remaining animals were placed in ice-cold PBS and used to isolate mammary epithelial cells.

Isolation of Mammary Epithelial Cells

Fresh mammary glands were finely minced over ice and incubated in PBS containing 9 mg/g collagenase Type III (Worthington Biochemicals, Freehold, NJ) for 2 hours at 37°C as described (Fong et al. 1990). After removal of the adipocytes and incubation with 0.05% pronase, stromal and epithelial cells were separated over a Percoll gradient (1.03 g/ml).

Enzyme activity

HMG-CoA reductase enzyme activity was determined in isolated mammary epithelial cells using a radiochemical assay as described in Chapter 3.

Immunoblotting

Plasma membrane fractions were isolated by homogenizing tissues in buffer A (250 mM Tris-maleate (pH 6.5), 150 mM NaCl, 2 mM CaCl₂, 1 mM PMSF and 2 mM leupeptin). Homogenates were centrifuged at 9,000xg for 15 min and the supernatant centrifuged at 40,000xg for 40 min. Membrane pellets were resuspended in buffer A containing 30 mM CHAPS and centrifuged at 40,000xg for 40 min. Total membrane protein was determined using a protein dye binding assay (Bio-Rad) according to the manufacturer's protocol with bovine serum albumin as a standard. The 135 kDa rat LDL-R was detected using a rabbit polyclonal antibody as described previously (Ellsworth et al. 1987). Immunodetection was determined as described in Chapter 4, except that equivalent amounts of protein were applied to 6% SDS-polyacrylamide

gels under non-reducing conditions and the LDL-R was detected by incubating with the anti-LDL-R antibody (1:5000) for 2 h at room temperature.

RT-PCR Analysis

Total RNA was isolated from frozen tissues or isolated mammary epithelial cells using the Trizol® reagent (Gibco, Life Technologies). Detection of mRNA levels by RT-PCR was carried out as described by the manufacturers of the reagents (Pharmacia & Boehringer Mannheim). Total RNA (5 µg) was reverse transcribed into single-stranded cDNA using 50U of MMLV reverse transcriptase. Equal amounts of template (2 µl) were amplified with a Perkin Elmer DNA thermal cycler using PCR beads (Pharmacia, Baie d'Urfe, QC), 1.5 mM Mg²⁺ and 2 µM primers. For HMG-CoA reductase, the 3' primer (5'-ATGCTCCTTGAACACCTAGCATCT-3') and 5' primer (5'-AGGTTCCAATGGCAACAACAGAAG-3') were chosen from the human cDNA sequence and yield a 872 bp product (Chin et al. 1984). For the LDL-R, the 3' primer (5'-GCCTTGACTTAGGCAGGTCAT-3') and 5' primer (5'-ACGCTACCGGGATTGTTTAAG-3') were chosen from the rat cDNA sequence and yield a 667 bp product (Lee et al. 1989). The expression of β-actin was determined as a control using the 3' primer (5'-CGTAGCCATCCAGGCTGTGTT-3') and 5' primer (5'-GCATCCTGTCAGCGATGCCTG-3') from the rat sequence to yield a 570 bp product (Nudel et al. 1996). The PCR conditions for HMG-CoA reductase and the LDL-R were an initial denaturing step for 5 minutes followed by 35 cycles of denaturing (95°C for 45 seconds), annealing (60°C for 30 seconds) and extension (72°C for 90 seconds)

with a 10 minute final extension at 72°C. Conditions were similar for β -actin except that 25 cycles of amplification were used, the denaturing and annealing steps were one minute each and the extension was 2 minutes. Control PCR reactions using no template were used to rule out contamination. PCR products were separated over 2% agarose gels and visualized with ethidium bromide. The size of the PCR products were confirmed by comparing their migration with ϕ X174 *Hae*III markers. Since expression of HMG-CoA reductase in the mammary gland was very low, further steps were required to detect the PCR products using radio-labeled primers. Amplified cDNA was denatured in the gel using 0.5 M NaOH and 0.15 M NaCl for 30 minutes followed by 30 minutes with 1.5 M Tris-HCl (pH 7.5) and 0.15 M NaCl to neutralize the gel. PCR products were transferred onto Zeta-probe membranes which were then X-linked using a UV Stratalinker 1800 (Stratagene, Richmond, BC). Membranes were pre-hybridized at 55°C for 4 hours in 6X SSC, 10 mM EDTA, 2X Denhardt's solution, 100 mg/ml calf thymus DNA and 1% SDS followed by overnight hybridization with primers for HMG-CoA reductase that were end-labeled with γ -³²P-ATP and T₄ DNA kinase. Membranes were washed repeatedly in 2X SSC and 0.05% SDS for 30 minutes at room temperature and signals were detected by exposing the membranes to X-ray film.

5.4 RESULTS

After feeding diets rich in either n-3 or n-6 PUFAs for one week, there were no differences in food intake or body weight gains. In these experiments, the effects of n-3 PUFAs were determined relative to the effects of n-6 PUFAs.

The first step was to determine whether the decrease in HMG-CoA reductase observed previously in whole mammary glands of rats fed n-3 PUFAs occurs in the epithelial cell fraction. Adipocytes, stromal and epithelial cells were separated from fresh mammary glands and assayed for HMG-CoA reductase activity together with samples prepared from frozen whole mammary glands that were used as a positive control. As expected, n-3 PUFAs decreased HMG-CoA reductase activity in whole mammary gland samples but enzyme activity was not detectable in any of the cell fractions, regardless of the dietary group (data not shown). There was also extensive RNA degradation that occurred during the isolation procedure that precluded analysis by RT-PCR. The remainder of the analyses, therefore, were carried out using preparations from whole mammary glands.

Figure 5-1 is a representative Western blot of the LDL-R in the liver and mammary gland. No differences were observed in the levels of immunodetectable protein between the dietary groups in either the liver or the mammary gland. However, n-3 PUFAs caused a marked induction of a protein in the mammary gland with an apparent molecular weight of ~300 kDa.

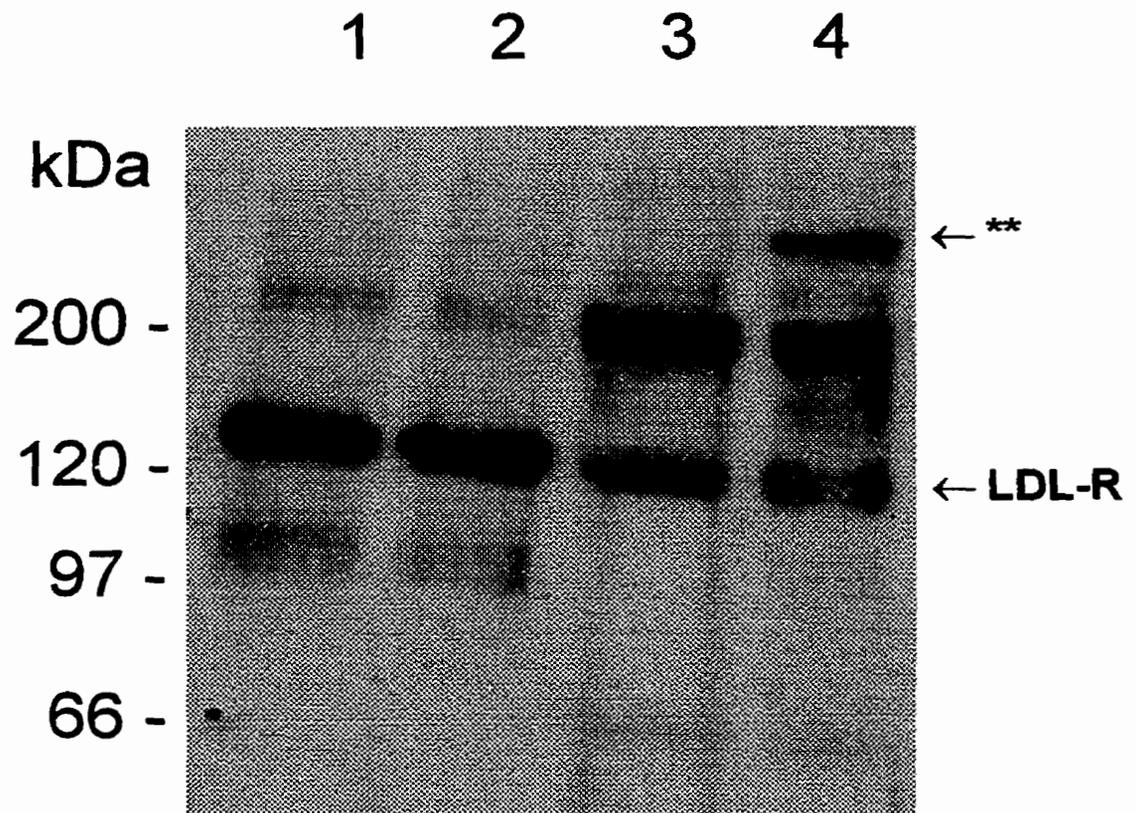


Figure 5-1. Representative Western blot of LDL-R from liver (lanes 1 and 2) and mammary glands (lanes 3 and 4) of rats fed either n-6 (lanes 1 and 3) or n-3 (lanes 2 and 4) PUFAs. (**) Indicates the unknown protein (~300 kDa).

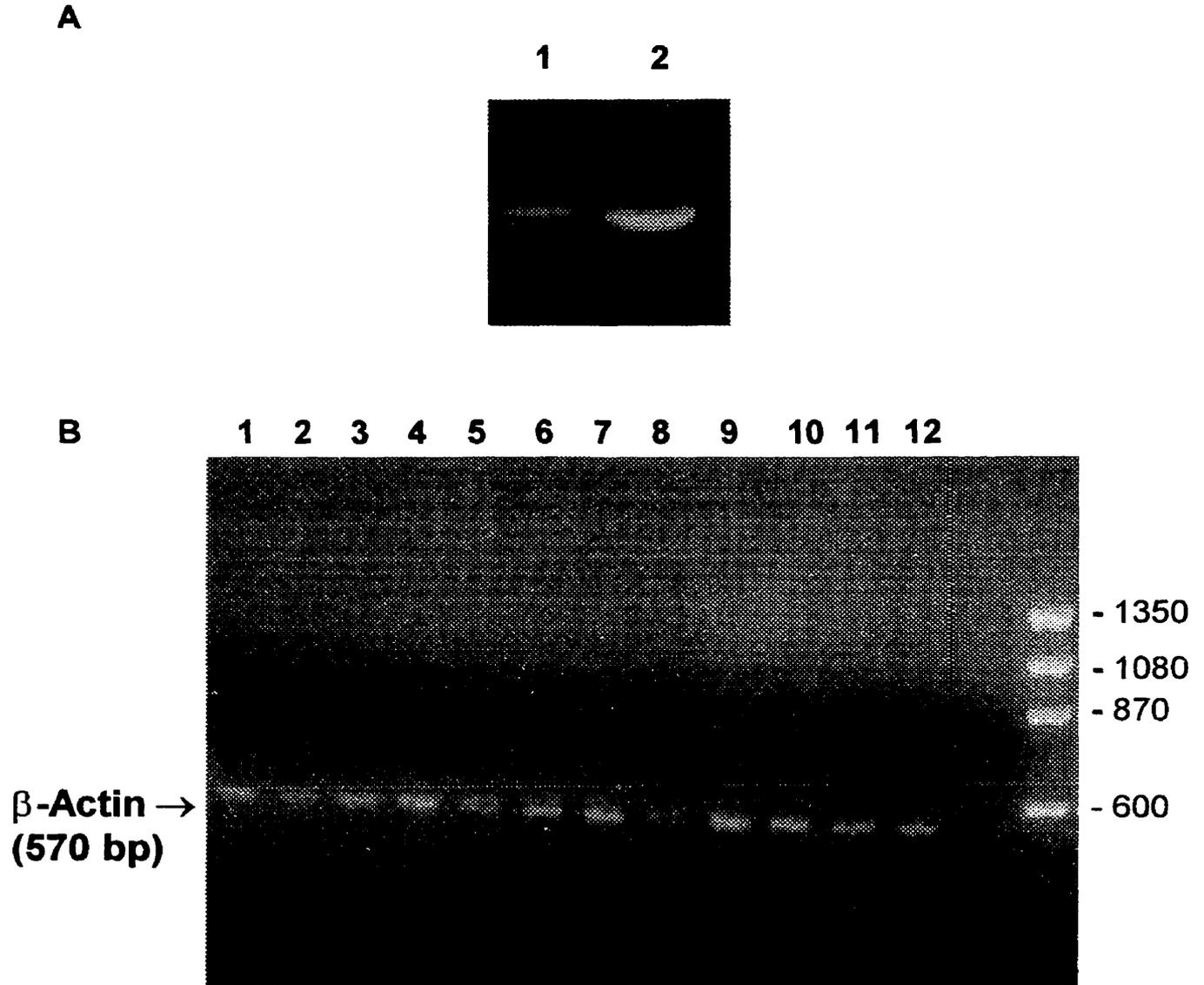


Figure 5-2. (A) RT-PCR products from liver amplified with primers for HMG-CoA reductase using 1 μ g (lane 1) or 5 μ g (lane 2) RNA. (B) RT-PCR products from mammary gland (lanes 1-6) and liver (lanes 7-12) RNA of rats fed either n-3 (lanes 1, 3, 5, 7, 9 and 11) or n-6 (lanes 2, 4, 6, 8, 10 and 12) PUFAs using primers for β -actin.

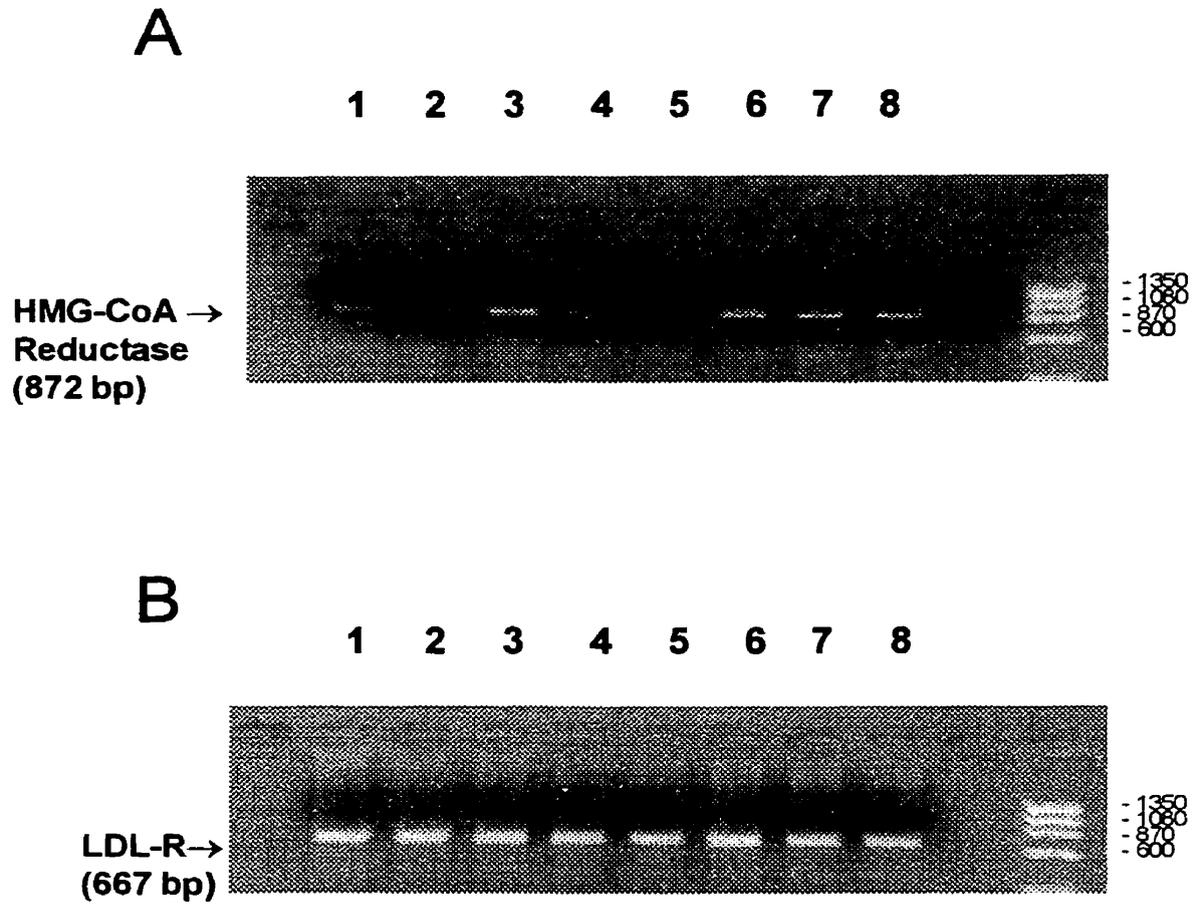


Figure 5-3. RT-PCR products from liver RNA of rats fed either n-3 (lanes 1,3,5 and 7) or n-6 (lanes 2,4,6 and 8) PUFAs using primers for HMG-CoA reductase (A) or LDL-R (B).

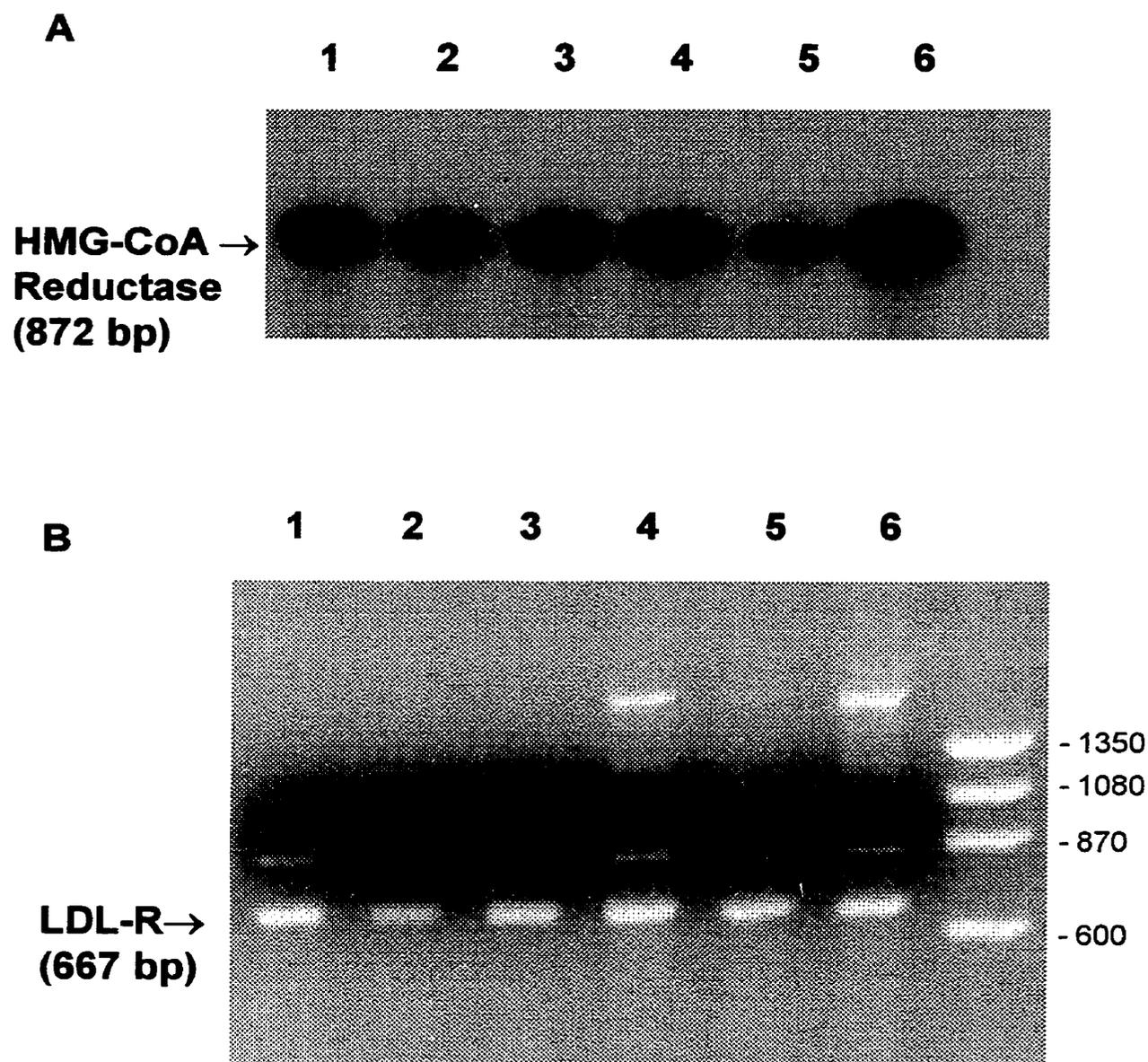


Figure 5-4. RT-PCR products from mammary gland RNA of rats fed either n-6 (lanes 1-3) or n-3 (lanes 4-6) PUFAs using primers for HMG-CoA reductase (A) or LDL-R (B).

Since HMG-CoA reductase and LDL-R are low abundance genes and their transcripts were undetectable in the mammary gland by Northern blotting, a more sensitive semi-quantitative RT-PCR method was used to measure gene expression. Different amounts of liver RNA were first reverse-transcribed and amplified using primers for HMG-CoA reductase to ensure that the method being used is capable of detecting differences in mRNA (Figure 5-2). β -actin was amplified to ensure that expression levels were the same in all samples (Figure 5-2). Figure 5-3 is a representative blot showing the relative abundance of PCR products corresponding to the expression of HMG-CoA reductase and LDL-R in the liver. No differences were detected between the dietary groups in either of these genes. In the mammary gland, HMG-CoA reductase expression was much lower than in the liver and required radio-labeled primers for detection. n-3 PUFAs had no effect on the expression of either HMG-CoA reductase or LDL-R in the mammary gland (Figure 5-4). However, there was a marked induction of a large, unknown transcript in the mammary glands of rats fed n-3 PUFAs, with an apparent size of ~2 Kb.

5.5 DISCUSSION

This study was designed to investigate the mechanism by which n-3 PUFAs decrease HMG-CoA reductase. The effects of n-3 PUFAs were compared to n-6 PUFAs by feeding female Sprague-Dawley rats diets rich in either Menhaden oil (n-3 PUFAs) or Safflower oil (n-6 PUFAs) for one-week. The objective of this study was to determine whether up-regulation of the LDL-R mediates the down-regulation of HMG-

CoA reductase by n-3 PUFAs and whether this may be due to changes in gene expression.

The mammary glands of virgin female rats consist mostly of adipocytes and a small number of epithelial and stromal cells (Low et al. 1988). Since epithelial cells are the target for chemical carcinogens and ultimately develop into neoplasms, it was desirable to determine whether the effects observed on HMG-CoA reductase in the whole mammary gland also occur in the epithelial cells. Therefore, adipose, stromal and epithelial cells were purified from fresh mammary glands and HMG-CoA reductase enzyme activity was measured. In all of the samples tested, enzyme activity was virtually undetectable due to technical limitations caused by the long isolation procedure. There was also extensive degradation in the RNA that was isolated from these cells which prevented any analyses by RT-PCR. These effects were most likely to be due to the long isolation procedure that resulted in the down-regulation or degradation of HMG-CoA reductase. To overcome this problem, it may be necessary to measure HMG-CoA reductase protein using immunohistochemistry on frozen sections. Since n-3 PUFAs down-regulate HMG-CoA reductase in cultured epithelial cells (Murthy et al. 1988), it is reasonable to presume that the down-regulation in whole mammary glands also occurs in epithelial cells. For these reasons, the gene expression analyses were conducted using whole mammary glands.

LDL-R protein was measured using a polyclonal antibody that recognizes the 135 kDa rat LDL-R (Ellsworth et al. 1987). Although n-3 PUFAs did not alter the levels of this protein in either the mammary gland or liver, there was a significant increase in the levels of another protein with an apparent molecular weight of ~300 kDa that was

detected only in the mammary glands of rats fed n-3 PUFAs. This raised the possibility that another membrane-bound protein, possibly a receptor, that has some homology to the LDL-R is induced by n-3 PUFAs. This protein is not the VLDL receptor or the LDL-R related protein (LRP) since the antibody that was used in the present study has been previously shown not to cross-react with either of these receptors (Kraemer et al. 1994). Thus, it is possible that this high molecular weight protein represents another receptor that has not been previously described and may function as part of the LDL-R-independent pathway of LDL uptake (Spady et al. 1985).

Preliminary experiments revealed that HMG-CoA reductase and LDL-R mRNA levels are undetectable in the mammary gland by Northern blotting. Therefore, a more sensitive RT-PCR method was used to measure the expression levels of these genes. Originally, we used primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 'house-keeping' gene commonly used in studies that examine changes in gene expression to ensure equal loading of RNA. Surprisingly, a significant increase in GAPDH was observed in mammary glands of rats fed n-3 PUFAs (see Appendix B). This difference in GAPDH between n-3 and n-6 PUFAs was also detected by Northern blotting and gave us more confidence in the semi-quantitative RT-PCR method that was used. Although this observation was interesting and more in-depth analyses on GAPDH were carried out, we switched to using β -actin as a control for the present study and observed no changes in its expression levels. n-3 PUFAs had no effect on HMG-CoA reductase or LDL-R gene expression in either the liver or the mammary gland. This observation is consistent with studies by Ness and colleagues who showed that dietary cholesterol significantly reduces the levels of hepatic HMG-CoA reductase

protein and enzyme activity but does not alter the levels of mRNA (Ness et al. 1994a). Interestingly, a significant increase was observed in the abundance of a PCR product that was much larger in size than the product of the LDL-R, and this was found only in the mammary glands of rats fed n-3 PUFAs. This larger band may represent a different gene with homology to the LDL-R and may encode the larger protein that was observed by Western blotting. However, a role for the LDL-R in mediating the effects of HMG-CoA reductase can not be excluded since Spady *et al* (1995) showed that n-3 PUFAs enhance hepatic LDL-R activity without altering the amount of immunodetectable protein. They proposed that this may involve increased LDL-R cycling.

In summary, n-3 PUFAs do not appear to alter HMG-CoA reductase or LDL-R gene expression in either the liver or mammary gland. n-3 PUFAs cause a marked induction in the mammary gland of a gene and protein homologous to the LDL-R. A role for the LDL-R in mediating the effects of n-3 PUFAs on HMG-CoA reductase can not be determined definitively from the results of the present study.

CHAPTER SIX

EFFECTS OF n-3 AND n-6 PUFAs ON HMG-CoA REDUCTASE IN LDL-RECEPTOR-DEFICIENT MICE

6.1 ABSTRACT

Mice with a targeted disruption of the *LDL-R* gene were used to determine whether the LDL-R mediates the down-regulation of HMG-CoA reductase by n-3 PUFAs. Female mice lacking the LDL-R (-/-) and wild-type (+/+) mice were fed a 7% fat diet rich in either n-3 (Menhaden oil) or n-6 (Safflower oil) PUFAs for one week. Compared to n-6 PUFAs, n-3 PUFAs reduced serum levels of total and LDL cholesterol in both +/+ and -/- mice. HMG-CoA reductase activity and levels of immunodetectable protein were reduced by n-3 PUFAs in the livers of +/+ and -/- mice. In the mammary gland, n-3 PUFAs also decreased HMG-CoA reductase in the +/+ group, but increased the levels in the -/- group. These results show that the LDL-R is required for n-3 PUFAs to decrease mammary gland HMG-CoA reductase, but is not required for the decrease in hepatic HMG-CoA reductase.

6.2 INTRODUCTION

In Chapter 4 it was shown that HMG-CoA reductase is decreased in the mammary glands of rats fed n-3 PUFAs and it was proposed that this may be a possible mechanism by which tumorigenesis is inhibited. In Chapter 5, the hypothesis that an up-regulation of the LDL-R mediates the down-regulation of HMG-CoA reductase was tested, but the results were not conclusive.

Mice with a targeted disruption of the *LDL-R* gene were recently developed as a model of familial hypercholesterolemia (Ishibashi et al. 1993), and have been used to study atherosclerosis and xanthomatosis (Ishibashi et al. 1994). Deletion of the LDL-R

results in a 2-fold increase in total serum cholesterol (Ishibashi et al. 1993) and a 14-fold increase in LDL-cholesterol (Osono et al. 1995). Tissue cholesterol concentrations are slightly higher in some organs of LDL-R deficient mice compared to normal mice, but rates of cholesterol synthesis do not differ (Osono et al. 1995).

The purpose of this study was to determine whether the LDL-R is required for n-3 PUFAs to lower HMG-CoA reductase levels.

6.3 MATERIALS AND METHODS

Materials

Reagents for electrophoresis and immunodetection were purchased from Bio-Rad Laboratories (Richmond, CA), and all other reagents and chemicals were obtained from Sigma (St. Louis, MO).

Animals and Diets

Twenty virgin female C57Bl/6J mice that are homozygous for a deletion in the *LDL-R* gene (-/-) and twenty virgin wild type mice (+/+) were purchased from Jacksons Lab (Bar Harbour, ME) at 7 weeks of age. Animals were housed at 23°C and 50% humidity with a 12-hour light/dark cycle and acclimatized on the AIN-93G standard reference diet (Reeves et al. 1993)(Dyets, Bethlehem, PA) for one week. Knockout and wild type mice (10 per group) were fed one of 2 diets in which the 7% Soybean oil in the AIN-93G diet was replaced by 1% Soybean oil (to ensure adequate amounts of essential fatty acids) plus either 6% Menhaden oil (rich in n-3 PUFAs) or 6% Safflower oil (rich

in n-6 PUFAs) for a period of one week. Mice were anaesthetized with halothane, blood collected by cardiac puncture and animals sacrificed by cervical dislocation. The livers and mammary glands were dissected, immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Serum Lipids

Blood samples were allowed to coagulate at ambient temperature then centrifuged at 2,500xg for 10 min. Serum was assayed for total cholesterol using a kit obtained from Boehringer Mannheim (Laval, Quebec, Canada), and TGs and HDL cholesterol using a kit from Sigma (St. Louis, MO). LDL cholesterol was then calculated as described by Sigma.

Tissue Preparation

Microsomes were prepared as described in Chapter 3 and plasma membrane fractions were prepared as described in Chapter 5. Protein was determined using a protein dye binding assay (Bio-Rad) according to the manufacturer's protocol with bovine serum albumin as a standard.

Immunoblotting

The 130 kDa mouse LDL-R and 97 kDa HMG-CoA reductase proteins were detected using rabbit polyclonal antibodies as described in Chapters 4 and 5.

HMG-CoA reductase Enzyme Activity

Total enzyme activity was determined as described in Chapter 3.

Statistical Analyses

All values are shown as means \pm SEM. Data were analyzed by one-way ANOVA followed by Student's t-test with Bonferroni *post hoc* for multiple comparisons. Two-way ANOVA was used for diet-gene interactions (GraphPad Prism™, San Diego, CA).

6.4 RESULTS

Groups of wild type (+/+) and LDL-R knockout (-/-) mice were fed cholesterol-free, 7% fat diets containing either Menhaden oil (n-3 PUFAs) or Safflower oil (n-6 PUFAs). These are the same diets used in Chapters 4 and 5. After feeding the experimental diets for one week, there were no differences in food intake or body weight gains between any of the groups.

Since the effects of n-3 PUFAs on cholesterol metabolism in mice were not known, it was necessary to determine whether these fatty acids produce any changes in serum lipids or HMG-CoA reductase in +/+ mice. The results of serum lipid analyses are shown in Table 6-1. In +/+ mice, serum TGs, total-, LDL- and HDL-cholesterol levels were reduced by n-3 PUFAs. Figures 6-1 and 6-2 show the effects of n-3 PUFAs on HMG-CoA reductase activity in the livers and mammary glands. The activity of HMG-CoA reductase in +/+ mice was reduced by n-3 PUFAs in the liver from 336 ± 80 to 109 ± 25 pmol/min/mg ($P < 0.005$) (Figure 6-1), and in the mammary glands from

36 ± 10 to 20 ± 9 pmol/min/mg ($P < 0.01$) (Figure 6-2). To determine whether these changes in enzyme activity were due to changes in the levels of HMG-CoA reductase protein, microsomes from livers and mammary glands were subjected to SDS-PAGE and probed using an anti-HMG-CoA reductase antibody. Figure 6-3 is a representative Western blot showing that the changes observed in enzyme activity are due, at least in part, to changes in the amount of HMG-CoA reductase protein.

LDL-R protein levels were not different in the liver or mammary glands of +/+ mice fed either of the two diets and the receptor is clearly absent in the -/- mice (Figure 6-4). However, n-3 PUFAs caused an up-regulation of a protein only in the mammary gland with an apparent molecular weight of ~300 kDa. This protein was also highly expressed in the mammary glands of -/- mice, regardless of the type of PUFAs in the diet.

Next, the effects of n-3 PUFAs were compared to n-6 PUFAs in -/- mice to determine the role of the LDL-R in mediating the effects observed in +/+ mice. In -/- mice, n-3 PUFAs lowered serum TGs, total- and LDL-cholesterol levels (Table 6-1). HDL-cholesterol levels were not altered and were between the values seen in +/+ mice fed the two diets (Table 6-1). n-3 PUFAs reduced hepatic HMG-CoA reductase activity from 123 ± 27 to 65 ± 38 pmol/min/mg ($P < 0.05$) (Figure 6-1), but unexpectedly increased activity in the mammary gland from 10 ± 2 to 19 ± 6 pmol/min/mg ($P < 0.05$) (Figure 6-2). The changes in enzyme activity corresponded with changes in the levels of HMG-CoA reductase protein (Figure 6-3). It appears, therefore, that the decrease in mammary gland HMG-CoA reductase that was observed in +/+ mice is mediated by the LDL-R.

The design of the present study also allowed us to determine the effects of LDL-R deletion on serum lipids and HMG-CoA reductase. A two-way ANOVA revealed that LDL-R status has an independent effect on these parameters, but a diet-gene interaction determines the magnitude of the responses. Deletion of the LDL-R resulted in a 27-fold increase in serum LDL-cholesterol in mice fed n-3 PUFAs, but only a 10-fold increase in mice fed n-6 PUFAs (Table 6-1). When mice were fed n-6 PUFAs, deletion of the LDL-R resulted in higher levels of serum TGs and lower levels of HDL-cholesterol. No significant effects on TG or HDL-cholesterol were observed, however, when mice were fed n-3 PUFAs. Total serum cholesterol was approximately 4-fold greater in *-/-* than in *+/+* mice, regardless of whether the animals were fed n-3 or n-6 PUFAs. Dietary PUFAs also modulated the effects of LDL-R deletion on HMG-CoA reductase. Compared to *+/+* mice, hepatic HMG-CoA reductase was 1.7-fold lower in *-/-* mice when the groups were fed n-3 PUFAs, but was 2.7-fold lower when fed n-6 PUFAs (Figure 6-1). Deletion of the LDL-R did not alter mammary gland HMG-CoA reductase when n-3 PUFAs were fed, but resulted in a significant (3.6-fold) decrease in enzyme activity and protein levels when mice were fed n-6 PUFAs (Figure 6-2). These analyses show that the composition of PUFAs in the diet can profoundly influence the effects of deleting the LDL-R on serum lipids and HMG-CoA reductase.

Table 6-1. Serum lipid analyses of LDL-R^{+/+} and LDL-R^{-/-} mice fed either n-3 or n-6 PUFAs.

	Total Cholesterol (mmol/L)	LDL (mmol/L)	HDL (mmol/L)	Triglycerides (mmol/L)
<u>LDL-R^{+/+}</u>				
n-6	2.94 ± 0.25 ^a	0.84 ± 0.14 ^a	1.79 ± 0.15 ^a	1.02 ± 0.15 ^a
n-3	1.68 ± 0.23 ^b	0.15 ± 0.06 ^b	1.27 ± 0.13 ^b	0.67 ± 0.10 ^b
<u>LDL-R^{-/-}</u>				
n-6	10.62 ± 1.16 ^c	7.94 ± 0.73 ^c	1.45 ± 0.12 ^b	1.75 ± 0.22 ^c
n-3	6.24 ± 1.34 ^d	4.09 ± 0.96 ^d	1.38 ± 0.25 ^b	0.87 ± 0.14 ^{a,b}

Values within a column not sharing the same letters differ significantly from each other ($P < 0.05$).

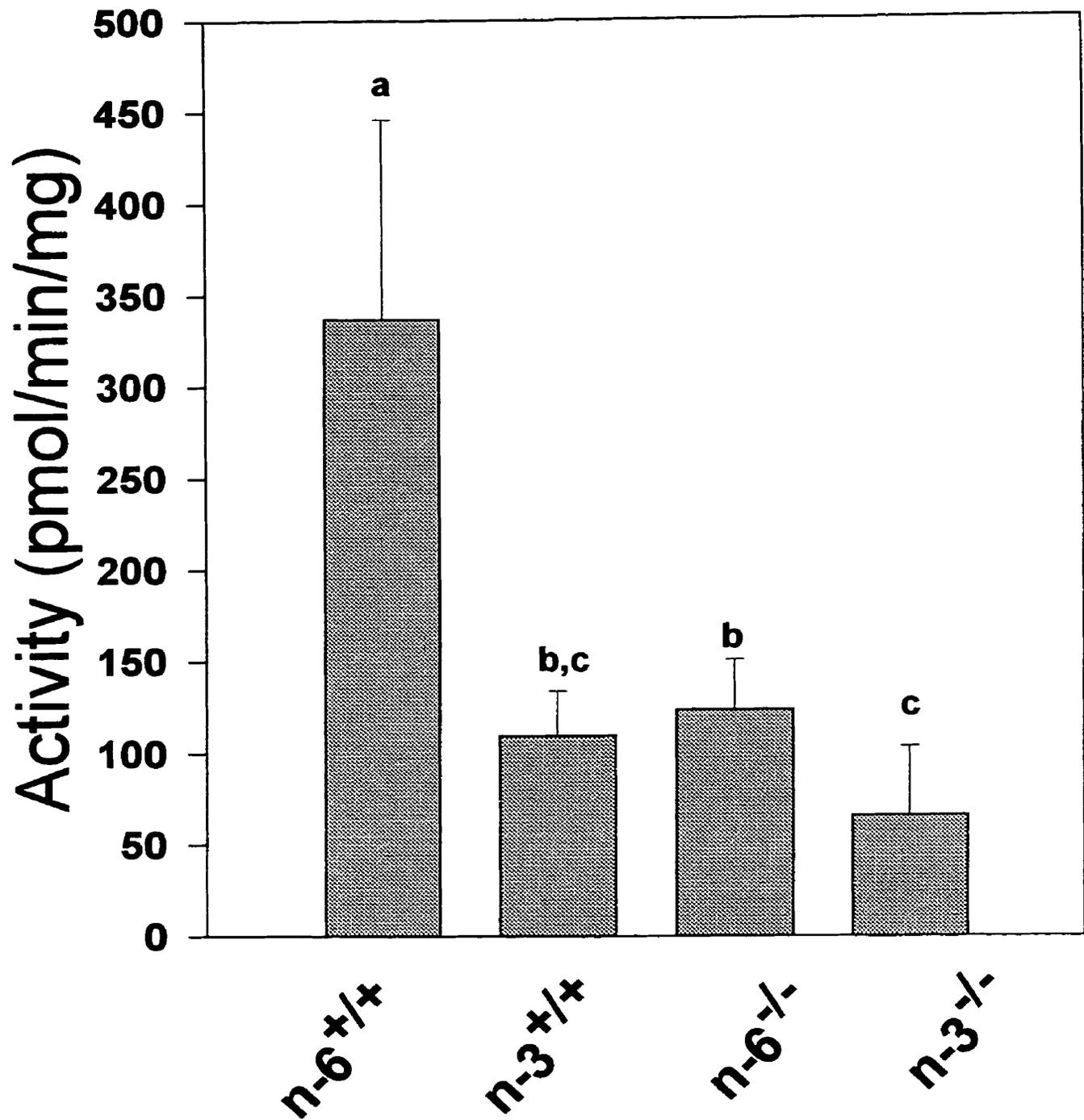


Figure 6-1. HMG-CoA reductase enzyme activity in liver of knockout (-/-) and wild-type (+/+) mice fed either n-3 or n-6 PUFAs. Different letters indicate groups are significantly different ($P < 0.05$).

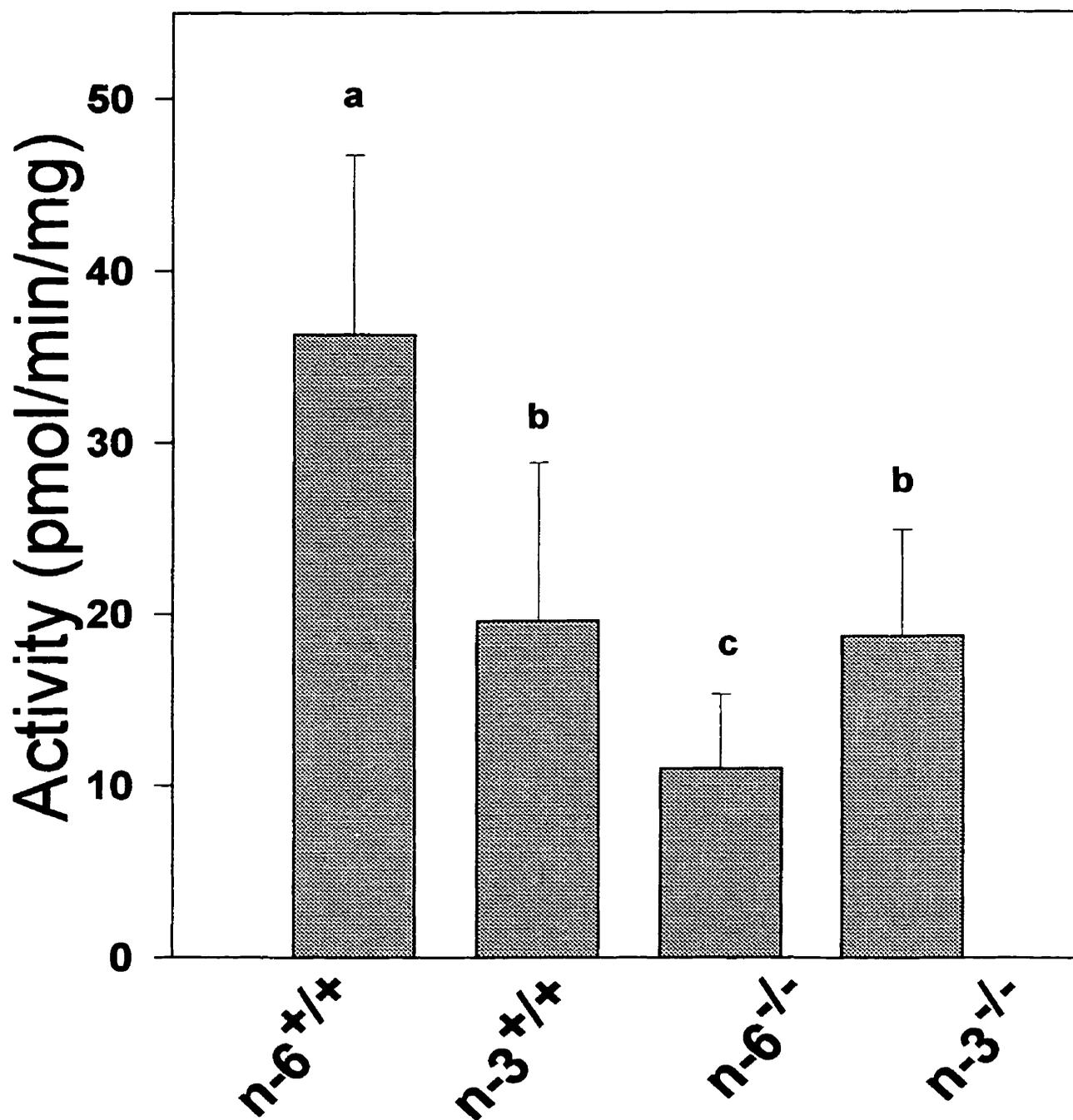


Figure 6-2. HMG-CoA reductase enzyme activity in mammary glands of knockout (-/-) and wild-type (+/+) mice fed either n-3 or n-6 PUFAs. Different letters indicate groups are significantly different ($P < 0.05$).

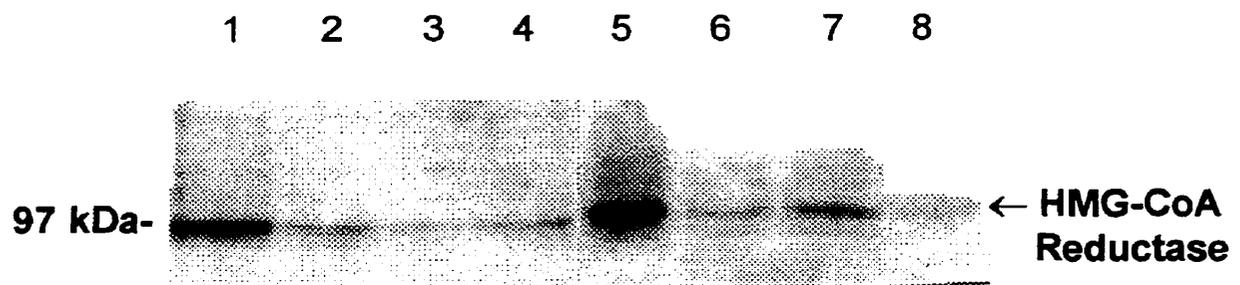


Figure 6-3. Representative Western blot of HMG-CoA reductase from mammary gland (lanes 1-4) and liver (lanes 5-8) or +/+ (lanes 1,2,5 and 6) and -/- (lanes 3,4,7, and 8) mice fed either n-6 (lanes 1,3,5 and 7) or n-3 (lanes 2,4,6 and 8) PUFAs.

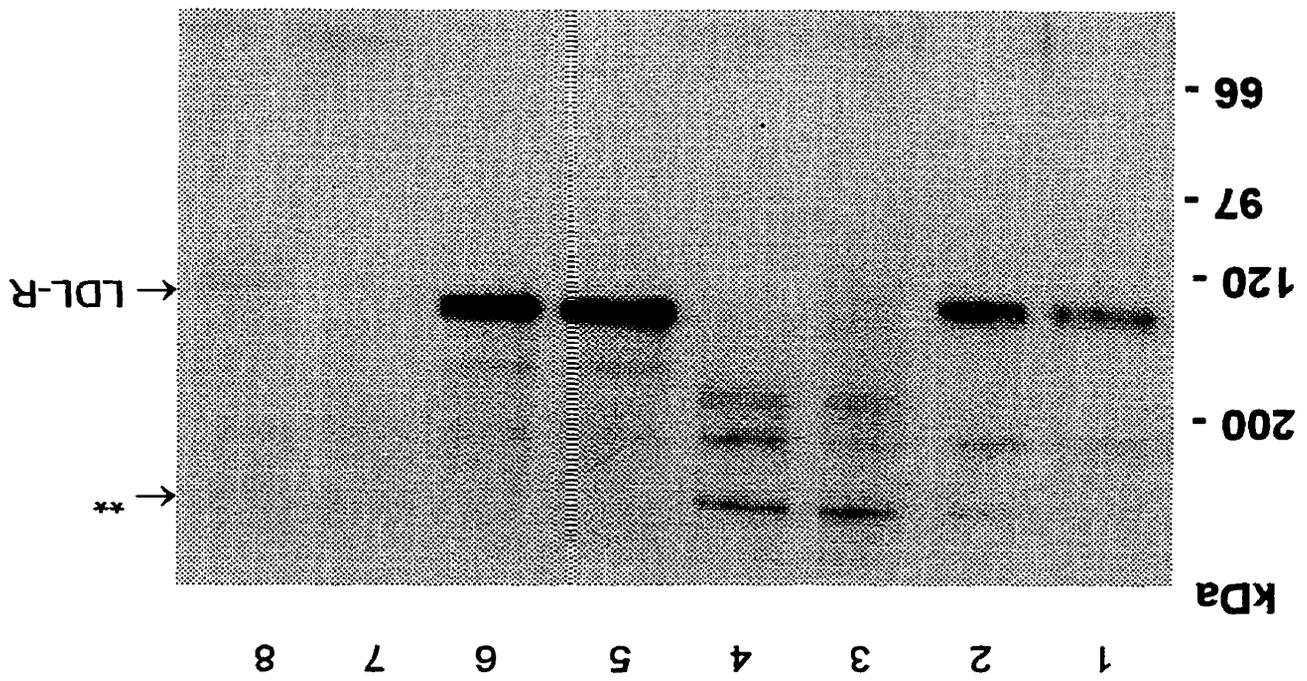


Figure 6-4. Representative Western blot of LDL-R from mammary gland (lanes 1-4) and liver (lanes 5-8) or +/+ (lanes 1,2,5 and 6) and -/- (lanes 3,4,7, and 8) mice fed either n-6 (lanes 1,3,5 and 7) or n-3 (lanes 2,4,6 and 8) PUFAs. (**) Indicates protein with apparent molecular weight of ~300 kDa.

6.5 DISCUSSION

In Chapter 4, it was shown that n-3 PUFAs decrease HMG-CoA reductase in rat mammary glands and this was proposed as a possible mechanism by which these fatty acids inhibit mammary tumorigenesis. To test the hypothesis that this decrease in HMG-CoA reductase is mediated by receptor-dependent uptake of LDL-cholesterol, knockout mice that lack the LDL-R were used. Previous studies by us (El-Sohemy and Archer 1997) and others (Spady 1993; Spady et al. 1995; Ventura et al. 1989) on the regulation of HMG-CoA reductase or cholesterol biosynthesis by n-3 PUFAs used rats. Since cholesterol metabolism is known to differ between species (Dietschy et al. 1993) and the effects of n-3 PUFAs in mice were not known, it was necessary to establish first whether the mouse responds like the rat to the effects of n-3 PUFAs on serum lipids or HMG-CoA reductase.

The results confirmed that mice respond like rats to the effects of dietary n-3 PUFAs with lowered levels of serum TGs, total-, LDL- and HDL-cholesterol and decreased HMG-CoA reductase in the liver and mammary glands. To determine whether any of these effects are mediated by the LDL-R, the experimental diets were fed to mice lacking a functional LDL-R. In these animals, n-3 PUFAs effectively reduced serum TG and total-cholesterol levels. LDL-cholesterol was reduced to a lesser extent in *-/-* (2-fold) than in *+/+* (6-fold) mice. Hepatic HMG-CoA reductase was also decreased by n-3 PUFAs in *-/-* mice, but to a lesser extent than in *+/+* mice. Although the rate of LDL uptake was not measured directly in these experiments, a reduction in serum LDL-cholesterol and a down-regulation of hepatic HMG-CoA

reductase are consistent with enhanced LDL uptake by the liver (Ventura et al. 1989). In the mammary gland, n-3 PUFAs did not decrease HMG-CoA reductase in *-/-* mice but, surprisingly, up-regulated the enzyme. This unexpected increase may have been a response to the drop in serum LDL-cholesterol levels that was caused by LDL-R-independent uptake of LDL in the liver. Importantly, this observation demonstrates that the LDL-R is required for n-3 PUFAs to decrease mammary gland HMG-CoA reductase but is not required to decrease hepatic HMG-CoA reductase or lower serum lipids.

Spady and colleagues conducted a series of experiments to elucidate the mechanisms by which n-3 PUFAs regulate cholesterol metabolism (Spady 1993; Spady et al. 1995; Ventura et al. 1989). They observed that dietary n-3 PUFAs enhanced the internalization of LDL by the liver and various extrahepatic tissues in the rat (Spady 1993; Spady et al. 1995; Ventura et al. 1989) but not the hamster (Spady et al. 1995). In the rat, enhanced uptake of LDL by the liver appears to be mediated predominantly by the LDL-R and in extrahepatic tissues by a LDL-R-independent pathway (Ventura et al. 1989). This mechanism may explain how n-3 PUFAs lower serum cholesterol and regulate HMG-CoA reductase. It appears from the present study that mice respond like rats to n-3 PUFAs by enhancing hepatic LDL uptake, but unlike rats, this effect occurs independently of the LDL-R. Since the mammary gland was the only extrahepatic tissue examined, it is not clear how the mechanisms of regulation compare between the rat and mouse in other extrahepatic tissues. Indeed, the mammary gland is capable of synthesizing cholesterol for secretion during lactation and may be subject to unique regulatory mechanisms (Gibbons et al. 1983).

In the present study, n-3 PUFAs had no effect on the levels of LDL-R in either the liver or mammary glands of wild-type mice. Although the results suggest that the LDL-R mediates the effects of n-3 PUFAs in the mammary gland, this effect does not involve a change in immunodetectable levels of the receptor. This result is consistent with previous findings by Spady *et al* (1995) who showed that n-3 PUFAs enhance LDL-R-mediated uptake of LDL in the liver of rats without altering the levels of LDL-R protein. Interestingly, an up-regulation of a ~300 kDa protein was observed in the mammary glands of wild-type mice fed n-3 PUFAs and an even greater up-regulation in the mammary glands of knockout mice fed either diet. This protein appears to be similar to the one observed in the previous chapter and its expression is consistent with increased uptake of LDL.

The first report on the LDL-R knockout mouse showed a 2-fold increase in total cholesterol, a 7- to 9-fold increase in IDL and LDL, and no significant changes in HDL or TG levels compared to wild-type littermates (Ishibashi *et al.* 1993). Osono *et al* (1995) were the first to examine the effects of LDL-R deletion on rates of cholesterol synthesis and cholesterol balance across the plasma space. They observed that deletion of the LDL-R results in a 14-fold increase in serum LDL and a significant increase in VLDL and HDL (Osono *et al.* 1995). There were no changes in the rates of cholesterol synthesis in the liver or extrahepatic tissues. The mice in these two studies (Ishibashi *et al.* 1993; Osono *et al.* 1995) were fed chow diets that contain low levels of cholesterol and undefined fatty acids. The results of the present study shows that the type of PUFAs in a semi-synthetic diet profoundly influence the effect of deleting the LDL-R on serum lipids. For example, LDL-cholesterol was increased 27-fold in mice

fed n-3 PUFAs but was increased only 10-fold in mice fed n-6 PUFAs. Interestingly, HDL-cholesterol levels were slightly higher in *-/-* than in *+/+* mice when both groups were fed n-3 PUFAs, but were significantly lower when they were fed n-6 PUFAs (Table 6-1). The type of dietary PUFAs were also important determinants of the effects of deleting the LDL-R on HMG-CoA reductase. In the liver, deletion of the LDL-R decreased HMG-CoA reductase to a greater extent in mice fed n-6 PUFAs than those fed n-3 PUFAs. These decreases in HMG-CoA reductase are likely due to LDL-R-independent uptake of LDL-cholesterol that is stimulated by its extremely high circulating levels in these animals. A more striking effect was observed in the mammary gland where deletion of the LDL-R had no effect on HMG-CoA reductase activity or protein levels when n-3 PUFAs were fed, but resulted in a marked (3.6-fold) decrease when n-6 PUFAs were the major source of dietary fat. These observations suggest that studies examining the effects of gene deletion or overexpression on lipid metabolism should take into account the possible interactions with dietary lipids.

In addition to dietary differences, the background strain of LDL-R knockout mice used in previous studies also differs from the one used in the present study. Earlier studies used the B129 strain (Ishibashi et al. 1993; Ishibashi et al. 1994; Osono et al. 1995) which is a cross between the 129Sv and C57Bl/6J strains. The LDL-R deficient mice used in the present study are in a C57Bl/6J background and were developed by Jackson Labs by backcrossing the B129 with the C57Bl/6J strain. Mouse strains are known to differ in their metabolism of cholesterol (Paigen et al. 1985) and expression levels of HMG-CoA reductase (Hwa et al. 1992). Comparisons between studies may, therefore, be further confounded by these genetic differences.

In summary, the LDL-R mediates the decrease in HMG-CoA reductase in the mammary gland but is not required for n-3 PUFAs to lower serum lipids or decrease hepatic HMG-CoA reductase. The diet-gene interactions observed show that the composition of dietary PUFAs is a major determinant of the effects of LDL-R deletion on measures of lipid metabolism and may alter the interpretation of previous studies using LDL-R knockout mice. These results may enable us to understand more fully the mechanisms by which dietary PUFAs modulate biochemical pathways that are involved in mammary carcinogenesis and possibly cardiovascular disease.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 OVERVIEW AND EXPERIMENTAL CONSIDERATIONS

The objective of this thesis was to investigate a mechanism by which dietary PUFAs and cholesterol could modulate experimental breast cancer development. Established rodent models were used since they allowed studies to be carried out that could not be conducted in humans. The first part of this thesis (Chapters 2 & 3) focused on the effects of cholesterol while the second part (Chapters 4, 5 & 6) focused on PUFAs. The hypothesis for this thesis has been: The mevalonate pathway mediates the effects of dietary cholesterol and fat on mammary tumorigenesis. Based on the results, the hypothesis is supported.

Two experiments were conducted using the carcinogen-induced rat mammary tumor model. The purpose of Chapter 2 was to determine the effects of oxidized cholesterol on MNU-induced mammary tumor development. A group of animals were also fed cholesterol that had not been oxidized. Unexpectedly, cholesterol was found to inhibit the development of rat mammary tumors compared to controls fed a cholesterol-free diet while oxidized cholesterol had no effect. To explain this observation, I hypothesized that cholesterol decreases mevalonate synthesis by down-regulating HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis. In addition to being a precursor of cholesterol, mevalonate plays a key role in regulating cell proliferation. The requirement for mevalonate in cell proliferation could be due, at least in part, to providing farnesyl-PPi that is required for the post-translational modification of H-Ras proteins. Farnesylation of H-Ras enables it to become membrane-bound and functional. The objective of Chapter 3, therefore, was

to determine whether the inhibitory effects of cholesterol on tumorigenesis are dependent on the frequency of *H-ras* mutations in preneoplastic cells that develop into tumors. In this experiment, the effects of cholesterol were assessed using two different carcinogens that produce tumors with either a high (MNU) or low (DMBA) frequency of *H-ras* mutations. Regardless of the carcinogen used, cholesterol significantly inhibited tumor development suggesting that the effects are not dependent on *H-ras* mutations. Since HMG-CoA reductase activity was down-regulated by dietary cholesterol in mammary glands, a decrease in mevalonate synthesis in preneoplastic mammary epithelial cells may be a mechanism by which tumorigenesis is inhibited.

In view of the role of cholesterol in cardiovascular disease, clearly using dietary cholesterol to prevent breast cancer is not practical. Nevertheless, the observations made in Chapters 2 and 3 pointed to the importance of the mevalonate pathway in mammary tumorigenesis and there was indirect evidence to suggest that this pathway may mediate the anti-tumorigenic effects of n-3 PUFAs. The results from Chapter 4 provided more direct evidence to support this hypothesis by showing that the levels of HMG-CoA reductase protein and enzyme activity were lower in mammary glands of rats fed n-3 PUFAs compared to those fed n-6 PUFAs. Extrahepatic HMG-CoA reductase is believed to be regulated by circulating cholesterol levels and the liver is a major organ regulating serum cholesterol. Therefore, serum cholesterol and hepatic HMG-CoA reductase were measured and both were lowered by n-3 PUFAs. Taken together with the results from Chapters 2 and 3, these findings suggest that changes in circulating cholesterol levels do not solely determine the activity of extrahepatic reductase. In the first two studies (Chapter 2 & 3), an increase in serum cholesterol

was associated with a decrease in tumor development, whereas in the next study (Chapter 4) a decrease in serum cholesterol was associated with a diet that decreases tumor development. The discordance between these studies relating serum cholesterol to tumorigenesis can be reconciled by the mevalonate pathway. Although dietary cholesterol increases serum cholesterol while n-3 PUFAs decrease it, both inhibit mevalonate synthesis in the mammary gland and both inhibit mammary tumorigenesis. Thus, it appears that changes in serum cholesterol alone, can not predict changes in mevalonate synthesis or tumor development. However, changes in mevalonate synthesis correlate well with tumorigenesis. These observations have important implications for epidemiological studies that attempt to correlate serum cholesterol with breast cancer development.

Rodents are not the preferred model to study cholesterol metabolism since they are thought to be resistant to changes in serum cholesterol and thus respond differently from humans. While this may be true for some strains, both rats and mice display wide variabilities in serum cholesterol among different strains in response to dietary cholesterol (Paigen et al. 1985; Van Zutphen and Den Bienman 1981). The same is also true among humans (Ginsberg et al. 1994). The studies described in this thesis used the Sprague-Dawley rat and the C57Bl/6J mouse. Both of these strains respond to dietary cholesterol with increased serum levels (see Appendix A and Chapter 2). Thus, despite differences in cholesterol metabolism between rodents and humans (Spady and Dietschy 1983), the effect of various dietary treatments on cholesterol biosynthesis appear to be similar (Jones 1997).

Studies that have examined the effects of n-3 PUFAs on HMG-CoA reductase have been very limited and the mechanisms remain unclear. More studies, however, have examined the effects of n-3 PUFAs on hepatic cholesterol metabolism. Hypotheses regarding the effects of n-3 PUFAs on HMG-CoA reductase were, therefore, formulated based on the findings of the effects of these fatty acids on cholesterol metabolism. Studies by Spady and colleagues have suggested that n-3 PUFAs lower serum cholesterol in rats by increasing the uptake of LDL in both the liver and extrahepatic tissues (Spady 1993; Ventura et al. 1989). Uptake in the liver occurs predominantly through the LDL-R whereas uptake in a number of extrahepatic tissues has been shown to occur mostly through a LDL-R-independent pathway (Spady et al. 1995). The mammary gland, however, is capable of synthesizing cholesterol for secretion during lactation and may be subject to different regulatory mechanisms (Gibbons et al. 1983).

The objective of Chapter 5 was to determine whether an up-regulation of the LDL-R by n-3 PUFAs may mediate the down-regulation of HMG-CoA reductase and whether any effects are due to changes in the expression of the *LDL-R* or *HMG-CoA reductase* genes. n-3 PUFAs did not alter the expression levels of these genes in either the mammary gland or liver and there were no changes in the levels of LDL-R protein. There was, however, an increase in the levels of an unidentified protein (~300 kDa) and partial mRNA transcript (~2 kb) in the mammary glands of rats fed n-3 PUFAs that may be involved in the uptake of LDL. This protein appears to have some homology with the LDL-R since it was recognized using an antibody raised specifically against a peptide corresponding to a region of the rat LDL-R (Ellsworth et al. 1987).

Similarly, the mRNA transcript was amplified using primers that correspond to the cDNA sequence of the rat LDL-R (Lee et al. 1989). Since there is evidence that increased uptake of LDL through the LDL-R can occur in the absence of changes in the levels of LDL-R protein (Spady et al. 1995), the results from this study can not rule out a possible role for the LDL-R in mediating the effects of n-3 PUFAs on HMG-CoA reductase.

To determine definitively whether the LDL-R is required for n-3 PUFAs to decrease HMG-CoA reductase, we made use of mice with a targeted disruption of the *LDL-R* gene that were available commercially. In view of the known species differences in cholesterol metabolism and since the work done in the previous chapters was carried out using rats, there was a need to establish first whether mice respond like rats to the effects of n-3 PUFAs. Chapter 6 confirmed that C57Bl/6J mice respond similarly to Sprague-Dawley rats by decreasing mammary gland and hepatic HMG-CoA reductase when fed n-3 PUFAs. In knockout mice lacking the LDL-R, n-3 PUFAs did not decrease mammary gland HMG-CoA reductase. This result demonstrates that the LDL-R is required for n-3 PUFAs to decrease HMG-CoA reductase in mouse mammary glands. Interestingly, a ~300 kDa protein was induced in the mammary glands of wild-type mice fed n-3 PUFAs and knockout mice fed either n-3 or n-6 PUFAs. This protein appears to be similar to the one observed in Chapter 5 that was highly expressed in the mammary glands of rats fed n-3 PUFAs. Moreover, the expression of this protein is consistent with enhanced uptake of LDL by mammary glands in both rats and mice and may represent a novel lipoprotein receptor.

Although the focus of the second part of this thesis was on the inhibitory effects of n-3 PUFAs as opposed to the promoting effects of n-6 PUFAs, all of the effects reported are relative. The issue of whether n-3 PUFAs decrease or n-6 PUFAs increase HMG-CoA reductase is moot. This is because there is no universal 'control' diet with well defined effects on biochemical pathways and/or tumorigenesis. In the case of AIN-93G, the current standard reference diet for rodents (Reeves et al. 1993), the source of fat is Soybean oil and its effects on carcinogenesis and cholesterol metabolism have not yet been thoroughly investigated. Another difficulty in designing nutrition experiments is that changing one component of a diet necessarily changes the proportion of the other components. Thus, it is usually not clear what dietary change caused any of the changes in the parameters measured. Nevertheless, the effects of n-3 PUFAs on carcinogenesis have been demonstrated in numerous studies (see Chapter 1).

Although it is not clear which fatty acids in the fish oil are responsible for these effects, there is evidence that the long chain n-3 PUFAs EPA and DHA are responsible for a major part of the effects on tumor development. There is also evidence that these fatty acids are responsible for the effects of fish oils on cholesterol metabolism (Spady 1993). For example, *in vitro* studies show that the long chain n-3 PUFAs decrease HMG-CoA reductase (Murthy et al. 1988) and inhibit cell proliferation (Rose and Connolly 1990). Low doses of EPA and DHA given by gavage inhibit the development of mammary tumors in rats fed high-fat vegetable oil diets and administering these n-3 PUFAs intravenously increases the uptake of LDL and decreases cholesterol biosynthesis (Spady et al. 1995). Therefore, it is reasonable to

presume that the effects that were observed are due to n-3 PUFAs derived from fish oil. Another reason for directly comparing the effects of n-3 to n-6 PUFAs is that previous studies on the effects of these fatty acids on either carcinogenesis or cholesterol metabolism have made similar comparisons. Hence, we are able to obtain mechanistic insights and formulate hypotheses based on the results of these other studies.

The diets used in Chapters 4, 5 and 6 contained 7% fat (w/w) and are considered low-fat diets. In carcinogenesis studies, the n-3 diet used would contain insufficient amounts of LA required for tumor growth (Ip et al. 1985). Since our studies examined biochemical and molecular changes in normal tissues, the formulation of the diets were such that there was an adequate amount of essential fatty acids (both LA and ALA) required for normal growth and development and the level of fat was kept the same as the standard reference diet. The diets were formulated to provide the maximum difference in the n-3 and n-6 PUFAs content and resulted in a 100-fold difference in the n-3/n-6 ratio. A review of the animal studies investigating the effects of n-3 PUFAs on mammary tumorigenesis has suggested that there is an optimal n-3/n-6 ratio required for maximal tumor inhibition and this may be achieved by mixing equal parts of Menhaden oil with Corn or Safflower oil (Ip 1997). Another study, however, has shown that a higher n-3/n-6 ratio may be more effective (Bunce and Abou-El-Ela 1990). It is clear that there are still some unresolved issues regarding the most appropriate experimental design and the effects of different fatty acids on mammary tumorigenesis. Thus, it is difficult to ascertain what the most suitable

comparisons should be. Nevertheless, the results presented in this thesis are consistent with other known effects of n-3 PUFAs.

Although there is considerable evidence that n-3 PUFAs derived from fish oils inhibit mammary tumorigenesis, there are still some uncertainties regarding the optimum level of the different fatty acids and, once these are met, the optimum n-3/n-6 ratio. Furthermore, it is not entirely clear whether the tumor promoting effects of a high n-6 PUFAs diet are due to a deficiency in n-3 PUFAs. If tumorigenesis studies are going to be conducted in the future, however, these dietary issues need to be addressed. Preliminary experiments using newly developed diets with fatty acid profiles giving the greatest tumor inhibition will be needed to confirm the effects of n-3 PUFAs on HMG-CoA reductase.

7.2 FUTURE DIRECTION

Although the results from Chapter 2 suggest that oxidized cholesterol may enhance mammary tumor development when compared to unoxidized cholesterol, the observation was not pursued. Indeed, the role of cholesterol oxides has not been adequately investigated in carcinogenesis experiments and they may be an important contributing factor in human breast cancer development. Thus, it seems reasonable to suggest that future studies may wish to examine whether specific cholesterol oxidation products promote rat mammary tumor development.

Based on the findings presented in this thesis, a number of experiments need to be carried out to provide more definitive conclusions regarding the role of the

mevalonate pathway in mediating the effects of n-3 PUFAs on mammary tumorigenesis. The precise level of n-3 and n-6 PUFAs and the ratio between these fatty acids that produce the greatest inhibition of tumor development needs to be established. If a dose-response effect is observed with respect to n-3 PUFAs and tumor development, it would be of interest to determine whether a similar dose-response is observed with the rate of mevalonate synthesis. Furthermore, it will be important to establish whether the range of n-3/n-6 PUFAs ratios in the human diet leads to corresponding differences in mevalonate synthesis.

Many studies that examine the molecular mechanisms by which diet regulates breast cancer development only show correlations between molecular, biochemical or hormonal changes and tumor development, without demonstrating a causal relationship. Although this is often a necessary first step, such observations are not always followed-up by studies that elucidate the functional significance of a given mechanism. The results presented in this thesis also provide correlations between mevalonate and tumorigenesis. Future work in this area should focus on establishing whether the mevalonate pathway actually mediates the effects of dietary fat and/or cholesterol in breast carcinogenesis. This can be achieved using a number of different approaches. For example, in a cell culture experiment, the inhibition of cell proliferation by n-3 PUFAs along with a decrease in mevalonate synthesis in these cells should first be demonstrated. If this decrease in mevalonate synthesis is causally related to the decrease in proliferation, then restoring the levels of mevalonate, by adding it directly to the medium, should reverse the growth inhibition. This type of 'repletion' experiment can also be done *in vivo*. In this case the inhibitory effects of n-3

PUFAs on rat mammary tumor development can be compared directly with a group of rats fed n-3 PUFAs and given mevalonate in the drinking water. Mevalonate given in the drinking water is known to reach the liver (Chambers and Ness 1998), but its delivery to the mammary gland will first need to be established.

Based on the results from Chapter 6, a carcinogenesis experiment should be carried out using LDL-R knockout mice. Since n-3 PUFAs do not decrease mammary gland HMG-CoA reductase in these animals, it is important to determine whether n-3 PUFAs still inhibit tumor development in the absence of a decrease in mevalonate synthesis. If tumorigenesis is still inhibited by n-3 PUFAs in these mice, then the mevalonate pathway is unlikely to be an important mechanism by which the effects of these fatty acids are mediated.

Before a mouse tumorigenesis study can be conducted, however, there are some methodological limitations that need to be considered. Knockout mice were developed in a C57Bl/6J background. This strain is not commonly used in mouse mammary tumorigenesis studies because it is not as susceptible to tumor formation as the more commonly used Balb/c strain (Medina 1974). However, as an alternative, a colon carcinogenesis study can be designed to examine the effect of n-3 PUFAs on the formation of aberrant crypt foci (ACF), putative precancerous lesions in the colon (see Appendix B). Fish oils have been shown to inhibit colon tumorigenesis and the C57Bl/6J strain is highly susceptible to the formation of ACF and colon tumors. Furthermore, there is indirect evidence that the mevalonate pathway mediates the effects of n-3 PUFAs on colon tumorigenesis. Singh *et al* (1997) have shown that dietary Menhaden oil inhibits Ras membrane localization and hypothesized that this

may be due to a down-regulation of HMG-CoA reductase. In a follow-up study Singh *et al* (1998) observed a decrease in the expression of FPTase in the fish oil group compared to the corn oil group. In these studies, however, no measures of HMG-CoA reductase were made. If mevalonate is found to mediate the inhibitory effects of fish oils on mammary tumor development, this mechanism may also apply to colon cancer.

Competitive inhibitors of HMG-CoA reductase (i.e. statins) have been used for many years to treat cardiovascular disease. The safety and efficacy of statins from 16 trials has recently been reviewed (Hebert *et al.* 1997). A number of studies have found a significant reduction in overall mortality due mainly to a decrease in cardiovascular deaths and stroke. No effects on cancer mortality were observed. The average follow-up in these trials, however, was only 3 years and any effects on cancer development are unlikely to be observed over such a short period of time. Thus, it remains to be seen whether this class of drugs will reduce the prevalence of cancer.

It is well documented that a number of dietary factors and hormones/growth factors, many of which are known to affect cancer development, modulate the expression and/or activity of HMG-CoA reductase (Chapter 1). The effects of these factors on HMG-CoA reductase correlate well with their effects on tumorigenesis suggesting that the mevalonate pathway may have a broad role in regulating cancer development. As discussed in Chapter 1, cholesterol synthesis is sometimes only a crude measure of mevalonate synthesis. In the rat, for example, feeding a level of cholesterol that results in a 98% decrease in cholesterol synthesis (measured by incorporation of radio-labeled acetate into sterols) leads to only a 50% decrease in mevalonate synthesis (measured by conversion of radio-labeled HMG-CoA to

mevalonate) (Sinensky et al. 1990). Therefore, since mevalonate and not cholesterol is required by mammalian cells for proliferation, cholesterol synthesis may only be an approximate measure of mevalonate synthesis. The distinction between mevalonate synthesis and cholesterol synthesis is crucial to conducting and interpreting human trials. In humans, whole body cholesterol biosynthesis can be measured using stable isotope techniques (Jones et al. 1996), and the levels of circulating and urinary mevalonate correlate well with cholesterol biosynthesis (Lindenthal et al. 1996). The major limitation, however, is the inability to measure non-invasively mevalonate or cholesterol synthesis in a target organ such as the mammary gland.

The observations made in Chapters 5 and 6 showing that a high molecular weight protein (~300 kDa) may be involved in the uptake of lipoproteins in the mammary gland clearly warrants further investigation. A number of lipoprotein receptors have so far been identified (Brown et al. 1997) and it is believed that there are 'receptor-independent pathways' of lipoprotein uptake (Spady et al. 1985). 'Receptor-independent pathways' may simply be receptors that are not readily saturable and have not yet been identified. The sequencing of the cDNA product that was amplified by PCR in Chapter 5 using primers for the LDL-R may lead to the identification of a novel gene that encodes a receptor involved in regulating the uptake of lipoproteins.

The association between dietary fat and breast cancer remains controversial and is unlikely to be resolved by any single experiment. At least one of the major reasons for the inconsistent findings among epidemiological studies may be related to the intake of different types of fatty acids in the populations studied. The observation

that some dietary fatty acids can be potent inhibitors of mammary tumorigenesis (e.g. EPA, DHA, CLA) while others significantly enhance, lends further support to the idea that fat can no longer be considered a single dietary factor in any study. Moreover, dietary recommendations on fat must take this into account. Until methodological issues are resolved, the involvement of different types of fatty acids in human breast cancer will remain unclear.

Researchers from the American Health Foundation have recently suggested that further studies using humans to study the fat-breast cancer connection are unlikely to provide new insights and that emphasis in this area should be directed at elucidating the molecular mechanisms of action (Wynder et al. 1997). A possible protective effect of long-chain n-3 PUFAs derived from fish oil suggests that fish oil supplements may be useful in the treatment and/or prevention of breast cancer. It is noteworthy that fish oil supplements reduce colonic cell proliferation in humans, a risk factor for colon cancer (Bartoli et al. 1993). Given that colon and breast cancer share similar dietary risk factors (Willet 1989), the potential use of fish oils to reduce the prevalence of breast cancer warrants further consideration. A recent intervention study has already shown that fish oil supplements can effectively increase the n-3/n-6 PUFA ratio in the plasma and breast adipose tissue of breast cancer patients (Bagga et al. 1997). This ongoing study should reveal, over the next few years, whether n-3 PUFAs derived from fish oils protect against the development of this disease in women.

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APPENDIX A

Reports

Dietary Cholesterol Inhibits the Development of Aberrant Crypt Foci in the Colon

Ahmed El-Soheby, Cyril W. C. Kendall, A. Venket Rao, Michael C. Archer, and W. Robert Bruce

Abstract

We evaluated the effect of dietary cholesterol and oxidized cholesterol on the promotion of aberrant crypt foci (ACF), which are putative precancerous lesions in the colon. Sixty female C57BL/6J mice were given four weekly injections (ip) of azoxymethane (AOM); then fed either a control AIN-76 diet or the control diet supplemented with 0.3% cholesterol or 0.3% oxidized cholesterol for 100 days. The oxidized cholesterol was prepared by heating cholesterol at 110°C for 48 hours. Gas chromatographic analysis of the oxidized cholesterol showed that 96% of the cholesterol was unchanged and less than 2% of the cholesterol was oxidized. The remaining 2% impurities were unidentified and present in both the cholesterol and heated cholesterol. The number of ACF in the group fed cholesterol was significantly lower than the control group (7.9 ± 1.0 vs. 12.5 ± 1.2, $p < 0.01$). The number of ACF in the group fed oxidized cholesterol (10.1 ± 1.1) was not different from the control or cholesterol groups. The size of the ACF (no. of crypts per focus) did not differ between the three dietary groups. Serum low-density lipoprotein (LDL) cholesterol was greater in the cholesterol-fed group than the control group (40.5 ± 4.6 vs. 24.3 ± 3.6 mg/dl, $p < 0.05$). LDL cholesterol from the animals fed oxidized cholesterol (37.7 ± 4.7 mg/dl) was not different from the control or cholesterol-fed animals. Total and high-density lipoprotein (HDL) cholesterol did not differ between the groups. The results show that dietary cholesterol significantly inhibits the promotion of ACF in the colon. The elevated LDL cholesterol may inhibit de novo cholesterol synthesis in the preneoplastic colonic epithelial cells, thereby inhibiting DNA synthesis and cell proliferation.

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Introduction

The association between dietary cholesterol and human colon cancer (1) has led to numerous animal studies that have examined the role of cholesterol in colon carcinogenesis (2-11). Although many of these studies have shown that cholesterol enhances carcinogenesis (2-9),

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the results have not been consistent (10,11). This may be due to differences in the experimental protocols employed. In studies where cholesterol was fed throughout the initiation and promotion (postinitiation) stages of carcinogenesis (2-9), there was an increase in the number of tumors or precancerous lesions in the colon. Such a protocol, however, does not give a clear measure of the promoting activity of the test compound because it may alter the metabolism of the carcinogen and/or the sensitivity of target cells to the carcinogen (12). When cholesterol was fed only during the promotion stage, it either enhanced (2), inhibited (11), or had no effect (9) on the development of colonic tumors or precancerous lesions. The striking differences in the experimental designs of these studies (2,9,11) may account for these apparently inconsistent results.

Recently, we have shown that oxidized cholesterol has a greater effect than cholesterol at enhancing the development of aberrant crypt foci (ACF) (4), which are putative precancerous lesions in the colon (13-16). Indeed, products of cholesterol oxidation may be cytotoxic, mutagenic, and carcinogenic (17,18). However, the oxidized cholesterol in this study (4) was fed throughout the initiation and promotion stages and thus its effect on promotion alone is not known. The present study was designed to examine the effect of oxidized cholesterol with a carcinogen and diet schedule that assessed the promotion stage of colon carcinogenesis. Because the role of cholesterol during promotion is unclear, the effect of oxidized cholesterol was compared to that of cholesterol using the same protocol. Unexpectedly, we found that cholesterol inhibited the development of ACF, whereas oxidized cholesterol had no effect. We speculate that the elevated levels of serum LDL cholesterol may have inhibited endogenous cholesterol biosynthesis, which is required for DNA synthesis and cell proliferation (19-21).

Materials and Methods

Animals

Pathogen-free female C57BL/6J mice (5 wks old) purchased from Jackson Laboratory (Bar Harbor, ME) were housed five to a cage in a temperature- ($24^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and humidity- (50%) controlled room, 12:12-hour light-dark cycle. They were acclimatized for seven days before the start of the experiment with food and water provided ad libitum.

Diets

Cholesterol [5-(6)-cholesten-3-ol] was purchased from Sigma Chemical (St. Louis, MO) and stored in a dark, sealed container at -20°C to prevent oxidation. Oxidized cholesterol was prepared by the method of Kendall and co-workers (4) by spreading cholesterol evenly to a thickness of about 5 mm on the surface of a glass tray and heating at 110°C for 48 hours in an electric oven. One batch of cholesterol and oxidized cholesterol were used throughout the experiment. The control diet consisted of 50% sucrose, 20% casein, 15% starch, 5% Alphacel, 5% corn oil, 3.5% AIN-76 mineral mix, 1% AIN-76 vitamin mix, 0.3% DL-methionine, and 0.2% choline. Cholesterol or oxidized cholesterol was added to the control diet at a level of 0.3% at the expense of sucrose. Fresh diet was replaced three times per week.

Experimental Protocol

Azoxymethane (AOM, Sigma Chemical) was dissolved in a 0.9% NaCl solution and administered within one hour of preparation. Each animal received four weekly injections (ip) of AOM (5 mg/kg). One week after the final carcinogen treatment they were randomized into three groups (20/group) and fed either the control diet or the control diet supplemented with 0.3% cholesterol or 0.3% oxidized cholesterol for 100 days. Animals were sacrificed by cervical dislocation, the colons were excised, and blood samples were collected from the venae cavae.

Analyses

Gas chromatographic analyses of cholesterol and oxidized cholesterol were performed on a Varian 3400 gas chromatograph with a flame ionization detector. Cholesterol, 5 α -cholestane, 3 β ,5 α ,6 β -cholestanetriol, 7 β -hydroxycholesterol, 7-ketocholesterol, and 5 α ,6 α -epoxide, purchased from Sigma Chemical, were analyzed as their trimethylsilyl ether derivatives on a fused silica capillary SE-30 column (0.25 mm \times 30 m, Altech, Guelph, Canada) with a liquid phase thickness of 0.25 μ m. 5 α -Cholestane was used as an internal standard. Samples were injected at a column temperature of 270°C that was raised to 300°C at a rate of 10°C/min. The injector and detector temperatures were 275 and 320°C, respectively. Nitrogen was used as the carrier gas with a flow rate of 0.4 ml/min. All samples were analyzed in triplicate. For determination of ACF, the colons were cut open longitudinally, rinsed with saline, and fixed flat between filter paper in 10% phosphate-buffered formalin. The colons were then stained with 0.1% methylene blue for 10 minutes, and ACF were scored under a light microscope at 40 \times magnification. The number of ACF observed per colon and the size of the ACF (no. of aberrant crypts per focus) were scored blindly by a single observer. Aberrant crypts are distinguished from the surrounding normal crypts by their increased size, darker staining, enlarged pericryptal zone, and the slit-like shape of the lumen (16). Serum was collected from blood samples and stored at -70°C prior to analysis for total, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol by enzymatic methods (Vita Tech Laboratories, Toronto, Canada). HDL cholesterol was precipitated and LDL was calculated based on the Friedewald factor. All chemicals for this determination were obtained as a test kit (Boehringer, Mannheim, West Germany).

Statistical Methods

All values for number and size of ACF and serum cholesterol levels are means \pm SEM. Analysis of variance followed by the Duncan's multiple-range test were used to detect differences between groups (SAS, Release 6.03, Cary, NC: SAS Institute, 1988).

Results

To assess the effects of cholesterol and oxidized cholesterol on the promotion stage of colon carcinogenesis, we injected C57BL/6J mice with AOM and randomized them to a control diet or to diets supplemented with 0.3% cholesterol or 0.3% oxidized cholesterol. Gas chromatography was used to measure the purity of the cholesterol sample, to measure the degree of oxidation in the oxidized cholesterol sample, and to identify the major cholesterol oxidation products. The purity of the cholesterol sample was >98% with the remaining 2% impurities unidentified. These impurities eluted after cholesterol and are not likely oxidation products that eluted before. Analysis of the oxidized cholesterol sample showed that 96% of the cholesterol was unchanged and less than 2% of the cholesterol was oxidized. The major cholesterol oxidation products were 7-ketocholesterol, 3 β ,5 α ,6 β -cholestanetriol, 7 β -hydroxycholesterol, and 5 α ,6 α -epoxide in addition to a few minor unidentified peaks.

The final body weights of the animals in the three groups did not differ (data not shown). The average number of ACF per animal from each of the three groups after 100 days on the diets is shown in Figure 1. Animals fed cholesterol had a significantly lower number of ACF compared with the control group (7.9 \pm 1.0 vs. 12.5 \pm 1.2, p < 0.01). The group fed oxidized cholesterol (10.1 \pm 1.1) had an intermediate number of ACF that was not significantly different from either the control or cholesterol-fed groups (p > 0.15). The size of the ACF (no. of crypts per focus) in the control (1.38 \pm 0.08), cholesterol (1.39 \pm 0.15), and oxidized cholesterol groups (2.00 \pm 0.15) did not differ.

The effects of cholesterol and oxidized cholesterol on serum cholesterol levels are shown in

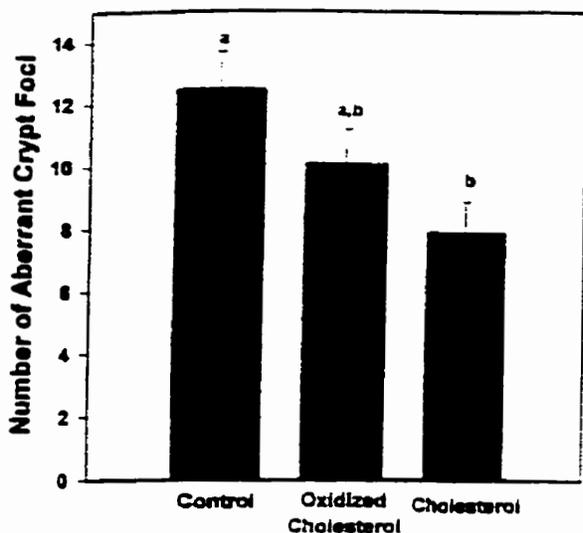


Figure 1. Effect of cholesterol and oxidized cholesterol on avg no. of aberrant crypt foci (ACF) per animal. Groups not sharing same letter (a,b) differ significantly, $p < 0.01$.

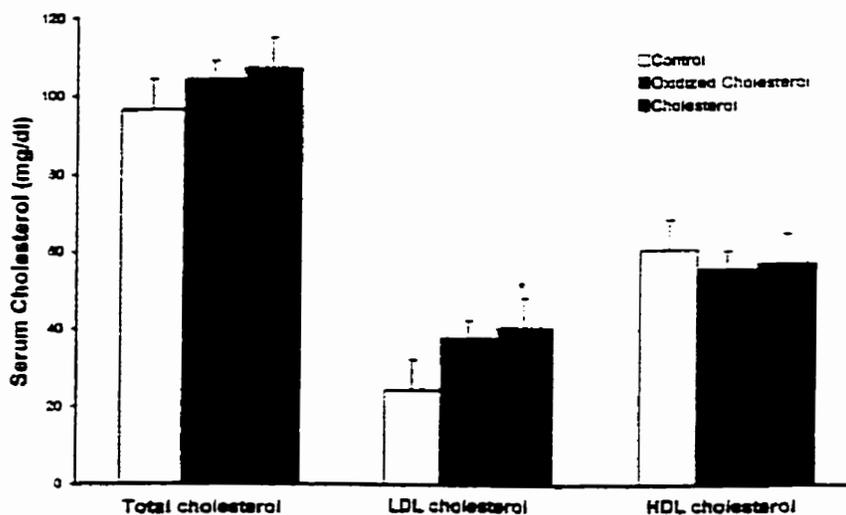


Figure 2. Effect of dietary cholesterol and oxidized cholesterol on serum total, LDL, and HDL cholesterol. *, Significantly different from controls, $p < 0.05$.

Figure 2. Serum LDL cholesterol was greater in the cholesterol group than in the control group (40.5 ± 4.6 vs. 24.3 ± 3.6 mg/dl, $p < 0.05$), whereas LDL cholesterol in the oxidized cholesterol group (37.7 ± 4.7 mg/dl) was intermediate and did not differ significantly from either the control or cholesterol groups. Total and HDL cholesterol in the control (96.5 ± 7.9 and 60.9 ± 4.4 mg/dl, respectively), cholesterol (107.1 ± 7.9 and 57.3 ± 4.1 mg/dl), and oxidized cholesterol groups (104.3 ± 7.4 and 55.9 ± 1.9 mg/dl) did not differ.

Discussion

The purpose of our study was to determine the effect of oxidized cholesterol on the promotion (postinitiation) stage of colon carcinogenesis using the ACF assay and to compare it with the effects of cholesterol and a cholesterol-free diet. Surprisingly, we found that dietary cholesterol at a concentration of 0.3% significantly inhibited the development of ACF and increased the

level of serum LDL cholesterol. Interestingly, both of these effects were reduced by oxidized cholesterol that contained only 2% oxidation products.

The results of this study are consistent with a previous study (11) which showed that dietary cholesterol given during the promotion stage of colon carcinogenesis inhibits tumor formation in Fischer rats. On the other hand, Cruse and colleagues (9) fed a liquid diet containing cholesterol to carcinogen-treated rats and reported no effect on the promotion of colon tumors. It is possible, however, that an inhibitory effect of cholesterol during the promotion stage was masked by the liquid diet, which is known to inhibit the development of colon tumors (5). In contrast, Rao and others (2) showed that cholesterol fed during the promotion stage increases the number of ACF in mice. In that study, cholesterol was fed after carcinogen treatment for only 28 days. It is possible that this period is not sufficiently long to predict accurately the long-term effects of cholesterol. In fact, studies by Bird (22) showed that early growth patterns of ACF may not reflect the long-term growth of these lesions and subsequent tumor development.

Several studies, including those from our research group, have shown that cholesterol enhances colon carcinogenesis when fed during the time of carcinogen treatment (2-9). It is not known why cholesterol enhances colon carcinogenesis when given during the initiation stage but inhibits when fed during the promotion stage. However, this phenomenon is not unique to cholesterol. Bird (22) recently reported that cholic acid enhanced the growth of ACF when given throughout initiation and promotion but inhibited ACF growth when given only during promotion. A similar mechanism of action may explain this phenomenon because cholesterol is the ultimate precursor of cholic acid.

In view of the results of previous studies, there appears to be a difference in the effect of cholesterol depending on whether it is given during initiation or promotion. We do not know how cholesterol enhances carcinogenesis during the initiation stage. However, the inhibitory effect of cholesterol during promotion may be a consequence of the increased serum LDL cholesterol. This cholesterol can enter the cell via the LDL receptor; it can act as a negative feedback inhibitor of endogenous cholesterol biosynthesis by reducing the level of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (21,23). This rate-limiting enzyme in the cholesterol biosynthesis pathway converts HMG-CoA to mevalonate, which is required for DNA synthesis and cell proliferation (19,20).

The growth of ACF in the oxidized cholesterol group was slightly greater than the cholesterol group and slightly less than the control group, although these differences were not significant. Oxidized cholesterol has been shown to inhibit the absorption of cholesterol in rodents (24). The attenuated rise in serum LDL cholesterol may have led to a reduced feedback inhibition of endogenous cholesterol biosynthesis. This could explain the intermediate response of oxidized cholesterol on both serum LDL cholesterol levels and the number of ACF. Alternatively, the small amount (2%) of cholesterol oxidation products may act as a promoter of ACF development, although the effect may have been masked by the relatively large amount of cholesterol that inhibited promotion. We were unable to show a significant effect by oxidized cholesterol, but this is likely because of the low level of oxidation products produced by the heating conditions employed. Thus it would be of interest to examine the effects of cholesterol oxidation products containing little or no cholesterol on colon carcinogenesis. A previous study (10), however, reported that cholesterol as well as two cholesterol oxidation products, 3 β ,5 α ,6 β -cholestanetriol and 5 α ,6 α -epoxide, do not enhance the promotion of colon tumors when administered intrarectally.

We have recently shown that dietary cholesterol at a level of 0.3% elevates serum LDL cholesterol and inhibits rat mammary tumor development (25). This observation supports the hypothesis that dietary cholesterol modulates the development of colon carcinogenesis by altering blood levels and not luminal concentrations of cholesterol, as previously believed (4,7,8,10).

In summary, dietary cholesterol inhibited the promotion of ACF in mice treated with the colon carcinogen AOM. The elevated LDL cholesterol in the serum of these animals may have modulated this effect by inhibiting *de novo* cholesterol synthesis, which is required for cell growth. We have also shown that cholesterol heated to produce approximately 2% oxidation products neither elevates serum LDL cholesterol appreciably nor inhibits ACF growth. The results of this study also underscore the importance of isolating the initiation and promotion stages of colon carcinogenesis in dietary studies. Any interactions between carcinogens and dietary factors may confound results and make it difficult to determine the underlying mechanisms of action. The mechanisms by which cholesterol modulates tumorigenesis when fed during the initiation or promotion stages of colon carcinogenesis warrant further investigation.

Acknowledgments and Notes

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APPENDIX B

**Tissue-Specific Alterations of Glyceraldehyde-3-phosphate Dehydrogenase in
Rats by Dietary (n-3) and (n-6) Polyunsaturated Fatty Acids^{1,2}**

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**Running Title: Polyunsaturated Fatty Acids and Glyceraldehyde-3-phosphate
Dehydrogenase**

ABSTRACT

Dietary (n-3) and (n-6) polyunsaturated fatty acids (PUFAs) regulate the expression of a variety of genes involved in glucose and lipid metabolism. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme of glycolysis and is widely used as a loading control in studies that measure gene expression. While investigating the effects of PUFAs on gene expression in rat mammary glands, we observed significant changes in the expression of GAPDH but not β -actin. The purpose of this study was to investigate this effect in more detail and to determine whether similar changes occur in other tissues involved in energy metabolism. Female Sprague-Dawley rats were fed 7% fat diets that were rich in either (n-3) (menhaden oil) or (n-6) (safflower oil) PUFAs for 1 week. In mammary glands, GAPDH mRNA, immunodetectable protein and enzyme activities were higher in the (n-3) group compared to the (n-6) group. In the liver, however, GAPDH protein and enzyme activities were lower in the (n-3) group compared to the (n-6) group, although no differences in mRNA were detected. In muscle, there were no differences in GAPDH between (n-3) and (n-6) PUFAs. These results show that (n-3) and (n-6) PUFAs have divergent effects on GAPDH in the mammary gland and liver. Since the mammary gland is largely composed of adipose tissue, the changes in both mammary gland and liver GAPDH may be related to the effects of PUFAs on energy metabolism.

KEY WORDS: rats, dietary fat, glyceraldehyde-3-phosphate dehydrogenase, mammary gland, liver.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), a key enzyme of glycolysis, is expressed ubiquitously at levels that are usually constant under a variety of physiological conditions (Fort et al. 1985). For this reason, it is commonly used as a loading control in studies that measure the expression levels of genes (Goldsworthy et al. 1993). There is evidence, however, that expression levels of GAPDH can be altered in cultured cells by hypoxia, insulin, or epidermal growth factor (Graven et al. 1994, Alexander et al. 1988, Matrisian et al. 1985). Moreover, GAPDH expression is elevated in proliferating rat liver, human lung cancer tissues, and in *ras*- and *mos*-transformed NIH 3T3 cells (Goldsworthy et al. 1993, Tokunaga et al. 1987, Persons et al. 1989).

Dietary (n-3) and (n-6) polyunsaturated fatty acids (PUFAs) have differential effects on glucose and lipid metabolism in both humans and rodents (Fickova et al. 1998, Harris et al. 1990, Delarue et al. 1996). These effects may be caused by alterations in the expression of genes in the liver that encode key metabolic enzymes (Jump et al. 1996). For example, compared to (n-6) PUFAs, (n-3) PUFAs decrease hepatic mRNA levels of fatty acid synthase, pyruvate dehydrogenase, pyruvate kinase and glucose-6-phosphate dehydrogenase while increasing the mRNA levels of fatty acyl-coA oxidase and mitochondrial 2,4-dienoyl-CoA reductase (Clark et al. 1990, Da Silva et al. 1993, Frøyland et al. 1997, Jump et al. 1994, Salati et al. 1988). The effects of dietary (n-3) and (n-6) PUFAs on the expression of GAPDH in hepatic or extrahepatic tissues, however, appear not to have been investigated.

While examining the effects of dietary (n-3) and (n-6) PUFAs on tumorigenesis in the rat mammary gland, we observed unexpected differences in GAPDH gene expression in this tissue. Because of the metabolic consequences and implications for mRNA analysis, we have explored these effects in more detail, including measurements of GAPDH protein levels and enzyme activity. Furthermore, we extended our studies to examine the effects of (n-3) and (n-6) PUFAs on GAPDH in liver and muscle, two major sites of energy metabolism.

MATERIALS AND METHODS

Animals and diets. Female Sprague-Dawley rats (43 days old) purchased from Charles River Laboratories (St. Constant, Quebec, Canada) were housed at $24 \pm 2^\circ\text{C}$ and 50% humidity, with a 12-h light/dark cycle. They were acclimatized for one week on the standard AIN-93G diet (Dyets, Bethlehem, PA) with free access to food and water. Animals were then randomized into two groups (six per group) and fed one of two experimental diets in which the 7% soybean oil in the AIN-93G diet was replaced with 1% soybean oil (to ensure adequate amounts of essential fatty acids) plus either 6% menhaden oil (rich in (n-3) PUFAs) or 6% safflower oil (rich in (n-6) PUFAs) for a period of one week (Reeves et al. 1993). All diets were stored in dark, air-tight containers at 4°C . Animals were anaesthetized and killed by cervical dislocation. Mammary glands, liver and muscle (quadriceps) were dissected, immediately frozen in liquid nitrogen and stored at -70°C . Care of the animals conformed to the guidelines of the Canadian Council on Animal Care, and the experimental protocol was approved by the University of Toronto Animal Care Committee.

RNA isolation. Total RNA was extracted from tissues using the Trizol® reagent (Life Technologies, Grand Island, NY) as described by the manufacturer.

mRNA analysis by RT-PCR. Total RNA (5 μg) was reverse transcribed into single-stranded cDNA using 50 U of MMLV-reverse transcriptase with 2.5 μM random hexamers (Boehringer Mannheim, Laval, Quebec, Canada). For GAPDH, the sense primer (5'-GTGGAGTCTACTGGCGTCTTC-3') and antisense primer (5'-CATGCCAGTGAGCTTCCCGTT-3') were selected from the rat cDNA sequence (Tso et al. 1985). For β -actin, the housekeeping gene we used as a loading control, the sense primer (5'-GCATCCTGTCAGCGACGATGCCTG-3') and antisense primer (5'-CGTAGCCATCCAGGCTGTGTT-3') were selected from the rat cDNA sequence (Nudel et al. 1983). These primer pairs yield amplified products of 408 bp for GAPDH and 570 bp for β -actin. Since the abundance of β -actin is comparable to that of GAPDH we developed a semi-quantitative method

of co-amplifying these two genes in a single reaction similar to a method recently described (Reue 1998). 25 cycles of amplification were used for all PCR reactions. This number showed a linear response of PCR products with varying amounts of RNA. The relative amount of PCR products for these two genes was the same when they were co-amplified or amplified separately. Reaction mixtures contained 2 μ l cDNA template and 0.2 μ M primers using Ready-To-Go® PCR beads (Pharmacia Biotech, Baie d'Urfe, Quebec). The cycling program was an initial 5 min for melting (95°C) followed by 25 cycles of melting (95°C, 1 min), annealing (60°C, 1 min) and extension (72°C, 2 min), and a final extension for 10 min at 72°C. Control PCR reactions were carried out using reaction mixtures containing no cDNA. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining. The amplified cDNA was denatured in the gel using 0.5 M NaOH/0.15 M NaCl for 30 min, then neutralized in 1.5 M Tris-HCl (pH 7.5)/0.15 M NaCl for 30 min. The amplified cDNA was transferred overnight onto Zeta-Probe GT Membranes (Bio-Rad, Hercules, CA) which were air dried for 30 min and crosslinked using a UV Stratalinker 1800 (Stratagene, Richmond, BC, Canada). Prehybridization at 55°C for 4 h in 6x SSC, 10 mM EDTA (pH 7.5), 2x Denhardt's solution, 100 mg/ml sheared and denatured calf thymus DNA and 1% SDS was followed by overnight hybridization with primers for GAPDH and β -actin that were end-labeled with γ -³²P-ATP and T₄ DNA kinase (Sambrook et al. 1989). Membranes were washed repeatedly in 2x SSC and 0.05% SDS for 30 min at room temperature followed by 20 min at 55°C. Bands were detected by exposing the membranes to X-ray film at room temperature.

mRNA analysis by Northern blotting. Total RNA (20 μ g) was subjected to Northern blot analysis using a standard protocol (Sambrook et al. 1989). The probe for GAPDH was a 1.1 kb human GAPDH cDNA fragment and the probe for β -actin was a 1.8 kb human β -actin cDNA fragment (Clontech Laboratories, Palo Alto, CA). The hybridization solution contained 100 ng of the corresponding probes labeled with α -³²P-dCTP using a Random Primed DNA labelling kit

(Boehringer Mannheim, Laval, Quebec, Canada). Following hybridization with the GAPDH probe and autoradiography, the membranes were stripped and rehybridized with the β -actin probe to ensure equal loading of RNA.

Protein analysis by Western blotting. Tissues were homogenized in ice-cold PBS (pH 7.2) with 0.1 mM leupeptin using a Polytron homogenizer at high speed for 10s. Homogenates were centrifuged at 500 xg for 10 min, and supernatants were collected and measured for protein concentration using the Bio-Rad protein assay with bovine serum albumin as a standard. Equal amounts of protein from mammary glands (2.0 μ g), liver (0.75 μ g) or muscle (0.1 μ g) were applied to 12% SDS-polyacrylamide gels, separated by electrophoresis and electrotransferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat dry milk overnight at 4°C and probed with a monoclonal mouse anti-GAPDH antibody (1:500) (Advanced Immunochemical, Long Beach, CA) in 1% non-fat dry milk for 2 h. After incubation with a peroxidase-conjugated anti-mouse antibody (Sigma Chemicals, Oakville, ON), signals were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and bands were compared with the migration of pre-stained molecular weight markers (Bio-Rad, Hercules, CA).

GAPDH enzyme activity. Protein samples prepared for Western blotting were also used to measure GAPDH enzyme activity using a colorimetric assay. The assay buffer contained 0.1 M Tris-HCl (pH 8.0), 0.5 mM EGTA, 10 mM MgCl₂, 0.2 mM NADH, 8 mM ATP and 5 U/ml phosphoglycerate kinase (Schraufstätter et al. 1988). Different amounts of protein were assayed for the different tissues (20.0 μ g mammary glands, 6.0 μ g liver or 0.5 μ g muscle) to ensure that activity was within the linear range. After a preincubation for 2 min, the reaction was initiated by the addition of D (-) 3-phosphoglyceric acid to a final concentration of 10 mM and continued for 10 min at 37°C. NADH oxidation was measured at 340 nm.

Statistical analysis. All values are shown as means \pm SEM. Data were analyzed by Student's *t*-test. Differences of $P < 0.05$ were considered significant.

RESULTS

This study was designed to determine the effects of (n-3) and (n-6) PUFAs on GAPDH gene expression, protein levels and enzyme activity in rat mammary glands, liver and muscle. In these experiments, the effects of (n-3) and (n-6) PUFAs were determined relative to each other.

GAPDH gene expression. Figure 1 shows representative blots of GAPDH and β -actin mRNA reverse transcribed then co-amplified by PCR. In mammary glands, the expression of GAPDH was clearly higher in the (n-3) than in the (n-6) group. No differences, however, were observed in either the liver or muscle. Furthermore, no differences were detected in the expression levels of β -actin in any of the tissues.

We next determined whether the differences in mammary gland GAPDH mRNA that we observed by PCR could be confirmed without amplification. Consistent with the changes detected by RT-PCR, the expression of GAPDH by Northern blot analysis was also higher in the (n-3) than the (n-6) group (Figure 2). The differences observed by RT-PCR, however, were more striking than those shown by Northern blotting, suggesting that the RT-PCR method augments the differences in mRNA levels.

GAPDH protein levels and enzyme activities. To determine whether the effects of dietary (n-3) and (n-6) PUFAs on GAPDH mRNA lead to similar effects on protein levels, we measured GAPDH by Western blotting. In the mammary gland, GAPDH protein was higher in rats fed (n-3) PUFAs compared to those fed (n-6) PUFAs (Figure 3). In contrast, the level of GAPDH in the liver was lower in the (n-3) group than the (n-6) group. There were no differences in muscle GAPDH between the two dietary groups. We next measured GAPDH enzyme activity in the different tissues. Table 1 shows that changes in GAPDH enzyme activity are consistent with the changes we observed in the immunodetectable levels of the protein. Compared to the (n-6) group, rats fed (n-3) PUFAs had significantly higher GAPDH activity in the mammary gland, lower activity in the liver, and the same activity in the muscle.

DISCUSSION

GAPDH is considered to be a 'housekeeping' gene that is commonly used as a loading control in studies that examine gene expression. While we were investigating the effects of dietary (n-3) and (n-6) PUFAs on a low abundance gene in the mammary gland by RT-PCR, we observed a significant up-regulation in GAPDH that had been used as a control in rats fed (n-3) compared to (n-6) PUFAs. There were, however, no changes in β -actin, another commonly used housekeeping gene. In the present study we document these effects and extend our observations to include measurements of GAPDH mRNA in liver and muscle as well as GAPDH protein and enzyme activity.

The differences in mammary gland GAPDH expression between rats fed (n-3) or (n-6) PUFAs from our preliminary experiments were confirmed in the present study by both RT-PCR and Northern blotting. We next showed that the levels of immunodetectable protein and enzyme activities of GAPDH were also higher in the (n-3) PUFA group and were consistent with the changes in gene expression. Mammary glands consist of different cell types, most of which are adipocytes with a small fraction of epithelial and stromal cells (Low et al. 1988). Thus, the changes in GAPDH that we observed probably occur in the adipose tissue and may be related to the effects of PUFAs on energy metabolism.

In a previous study from our laboratory (Badawi et al. 1998), we fed rats the same two experimental diets used in the present study, but for 3 weeks instead of one week. In that study, however, we observed no differences in the expression of mammary gland GAPDH when it was used as a control. A direct comparison between samples verified that (n-3) PUFAs increase GAPDH expression, compared to (n-6) PUFAs, after one week of feeding but not after 3 weeks (data not shown). Although the differences may be due to the duration of feeding, it is also possible that the age of the animals plays a role, since the mammary glands of rats that are about 50 days old are undergoing rapid differentiation that is complete by approximately 65 days of age (Russo et al. 1982). In the present study, we fed the rats for one week beginning at 50 days of

age when they are highly susceptible to mammary carcinogenesis (Russo et al. 1982).

Since the liver plays a central role in regulating glucose and lipid metabolism, we wondered whether dietary PUFAs also regulate GAPDH in this tissue. We showed that the levels of hepatic GAPDH protein and enzyme activity are lower in rats fed (n-3) PUFAs than those fed (n-6) PUFAs, but the levels of GAPDH mRNA do not differ. This observation suggests that hepatic GAPDH may be regulated post-transcriptionally by these fatty acids. Previous studies have shown that (n-3) PUFAs down-regulate other hepatic glycolytic enzymes (Da Silva et al. 1993, Jump et al. 1994, Salati et al. 1988), but these effects appear to occur at the level of transcription. The decrease in liver GAPDH by (n-3) PUFAs may be related to their ability to reduce fatty acid synthesis via the production of acetyl CoA (Clarke et al. 1990, Salati et al. 1986).

GAPDH is abundant in skeletal muscle and is important for anaerobic energy production (Edwards et al. 1985). Certain physiological conditions are known to alter muscle GAPDH. For example, the levels of GAPDH mRNA, protein and enzyme activity are decreased in induced hibernating jerboa (Soukri et al. 1996). There have been no studies of dietary effects on muscle GAPDH. Our results show, however, that muscle GAPDH does not differ between rats fed (n-3) or (n-6) PUFAs.

Recent studies have shown that GAPDH is a multifunctional protein (Engel et al. 1998). In addition to its classical role in glycolysis, GAPDH is involved in binding cytoskeletal proteins such as tubulin and actin (Muronetz et al. 1994, Méjean et al. 1989), regulating gene expression by binding to AU-rich 3'-untranslated regions of mRNA (Nagy et al. 1995), and participating in DNA repair by acting as a uracil DNA glycosylase (Mansur et al. 1993). Thus, the changes in GAPDH observed by us and by others may be associated with alterations in glycolysis or with these other biological effects mediated by GAPDH.

In summary, we have shown that dietary (n-3) and (n-6) PUFAs have divergent effects on GAPDH in the mammary gland and liver but no effect in

muscle. Compared to (n-6) PUFAs, (n-3) PUFAs increased mammary gland GAPDH and decreased hepatic GAPDH. These results indicate that GAPDH is regulated by dietary (n-3) and (n-6) PUFAs in a tissue-specific manner, and changes in the levels of this protein may mediate some of the biological effects of fatty acids. Furthermore, the changes in mammary gland GAPDH mRNA that we observed indicate that use of this gene as a loading control may not always be appropriate for measuring the expression levels of other genes.

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TEXT FOOTNOTES**Footnotes to the title:**

¹ Presented in part at the Third Congress of the International Society for the Study of Fatty Acids and Lipids, June 1-5, 1998, Lyon, France [Lu, S., El-Sohemy, A. and Archer, M.C. (1998) Differential expression of glyceraldehyde-3-phosphate dehydrogenase by n-3 and n-6 polyunsaturated fatty acids in rat mammary glands. Proc. 3rd Cong. Int. Soc. Study Fatty Acids Lipids, 3:221 (Abs.)].

² MCA is the recipient of a Natural Sciences and Engineering Research Council of Canada Industrial Research Chair and acknowledges support from the member companies of the Program in Food Safety (University of Toronto). AE-S is the recipient of a Natural Sciences and Engineering Research Council of Canada Postgraduate Scholarship.

Footnotes to author:

³ To whom correspondence should be addressed at: Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, 150 College St., Toronto, Canada, M5S 3E2. Tel: 416-978-8195; Fax: 416-978-5882; E-mail: m.archer@utoronto.ca.

Table 1*The effects of dietary (n-3) and (n-6) PUFAs on GAPDH enzyme activity*

Group	GAPDH Activity ^a (IU/mg protein)		
	Mammary gland	Liver	Muscle
(n-3)	0.79 ± 0.10	1.40 ± 0.07	14.08 ± 2.42
(n-6)	0.52 ± 0.03 ^b	1.70 ± 0.11 ^b	13.82 ± 1.77

^a Values are means ± SEM for six animals per group.

^b P<0.05 between dietary groups for each tissue by Student's *t*-test.

FIGURE LEGENDS

FIGURE 1 Representative RT-PCR for GAPDH expression in mammary gland (A), liver (B) and muscle (C) of rats fed diets rich in either (n-6) (lanes 1, 3, 5 and 7) or (n-3) (lanes 2, 4, 6 and 8) PUFAs. Total RNA was reverse transcribed into single stranded cDNA and co-amplified by PCR using primers for GAPDH and β -actin. Each lane is a sample from an individual animal.

FIGURE 2 Northern blot analysis of total RNA for GAPDH and β -actin in mammary gland (A), liver (B) and muscle (C) of rats fed (n-6) (lanes 1, 3, 5 and 7) or (n-3) (lanes 2, 4, 6 and 8) PUFAs. Each lane is a sample from an individual animal.

FIGURE 3 Representative Western blot analysis of GAPDH protein in mammary gland (A), liver (B) and muscle (C) of rats fed (n-6) (lanes 1, 3, 5 and 7) or (n-3) (lanes 2, 4, 6 and 8) PUFAs using a mouse monoclonal anti-GAPDH antibody. Each lane is a sample from an individual animal.

Figure 1

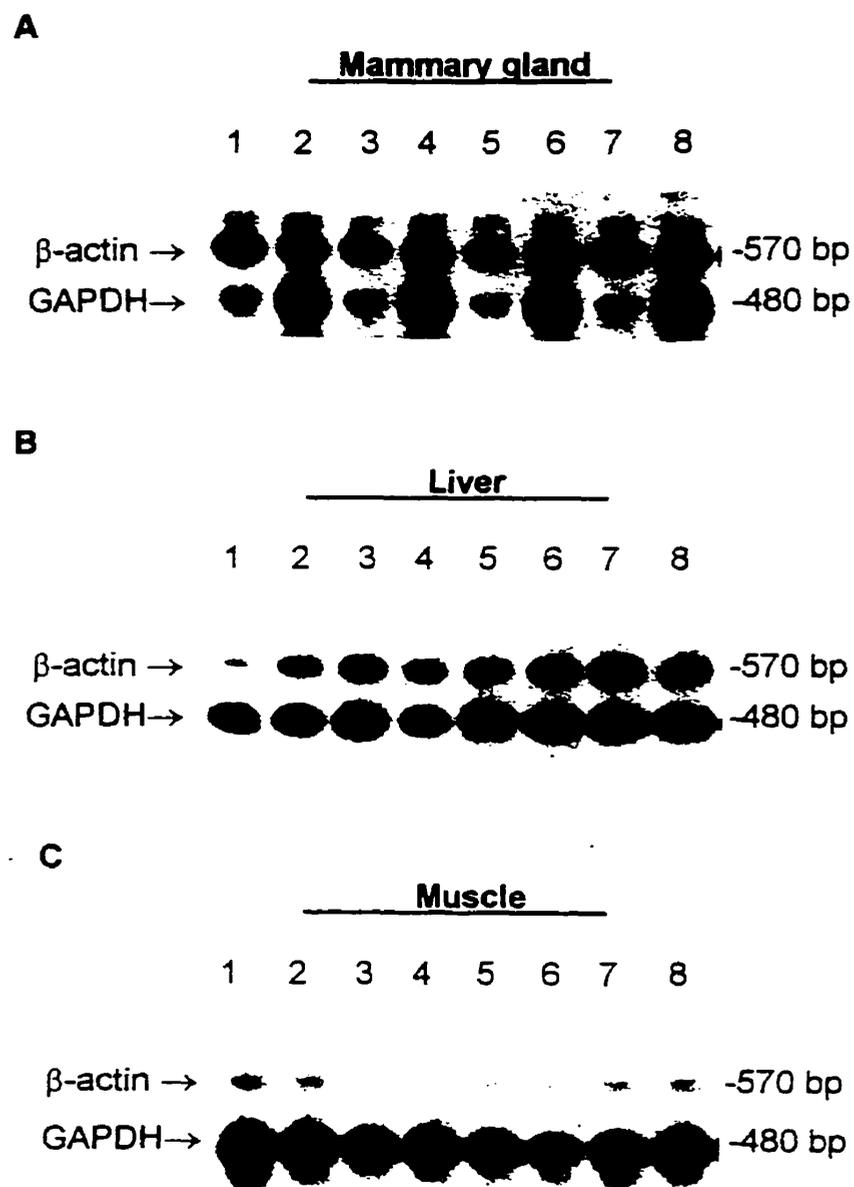


Figure 2

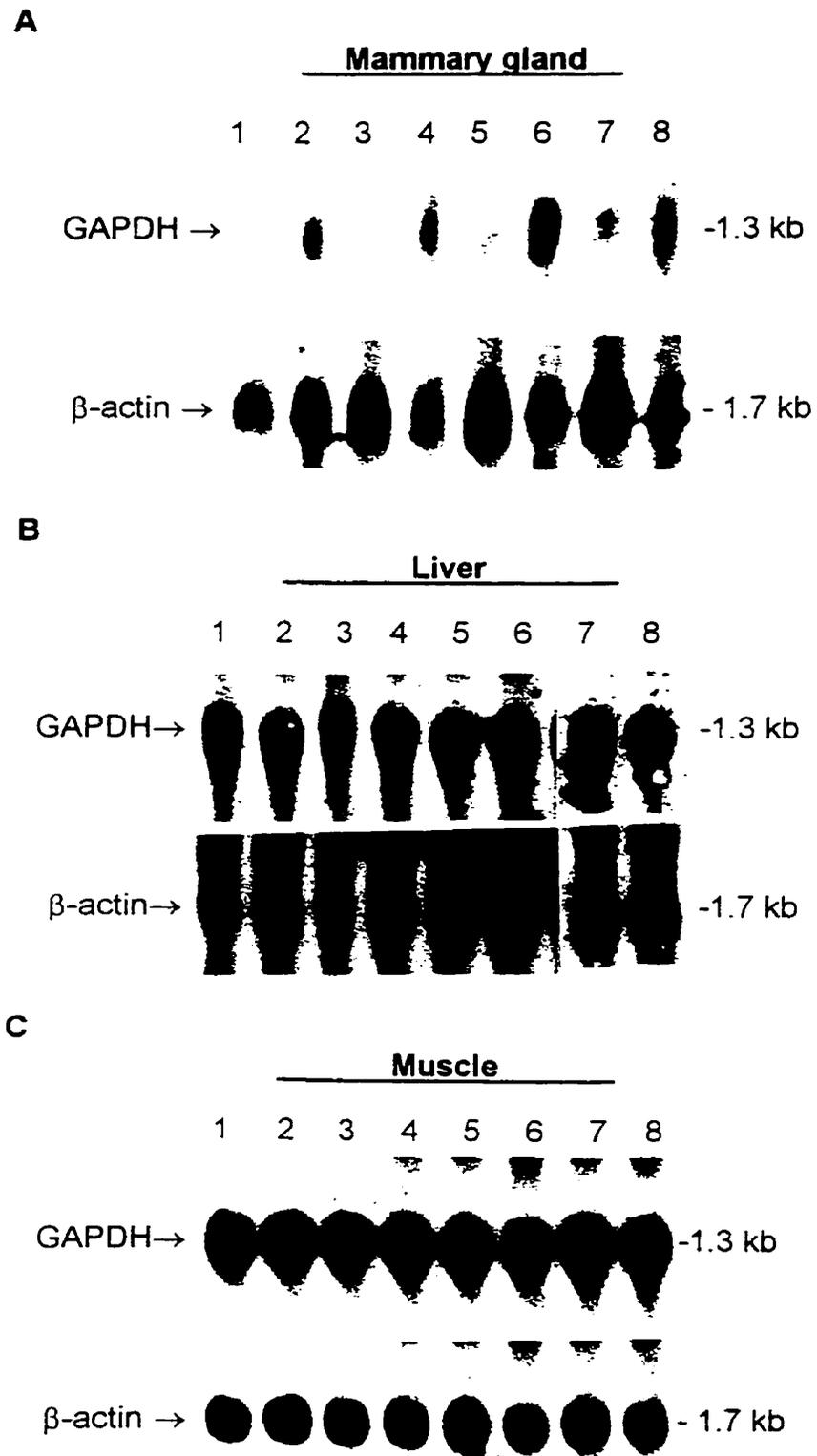
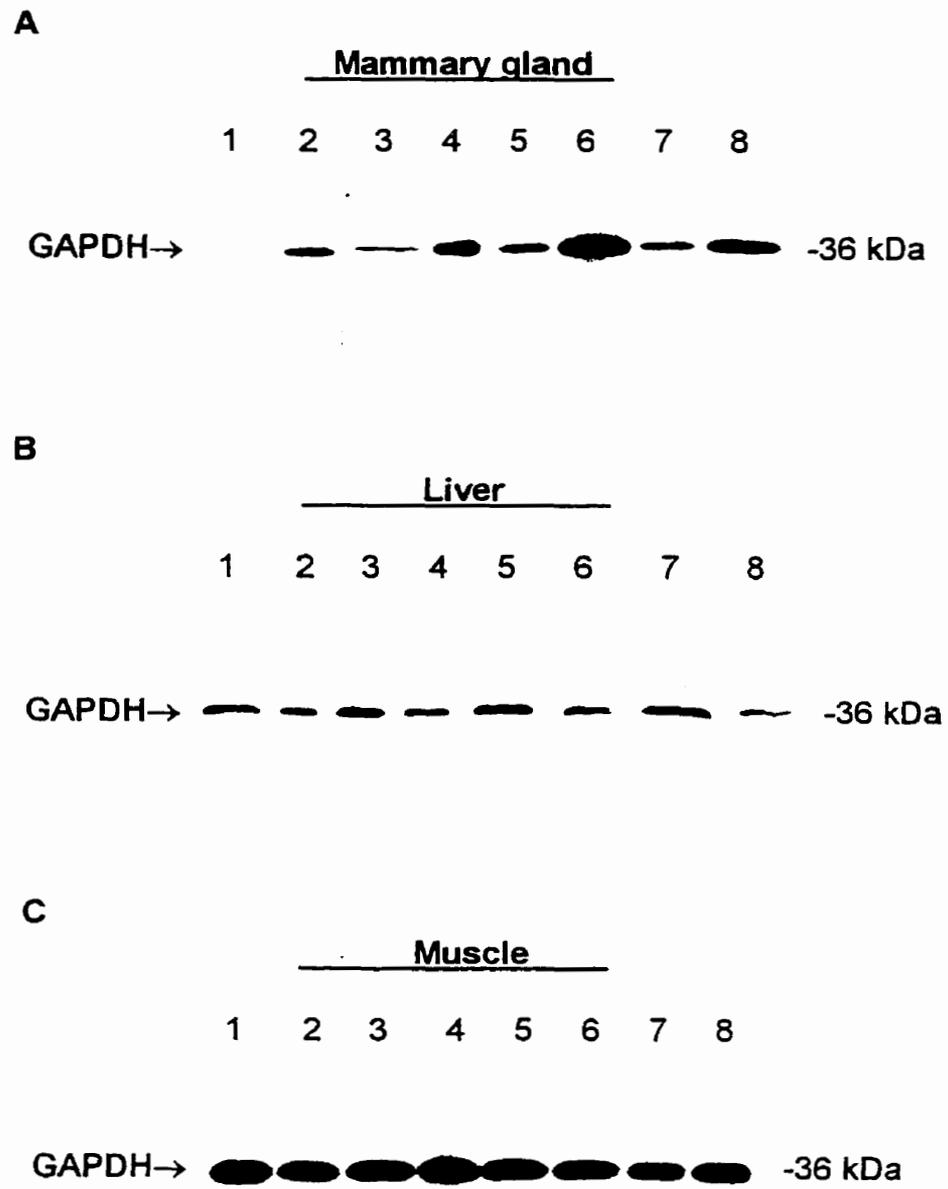


Figure 3



APPENDIX C



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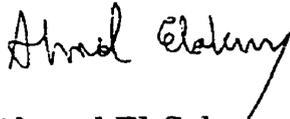
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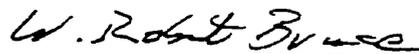


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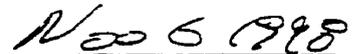
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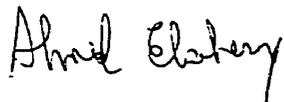
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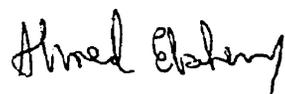
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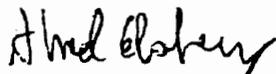
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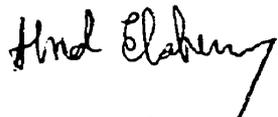
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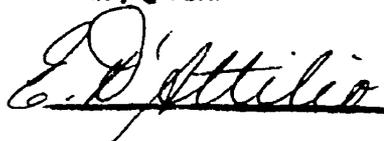
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Department of Nutritional Sciences
University of Toronto
150 College Street
Toronto, ON
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November 5, 1998

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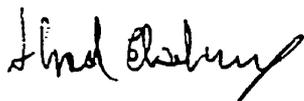
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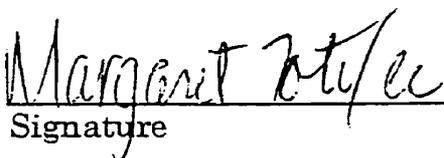
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