**Glutathione protects PC12 cells from
ascorbate- and dopamine-induced apoptosis**

by

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in conformity with the requirements for the degree of
Master of Science

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Abstract

Parkinson's disease is characterized by degeneration of central dopaminergic neurons which undergo apoptosis. The cause of neural cell loss is unclear but there is increasing evidence to show that oxidizing agents and depletion of reduced glutathione may play significant roles. Dopamine is an established oxidative agent on dopaminergic neurons. Therefore, we have further examined the effect of dopamine in PC12 cells, an experimental model for the study of dopamine producing substantial nigra neurons. We observed that dopamine induced cell death. Ascorbate (vitamin C) is a potent reducing agent which protects dopamine from oxidation and thus, ascorbate is expected to protect against dopamine-induced apoptosis. However, we demonstrated that physiological concentrations (1 mM) of ascorbate killed PC12 cells and subthreshold concentrations (0.1 mM) of ascorbate enhanced dopamine-induced toxicity instead of protecting it. We also showed that both ascorbate- and dopamine-induced apoptosis was protected by glutathione, an reducing agent. Besides, when PC12 cells were treated with the glutathione synthesis inhibitor, DL-buthionine-(S,R)-sulfoximine (BSO), BSO itself induced cell toxicity and potentiated the toxic effects of dopamine. These observations suggested that endogenous glutathione participated in protecting against basal oxidative stress. In summary, we observed that both ascorbate and dopamine induced apoptosis in PC12 cells and glutathione protected them from apoptosis. This study concluded that the toxic effects of ascorbate were due to an oxidative stress.

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Chapter 1. General Introduction

(I) Overview

Oxidative stress may be an important primary or secondary cause of degeneration of dopaminergic neurons in the substantia nigra pars compacta in Parkinson's disease (PD) (Olanow, 1990; Jenner et al., 1992; Jenner and Olanow, 1996). The concept of oxidative stress as being important in the pathogenesis of PD has been based on several lines of evidence from both human and experimental studies. Dopamine (3,4-dihydroxyphenylethylamine, DA), an endogenous neurotransmitter, or DA derivatives produced in the brain upon oxidation or enzymatic catabolism have a damaging effect on dopaminergic neurons (Ambani et al., 1975; Graham et al., 1978; Slivka and Cohen, 1985; Zigmond et al., 1992; Gabbay et al., 1996).

Neurons are normally equipped with defense mechanisms against oxidative stress which include catalytic enzyme systems (e.g. superoxide dismutase, glutathione peroxidase, catalase) and radical scavengers (e.g. glutathione, ascorbate and vitamin E) (Wefers and Sies, 1988; Sian et al., 1994; Jenner and Olanow, 1996; Augustin et al., 1997). Nigral cell loss based on oxidative stress implies that antioxidant defense mechanisms in the PD brain may be impaired or inadequate.

Reduced glutathione (GSH) functions as a major antioxidant in the cellular defense against oxidative stress (Meister, 1983; Cohen and Spina, 1989). Several studies using cultured cells demonstrate that GSH is involved in protecting dopaminergic neurons from the neurotoxic effects of DA or 6-hydroxydopamine (6-OHDA) (Spina et al., 1992;

Walkinshaw and Waters, 1994; Offen, 1996; Gabbay et al., 1996). There is a general agreement that the levels of total and reduced GSH in the substantia nigra of PD are decreased (Perry et al., 1982; Sofic et al., 1992). However, there is little direct evidence about the role of the anti-oxidant ascorbate (AA) in PD. Riederer et al. (1989) report that the concentration of AA in PD is normal.

The work presented in this thesis was originally designed to explore the protective effect of GSH and AA on DA-induced apoptosis in PC12 cells. However, we found that physiological concentration of AA (1 mM) killed PC12 cells instead of protecting them and subthreshold concentration of AA (0.1 mM) enhanced DA-induced toxicity. Nevertheless, the AA- and DA-induced cell death was protected by exogenous GSH. The toxic effect of DA was enhanced by DL-buthionine-(S,R)-sulfoximine (BSO), the GSH synthesis inhibitor (Owen et al., 1996; Pileblad et al., 1989; Gabbay et al., 1996), suggesting that endogenous GSH participates in protecting against basal oxidative stress.

Our observations indicated that toxic effects of AA were potentially due to an oxidative mechanism, similar to that induced by DA. This mechanism of AA-induced oxidative stress is a new and original concept based on research in our lab.

(II) Dopamine (DA)

DA is an important neurotransmitter in the central nervous system and highly concentrated in the basal ganglia (Adolfsson et al., 1979). DA from the nigrostriatal pathway normally acts to inhibit acetylcholine-producing neurons or in some other ways to counter the activity of the excitatory transmitters (Rhoades and Pflanzner, 1996). In the

absence of DA secretion, the excitatory signals to the corticospinal motor control system become overly active which presumably is the basis of the symptoms of PD (Rhoades and Pflanzner, 1996), such as slowness of movement, rigidity of musculature and tremor at rest.

DA is also an apoptotic agent on dopaminergic neurons (Graham et al., 1978; Gabbay et al., 1996; Simantov et al., 1996; Jenner and Olanow, 1996). It can readily be oxidized to toxic products, such as 6-OHDA, 5-hydroxydopamine (5-OHDA), 2-hydroxydopamine (2-OHDA) (Slivka and Cohen, 1985; Zigmond et al., 1992; Graham et al., 1978). Fig. 1-1 illustrates the three possible ring-monohydroxylated products of DA (Slivka and Cohen, 1985). DA or DA derivatives produced tissue damage upon oxidation is similar to that seen in Parkinson's disease (Hastings et al., 1996, Slivka and Cohen 1985).

DA is the prolactin-release inhibiting hormone (PIH) as well (MacLeod 1976). However, we have established that a cofactor is required for DA to be the PIH, and this cofactor is AA (Shin et al., 1990). Therefore, we conclude that the PIH is DA plus AA (Shin et al., 1990; Shin et al., 1997). AA is also a cofactor for DA-hydroxylase (Kaufman, 1966) and thus enhances noradrenaline synthesis in PC 12 cells (Green and Rein, 1978).

(III) Ascorbate (AA)

An important chemical property of AA is its ability to act as a reducing agent (electron donor) (Bielski et al., 1975; Shin et al., 1990). This property allows AA to protect against superoxide radical anions, free hydroxyl radicals (Bielski et al., 1975; Nishikimi et al.,

Fig. 1-1. The products of DA (3,4-dihydroxyphenylethylamine) oxidation, such as 2-OHDA, 5-OHDA and 6-OHDA.

1975) and oxidation of catechols (Sachs and Jonsson, 1975; Tse et al., 1976; Shin et al., 1990; Lai and Yu, 1997).

A high concentration of AA is found in the adrenal glands, pituitary, liver and brain (Hornig 1975). The concentration of AA is 1-2 mM in brain tissues, 0.25 mM in extracellular fluid of brain and 0.023-0.085 mM in the peripheral blood (Schenk et al., 1982; Scully, 1986). The high concentration of AA in brain tissue suggests that AA plays an important physiological role.

AA also acts as a pro-oxidant in certain conditions, such as inducing peroxidation of lipids (Pileblad et al., 1988). AA can not protect ferrocene-mediated lipid peroxidation (Velez-Pardo et al., 1997). AA causes apoptosis in leukemic cell lines (Kuribayashi et al., 1994; Sakagami et al., 1996) and it undergoes autoxidation in presence of iron salt (Mohanakumar et al. 1994; Ramassamy et al., 1994). The oxidative action of AA is the greatest in concentrations of 0.06-0.6 mM (Heikkila et al., 1982; Heikkila and Cabbat, 1983).

We observed that AA caused apoptosis in PC12 cells and GSH inhibited the AA-induced apoptosis, suggesting that the AA-induced apoptosis in PC12 cells was caused by oxidative stress. When we tested the effect of AA on another cell line (GH₄ZR₇, a prolactin secreting rat pituitary cell line), AA (1 mM) did not show the apoptotic effect on GH₄ZR₇ cells (unpublished observation), indicating that AA was not a general apoptotic agent but selectively acts on particular types of cells such as PC 12 cells.

(IV) Glutathione (GSH)

Glutathione, a tripeptide, is a highly distinctive amino acid derivative. Glutathione cycles between a reduced thiol form (GSH) and an oxidized form (GSSG) in which two tripeptides are linked by a disulfide bond (Stryer, 1995). GSSG is reduced to GSH by glutathione reductase (Fig. 1-2). The ratio of GSH to GSSG in most cells is greater than 500 (Stryer, 1995).

GSH is present at high levels (about 5 mM) in animal cells and plays several important roles (Stryer, 1995). For example, reduced GSH with a free sulfhydryl group serves as a sulfhydryl buffer which is essential for maintaining the normal structure of red blood cells and protects them from oxidative damage (Stryer, 1995). GSH also plays a role in detoxification by reacting with hydrogen peroxide and organic peroxides as a reducing agent (Stryer, 1995). The oxidation of reduced GSH to GSSG by glutathione peroxidase is the normal process for the decomposition of hydrogen peroxide in brain (Sian, 1994).

Two steps are required in GSH synthesis (Stryer, 1995, Fig. 1-3). The first step is the formation of a peptide linkage between the γ -carboxyl group of glutamate (Glu) and the amino group of cysteine (Cys) in a reaction catalyzed by γ -glutamylcysteine synthetase. Formation of this peptide bond requires activation of the γ -carboxyl group, which is achieved by ATP. In the second step, which is catalyzed by glutathione synthetase, ATP activates the carboxyl group of cysteine to enable it to condense with the amino group of glycine (Gly).

Fig. 1-2. GSSG is reduced to GSH catalyzed by glutathione reductase (Glu: glutamate; Cys: cysteine; Gly: glycine).

Fig. 1-3. Synthesis of glutathione. BSO is an inhibitor of a γ -glutamyl cysteine synthetase.

GSH in the substantia nigra may play a pivotal role in the pathogenesis of PD (Jenner and Olanow, 1996). DA alters GSH homeostasis and decreases intracellular GSH levels in neuronal cells (Gabbay et al., 1996). The decrease in GSH levels in the brain appear to be an early marker of Parkinson's disease (Jenner et al., 1992). GSH depletion initiates a cascade of events leading to oxidative stress, destruction of the nigrostriatal pathway, and also renders the nigrostriatal pathway susceptible to a toxic insult (Jenner and Olanow, 1996).

BSO is an inhibitor of a γ -glutamyl cysteine synthetase (Pileblad et al. 1989; Seaton et al., 1996; Owen et al., 1996; Gabbay et al., 1996) (Fig. 1-3). The administration of BSO leads to an irreversible inhibition of the rate limiting enzyme for GSH synthesis (Meister, 1991) inducing a reduction in GSH levels and thus, degeneration of cultured dopaminergic neurons (Jenner and Olanow, 1996). BSO administration to rats or mice causes a 40 - 60 % decline in basal ganglia GSH levels and mimics the degree of GSH loss that occurs in PD. Also, the ability of 6-OHDA to deplete brain GSH level or to destroy the nigral cells is enhanced following the administration of BSO (Pileblad et al., 1989). Similarly, we demonstrated that exogenous GSH protected DA- and AA-induced apoptosis and depletion of intracellular GSH levels with BSO enhanced DA neurotoxicity which were consistent with the previous work (Owen et al., 1996; Gabbay et al., 1996).

(V) Apoptosis

Usually, cells die in two different ways: necrosis or apoptosis. Apoptosis or programmed-cell-death (PCD) is a mode of genetically-controlled active cellular death

(Ziv, 1997; Walkinshaw and Waters, 1994). It is a major physiological mode of selective cell death employed during embryogenesis, normal development, evolution of the nervous system, pathological atrophy, regression of hyperplasia, mild injury, and malignant tumors (Ellis et al., 1991; Schwartzman and Cidlowski, 1993; Wyllie et al., 1980). The apoptotic cell death may act to create order out of the diversity of cell types during embryonic life (Michaelson, 1991). In contrast, necrosis is a passive form of cell death observed in response to many acute noxious stimuli, such as burns, radiation or toxic chemicals (Walkinshaw and Waters, 1994). The necrotic cells release toxic chemicals into the body causing inflammation and production of immune responses (Michaelson, 1991).

Apoptotic cell death is characterized by classical morphological and biochemical hallmarks (Walkinshaw and Waters, 1994). The morphological changes include cell shrinkage, plasma membrane blebbing, cytoplasmic vacuolization and chromatin condensation. This is contrasted to necrosis which is associated with an increase in cell volume, swelling of intracellular organelles, membrane blebbing, and the eventual cell lysis. The biochemical hallmark of apoptosis is the oligonucleosomal laddering of DNA, a phenomenon not usually seen in necrotic cell death. The DNA "ladder" of oligonucleosomal fragments can be visualized when separated by conventional gel electrophoresis (Walkinshaw and Waters, 1994; Wyllie, 1980). These internal changes of apoptosis occur before the plasma membrane becomes permeable, and thus the apoptotic cell would be phagocytosed before its contents are spilled. Thus, apoptosis results in a less traumatic type of cell death (Michaelson, 1991).

Increasing evidence suggests that dopaminergic neurons are undergoing apoptosis in the substantia nigra of PD (Ziv et al., 1997; Mochizuki et al., 1997). Since apoptosis does not cause either inflammation, an immune response or harmful effects on the body, no sign or symptom becomes apparent in PD until a major portion (60-70%) of the pars compacta neurons are lost (Youdim and Riederer, 1997).

(VI) PC12 cells

PC12 cells originate from pheochromocytoma cells of rat adrenal medulla (Greene and Tischler, 1976) and share many common characteristics with substantia nigra cells. They produce DA (Greene and Rein, 1978), GSH (Sampath 1994), several growth factors, such as nerve growth factor, fibroblast growth factor and transforming growth factor, and express DA receptors (Sampath et al., 1994; Ohmoto, 1996). Further, PC12 cells are very sensitive to an oxidizing environment and undergo a characteristic apoptotic pathway when they are exposed to DA or 6-OHDA (Walkinshaw and Waters, 1994). Therefore, PC12 cells have been widely used as a model system for the study of DA producing substantia nigra neurons (Snyder et al., 1986; Offen et al., 1996).

(VII) The methods used in this study

A simple and reliable method to accurately estimate cell numbers is needed for studying cell death. Acid phosphatase assay (Connolly et al., 1986; Ueda et al., 1994) and tetrazolium (MTS or MTT) assay (Mosmann, 1983; Cory et al, 1991; Riss and Moravec, 1992) are now the most frequently used methods. The former measures lysosomal acid

phosphatase activity while the latter measures the mitochondrial dehydrogenase activity.

We used tetrazolium assay at the beginning of the project but later we found that this assay was interfered by DA (Si et al., under review). Thus, acid phosphatase assay was chosen in the subsequent study to evaluate cell numbers.

Chapter 2

**Estimation of number of PC12 cells with
acid phosphatase assay and mitochondrial dehydrogenase assay:
dopamine interferes with assay based on tetrazolium**

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Abstract

Both acid phosphatase assay and mitochondrial dehydrogenase assay have been used to quantify number of cells. The commonly used acid phosphatase assay uses *p*-nitrophenyl phosphate as a substrate while the mitochondrial dehydrogenase assay is based on the conversion of tetrazolium to formazan. Our experimental results showed that the former assay was more sensitive in detecting small numbers of PC12 cells (200-10,000 cells/well) whereas the latter was useful for larger numbers of cells (2,000-40,000 cells/well). The number of PC12 cells were decreased after dopamine treatment according to the acid phosphatase assay and by direct cell counting under a light microscope. However, the optical densities measured by mitochondrial dehydrogenase assay were increased after dopamine treatment. We tried to clarify discrepancies between the two assays since dopamine is an important neurotransmitter and both assays are commonly used to estimate numbers of cells. To elucidate the interference between dopamine and tetrazolium salt, cell free control experiments were performed. It was found that dopamine reacted with tetrazolium and thus produced false positive reaction in the assay. We therefore conclude that the tetrazolium assay is not a suitable method for evaluating the number of dopamine-treated cells while acid phosphatase assay is reliable and sensitive.

Introduction

PC12 cells originate from rat pheochromocytoma cells (Greene and Tischler, 1976). They share many common characteristics with dopaminergic neurons and thus have been widely used as an experimental model for the study of dopamine (DA) producing substantia nigra neurons (Snyder et al., 1986).

DA is oxidized by oxygen and the oxidative products covalently bind to vital proteins (Ross et al., 1993; Ross et al., 1995) which may be one mechanism of DA-induced apoptosis. Lack of DA due to the destruction of DA neurons causes Parkinson's disease (Jenner et al., 1992). We have been investigating DA-induced apoptosis in PC12 cells in order to further characterize the mechanisms of etiology of Parkinson's disease.

A simple and reliable method to accurately estimate cell numbers is needed for studying cell death. Colorimetric tetrazolium assay (MTT) is one of the methods which has been used for several decades (Slater et al., 1963; Mosmann, 1983). The tetrazolium salts are converted into formazan by mitochondrial dehydrogenase in the cells. The formazan product of MTT is a crystalline precipitate and an additional step in the procedure is required to dissolve the crystals before reading the absorbance of formazan. A new tetrazolium compound, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], has been introduced whose formazan product is soluble in tissue culture medium and thus simplifies the assay (Cory et al, 1991; Riss and Moravec, 1992).

In our experiment, we found that the optical densities (OD) in MTS assay were proportional to the cell numbers. However, the OD was increased after DA treatment even though DA is known to kill cells (Velez-Pardo et al., 1997). We hypothesized that the *ortho*-catechol group of DA may interfere with the tetrazolium assay system. Therefore, we compared the MTS assay to an acid phosphatase assay (using *p*-nitrophenyl phosphate as a substrate) which showed close correlation with numbers of cells (Connolly et al., 1986; Ueda et al., 1994). We conclude that the mitochondrial dehydrogenase assay is not a suitable method for evaluating the number of DA-treated cells whereas acid phosphatase assay is reliable and sensitive.

Materials and Methods

PC 12 cell culture and treatments

PC 12 cells were obtained from American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagles' medium (DMEM, Gibco, Grand Island, NY, USA) containing 12.5% horse serum, 3% fetal calf serum and penicillin (50 IU/mL) in tissue culture flasks. The flasks were placed in a water-jacketed incubator at 37 °C under an atmosphere of 5 % CO₂ and 95 % air. Cells were maintained in culture medium until they were confluent after which they were incubated in calcium-magnesium free Gey's balanced salt solution (pH 7.4, NaCl 8.6 g/L, KCl 0.37 g/L, Na₂HPO₄·2H₂O 0.15 g/L, KH₂PO₄ 0.03 g/L, NaHCO₃ 0.227 g/L, Glucose 1 g/L) at 37 °C for 30 min to harvest them. The suspended cells were pelleted by centrifugation (500 x g for 3 min) and the pellet was resuspended in culture medium. The number of cells in suspension was quantified with a hemocytometer and the cell suspension was diluted to make a density of $3 - 4 \times 10^4$ cells/mL. A 100 µL aliquot of cell suspension was distributed to each well of Microtest tissue culture plates (96-well, flat) using a multichannel pipette. The doubling time of PC12 cells in our experimental conditions was about 36 h. After 36 h, the conditioned medium were replaced with fresh culture medium containing DA (0 - 1 mM, Sigma Chemical Co., ST. Louis, MO, USA) for 3 or 24 h. At the end of treatment with DA, the medium was removed gently and the cells were washed once with 200 µL of phosphate buffered saline (PBS; NaCl 0.15 M, Na₂HPO₄ 0.01 M; pH 7.6). Then both acid

phosphatase assay and mitochondrial dehydrogenase assay were performed for estimating the number of cells.

Acid phosphatase assay

A 100 μ L aliquot of buffer containing 0.1 M sodium acetate (pH 5.5, J. T. Baker Chemical Co., Phillipsburg, NJ, USA), 0.1 % (vol/vol) Triton X-100 (BDH Chemicals) and 10 mM *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate, Sigma Chemical Co.) was added to each well. The plates were placed in the incubator at 37 °C for 2 h. The reaction was stopped by the addition of 10 μ L of 1 M NaOH to each well and the wells were measured at wavelength of 405 to 590 nm using a microplate reader (Titertek Multiskan, MCC/340, Finland).

Mitochondrial dehydrogenase assay

The MTS solution (Promega, Madison, WI, USA) and phenazine methosulfate solution (PMS, Promega), an electron coupling reagent, were thawed 90 min before assay, then 2 mL MTS solution was mixed with 100 μ L PMS. Subsequently 20 μ L of freshly prepared MTS/PMS solution was added into each well. The plates were incubated for 1 - 2 h and then OD at wavelength between 405 and 595 nm was read on the microplate reader.

Control experiments

Wells without cells were loaded with DA or ascorbate (AA, Sigma Chemical Co.) and which were scanned at wavelength between 340 and 595 nm after 0 - 48 h incubation at 37°C. In different experiments, the medium containing DA or AA was removed after 24 h incubation, the wells were washed once with 200 μ L PBS and then the acid phosphatase assay or MTS assay was performed. The OD was obtained before (as control) and after the assays. Different concentrations of DA (1 nM - 1 mM) were also scanned in the absence or presence of MTS compound for investigating the reaction between DA and MTS.

Statistic analysis

Data were expressed as mean $\pm$ SEM. Statistical significance was assessed with a one-way analysis of variance followed by Bonferroni's modified t-test (Instat, GraphPad, San Diego, CA). A difference of $p < 0.05$ was considered statistically significant.

Results

Relationships between numbers of cells and acid phosphatase activities or mitochondrial dehydrogenase activities

A direct linear relationship between the number of PC12 cells and acid phosphatase activity was shown in the range of 200 - 10,000 cells in each well (upper panel, Fig. 2-1). When there were more than 10,000 PC12 cells in each well, the relationship became nonlinear (upper panel, Fig. 2-1) indicating that the high OD distorted the direct relationships. The reaction products of acid phosphatase assay showed that 405 nm was the optimal wave length between 405 and 595 nm (upper panel, Fig. 2-1).

The OD of mitochondrial dehydrogenase assay was also directly proportional to the number of PC12 cells in a wide range of 1,000 - 40,000 cells per well (lower panel, Fig. 2-1). Good correlations with number of PC12 cells and the OD were shown at wavelength of 490, 540 and 595 nm, but 490 nm was the most sensitive among them (lower panel, Fig. 2-1).

Acid phosphatase assay and mitochondrial dehydrogenase assay after DA treatment

The acid phosphatase activities of PC12 cells were decreased in proportion to the decreased number of cells after they were treated with 1 mM DA for 24 h (upper panel, Fig. 2-2). DA (1 mM) decreased the numbers of cells by 30 ± 2 % and 95 ± 4 %, respectively, after a 3 h and 24 h treatment period (lower panel, Fig. 2-2). These observations indicated that the numbers of cells were decreased in a time- and

Fig. 2-1. The relationship between numbers of PC12 cells and the acid phosphatase activities (upper panel) or mitochondrial dehydrogenase activities (lower panel). Different numbers of PC12 cells were loaded into 96-well plates and incubated for 36 h and then acid phosphatase assay (AP) and MTS assay were performed. Optical densities were measured at different wavelengths. Each point shows the mean $\pm$ SEM of 4 replicates.

Fig. 2-2. The effect of DA on PC12 cells measured by acid phosphatase assay. Different numbers of cells were treated with 1 mM DA for 24 h (upper panel). PC12 cells (5,000 cells/well) were treated with 0 - 1 mM DA for 3 h and 24 h (lower panel). Each point shows the mean $\pm$ SEM of 4 replicates. Difference between DA treatment and control was shown as * $P < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

dose-dependent manner after DA treatment which were consistent with our estimation under a light microscopy.

In contrast to the acid phosphatase assay, mitochondrial dehydrogenase assay showed that OD of PC12 cells after DA treatment was increased (upper panel, Fig. 2-3). During 3 h and 24 h treatments with 1 mM DA, the OD was increased by more than two- and seven-fold, respectively (lower left panel, Fig. 2-3), suggesting that the increase of OD of DA-treated cells was time- and dose-dependent (lower panel, Fig. 2-3). When MTT instead of MTS was used, the results were the same, showing that the OD was increased after DA treatment (data not shown).

However, it was unlikely that the cell numbers or the mitochondrial dehydrogenase enzyme activities were increased several times after DA treatment. The only logical explanation of the increased OD in the tetrazolium assay was an experimental artifact. We therefore ran the following control experiments to examine the possible interference of DA on mitochondrial dehydrogenase assay as well as acid phosphatase assay.

Control experiments

The wells without cells were treated with DA for 24 h and scanned before and after the assays (mitochondrial dehydrogenase assay and acid phosphatase assay). After the wells containing DA were washed once, no absorbance was observed (Fig. 2-4a, b). OD was not detectable after the reagents of acid phosphatase assay were added to the washed wells containing the residual DA, indicating that acid phosphatase assay did not interfere with DA (Fig. 2-4a). However, when the MTS/PMS mixture was added to the washed

Fig. 2-3. The effect of DA on PC12 cells measured by MTS assay. Different numbers of cells were treated with 1 mM DA for 24 h (upper panel). PC12 cells (5,000 cells/well) were treated with 0 - 1 mM DA for 3 h and 24 h (lower panel). Each point shows the mean $\pm$ SEM of 4 replicates. Difference between DA treatment and control was shown as * $P < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

Fig. 2- 4. Control experiments. Wells without cells were treated with 1 mM DA for 24 h and then the OD was measured before (DA) and after the wells were washed (wash), and further the reaction between the residual dopamine and the reagents of acid phosphatase assay (a) or MTS assay (b) was obtained. Wash-AP: The washed wells were treated with AP reagents; AP: AP reagents were added in fresh wells; Wash-MTS: The washed wells were treated with MTS; MTS: MTS was added in fresh wells. (c) 24 h-old DA solutions (1 nM - 1 mM) were scanned in the absence (□) or presence (■) of MTS. Difference of OD between control and treatment group was shown as * $P < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

well, the OD was more than double the value of 1 mM DA alone (Fig. 2-4b), suggesting that the small amount of residual DA produced a chemical reaction with the MTS/PMS mixture. A dose-response relationship between OD and the reaction of DA with the MTS/PMS mixture was shown in Fig. 2-4c, showing that OD values of different concentrations of DA were increased several times in the presence of MTS/PMS mixture.

Physio-chemical properties of DA and AA

Since DA is easily oxidized and AA is a reducing agent (Graham et al., 1978; Shin et al., 1990), we tested the autoxidation of DA and also if the oxidation of DA was protected by AA.

DA (1 mM) and AA (1 mM) solutions were scanned at wavelength between 340 and 750 nm after 24 h incubation. The OD of AA was independent of the wavelengths (Fig. 2-5a) and the incubation period (Fig. 2-5b). The OD of DA was decreased when wavelengths were increased (Fig. 2-5a), but it was increased when the incubation time was prolonged indicating that DA oxidation was time-dependent (Fig. 2-5c). AA (1 mM) prevented the OD increment (1 mM DA) by 90 % within a 18 h incubation period while about 50 % inhibition was observed when the incubation time was more than 24 h (Fig. 2-5d). The effect of 0.1 mM AA on DA was smaller than that of 1 mM AA (Fig. 2-5d). The increase of OD of DA during the incubation and the suppression of the increase by AA suggested that oxidized DA was responsible to the OD increase.

Fig. 2-5. The physio-chemical properties of DA and ascorbate (AA). (a) Relationship between wavelengths and OD of 1 mM DA and 1 mM ascorbate after 24 h incubation. (b) Relationship between ascorbate concentrations (0 - 10 mM) and OD after different incubation periods (0-48 h). (c) Relationship between DA concentrations (0 - 10 mM) and OD after different incubation periods (0 - 48 h). (d) Relationship between the incubation time (0 - 48 h) and OD of 1 mM DA in the presence of absence of ascorbate (0.1 or 1 mM). Each point shows the mean $\pm$ SEM of 8 replicates.

Discussion

We have confirmed that both acid phosphatase assay and mitochondrial dehydrogenase assay were good methods to estimate the number of cells since OD was proportional to the numbers of PC 12 cells (Slater et al., 1963; Mosmann, 1983; Connolly et al., 1986; Ueda et al., 1994). But both assay systems had advantages and disadvantages. The phosphatase assay was more sensitive than the mitochondrial dehydrogenase assay in detecting small numbers of cells (200 - 10,000 cells/well) while the latter could handle a wider range of numbers of PC 12 cells (2000 - 40,000 cells/well). This observation is consistent with the work done by Ueda et al. (1993) that acid phosphatase assay is much more sensitive than MTT assay. We also demonstrated that DA killed PC12 cells using the acid phosphatase assay (Fig. 2-2), confirming the results of Velez-Pardo et al. (1997). However, mitochondrial dehydrogenase assay showed an entirely different picture (Fig. 2-3). We demonstrated that the OD of DA-treated PC12 cells was increased in MTS assay (Fig. 2-3) which could easily be interpreted as an increase in cellular mitochondria activity after treatment with DA.

Despite the widespread use of mitochondrial dehydrogenase assay (MTT or MTS) to measure the cell viability and proliferation, the mechanism of tetrazolium reduction is poorly understood (Liu et al., 1997). An earlier study by Slater et al. (1963) using respiratory chain inhibitors, shows that succinate-dependent MTT reduction by rat liver homogenates occurs at two sites along the mitochondrial electron transport chain (coenzyme Q and cytochrome c) and this finding is often taken as evidence that MTT is

reduced by mitochondrial dehydrogenase in live cells. However, the exclusive role of mitochondria in cellular MTT reduction is questioned (Berridge and Tan, 1993; Shearman et al., 1995). Many non-mitochondrial dehydrogenases or flavin oxidases reduce tetrazolium (Altman, 1976; Burdon et al., 1993). Cellular MTT reduction also occurs within the endosomal/lysosomal compartment (Liu et al., 1997). In addition, mitochondrial dehydrogenase can be altered without changes of number of cells since the mitochondrial activities can be changed during pharmacological treatment (Katyare et al. 1994).

It is important to clarify the interactions between DA and tetrazolium since tetrazolium assay is a commonly used method and DA is a major neurotransmitter in the central nervous system. By performing parallel experiments, we found that the residual DA did not absorb light at 490 nm but the small amount of residual DA reduced tetrazolium to form formazan (Fig. 2-4b, c). Therefore, the increased OD after DA treatments (Fig. 2-3) was not due to an increase of mitochondria activities but a reaction between tetrazolium and DA. Thus the linear increase of OD with the increase of cell numbers (upper panel, Fig. 2-3) was likely due to a constant mitochondria activity. In contrast, the reagents of acid phosphatase assay were not interfered by DA (Fig. 2-4a), thus acid phosphatase assay could be a choice for estimating number of DA-treated cells.

Spectrophotometry can be easily interfered since it is reliant solely on OD at a chosen wavelength. If any pharmacological agent or its reaction product has a substantial effect on the absorption of light at the same wavelength, the pharmacological agent will obviously generate false positive reading. DA was oxidized by oxygen in a time-

dependent manner (Fig. 2-5c) and these oxidized products have their own absorption spectrum (Fig. 2-5a). The absorbance of DA was low at a longer wavelength (Fig. 2-5a) suggesting a longer wavelength should be used to avoid the interference of DA in mitochondrial dehydrogenase assay, but the sensitivity of the assay would be lost if more than 540 nm of wavelength was used (lower panel, Fig. 2-1).

We have shown that DA is a prolactin-release inhibiting hormone (PIF) (Shin et al, 1990) and DA requires AA to be the PIF (Shin et al., 1997). AA has a relative short half-life (5 - 30 min) (Feng et al., 1977; Sakagami et al., 1996). The half-life of DA in culture medium is 36 min and is extended to 792 min in the presence of 0.1 mM AA (Shin et al., 1990). In this study, we also showed that oxidation of DA was suppressed by AA (Fig. 2-5d). In spite of the short half-life of AA, it still showed a long-lasting protective effect on DA up to 48 h (Fig. 2-4d), suggesting that the oxidized AA may still protect DA from oxidation.

We conclude that tetrazolium assay cannot be used in estimating number of cells after DA treatment whereas acid phosphatase assay can be employed.

Chapter 3

Glutathione protects PC12 cells from ascorbate- and dopamine- induced apoptosis

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Abstract

The role of reduced glutathione on ascorbate- and dopamine-induced apoptosis in PC12 cells was investigated. Ascorbate is a potent reducing agent and is thus expected to protect against dopamine-induced apoptosis. However, we found that both ascorbate (1 mM) and dopamine killed PC12 cells and ascorbate (0.1 mM) enhanced dopamine toxicity. When the medium contained 10 mM glutathione, both dopamine and ascorbate toxicity were partially prevented. DL-buthionine-(S,R)-sulfoximine (BSO), glutathione synthesis inhibitor, induced cell toxicity and potentiated the toxic effects of dopamine suggesting that endogenous glutathione participates in protecting against basal oxidative stress. We conclude that both ascorbate and dopamine induce apoptosis in PC12 cells and further that glutathione protects them from apoptosis. This study indicates that the toxic effects of ascorbate are potentially due to an oxidative mechanism, similar to that induced by dopamine.

Introduction

Parkinson's disease is characterized by the degeneration of central dopaminergic neurons which undergo programmed cell death (PCD, also called apoptosis). The cause of neural loss is unclear but there is increasing evidence that oxidizing agents, free radicals, loss of the antioxidant glutathione (GSH), increased iron levels and mitochondrial complex I deficiencies may play significant roles (Jenner et al., 1992; Owen et al., 1996). Under normal conditions, cells possess defense mechanisms which include catalytic enzyme systems (e.g. superoxide dismutase, glutathione peroxidase and catalase) and radical scavengers (e.g. vitamin E, ascorbate and GSH). Dopamine (DA), the endogenous neurotransmitter of nigrostriatal neurons, is an established apoptotic agent on dopaminergic neurons (Graham et al., 1978; Gabbay et al., 1996). Oxidation of DA produces tissue damage similar to that seen in Parkinson's disease (Hastings et al., 1996). PC12 cells, originated from rat pheochromocytoma cells (Greene and Tischler, 1976), secrete DA (Greene and Rein, 1978), produce GSH and express DA receptors (Sampath et al., 1994). PC12 cells share many common characteristics with substantia nigra cells and therefore have been widely used as a model system for the study of DA producing substantia nigra neurons (Snyder et al., 1986).

Ascorbate (AA) is a well-known reducing agent and is involved in several types of protective mechanisms. The brain contains much higher concentration of AA (1-2 mM) than peripheral blood (0.02-0.08 mM) (Hornig, 1975; Schenk et al., 1982) suggesting that AA plays an important physiological role in the CNS. However, we observed that the

physiological concentration of AA (1 mM) is toxic to PC12 cells and a subthreshold concentration of AA (0.1 mM) potentiates DA-induced toxic effects via apoptotic mechanisms (Si et al., under review).

GSH is a major scavenging compound of oxidative chemicals. GSH levels are decreased selectively in the substantia nigra in Parkinson's disease and not in other brain areas or in other neurodegenerative diseases (Jenner et al., 1992). The changes in GSH levels in the brain thus appear to be an early marker of Parkinson's disease (Jenner et al., 1992). Several studies using cultured cells also demonstrate that GSH is involved in protecting dopaminergic neurons from the neurotoxic effects of 6-OHDA and DA (Spina et al., 1992; Walkinshaw and Waters, 1994), and human neuronal cells from DA-induced apoptosis (Gabbay et al., 1996). We tested the effect of GSH on AA- and DA-induced apoptosis in PC12 cells and found that it protected against the toxic effects of both agents.

BSO, an irreversible inhibitor of γ -glutamylcysteine synthetase, decreases GSH levels in rat brain (Owen et al., 1996) and in cultured cells (Gabbay et al., 1996). The toxicity of 6-OHDA and DA is potentiated when endogenous GSH is depleted by BSO (Pileblad et al., 1989; Gabbay et al., 1996). In the present study we demonstrated that BSO induced toxic effects in PC12 cells and potentiated the DA-induced apoptosis.

Materials and Methods

Materials

AA, DA, GSH, BSO, *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate) and Trypan Blue were purchased from Sigma Chemical Co.. Dulbecco's modified Eagles' medium (DMEM) was purchased from Gibco. Sodium acetate was purchased from J. T. Baker Chemical Co. Phillipsbur and Triton X-100 from BDH Chemicals.

Cell Cultures and treatments

PC12 cells were obtained from American Type Culture Collection (ATCC) and grown in DMEM containing 12.5 % horse serum and 5 % fetal calf serum in tissue culture flasks. The flasks were placed in a water-jacketed incubator at 37°C under a water saturated atmosphere of 5 % CO₂ and 95 % air. Cells were maintained in culture medium until they were confluent after which they were incubated in a calcium-magnesium free Gey's balanced salt solution (pH 7.4, NaCl 8.6 g/L, KCl 0.37g/L, Na₂HPO₄·2H₂O 0.15 g/L, KH₂PO₄ 0.03 g/L, NaHCO₃ 0.227 g/L, Glucose 1 g/L) at 37°C for 30 min to harvest them. The suspended cells were pelleted by centrifugation (500 × g for 3 min) and the pellet was resuspended in culture medium. At a concentration of 3-4 × 10⁴ cells/mL, a 100 µL aliquot of cell suspension was distributed to each well of Microtest tissue culture plates (96-well, flat bottom). The doubling time of PC12 cells in our experimental condition was about 36 h. After 36 h, the monolayered cells were treated with pharmacological agents by replacing fresh culture medium containing AA (1 µM - 1 mM)

and DA (1 μ M - 1 mM) with or without 0.1 mM AA and incubated for 1 to 72 h as controls; The effect of GSH on DA and AA was tested by treating the cells the same way as controls except the medium contained 10 mM GSH. The effect of GSH (2.5 - 20 mM) alone on PC12 cells was tested by incubating cells for 24 h. The pH of all AA and GSH solutions was adjusted to 7.4 with 1 M NaOH. For investigating the effect of BSO, cells were treated with 1.5 - 6 mM of BSO with or without 1 mM of AA or DA for 24 h. In another experiment, the cells were treated with 0.01 - 1 mM DA with or without 3 mM BSO for 6 h. Cell numbers were evaluated by assaying acid phosphatase activity (Connolly et al., 1986; Ueda et al., 1994).

Acid phosphatase assay

At the end of treatment with the pharmacological agents, the medium was removed gently and the cells were washed once with 200 μ L of phosphate buffered saline (PBS; NaCl 0.15 M, Na_2HPO_4 0.01 M; pH 7.6), then 100 μ L aliquot of buffer containing 0.1 M sodium acetate (pH 5.5), 0.1 % (vol/vol) Triton X-100 and 10 mM *p*-nitrophenyl phosphate were added to each well. The plates were placed in a water-jacketed incubator at 37°C for 2 h. The reaction was stopped by the addition of 10 μ L 1 M NaOH to each well and the optical densities (OD) were measured at 405 nm using a microplate reader (Titertek Multiskan, MCC/340, Finland).

In order to establish the relationship between acid phosphatase activities and the number of living cells, a wide range of number of PC12 cells (100 - 20,000 cells) was seeded in each well for 36 h. A direct linear relationship between the number of cells and OD was shown in the range of 200-8,000 cells per well (inset, Fig. 3-1) (correlation

Fig. 3-1. The relationship of PC12 cell number and acid phosphatase activities. Different numbers of PC12 cells (100-200,000 per well) were loaded in 96-well plates and incubated for 36 h. The cells were then assayed for acid phosphatase activities. The color development was determined using a microplate reader at 405 nm. Each point shows the mean $\pm$ SEM of 12 replicates.

coefficient $r = 0.989$). When more than 8,000 cells were in one well, the relationship became non-linear (Fig. 3-1) indicating that the high OD values distorted the cell numbers. Therefore, 3,000 - 4,000 cells per well were seeded in all subsequent experiments.

Trypan Blue exclusion test was performed for confirming that the changes in acid phosphatase activity after treatments were correlated with changes in cell numbers. Cells were grown and treated exactly the same as in the previous experiments. The cells were treated with 1 mM DA or 1 mM AA. After 24 h, the old medium was removed and the wells were washed by 200 μ L PBS. Then 0.4 % Trypan Blue solution was added to the wells and which were incubated for 10 min. Then the solution was removed and the cells in the wells were counted directly under a microscope. There were 5 % live cells left in the wells after DA treatment and about 60 % live cells in the AA treatment group relative to controls. These observations were consistent with the results measured by acid phosphatase assay, confirming the changes in acid phosphatase activities indicated changes in cell numbers (see Results).

Statistic analysis

Differences between groups were evaluated by an one-way analysis of variance followed by Bonferroni's modified t-test (Instat, GraphPad, San Diego, CA). A difference of $p < 0.05$ was considered to be statistically significant. Data were expressed as the means $\pm$ SEM of the indicated number of replicates performed.

Results

Protective effect of GSH on DA- and AA-induced apoptosis in PC12 cells

PC12 cells were treated with different concentrations of AA, DA and DA plus 0.1 mM AA with or without GSH during a period of 1 to 72 h. One hour treatments with AA, DA and DA plus AA did not change acid phosphatase activities (data not shown) in any of the three groups. After a 3 h treatment period, the highest concentration (1 mM) of AA, DA or DA plus 0.1 mM AA significantly depressed acid phosphatase activities to $87 \pm 3 \%$ ($p < 0.05$), $74 \pm 4 \%$ ($p < 0.01$) and $74 \pm 3 \%$ ($p < 0.01$), respectively (Fig. 3-2a). GSH itself up to 20 mM had no effect on acid phosphatase activity in PC12 cells (Fig 3-3). When 10 mM GSH was introduced in the culture medium, the toxic effect of AA and DA was completely blocked during a 3 h period in all three groups (Fig. 3-2a).

After 24 h treatments, AA-treated groups (up to 0.1 mM) did not exhibit altered viability, but 1 mM AA depressed the phosphatase activities to $48 \pm 2 \%$ ($p < 0.001$) while $71 \pm 7 \%$ ($p < 0.001$) in the presence of 10 mM GSH (upper panel, Fig. 3-2b). In DA-treated groups, 0.01, 0.1 and 1 mM DA significantly inhibited the phosphatase activities to $89 \pm 5 \%$ ($p < 0.05$), $74 \pm 3 \%$ ($p < 0.01$) and $5 \pm 0.7 \%$ ($p < 0.001$), respectively (middle panel, Fig. 3-2b). GSH completely prevented the toxicity of 0.01 - 0.1 mM DA but partially (48 %) prevented the decrease of acid phosphatase activity induced by 1 mM DA. Even though 0.1 mM AA itself did not change the phosphatase activity, when it was added to DA treated groups, 0.01, 0.1 and 1 mM DA inhibited the phosphatase activities to $85 \pm 6 \%$ ($p < 0.05$), $61 \pm 6 \%$ ($p < 0.01$) and $5 \pm 1.4 \%$ ($P < 0.001$), respectively (lower panel, Fig. 3-2b). When DA plus AA-treated and DA-treated groups

Fig. 3-2. The protective effect of GSH on AA- and DA-induced apoptosis. PC12 cells were treated with AA (1 μ M -1 mM), DA (1 μ M -1 mM) or DA (1 μ M -1 mM) plus AA (0.1 mM) in the presence or absence of 10 mM GSH for 3 h, 24 h and 48 h (columns). Each point represents mean $\pm$ SEM of 6 replicates. Each panel shows a representative experiment of four. The symbols *, ** and *** on the graphs refer to the difference between the treatment with or without GSH as $p < 0.05$, $p < 0.01$ or $p < 0.001$.

Fig. 3-3. The effect of GSH on PC12 cells. PC12 cells were treated with 2.5 - 20 mM GSH for 24 h and then acid phosphatase activities were assayed. Each point represents mean $\pm$ SEM of 16 replicates.

were compared, the phosphatase activities of the former group were significantly lower at 0.1 mM DA indicating the potentiating effect of AA on DA-induced toxicity. The protective effect of GSH on DA plus 0.1 mM AA was the same as that of DA treatment alone. Namely, GSH completely blocked the toxic effect of AA plus low concentrations of DA (up to 0.1 mM) and partially prevented the cell toxicity induced by AA plus 1 mM DA.

When incubation periods were extended to 48 h (Fig. 3-2c) or up to 72 h (data not shown), general trends of the inhibitory actions on acid phosphatase activities induced by AA, DA or DA plus AA were similar to that of the 24 h treated groups. The protective effect of GSH exhibited the same extent on AA as that of 24 h treatment but only slightly at 0.1 mM DA and no effect on 1 mM DA (Fig. 3-2c).

Effect of BSO on DA- and AA-induced apoptosis in PC12 cells

PC12 cells were treated with BSO with or without 1 mM AA or DA for 24 h. BSO at concentrations of 1.5, 3 and 6 mM decreased the acid phosphatase activities to $78 \pm 2\%$ ($p < 0.001$), $68 \pm 8\%$ ($p < 0.001$) and $57 \pm 5\%$ ($p < 0.001$), respectively (upper panel, Fig. 3-4). The acid phosphatase activities at 3 mM and 6 mM of BSO treatment were significantly lower than that at 1.5 mM, but there was no difference at 3 mM and 6 mM concentration. These data indicated that BSO itself killed PC12 cells. By 24 h incubation, 1 mM AA significantly inhibited acid phosphatase activity to 74 % and the acid phosphatase activities inhibited by BSO plus AA were significantly lower than both BSO- and AA-treated alone, suggesting that BSO and AA had an additive toxic effect on PC12 cells (upper panel, Fig. 3-4). However, during a 24 h treatment with DA, the effect of

Fig. 3-4. BSO killed PC12 cells and potentiated the toxic effect of AA. PC12 cells were treated with 1.5 - 6 mM BSO with or without 1 mM AA (upper panel) or DA (lower panel) for 24 h. Each bar represents mean $\pm$ SEM of 6 replicates. Difference between control and the treatment with BSO was shown as $^{\circ\circ}p < 0.01$ and $^{\circ\circ\circ}p < 0.001$; difference between the treatment with BSO and BSO plus AA or DA was shown as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$; difference between treatment with AA and BSO plus AA was expressed as $^+p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$.

BSO on DA could not be observed since 1 mM DA alone has already reached the maximal toxic effect on the cells (lower panel, Fig. 3-4). When PC12 cells were treated with 0.01-1 mM DA with or without 3 mM BSO for 6 h, BSO did not change the acid phosphatase activity, but potentiated the toxicity of DA in all dose ranges (Fig. 3-5).

Fig. 3-5. BSO potentiated the toxic effect of DA. The PC12 cells were treated with 0.01 - 1 mM DA with or without 3 mM BSO for 6 h. Each point represents mean $\pm$ SEM of 6 replicates. Difference between control and treatment with DA was shown on the graph as $^{**}p < 0.01$. Difference between the treatment of DA with or without BSO was shown on the graph as $*p < 0.05$.

Discussion

The major mechanisms of DA-induced apoptosis are oxidative stress and covalent binding of DA's oxidative products to vital proteins (Graham et al., 1978; Ross et al., 1993; Ross et al., 1995). Monoamine oxidase inactivates DA by oxidative deamination which produces 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL is taken up by the DA uptake system and selectively accumulates in and damages cells of the substantia nigra (Mattammal et al., 1995). DOPAL is further oxidized to 3,4-dihydroxyphenyl acetic acid (DOPAC). During the oxidative deamination processes, other cytotoxic products are also generated, such as hydrogen peroxide and superoxide radicals (Olanow, 1990). Our observations that the reducing agent GSH inhibits DA toxicity support that DA induces apoptosis through an oxidative stress mechanism.

AA has many different biological actions. AA potentiates DA's inhibitory action on prolactin release (Shin et al., 1990). AA is an important reducing agent and thus defends against singlet oxygen, superoxide anions, free hydroxyl radicals (Bielski et al., 1975; Nishikimi, 1975; Bodaness and Chan, 1979) and inhibits catechol oxidation (Shin et al., 1990). However, we found that a physiological concentration of AA (1 mM) caused apoptosis in PC12 cells and a subthreshold concentration of AA (0.1 mM) potentiated DA's apoptotic action. These observations are consistent with previous work that AA acts as a pro-oxidant for the induction of apoptosis in human leukemic HL-60 cells (Sakagami and Satoh, 1997) and 0.5 - 2 mM AA potentiates DA-induced apoptosis in a

human neuronal cell line (Gabbay et al., 1996). Offen et al. (1996) also demonstrate that AA can not rescue PC12 cells from DA-induced apoptosis.

A biphasic effect of AA, anti-oxidant or pro-oxidant, has been shown by Wefers and Sies (1988) and AA depends on vitamin E in switching the role of a potentially damaging agent to a protective agent. AA exhibits an additional anti-lipoperoxidant effect against oxidized lipoproteins on lymphoid cell lines when AA is used together with vitamin E and rutin (Negre-Salvayre et al., 1991). In vitamin E deficiency the pro-oxidant effect of AA is abolished when GSH is present (Wefers and Sies, 1988). Taken together, the balance between anti- and pro-oxidant effect of AA is dependent on vitamin E, GSH or other reducing agents.

While the molecular details of the effects of AA on apoptosis are still not established, we propose that AA toxicity is due to a serial redox reaction. It is known that AA is oxidized to dehydroascorbic acid (DHA) and rapidly ferried into cells by glucose transporters in the form of DHA, and then the accumulated DHA is reduced back to AA in cytosol by the action of DHA reductase (Vera, 1995). The reduction of DHA to AA means that other cytosolic component(s) should be oxidized since oxidation-reduction reactions transfer electron from one compound to another. This oxidation may be a potential cause of cell death. In essence, GSH, a major antioxidant in the cellular defense system, can inhibit the oxidation reaction from AA to DHA or from the oxidation of some cytosolic components thus prevent AA-induced apoptosis. This proposed mechanism is supported by our observations that GSH inhibited AA-induced oxidation.

DA toxicity was enhanced in the presence of AA (Fig. 3-2). The potentiation effect of AA may be due to an enhanced oxidation during conversion processes from DHA to AA in cytosol, and/or more DA is available due to increased DA half-life in presence of AA. The half-life of DA in DMEM is 36 min and it is extended to 792 min in the presence of 0.1 mM AA (Shin et al., 1990). However, the second possibility is excluded because GSH which increases the half-life of DA, decreased apoptotic action of DA and AA (Fig. 3-2). Therefore, DA is likely exposed to more oxidation stress caused by DHA than an increase of stored DA in granules during uptake processes.

Both AA and DA reached their maximal toxic effect at around 24 h and no further toxic effect was observed when the incubation period was prolonged to 48 h (Fig. 3-2), suggesting that the pharmacological agents were no longer active after 24 h. GSH had the same protective effect on AA during a 24 h or 48 h treatment period while much less effect on DA when the cells were incubated for 48 h.

DA decreases intracellular GSH levels in human neuronal cells (Gabbay et al., 1996) and the intracellular GSH levels fall concomitant with the onset of apoptosis (van den Dobbelen et al., 1996). BSO, an inhibitor of GSH formation, potentiated the neurotoxic effect of DA in PC12 cells in our experiments, suggesting that endogenous GSH participates in protecting against DA neurotoxicity. These observations confirm that PC12 cells synthesize GSH (Sampath et al., 1994). We also observed that BSO itself killed PC12 cells. However, Owen and coworkers (1996) demonstrate that the loss of GSH caused by BSO is not sufficient to produce degeneration of the nigrostriatal pathway in rat brain. Gabbay et al (1996) reports that 1 - 6 mM of BSO has no effect on human

neuronal cells during a 24 h treatment. Our studies suggest that PC12 cells are more vulnerable to BSO than human neuronal cells and rat brain cells. We will investigate the intracellular GSH levels in PC12 cells in future studies.

Some mammalian species, such as humans, monkeys and guinea pigs, have lost their ability to synthesize AA (Chatterjee et al., 1961). AA production is accompanied by GSH consumption in the liver with transfer of GSH to GSSG (Banhegyi et al., 1996). Our observations that AA kills PC12 cells and potentiates DA's toxic effects may be one of the reasons why the loss of the AA synthesizing ability can be advantageous. The levels of AA in Parkinson's disease are unchanged (Riederer et al., 1989). AA acts as an anti-oxidant or pro-oxidant in different situations and therefore the role of AA in Parkinson's disease needs further investigation.

Chapter 4. General Discussion

Oxidative stress is a major causal factor of apoptotic cell death

Current concepts of the pathogenesis of Parkinson's disease center on the formation of reactive oxygen species and the oxidative stress leading to oxidative damage to pars compacta of substantia nigra (Olanow, 1990; Jenner and Olanow, 1996). Metabolic products of DA are one of the important causes to induce substantia nigral degeneration (Ambani et al., 1975; Graham et al., 1978; Cohen, 1983; Gabbay et al., 1996). Three different mechanisms of actions for the metabolites are involved in the degeneration of dopaminergic neurons (Fig. 4-1): (a) Oxidation of DA produces a toxic semiquinone. The electron-deficient semiquinone reacts readily with nucleophilic compounds such as sulfhydryl (-SH) groups (Scheulen, et al., 1975; Graham et al., 1978; Ito, 1988; Fornstedt and Carlsson, 1991). The chemical reaction may lead to inhibition of critical enzymes and then result in cell death; (b) Monoamine oxidase B (MAO-B) inactivates DA by oxidative deamination which produces 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL can damage substantia nigra neurons, cultured fetal mesencephalon cells and PC12 cells by chemically binding to vital proteins (Mattammal et al., 1993; Mattammal et al., 1995). DOPAL is further oxidized to 3,4-dihydroxyphenyl acetic acid (DOPAC); (c) During the oxidative deamination process, other cytotoxic products (such as free oxygen radicals, superoxide radical anion or hydrogen peroxide) are also generated which may catalyze catechol oxidation (Misra, 1972; Olanow, 1990; Olanow, 1993). These free radicals are destructive because they possess an unpaired electron, and thus oxidize other molecules by

Fig. 4-1. A potential model of the oxidative stress-induced apoptosis and the protective mechanisms.

MAO-B inactivates DA which produces DOPAL. DOPAL is further oxidized to DOPAC. During the oxidative deamination processes, other cytotoxic products are also generated. The covalent binding of DA's oxidative products to vital proteins induce cell death. Selegiline inhibits MAO-B and thus protect against formation of some toxins and free radicals from the deamination of DA.

AA toxicity is due to a serial redox reaction. AA is oxidized to DHA and rapidly ferried into cells by glucose transporters in the form of DHA, and then the accumulated DHA is reduced back to AA in cytosol by the action of DHA reductase. The reduction of DHA to AA means that other cytosolic components are oxidized which causes cell death. The toxic effect of AA is inhibited by blocking the glucose transporters, wortmannin and genistein, or by 2-Deoxy-D-glucose which competitively enter the cells through the glucose transporters.

GSH can inhibit the oxidation reaction from AA to DHA or from the oxidation of some cytosolic components thus prevent DA- and AA-induced apoptosis.

(Abbreviations: AA, ascorbate; DA, dopamine; DHA, dehydroascorbic acid; DOPAC, 3, 4-dihydroxyphenyl acetic acid; DOPAL, 3, 4-dihydroxyphenylacetaldehyde; GSH, reduced glutathione; MAO-B, monoamine oxidase-B)

snatching an electron and injure vital subcellular organelles (Wallace, 1992; Yu, 1994; Augustin et al., 1997). The oxidative stress and covalent binding of DA's oxidative products to vital proteins are the major mechanisms of DA-induced apoptosis (Graham et al., 1978; Ross et al., 1993; Ross et al., 1995). GSH, the major antioxidant, prevents the redox cycling and thus protects neuronal cells from DA neurotoxicity (Sian, 1994; Jenner and Olanow, 1996).

Selegiline, an inhibitor of MAO-B, may protect against activation of some toxins and free radicals formed from oxidation of DA (Jenner and Olanow, 1996, Fig. 4-1). In addition, selegiline may also act through a mechanism unrelated to MAO-B to increase neurotrophic factor activity and unregulated molecules such as glutathione, superoxide dismutase and catalase, which protect against oxidant stress and apoptosis (Jenner and Olanow, 1996). Consequently, selegiline may be advantageous in the long-term treatment of Parkinson's disease (Jenner and Olanow, 1996).

AA induces apoptosis through an oxidative stress mechanism

AA is an important reducing agent (Bielski et al., 1975; Tse et al., 1976 Shin et al., 1990). Drehmer et al. (1997) demonstrate that AA acts as an efficient antioxidant, such as maintaining GSH levels and abolishing lipid peroxidative in the hepatocytes and thus preventing hepatocytes from injury. However, we found that physiological concentrations of AA caused apoptosis in PC12 cells and the apoptotic action was inhibited by GSH, an endogenous reducing agent. Therefore, we proposed that AA toxicity was due to a serial

redox reaction during the reduction of DHA to AA intracellularly, then the cytosolic component(s) was oxidized and eventually the cells were killed (Fig. 4-1).

There are a few pieces of evidence supporting our research hypothesis that AA induces oxidative stress: (a) AA is easily oxidized to DHA by oxygen in solution (Vera et al., 1995); (b) DHA is rapidly transported into cells via a GLUT transporter (Welch et al., 1995) while AA is slowly taken up through a Na^+ -dependent mechanism (Welch et al., 1995; Vera, 1995; Ngkeekwong and Ng, 1997); (c) DHA in the cytosol is rapidly reduced to AA by an enzymatic (Hughes, 1964; Bode et al., 1993; Paolicchi et al., 1996) or non-enzymatic process; and (d) Sakagami and Satoh (1997) show that AA induces cell death by increasing oxidation potential of human glioblastoma cells. These four pieces of evidence have been known individually, but we are the first to integrate the evidence and propose a new mechanism of AA-induced apoptosis in DA secreting neuronal cells. A schematic diagram of the proposed mechanisms of AA-induced oxidative stress in relation to neuronal cell death is presented in Fig. 4-1.

Since AA is transported into cells through glucose transporters via the form of DHA (Welch et al., 1995), we tried to block the glucose transporters with wortmannin and genistein, glucose transporter inhibitors (Clarke et al., 1994; Vera 1996). We found that AA toxicity was prevented by these glucose transporter inhibitors confirming that the oxidized AA (DHA) was transported into cells through glucose transporters (Si and Shin, manuscript in preparation). When we treated PC12 cells with 2-deoxy-D-glucose, an analogue of glucose (Vera et al., 1995), we found that the AA-induced apoptosis was also inhibited (Si and Shin, manuscript in preparation). These observations indicated that DHA

and 2-deoxy-D-glucose competitively enter the cells through the same pathway. We concluded that AA was transported into cells in the form of DHA through glucose transporters, and the toxic effect of DHA was inhibited either by blocking the glucose transporters or by competitively saturating the transporters (Si and Shin, manuscript in preparation) (Fig. 4-1).

Conclusion

AA is a potent reducing agent and is thus expected to protect against DA-induced apoptosis. However, we found that both AA and DA killed PC12 cells and AA enhanced DA-induced toxicity. GSH, an antioxidant, protected AA- and DA-induced apoptosis in PC12 cells. The GSH synthesis inhibitor, BSO, induced cell toxicity and potentiated the toxic effects of DA, suggesting that endogenous GSH participates in protecting against basal oxidative stress. Our observations that the reducing agent GSH inhibits DA and AA toxicity support the fact that DA and AA induce apoptosis through an oxidative stress mechanism.

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IMAGE EVALUATION TEST TARGET (QA-3)

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