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The Role of Astroglial Iron in the Pathogenesis of Parkinson's Disease

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

The excessive deposition of redox-active iron has been amply documented in the basal ganglia of subjects with Parkinson's disease (PD). Yet, much remains to be learned regarding the cellular and subcellular distribution of this metal and the precise role(s) it plays in the pathogenesis of PD. Cysteamine (CSH) induces the appearance of peroxidase-positive cytoplasmic granules in cultured neonatal rat astroglia which are identical to glial inclusions that progressively accumulate in the aging subcortical brain. These inclusions are derived from degenerate mitochondria which sequester iron and other transition metals before undergoing fusion with lysosomes in an autophagic process. Dr. Schipper has previously demonstrated that iron-mediated peroxidase activity in these cells is capable of oxidizing dopamine and other catechols to potentially neurotoxic semiquinone radicals (Schipper et al., 1991). In the present study, we co-cultured PC12 cells, a catecholamine-secreting cell line, atop confluent monolayers of either CSH pre-treated (iron-enriched) or control neonatal rat astroglia. We observed that the PC12 cells grown on the surface of iron enriched (senescent-like) astroglia were far more susceptible to dopamine / H_2O_2-related killing than PC12 cells cultured atop control glial substrata. Augmented killing of PC12 cells in the former paradigm was inhibited by the antioxidants, ascorbate, melatonin or resveratrol implicating a free radical mechanism of action. The aging-associated accumulation of iron in mitochondria of subcortical astroglia may facilitate the oxidation of dopamine to neurotoxic free radical intermediates and thereby predispose the senescent nervous system to PD and other neurodegenerative afflictions.

RÉSUMÉ

Le dépôt excessif de fer redox-actif au niveau des ganglions de la base des sujets atteints de la maladie de Parkinson, a été amplement documenté. Il y a, par contre, encore beaucoup à apprendre concernant la distribution cellulaire et subcellulaire de ce métal, ainsi que les rôles précis auxquels il contribue dans la pathogénèse de la maladie de Parkinson. La cystéamine (CSH) induit l'apparition de granules cytoplasmiques peroxydase positives dans les cultures de cellules astrogliales néonatales de rats, lesquelles sont identiques aux inclusions glials qui s'accumulent progressivement au niveau du cerveau subcortical viellissant. Ces inclusions sont dérivées à partir de mitochondries dégénérées qui séquestrent le fer et d'autre métaux de transition avant de se fusionner aux lysosomes lors d'un processus d'autophalgie. Dr. Schipper a précédemment démontré que l'activité peroxydase engendrée par le fer parmi ces cellules est capable d'oxyder la dopamine ainsi que d'autres catécholamines, potentialisant la présence de radicaux semiquinone neurotoxiques (Schipper *et al.*, 1991). Dans la présente étude, nous avons fait cultiver des cellules PC12, une lignée cellulaire sécrétant les catécholamines, sur une monocouche confluente de cellules astrocytaires néonatales de rats pré-traités à la CSH (enrichies en fer) ou non (contrôle). Nous avons observé que les cellules PC12 qui ont poussé à la surface des cellules astrcytaires enrichies en fer (senescent-like) étaient beaucoup plus susceptibles à une mort occasionnée par la dopamine / H_2O_2, que les cellules PC12 cultivées sur un substrat formé de cellules glials contrôles. L'accroissement de la mort cellulaire PC12 au centre de ce paradigme, a été inhibé par la mélatonine et l'acide ascorbique, impliquant donc des mécanismes d'action des radicaux libres.

L'accumulation de fer dans la mitochondrie, associée au viellissement des cellules astrogliales subcorticales, pourrait facilitier l'oxydation de la dopamine en radicaux libres intermédiaires neurotoxiques, et ainsi prédisposer le système nerveux sénescent à la maladie de Parkinson, tout autant qu'à d'autres afflictions neurodégénératives.

To my grandparents, parents, and siblings for their help and support.

To my dear wife, Atara, and children for their love, patience and confidence in me.

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I would like to take this opportunity to express my sincere gratitude to my Graduate Supervisor, Dr. Hyman M. Schipper, for his patience, support and guidance throughout my graduate studies and for giving me the opportunity to grow under his superior tutelage. Dr. Schipper has instilled in me a love for science and has helped broaden my understanding of free radical biology and neurodegenerative diseases.

I gratefully acknowledge Mrs. Adrienne Liberman and Dr. Lise Bernier for their expert technical support and valuable advice. I would also like to extend my gratitude to Dr. Khalil Mehindate for his technical guidance and for help with translation of the abstract. I am also very appreciative of all the former and present students in Dr. Schipper's laboratory and my colleagues at the Bloomfield Centre for Research in Aging for their help and for a providing an invigorating work environment.

Contributions to Original Knowledge

1) Dr. Schipper's laboratory has previously demonstrated that cysteamine (CSH) induces the formation of autofluorescent, peroxidase-positive (iron-rich) cytoplasmic inclusions in primary astroglial cultures akin to those which naturally accumulate in the aging brain. The results of the present study indicate that PC12 cells, a catecholamine-secreting cell line, are more vulnerable to oxidative challenge when cultured atop CSH-pretreated astroglial monolayers than when cultured atop control, healthy young astrocytes. The accumulation of redox-active iron in aging astroglia may predispose the senescent nervous system to the development of Parkinson's disease and other free radical-related neurodegenerative disorders.

2) Dr. Schipper has previously shown that CSH-pretreatment results in late up-regulation of manganese superoxide dismutase (MnSOD) mRNA and protein levels in astrocyte cultures. In the current study, we show that the up-regulation of the MnSOD gene in CSH- and dopamine-challenged astroglial cultures is dependent on the antecedent induction of heme oxygenase-1 (HO-1).

Preface

The author performed all of the experiments and analyzed the data presented in the main body of this thesis. The data were presented as a poster at the 27th Annual Meeting of the Society for Neurosciences (New Orleans, 1997). Frankel, D. and Schipper, H.M. Does astroglial senescence facilitate oxidative neuronal injury? Soc. Neurosci. Abstr. 23: 1371, 1997.

The author was responsible for astroglial cell culturing, pharmacological treatments, immunocytochemistry and confocal microscopy for the study on MnSOD expression presented in the Appendix. HO-1 transfection experiments and Northern Blot analyses were performed by Dr. Khalil Mehindate, a post-doctoral fellow in Dr. Schipper's laboratory.

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INTRODUCTION

Parkinson's Disease

Parkinson's disease (PD) or paralysis agitans refers to a movement disorder of uncertain etiology characterized by degenerative changes (neuronal degeneration, depigmentation and gliosis) in the pars compacta of the substantia nigra and other pigmented brainstem nuclei (Bugiani, *et al.*, 1980). Accelerated degeneration of dopaminergic neurons in the pars compacta of the substantia nigra results in a marked depletion of dopamine in the caudate nucleus and putamen (Cohen, 1983). Lesions or dysfunction of dopamine in this pathway results in an array of abnormal movements including: 1) disorders in the initiation of movement (akinesia), 2) difficulty continuing an ongoing movement (bradykinesia), 3) muscle tone abnormalities (rigidity), and 4) involuntary movements (tremor) (Greenfield, *et al.*, 1953; Duvoisin, 1982). These patients eventually present with gait disturbances and postural instability ultimately leading to loss of motion and the need for full-time medical assistance.

Although there currently exists no effective treatment for arresting the progression of dopaminergic cell loss in this disease, there are several treatments that offer temporary symptomatic relief. For the first few years of their disease, PD patients can alleviate some of their symptoms by implementing levodopa replacement therapy which enters the brain (in contrast to dopamine) and is metabolized to dopamine in the striatum where it activates dopamine receptors (Fahn, *et al.*, 1984). However, after about five years of treatment, 75% of patients exhibit troublesome dyskinesias and inadequate responses to the medication (Han, *et al.*, 1993). Surgical procedures are now under intensive study as a

means to reduce akinesia in PD. Many patients have regained control of their movements following lesioning of the internal segment of the globus pallidus (pallidotomy) (Yahr, 1990). Finally, excessive concentrations of hydrogen peroxide (H_2O_2) are produced by the accelerated deamination of dopamine (DA) by the enzyme monoamine oxidase B (MAO-B) in idiopathic PD and experimental parkinsonism. Therefore, deprenyl, an inhibitor of MAO-B, is now used clinically in attempt to slow the progression of the disease (Birkmayer, *et al.*, 1983). By inhibiting monoamine oxidase activity the production of dopamine-derived H_2O_2 is curtailed (Langston, *et al.*, 1984).

An attempt to develop effective therapies for the management of PD would be simplified by a deeper understanding of some of the mechanisms responsible for the pathogenesis of this disease. For example, although oxidative stress is believed to play an important role in PD, it is unclear whether this is a direct cause of neuronal degeneration or whether it represents the effects of a more basic disease process. The excessive accumulation of tissue iron have been reported in PD subjects. However, several important questions still remain unanswered concerning the sequestration of brain iron in PD (see below). In addition, a deficiency of mitochondrial activity, and therefore reduction in energy production and changes in neuronal metabolism, is hypothesized to contribute to the neurodegeneration in PD (DiMonte, 1991). Again it is not known to what extent the mitochondrial insufficiency represents a primary (causative) or secondary feature of the disease. Finally, reactive gliosis is a characteristic pathological property of neurodegenerative disorders including Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and PD (Beach, *et al.*, 1989; Duffy, *et al.*,

1980; Kushner, *et al.*, 1991; Schipper, *et al.*, 1993; Tomlinson, *et al.*, 1984). Whether the astrogliosis contributes to disease progression or is merely a passive response to neuronal degeneration remains to be determined.

Free Radicals and Oxidative Stress in PD

The term *free radical* refers to any molecule with free, or unpaired electrons in its outermost shell. This instability creates a highly reactive molecule that can either abstract or donate electrons from/to other molecules. There are two major classes of free radicals, the organic or carbon-centered radicals and the oxygen-centered radicals referred to as oxy-radicals. Some of these oxy-radicals include: superoxide (O_2^-) and hydroxyl radicals (OH^-) derived from molecular oxygen, and semiquinones which are derived from the auto-oxidation of catechols (Kalyanaraman, *et al.*, 1985; Kalyanaraman, *et al.*, 1984). In the presence of MAO-B, dopamine (a catecholamine) is metabolized to DOPAC, ammonia and the pro-oxidant species, H_2O_2. In the presence of ferrous iron, H_2O_2 can undergo a Fenton reaction leading to the production of hydroxyl radicals, one of the most damaging free radicals generated in living tissues (Goldfischer, et al., 1966; Graham, 1978; Schipper, et al., 1991).

A variety of free radical scavenging enzymes have evolved to protect mammalian cells against cytotoxic redox reactions. There exists two forms of antioxidant defense systems; 1) the enzymatic defenses which include: superoxide dismutase (SOD's), glutathione (GSH) peroxidase, glutathione reductase and catalase, and 2) the non-enzymatic defenses such as: ascorbic acid (vitamin C) and α-tocopherol (vitamin E).

Changes in cellular concentrations of reduced glutathione has been implicated as a major participant in the events leading to the degeneration of dopaminergic neurons in PD. Reduced glutathione (GSH) catalyzes the reduction of H_2O_2 to H_2O thereby preventing oxygen radical formation (Olanow, 1990). Reduced GSH is oxidized to GSSG and unless there is an excess production of H_2O_2, is immediately reduced back to GSH by glutathione reductase thereby maintaining optimal tissue ratios of GSH and GSSG (Olanow, 1990). In the PD nigra, overproduction of H_2O_2 arises from augmented oxidative deamination of dopamine by MAO-B, and levels of GSH are drastically reduced in the substantia nigra of PD subjects relative to age-matched controls (Spina, 1989). Although GSH is mainly found in the cytosolic fraction, about 10% is localized to the mitochondria (Reed, 1990). With glutathione peroxidase and glutathione reductase present in this organelle as well, a system is in place for the intramitochondrial detoxification of H_2O_2 (Reed, 1990). In addition, mitochondrial GSH plays an important role in maintaining mitochondrial thiols in their reduced states (Sandri, *et al.*, 1990). In PD, the excessive production of H_2O_2 may boost depletion of mitochondrial GSH, which, in turn, could lead to oxidation of protein thiols and progressive mitochondrial dysfunction. As a consequence of the latter, there may be increased infidelity of electron transport in the inner mitochondrial membrane resulting in augmented production of superoxide and H_2O_2, and further oxidative stress. Mitochondrial damage could conceivably be an initiating step in the degeneration of nigrostriatal projections in PD. Since GSH synthesis requires energy, a primary deficiency of ATP production by the mitochondria would likely affect cellular turnover of glutathione (Spina, 1989). Studies using the dopaminergic toxin, MPP+, a metabolite of MPTP, have

revealed that a decrease in GSH levels was not accompanied by commensurate increases in levels of GSSG and could be counterbalanced by the addition of substrates for glycolytic production of ATP (Mithofer, *et al.*, 1992). Since the results of MPP+ intoxication bear many clinical and pathologic features in common with idiopathic PD, the mechanism by which MPP+ reduces GSH levels may be analogous to that seen in PD brains and point to decreased production of ATP as an initiating factor (Bradbury, et al., 1986). Consequent impairment of GSH levels may then make dopaminergic neurons in the substantia nigra susceptible to oxidative stress, thereby leading to accelerated dopaminergic depletion and nigrostriatal damage. In neural and non-neural cells, SOD catalyzes the dismutation of superoxide to H_2O_2. There are presently three forms of SOD: a copper-zinc SOD (CuZnSOD) which is specific to the cytosol, a manganese SOD (MnSOD) in the mitochondrial compartment and an iron SOD found in bacteria (Fridovitch, 1976). Finally, ascorbic acid and α-tocopherol are present in high concentrations in the aqueous and lipid cellular compartments, where they respectively, function to reduce toxic quinones scavenge superoxide radicals.

The formation of hydroxyl radicals in response to oxidative stress can initiate a chain of events leading to the abstraction of hydrogen atoms from unsaturated lipids, a process referred to as lipid peroxidation (Gebicki, *et al.*, 1981). The excessive generation of free radicals in neurodegenerative diseases has been amply documented (Dexter, *et al.*, 1986; Fahn, *et al.*, 1992; Jenner, 1991). Although they are found in essentially all organs, the relative abundance of unsaturated fat in the brain makes this organ particularly

vulnerable to oxidative stress and free radical-related neurodegeneration (Cohen, *et al.*, 1994; Fahn, *et al.*, 1992; Jenner, 1992; Youdim, 1994).

There is currently a broad consensus implicating oxidative stress as a major factor in the pathogenesis of PD (Cohen *et al.*, 1994; Fahn *et al.*, 1992). This hypothesis is supported by reports that: a) excessive concentrations of hydrogen peroxide (H_2O_2) are produced by MAO-B-mediated deamination of dopamine (DA) in idiopathic PD and experimental parkinsonism, b) the neurotoxins 6-hydroxydopamine, manganese and MPTP, are all capable of producing parkinsonism in animals, at least in part, via the generation of free radicals (Barbeau *et al.*, 1984; Cadet *et al.*, 1989), c) lipid peroxidation in the substantia nigra is augmented in post mortem Parkinsonian brain relative to aged matched controls (Dexter et al., 1989), and d) free radical scavenging enzymes such as catalase and reducing substances such as reduced glutathione are deficient in the basal ganglia of PD subjects (Ambani et al., 1975; Perry et al., 1982). In contrast, manganese superoxide dismutase (MnSOD) is elevated in PD which has been interpreted as a compensatory response to oxidative stress (Karla *et al.*, 1992; Riederer *et al.*, 1989). A combination of both genetic and environmental factors that contribute to the production of free radicals and decreased antioxidant defense capabilities observed in PD leading to nigrostriatal degeneration and loss of dopaminergic input to the neostriatum. As discussed below, the accumulation of tissue iron observed in the brains of PD subjects may serve as an important source of reactive oxygen species in this condition.

The role of iron in PD

High levels of iron has been shown to accumulate in the substantia nigra and basal ganglia of PD subjects, and the metal has been implicated as a major generator of reactive oxygen species in this condition (Jellinger *et al.*, 1990; Jenner, 1992). Under normal conditions, there exists two iron binding proteins; ferritin and tranferrin. The former is the major iron storage protein in mammalian tissues and has been shown to be significantly increased in PD (Jellinger, *et al.*, 1990). Transferrin, the primary iron transport protein, is hypothesized to regulate the extracellular transport of ferric iron within pigmented nigral neurons (Aisen, 1992; Swaiman, and Machen, 1985). In order to maintain normal tissue iron homeostasis, the two proteins are regulated at both the transcriptional and posttranscriptional level by iron bioavailability and intracellular iron stores. Somewhat surprisingly, there are no reported differences in the number of transferrin binding sites in the basal ganglia of PD subjects relative to controls (Faucheux, *et al.*, 1993). Thus, in contradistinction to peripheral tissues, transferrin and its receptor may play a limited role in the sequestration of iron in PD and other neurodegenerative diseases. On the other hand, lactoferrin, another iron transport protein, and the lactoferrin receptor are reportedly increased in PD subjects suggesting another mechanism of tissue iron sequestration (Kawamata, *et al.*, 1993; Leveugle, *et al.*, 1994). It is interesting to note in this regard that the highest expression of lactoferrin receptors in human brain occurs within the substantia nigra, the region most profoundly affected in idiopathic PD (Agid, *et al.*, 1993).

With augmented iron sequestration in PD subjects, there are strong theoretical grounds to consider that this transition metal participates in promoting oxidative stress and lipid peroxidation and thereby directly contributes to the neuronal cell killing. Specifically, H_2O_2 may be reduced to the highly cytotoxic hydroxyl radical in the presence of iron (Gutteridge *et al.*, 1985; Youdim, 1994). Furthermore, in the presence of H_2O_2, ferrous iron (Fe^{2+}) may behave as a non-enzymatic peroxidase activity capable of converting catecholamines to neurotoxic quinones and ortho-semiquinone radicals (Goldfischer *et al.*, 1966; Graham, 1978; Schipper *et al.*, 1991). To better comprehend the role of iron-mediated neuronal injury in PD, we must first understand the mechanisms subserving iron metabolism and sequestration in the aging and degenerating nervous system. Several important questions in this regard include: (a) What is the role of heme versus non-heme iron in PD and other neurodegenerative diseases? (b) Which cell type(s) and subcellular compartments are responsible for the abnormal sequestration of brain iron in PD? (c) Does induction of a cellular stress (heat shock) response facilitate the accumulation of redox-active iron in neural tissue?, and what is the role of transferrin versus non-transferrin derived iron in this disease? As discussed below, the accumulation of iron in aging subcortical astrocytes may render the senescent nervous system prone to PD and other aging-related neurodegenerative disorders.

Astrocytes

Astrocytes represent the most numerous and diversified class of nonneuronal brain cells (neuroglia) in the fetal and mature CNS. At the light microscopic level, they are

characterized by their stellate perikarya and numerous processes. Ultrastructurally, astrocytes exhibit euchromatic nuclei and an electron-lucent cytoplasm containing relatively few ribosomes. Astrocytes also contain short strands of smooth endoplasmic reticulum (ER), well-developed Golgi bodies, multivesicular bodies, and, occasionally, lysosomal dense bodies (Mugnaini and Walberg, 1964; Braak, 1975; Montgomery, 1994). These cells are readily identified by using antibodies against cytoskeletal components such as vimentin and glial fibrillary acidic protein (GFAP) (Fedoroff and Vernadakis, 1986a; Schipper, 1991). GFAP, found exclusively in astrocytes and tanycyte processes is an intermediate filament protein initially isolated from multiple sclerosis plaques (Eng *et al.*, 1971). Although the precise functions of GFAP and vimentin remain poorly understood, another astroglial marker, S-100β protein is capable of inducing proliferation of primary astrocyte cultures (Takahashi, 1992; Fedoroff and Vernadakis, 1986a; Selinfreund *et al.*, 1991). Based on their morphological characteristics and location, astrocytes have been subdivided into protoplasmic and fibrous types in the CNS (Andriezen, 1893; Retzius, 1894; Azoulay, 1894; Kollicker, 1896; Cajal, 1909; Fedoroff and Vernadakis, 1986a; Miller *et al.*, 1989). Protoplasmic astrocytes are found in the gray matter and have short, sheet-like ramified processes and their cytoplasm contains relatively few intermediate filament bundles (Fedoroff and Vernadakis, 1986a). In contrast, the fibrous astrocytes contain numerous intermediate filaments associated with GFAP, are located in the white matter, are stellate in shape, and extend numerous end feet into the nearby gray matter.

Astrocyte functions in the CNS

Astrocytes perform a wide range of adaptive functions in the nervous system. Some astrocytes extend processes to the surface of the CNS to form a glial membrane (glia limitans) that serves as a protective covering for the CNS (Kandel and Schwartz, 1991). Astrocytic "end feet" also abut on blood vessels and induce tight junctions between adjacent endothelial cells thereby establishing the blood brain barrier. The latter limits the entry of many blood-borne substances into the CNS (Kandel and Schwartz, 1991). Astrocytes are thought to be involved in the guidance and migration of neuronal cells and growth cones during embryogenesis (Rakic, 1971; Silver and Shapiro, 1981; Silver et al., 1982). Finally, astrocytes are responsible for the maintenance of ion homeostasis, production of proinflammatory and immunomodulatory cytokines and metabolism of various neurotransmitters (Hertz, 1981; Fedoroff and Vernadakis, 1986a; Wilkin *et al.*, 1990; Plata-Salaman, 1991; Benveniste, 1992). Evidence suggests that astrocytes can act as antigen presenting cells and IFN-γ can induce MHC class II gene expression in primary astroglial culture (Benveniste, *et al.*, 1989). Moreover, astrocytes are thought to be instrumental in the recovery process after CNS injury. For example, following transection of the corpus callosum, local implantation of immature astroglia has been shown to promote axonal regeneration (Smith et al., 1986). Taken together, these data suggest that astrocytes subserve protective and restorative roles in the injured nervous system.

Dystrophic Effects of Astroglia

Under certain conditions, astrocytes may exert detrimental effects on surrounding neurons and thereby contribute to a decline in neurologic function. For example, in response to CNS injury, astrocytes may contribute to the formation of epileptogenic scar tissue. In the human CNS, astrocytes may undergo neoplastic transformation and exhibit malignant behaviour (Carpenter, 1983). The release of excitatory amino acids from astrocytes upon exposure of certain metals, in an hypoxic environment or in the presence of oxidative stress, may result in excitotoxic neuronal injury (Fedoroff and Vernadakis, 1986b). For example, under ischemic conditions, or in the presence of reactive oxygen species (ROS), astrocytes lose their ability to sequester excess glutamate released at excitatory synapses within the CNS (Volterra, *et al.*, 1994). Increased amounts of glutamate in the synaptic cleft can induce the activation of glutamate receptors in postsynaptic cell membranes causing intracellular biochemical changes leading to cell death (Wood, *et al.*, 1990). Astrocytes may also participate in the biotransformation of protoxins into potent neurotoxins. For example, astroglia contain MAO-B that bioactivates the protoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into the dopaminergic neurotoxin MPP+ (Ransom *et al.*, 1987; Brooks *et al.*, 1989). Upon its release from astrocytes, MPP+ is then actively taken up by surrounding dopaminergic neurons where it inhibits complex I of the mitochondrial respiratory chain resulting in nigrostriatal degeneration and parkinsonism (Javitch and Snyder, 1984; Nicklas et al., 1985; Ramsay and Singer, 1986; DiMonte and Smith, 1988).

As mentioned above, astrocyte hypertrophy and proliferation (reactive gliosis) is a characteristic pathological feature of CNS injury and disease. Reactive gliosis may produce excessive scarring at the site of the injury and thereby impede axonal regeneration and sprouting (Rudge *et al.*, 1990; Reir *et al.*, 1983). Reactive gliosis is followed by a significant increase in MAO-B activity in PD and in the normal aging brain (Beach *et al.*, 1989; Duffy *et al.*, 1980; Kushner *et al.*, 1991; Schipper *et al.*, 1993). As described above, the accelerated deamination of dopamine and other monoamines in the presence of MAO-B yields excess amounts of H_2O_2 perpetuating further neuronal injury. As discussed in the following sections, sub-populations of astroglia exhibit unique histochemical properties that may implicate these cells in the pathogenesis of PD and other aging-related neurodegenerative diseases.

The Peroxidase-positive Subcortical Glial System

A unique subpopulation of granule-laden neuroglia initially referred to as Gomori-positive astrocytes on the basis of their affinity for chrom-alum hematoxylin and aldehyde fuchsin (Gomori stains), resides in limbic and periventricular brain regions of all vertebrates studied to date including humans (Srebro *et al.*, 1971; Srebro, *et al.*, 1975; Wislocki *et al.*, 1954; Schipper *et al.*, 1991; Schipper *et al.*, 1995). The Gomori-positive gliosomes are replete with transition metals including iron, copper, and chromium (Brawer, *et al.*, 1994; Hill, *et al.*, 1984; McLaren, *et al.*, 1992; Srebro, *et al.*, 1972). These astrocytic inclusions possess a high sulfur content (Srebro *et al.*, 1971), emit

orange-red autofluorescence (Goldgefter *et al.*, 1980) and stain intensely with diaminobenzidine (DAB), a marker for endogenous peroxidase activity (Srebro *et al.*, 1971). DAB staining in these cells persists even after preheating the cells at a temperature of 95°-100°C, at extremes of pH (4-10.5) and in the presence of the catalase inhibitor, aminotriazole (Kumamoto, 1981; Schipper, *et al.*, 1995; Schipper, *et al.*, 1990). Therefore, the peroxidase activity in these cells is non-enzymatic in nature (pseudoperoxidase). Iron-catalyzed peroxidation has been implicated as the mechanism for non-enzymatic H_2O_2 reduction in these cells (Goldfischer, *et al.*, 1966). In both the human and rat, peroxidase-positive glial inclusions progressively accumulate with advancing age (Schipper, *et al.*, 1981). Despite their accumulation with aging, the glial inclusions are histochemically and structurally distinct from the aging pigment lipofuscin (Schipper, 1991). In addition, increases in numbers of Gomori-positive glial granules have been observed in the rodent periventricular brain following x-irradiation or chronic estrogen exposure (Brawer, *et al.*, 1980; Brawer, *et al.*, 1983; Schipper, *et al.*, 1990; Srebro, 1971). As discussed in the following section, Dr. Schipper has developed a model in which he can rapidly accelerate the aging-related accumulation of peroxidase-positive astroglia by treating neonatal rat astroglia with the sulfhydryl reagent, cysteamine (Schipper *et al.*, 1990). The ability to generate these primary cell cultures has considerably enhanced our understanding concerning the origin of these peroxidase-positive inclusions, the mechanism(s) responsible for their biogenesis, and the role these cells may play in brain aging and neurodegenerative diseases.

Peroxidase-Positive Astrocytes in Primary Culture

Dr. Schipper's laboratory has demonstrated that treatment of neonatal rat astroglia with the sulfhydryl agent, 2-mercaptoethylamine or cysteamine (CSH) (880μM in culture medium administered twice weekly from *in vitro* day 6-18) induces a massive accumulation of astrocytic inclusions in the context of a cellular stress (heat shock) response (Mydlarski *et al.*, 1993; Schipper *et al.*, 1990 Fig. 1a, 1b). These CSH-induced inclusions are structurally and histochemically identical to the Gomori-positive glial granules that progressively accumulate in the aging subcortical brain. As *in situ*, the CSH induced inclusions exhibit orange-red autofluorescence and non-enzymatic peroxidase activity (Schipper *et al.*, 1991; Schipper *et al.*, 1990). The gliosomes are membrane-bound, variable in size and shape, and exhibit an intensely electron-dense granular matrix (Brawer *et al.*, 1994a; McLaren *et al.*, 1992 Fig. 2a, 2b). Both electron microprobe analysis and DAB (peroxidase) staining were used to detect the presence and concentration of elemental iron in these inclusions (McLaren *et al.*, 1992). Many astroglial mitochondria exhibit progressive swelling, rearrangement of cristae, sequestration of redox-active iron, and, in some cases, fusion with the lysosomes after 24-72 hours of CSH exposure (Brawer *et al.*, 1994a). Subcutaneous injections of CSH (150-300 mg/kg biweekly for three weeks) in young adult rats induces a 2-3 fold increase in the number of peroxidase-positive astrocyte inclusions in many brain regions including the basal ganglia and hippocampus (Schipper *et al.*, 1993). As in the case of the CSH-pretreated cultures, peroxidase-positive glial inclusions in the intact rat and human brain invariably share mitochondrial epitopes (Brawer *et al.*, 1994b; Schipper *et al.*, 1995). Taken together,

these observations indicate that a) the iron-laden astrocyte granules are derived from abnormal mitochondria engaged in a complex autophagic process and b) CSH accelerates the appearance of a senescent phenotype in these cells. In the following section, the role of heme oxygenase-1 (HO-1) in the formation of peroxidase-positive glial inclusions is reviewed.

Figure 1: DAB staining for endogenous peroxidase activity.

CSH stimulates the appearance of peroxidase positive cytoplasmic inclusions in cultured astroglia (B). Few or no such inclusions are observed in untreated controls (A). (From Schipper *et al.*, 1990)

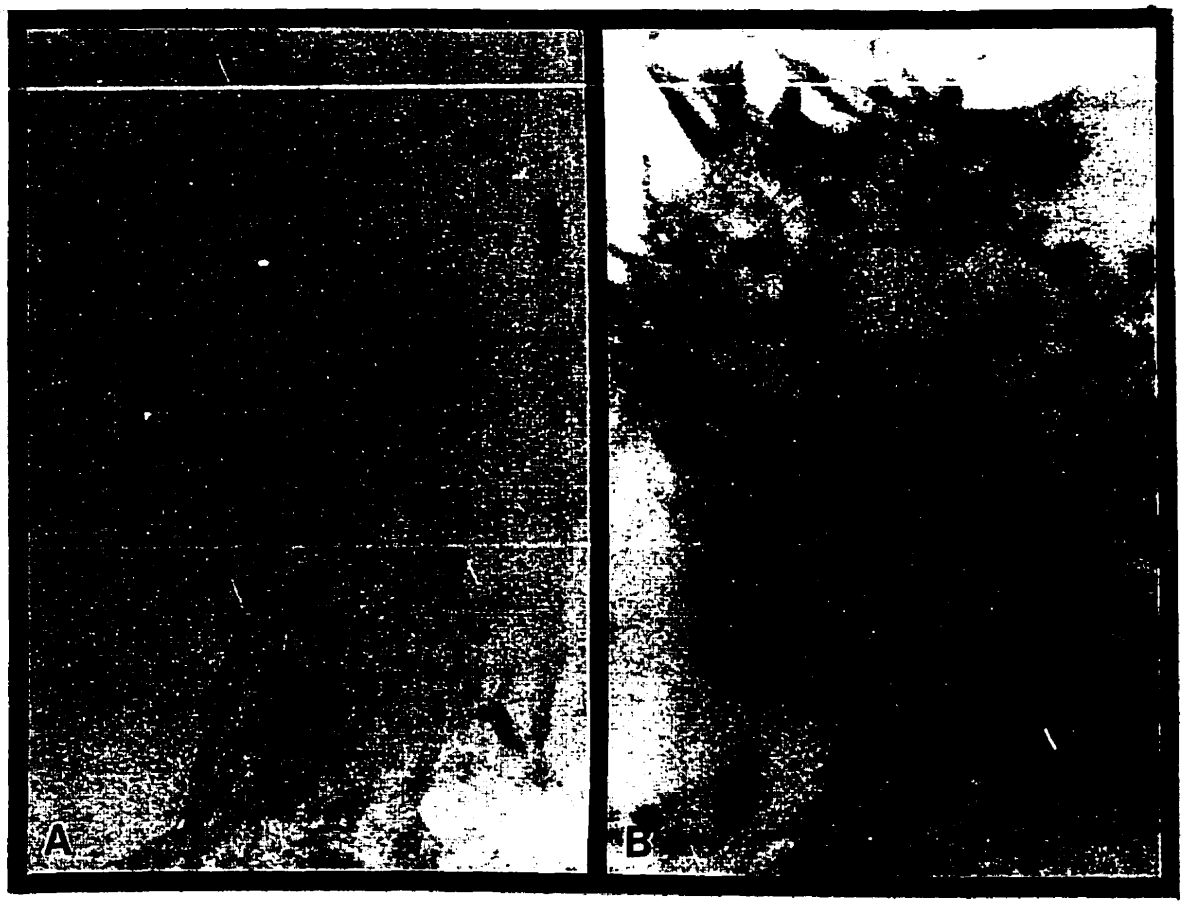
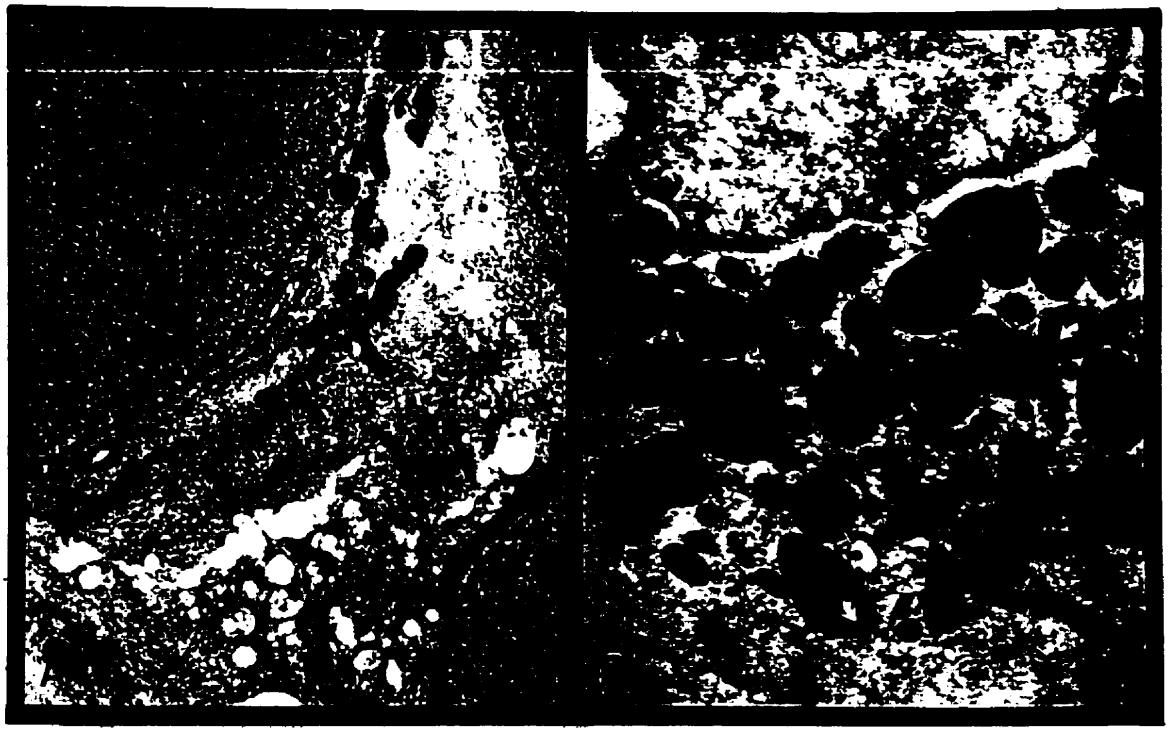

A
B

Figure 2: CSH-induced gliosomes are variable in size and shape, and exhibit an intensely electron-dense granular matrix. The peroxidase-positive astrocytic inclusions induced by CSH exposure (B) are osmiophilic, membrane-bound and derived from iron-laden mitochondria. (A) untreated controls.

(From McLaren *et al.*, 1992)



The Biogenesis of Astrocytic Inclusions: Role of Heme Oxygenase-1

HO-1 is a 32-kd member of the stress protein superfamily that catalyzes the rate limiting step in heme degradation (Ewing *et al.*, 1991; Willis *et al.*, 1996). The HO-1 protein has a heat shock element in its promoter region and is rapidly induced upon exposure to heme as well as metal ions, ultraviolet light, sulfhydryl compounds and various pro-oxidants (Dwyer *et al.*, 1992; Applegate *et al.*, 1991; Keyse *et al.*, 1989). Induction of HO-1 in response to oxidative stress has led to the prevailing belief that its expression is a fundamental component of the cellular anti-oxidant defense system (Applegate *et al.*, 1991; Abraham *et al.*, 1995). Heme contains a redox-active ferrous iron atom coordinated within a tetrapyrolle ring that can generate cytotoxic free radicals by participating in Fenton reactions ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + H_2O$). On the other hand, the bile pigment, bilirubin, the final product of heme catabolism, has potent free radical scavenging capabilities *in vitro* (Schacter, 1988). Stocker suggested that augmentation of HO-1 activity in oxidatively challenged cells may serve to normalize the redox microenvironment by converting pro-oxidant heme to anti-oxidant bilirubin (Stocker 1990). In contrast, ***HO-1 catalyzed heme degradation liberates carbon monoxide (CO) and free ferrous iron which may, under certain circumstances, potentiate oxidative damage within specific intracellular compartments.*** For example, exposure of human MCF-7 cells to menadione strongly induces HO-1, and blocking its activity does not enhance oxyradical-mediated DNA damage. In fact, HO-1 inhibition appeared to confer

protection to these cells relative to controls (Nutter et al., 1994). Most significantly, HO-mediated release of CO may exacerbate intracellular oxidative stress by stimulating oxyradical generation within the mitochondrial compartment (Zhang et al., 1992).

Recently, HO-1 has also been shown to be overexpressed in neurodegenerative diseases, possibly in response to chronic oxidative stress. Although the cause of progressive neural degeneration of AD remains enigmatic, it has been proposed that oxidative stress, possibly a consequence of amyloid deposition, may play an important role in the pathogenesis of this disorder. Recent studies have confirmed that, in comparison to normal senescent brains matched for age and post-mortem interval, AD patients demonstrate a marked overexpression of HO-1 in hippocampal neurons and astrocytes (Schipper *et al.*, 1995). As well, there is a greater percentage of GFAP-positive astrocytes in the PD substantia nigra expressing HO-1 compared to age-matched controls (Schipper *et al.*, 1998). Although HO-1 up-regulation in these conditions may confer some cytoprotection by degrading pro-oxidant heme to the antioxidant bile pigment, bilirubin, heme-derived ferrous iron and CO may contribute to the development of mitochondrial electron transport chain deficiencies and excess mitochondrial DNA mutations reported in the brains of Alzheimer and PD subjects (Beal, 1995; Reichmann *et al.*, 1994).

The astrocytic inclusions observed in senescent and CSH-pretreated astroglia may be due, in part, to the up-regulation of HO-1. After six hours of exposure to CSH, cultured astroglia exhibit a 4-10 fold increase in HO-1 mRNA and protein levels, as well as a three fold increase in HO enzymatic activity compared to controls (Chopra *et al.*, 1995; Manganaro *et al.*, 1995; Mydlarski *et al.*, 1995). Conceivably, induction of HO-1 in

CSH-pretreated astroglia may promote oxidative injury to mitochondrial membranes via the liberation of free ferrous iron and CO, and thereby facilitate the transformation of normal astrocytic mitochondria to peroxidase-positive cytoplasmic inclusions.

Mechanisms of Astroglial Iron Sequestration

As described above, CSH induces the appearance of iron-rich cytoplasmic granules in cultured neonatal rat astroglia that are identical to glial inclusions that progressively accumulate in the aging subcortical brain. Delineating the mechanism by which CSH promotes the sequestration of iron in the mitochondrial compartment of these cells may provide us with an opportunity to study the underlying fundamental processes subserving iron deposition in the aging and degenerating nervous system. Dr. Schipper has previously determined that CSH suppresses the incorporation of heme precursors, Δ-amino[^{14}C]-levulinic acid and [^{14}C]-glycine into astroglial porphyrin and heme in primary cell cultures preceding mitochondrial iron incorporation by the mitochondrial compartment (Wang *et al.*, 1995). Thus, in contrast to earlier conjecture, *de novo* biosynthesis of porphyrins and heme is <u>not</u> responsible for the increased mitochondrial iron, autofluorescence or peroxidase activity in Gomori-positive astrocytes (Graham, 1978; Schipper, 1991). After suppression of heme biosynthesis in these cells, CSH significantly increases the mitochondrial iron content without affecting the transfer of iron into total-cell and lysosomal compartments (Wang *et al.*, 1995). Furthermore, this CSH effect was observed when inorganic $^{59}FeCl_3$, but not ^{59}Fe-diferric transferrin, served as the metal donor. These findings are consistent with previous studies showing that inorganic, but not transferrin-

bound, iron readily taken up by certain tissues including melanoma cells (Richardson *et al.*, 1994), Chinese hamster ovary cells (Chan *et al.*, 1992), and K562 cells (Inman *et al.*, 1993). These observations also substantiate previous reports that inhibition of heme biosynthesis provokes the transport of low molecular weight iron from the cytoplasm into the mitochondrial compartment (Adams *et al.*, 1989).

Recent evidence from Dr. Schipper's laboratory suggests that like CSH, dopamine up-regulates HO-1 and stimulates the sequestration of nontransferrin bound ^{59}Fe in the mitochondrial compartment of neonatal rat astroglial cultures (Schipper, *et al.*, 1996 abstr.). These novel dopamine effects were abrogated by co-administration of ascorbate implicating a free radical mechanism of dopamine action (*ibid*). These findings recapitulate the discordant pattern of iron/tranferrin receptor localization observed in PD nigra (see above) and suggest that stress-induced trapping of non-transferrin-derived iron by astroglial mitochondria may be a key mechanism responsible for the pathological accumulation of this transition metal in the basal ganglia of PD subjects.

Pro-toxin Bioactivation by Peroxidase-positive Astrocytes in Primary Culture

Schipper and co-workers used electron spin resonance spectroscopy (ESR) to assess whether the peroxidase activity (mitochondrial iron deposition) in CSH-pretreated cells was capable of oxidizing catecholestrogens and catecholamines to their respective orthosemiquinone radicals (Schipper *et al.*, 1991). Little or no o-semiquinone spectra were observed when control astroglial homogenates were incubated with 2-hydroxyestradiol in the presence of H_2O_2 and NADPH co-factors. However, incubation of

equimolar concentrations of 2-hydroxyestradiol with CSH-pretreated astroglia homogenates produced intense o-semiquinone spectra indicative of catechol oxidation. In contrast, they observed a marked reduction in signal amplitude in the absence of H_2O_2 substrate attesting to the role of glial peroxidase activity in the oxidative metabolism of catecholamines and catecholestrogens (Schipper *et al.*, 1991).

Analogous to the case of 2-hydroxyestradiol, Schipper *et al*, demonstrated that the peroxidase activity induced by CSH-pretreatment significantly enhances the oxidation of the catecholamine, dopamine, into its dopamine-o-semiquinone radical in the presence of H_2O_2 (Schipper *et al.*, 1991). This latter observation is in accordance with others who have shown that dopamine and norepinephrine can be oxidized to semiquinones with proven neurotoxic capabilities via peroxidase-mediated reactions (Metodiewa *et al.*, 1989). Due to the fact that astrocytes in the aging subcortical brain are replete with mitochondrial iron and display enhanced MAO-B activity, it is possible that excess H_2O_2 produced from dopamine oxidation in the presence of MAO-B may serve as a co-factor for further dopamine oxidation (to neurotoxic semiquinone radicals) by peroxidase-mediated reactions. In addition to dopamine, redox-active glial iron may also facilitate the non-enzymatic oxidation of a) the protoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), to the dopaminergic toxin, MPP+, in the presence of MAO inhibitors (DiMonte *et al.*, 1995), and b) the dopamine precursor, DOPA, to 2,4,5-trihydroxyphenylalanine (TOPA) and the non-NMDA excitotoxin, TOPA-quinone (Newcomer *et al.*, 1995). In response to oxidative stress in CSH-pretreated (peroxidase-positive) cells, there is an up-regulation of various heat-shock proteins and a late up-

regulation of manganese superoxide dismutase (MnSOD) which are thought to protect the astroglia from oxidative injury (Manganaro *et al.*, 1995). Nonetheless, the dystrophic effects of reactive oxygen species need not be restricted to the cellular compartments in which they are formed. For example, H_2O_2, a by-product of dopamine oxidation, is lipid soluble and can readily cross the plasma membrane to the intercellular space, while superoxide (produced during semiquinone-quinone redox cycling or from the electron transport chain in damaged mitochondrial membranes) can be actively pumped out of cells via anion channels (Kontos *et al.*, 1985). On the basis of the ESR experiments described above, Schipper hypothesized that, in the intact basal ganglia, free radical leakage from peroxidase-positive astrocytes into the surrounding neuropil may promote lipid peroxidation and further degeneration of nearby dopaminergic neurons. He also conjectured that the progressive increase of peroxidase-positive astrocytes in the basal ganglia and other subcortical regions observed in aging rodents and human brain may predispose the latter to parkinsonism and other free radical-related neurodegenerative diseases (Schipper, 1996).

The major goal of this thesis is to determine whether senescence-related increases in astrocyte iron are detrimental to dopaminergic neurons in Parkinson's disease and other aging-associated neurodegenerative disorders. The hypothesis is that in *PD and other neurodegenerative disorders, sequestration of redox-active iron in "stressed" astroglial mitochondria promotes the bio-activation of dopamine to neurotoxic semiquinone radicals*. To test this hypothesis, PC12 cells, a catecholamine-secreting pheochromocytoma cell line, will be cultured atop confluent monolayers of either CSH-

pretreated (iron enriched) or control neonatal rat astroglia. We will attempt to show that the non-enzymatic (iron-mediated) glial peroxidase activity oxidizes dopamine into dopaminergic neurotoxins. Growth patterns and viability of PC12 cells atop control and CSH-pretreated monolayers will be monitored by vital dye exclusion (trypan blue and ethidium monoazide bromide (EMA)) and anti-tyrosine hydroxylase immunolabeling. Positive results in the proposed experiments would suggest a mechanism for the abnormal sequestration of redox-active iron, mitochondrial insufficiency and increased oxidative stress in Parkinson's disease. If peroxidase-positive (iron-rich) astrocytic inclusions promote oxidative damage within the aging CNS, delineation of the cellular and molecular processes governing their biogenesis may facilitate the design of neuroprotective strategies for the treatment of Parkinson's disease and other free-radical related neurodegenerations.

PC12 Cell Line

PC12 cells, a catecholamine secreting cell line, was originally cloned in 1975 and is derived from rat adrenal chromaffin cells (Green, et al., 1976). Using histochemical techniques, these cells exhibited yellow-green fluorescence confirming the presence of intact catecholamine biosynthetic pathways. Their cell bodies contain irregular round dense core granules that have previously been detected in the cytoplasm of rat adrenal chromaffin cells (Coupland, 1965). PC12 cells possess the ability to transform from a proliferating pheocromocytoma cell-type into a nonproliferating, sympathetic neuron-like cell in the presence of nerve growth factor (NGF) (Levi-Montalcini, 1987). The use of PC12 cells in studying neurodegenerative disorders is advantageous for several reasons.

Firstly, PC12 cells are a homogeneous cell line allowing for precise quantitative analyses. Secondly, PC12 cells contain large amounts of dopamine and norepinephrine making them more specific for investigating neurodegenerative diseases such as PD. Finally, as mentioned above, PC12 cells can extend their neurites, post NGF treatment, creating a sympathetic-like neuron. Taken together, the PC12 cell line is a useful source of neuron-like cells for investigating various issues concerning catecholamine metabolism / regulation, catecholaminergic cell aging, and nigrostriatal cell degeneration. As described in the following sections, PC12-astroglial co-cultures present a simplified, albeit biologically-relevant, model to determine whether the vulnerability of catecholaminergic cells to oxidative damage is enhanced when these cells are grown in close proximity to peroxidase-positive astroglia (a senescent glial phenotype).

MATERIALS AND METHODS

Materials

Neonatal Sprague-Dawley rats were obtained from Charles River Canada. Ham's F-12, high glucose Dulbecco's modified Eagle's medium (DMEM), and nerve growth factor (2.5s-NGF) were purchased from GIBCO BRL (Life Technologies, Burlington, Ontario). Horse serum and fetal bovine serum were purchased from WISENT (St-Bruno, Quebec). Cysteamine (CSH), dopamine, collagen, poly-D-lysine, anti-200kDa neurofilament antisera, anti-glial fibrillary acidic protein (GFAP) antisera, trypan blue and penicillin-streptomycin were obtained from Sigma Chemical Co. (St. Louis, MO.). HEPES, melatonin and rhodamine-conjugated goat anti-mouse secondary antibodies were obtained from ICN Biomedicals (Aurora, Ohio). Hydrogen peroxide (H_2O_2), ascorbic acid and 75 cm^2 Tissue culture flasks were obtained from Fisher Scientific Ltd. (Montreal, Qc, Canada). Ethidium monoazide bromide (EMA) was obtained from Molecular Probes, Inc. (Eugene, Or, USA). Eight-chamber culture slides (Lab-Tek) were purchased from Nunc (Napierville, IL, USA). PC12 rat pheochromocytoma cells were obtained from ATCC (Rockville, MD, USA). FITC anti-rabbit antisera and FITC conjugated goat anti-mouse antibody were purchased from Jackson Immunoresearch Lab (Baltimore, MD, USA). Anti-tyrosine hydroxylase (TH) antibody was obtained from Boehringer Mannheim (Laval, Qc. Canada). Resveratrol was a gift from Pharmascience Inc. (Montreal, Qc. Canada).

Primary Astrocyte Cultures

Primary neurological cell cultures were prepared by mechanoenzymatic dissociation of cerebral tissue obtained from 2 day old Sprague Dawley rat pups, as previously described (Schipper and Mateescu-Cantuniari, 1991): Rat pups were sacrificed by carbon dioxide inhalation, and sterilized by dipping them into 70% ethanol. The heads of the pups were decapitated using sterile operating scissors. The skin was then folded to the sides in order to expose the skull. Using a second pair of microdissecting scissors, the skull was cut along the midline fissure. The brain was then extracted from the head using sterile curved forceps and placed in DMEM where the cerebellum and the meninges were removed using sterile microdissecting forceps. The remaining brain material was cut into small pieces using a scalpel, and passaged successively through 10 ml, 5 ml, and fire-polished pasteur pipettes. The partially homogenized brain tissue was then incubated at 37^{o}C for 30 minutes in an equal volume of papain enzyme solution (7,4 mM $MgCl_2 \cdot 6H_2O$, 0.1% Papain, 0.01% deoxyribonuclease, 0.1% dispase II). The material was then resuspended 10 times through a fire-polished pasteur pipette and reincubated for an additional 30 minutes. After this time, brain tissue was pelleted by gentle centrifugation at 1000 rpm for 10 minutes. The tissue was then resuspended in an equal volume of DNAse solution (0.1% deoxyribonuclease), incubated for 30 minutes, and pelleted as before. Cells were grown in Ham's F12 and high glucose DMEM (50:50 v/v) supplemented with 10 mM HEPES, 5% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, and penicillin-streptomycin (50 U/mL and 50 µg/mL, respectively). The cells were plated in 75 cm^2 tissue culture flasks at a density of $1x10^6$ cells/mL. Cultures were incubated at

37°C in a humidified 95% air-5% CO_2 chamber for 6 hours after which time they were vigorously shaken 20-30 times and replaced with fresh media to remove adherent oligodendroglia and microglia from the astrocytic monolayers. The cultures were then incubated under the above-mentioned conditions for 6 days at which time more than 98% of the cells comprising the monolayer were astroglia as determined by immunohistochemical labeling for the astrocyte-specific marker, glial fibrillary acidic protein (GFAP) (Chopra *et al.*, 1995). Cultures were either left untreated (control) or received 880μM cysteamine with each change of culture medium (twice weekly) after 6 days *in vitro* (DIV). Stock solutions were freshly prepared by dissolving the CSH in DMEM and sterilized by filtering with a 0.22 μm Millipore membrane. The pH was adjusted between 7.2 and 7.4. This CSH regimen induces the accumulation of peroxidase-positive (iron-rich) cytoplasmic inclusions in cultured astroglia as previously described (Schipper *et al.*, 1990; Schipper and Mateescu-Cantuniari, 1991; Schipper *et al.*, 1991)

PC12 Cell Cultures

PC12 rat pheochromocytoma cells were obtained from ATCC and kept at -135°C in sterilized freezing media until plating. The cells were then thawed in warm H_2O for approximately 5 minutes and resuspended in 10 ml of fresh DMEM. Following resuspension, cells were centrifuged at 1000 rpm for 10 minutes and this step was repeated 3 times. Between each centrifugation, the test tube was repeatedly tapped for 1 minute to dissociate the cells from one another. When the tapping was not performed, the cells were clumped and were impossible to quantify. Subsequent to the last centrifugation,

the PC12 cells were plated on 35-mm-diameter Petri dishes pre-coated with 0.01% poly-D-lysine at a density of 10^5 cells/cm^2 (Letourneau, 1975; Yavin and Yavin, 1974) in DMEM as mentioned above. In order to control the proliferating state of the PC12 cells, the cells were sub-cultivated twice weekly and replated as mentioned above. As in the case of astroglial cultures, the PC12 cells were incubated at 37°C in a humidified 95% air-5% CO_2 chamber.

NGF Treatment

In some experiments, PC12 cells were treated with nerve growth factor (2.5s-NGF) in order to promote differentiation (neurite outgrowth). NGF has also been shown to confer protection to PC12 cells. The latter is thought to be due, in part, to a 2-3 fold induction of glutathione peroxidase and catalase expressed in these cells relative to untreated PC12 cell cultures (Sampath *et al.*, 1994; Jackson *et al.*, 1994). After resuspending the cells, they were plated at a density of 10^5 cells/cm^2 on collagen-coated 35-mm-diameter Petri dishes in DMEM as previously described (Greene and Tischler, 1976). When the cells were well dispersed and roughly 50% confluent, NGF was given with each change of medium at a concentration of 50 ng/mL for 6 days (Mobley *et al.*, 1976). After this time, neuritic extensions were observed using phase contrast and immunofluorescence microscopy (see below).

PC12 / Astrocyte Co-Cultures

Following 6 days of CSH treatment (DIV 12), both control and CSH pre-treated astrocytes were incubated for 24 hours in fresh DMEM at which time CSH can no longer be detected in cell homogenates by HPLC (Mydlarski *et al.*, 1995). The cells were subsequently trypsinized and plated on eight-chamber (LabTek) culture slides precoated with 0.01% poly-D-lysine at a density of $3.5x10^5$ cells/mL. The astrocytes were given an additional 24 hours to adhere to the bottom of the chamber slides before the PC12 cells were added. NGF-treated and non-NGF-treated PC12 cells were removed from the Petri dishes by gentle, repetitive pipetting of the culture media, concentrated by centrifugation (800 rpm for 10 mins.), resuspended in fresh DMEM, and plated on top of the astrocytes at a density of $8.7x10^3$ cells/mL. The co-cultures were incubated at 37^oC in a humidified 95% air-5% CO_2 chamber and allowed 48 hours to settle in preparation for the following cytotoxicity experiments.

Cytotoxicity Experiments

On DIV 16, PC12 cell cultures (non-pretreated and NGF-pretreated), astrocyte cultures (non-pretreated and CSH-pretreated), and PC12 / astrocyte co-cultures (combinations of the above pure cultures), were challenged with the following conditions: 1) dopamine (1μM) and H_2O_2 (1μM) (DH), 2) dopamine (1μM) alone, 3) H_2O_2 (1μM) alone, 4) DH with melatonin (100μM), 5) DH with ascorbic acid (200μM), 6) DH with

resveratrol (50μM), and DH with desferoxamine (400μM). All drugs were administered in pre-warmed culture media for 24 hours. The cultures were subsequently washed with 0.1*M* phosphate-buffered saline (PBS; pH 7.2) and stained with ethidium monoazide bromide (EMA;10μg/mL) for 10 minutes at room temperature under UV light exposure. Unlike standard ethidium bromide, EMA covalently binds to the DNA of dead nuclei thereby preventing post-fixation dye leakage (Martin *et al.*, 1994). Following further washes in PBS, the monolayers were fixed in 4% paraformaldehyde for 20 minutes at room temperature.

Immunofluorescence

After fixation, as described above, the cultures were washed in washing buffer (Sodium Phosphate 10mM, NaCl 0.5M, 0.2% Triton X and 0.5% BSA) for 10 minutes, rinsed three times in PBS, and incubated with 20μL of anti-tyrosine hydroxylase overnight. The secondary antibody consisted of rhodamine-conjugated anti-mouse antibody (diluted 1/50) incubated for one hour at 37^{o}C. Cultures were blocked with BSA for 30 minutes at room temperature. In some experiments, astrocytes were also immunolabeled with rabbit derived polyclonal anti-GFAP antiserum (1/50 dilution) over night at 4^{o}C followed by FITC goat anti-rabbit (1/50 dilution) for one hour at room temperature. When astrocytes were not stained, PC12 cells were identified with anti-200kDa neurofilament antisera and FITC goat anti-rabbit was used as the secondary antibody. In preparation for cell death quantification, the chamber slides were covered

with coverslips using a 50-50 mixture of glycerol and PBS and the coverslips were sealed with nail polish.

Cell Death Determination

Total PC12 cell numbers were determined in 25x fields under phase contrast microscopy. Dead cells were identified by the presence of bright red (EMA-positive) nuclear staining under epifluorescence microscopy (Leitz Diaplan Photomicroscope) using a rhodamine filter. The extent of cell death was expressed as the ratio of EMA-positive to total cells in each co-culture. In other experiments, we wanted to show the effect of clumped PC12 cells (as in the human brain) as opposed to dispersed PC12 cells. After challenging the culture, as described above, the media was replaced with an equal volume of trypan blue solution (0.4% trypan blue, 0.81% NaCl, and 0.06% K_2HPO_4). Due to the cell clumping, cells were not able to be quantified and data was only collected qualitatively.

Laser scanning confocal microscopy

The FITC-labeled slides were scanned using a Bio-Rad MRC-600 laser scanning confocal microscope. This system is equipped with a 15 mW krypton/argon laser which excites samples with lines at 488,568, and 647 nm. The excitation filter wheel was used in the dual excitator position which allows only the 488 and 568 nm lines of the laser to reach the sample. The emission wavelengths were detected from the samples using the K1 and K2 filter block set. The latter allows the detection of wavelengths between 522 and

554 nm on the green channel and 585 nm or greater on the red channel. Both channels were 70% open. Images scanned on the two channels (green and red) were merged to produce a single profile. After scanning, the images were collected using COMOS software package and stored on optical disk.

Statistics

Four sister cultures were evaluated for each experimental group. A minimum of 180 cells were assessed per culture. Statistical analysis was performed using a two-factor between subjects analysis of variance (ANOVA) test with $p<0.05$ indicating significance. A simple main effects test was performed to determine where the significance of the interaction term lies. A Tukey's post-hoc test was also used to assess the significant main effects within a given group.

RESULTS

Immunofluorescence and Confocal Microscopy

In the co-cultures, both control and CSH-pretreated astrocytes were stained with an antibody against GFAP (FITC-labeled) and PC12 cells were stained with an antibody against tyrosine hydroxylase (rhodamine-labeled; (Fig. 3a and 3b respectively). The astrocytes, which appeared green under fluorescence microscopy, were largely polygonal in shape and formed tight, "cobblestone"-like monolayers beneath the overlying PC12 cells. A minority of astroglia exhibited a stellate appearance and extended short, fine processes over adjacent cells in the glial substratum. The PC12 cells, which appeared red under fluorescence microscopy, were spherical in shape and occasionally elaborated short processes even in the absence of NGF-pretreatment. In cultures exposed to NGF for 6 days, a majority of PC12 cells elaborated long, thin processes which were usually branched and reminiscent of the dopaminergic neuronal phenotype. In the initial set of experiments, PC12 cells were added to the astroglial monolayers in a "dispersed" fashion at a relative ratio of 1 PC12 cell to 40 astrocytes. As depicted in figure 3, the morphology and growth characteristics of PC12 cells cultured atop control and CSH-pretreated astrocyte monolayers were indistinguishable from one another. Thus CSH-pretreatment of the astroglia, per se, has no appreciable effect on the growth of dispersed PC12 cells in this co-culture paradigm.

Cytotoxicity Experiments

(i) Qualitative Analysis

In order to visualize PC12 cell death in our co-cultures, nuclei of dead PC12 cells were stained with EMA which yields a strong red fluorescent signal. All PC12 cells were labeled with anti-TH using an FITC-conjugated secondary antibody (green fluorescence), and the astroglial substratum was left unstained. Thus, dead PC12 cells exhibited green cytoplasm and red nuclei, whereas live PC12 cells exhibited green cytoplasm and unstained (black) nuclei, under confocal microscopy (Fig. 4). When PC12 cells were grown atop control astroglia, and the co-cultures were left unchallenged, little PC12 cell death was observed (few EMA+ nuclei, Fig. 4, panel A). In panel B, when we cultured PC12 cells atop CSH-pretreated astrocytes and left the co-cultures unchallenged, we observed minimal PC12 cell killing (few EMA+ nuclei, Fig.4, panel B). As in panel A, when PC12 cells were cultured atop control astrocyte monolayers and challenged for 24 hours with dopamine and H_2O_2 (DH), little PC12 cell death was observed (few EMA+ nuclei, Fig. 4, panel C). However, when PC12 cells were cultured atop CSH-pretreated astroglia and challenged for 24 hours with DH, robust PC12 cell killing was observed as evidenced by the abundance of red, EMA+ nuclei (Fig. 4, panel D). In all cases of PC12 cell death, the EMA+ nuclei appeared large and round-to-ovoid in shape. Shrunken or compacted PC12 cell nuclei and apoptotic bodies were not encountered in these experiments. Thus the PC12 cell death following DH challenge is likely necrotic, rather than apoptotic, in nature.

In all of the aforementioned glial-neuronal co-cultures, astroglial nuclei did not stain red with EMA. Therefore, we can conclude based on the absence of EMA+

astrocytes, that little or no astroglial cell death occurred in these co-cultures even when subjected to DH challenge.

Figure 3: Representative micrographs of the PC12/astrocyte co-cultures.

In both control (A) and CSH-pretreated (B) co-cultures, GFAP-labeled astrocytes appear green and TH-labeled PC12 cells appear red. Growth characteristics of PC12 cells on control and CSH-pretreated astrocytes are indistinguishable from one another.

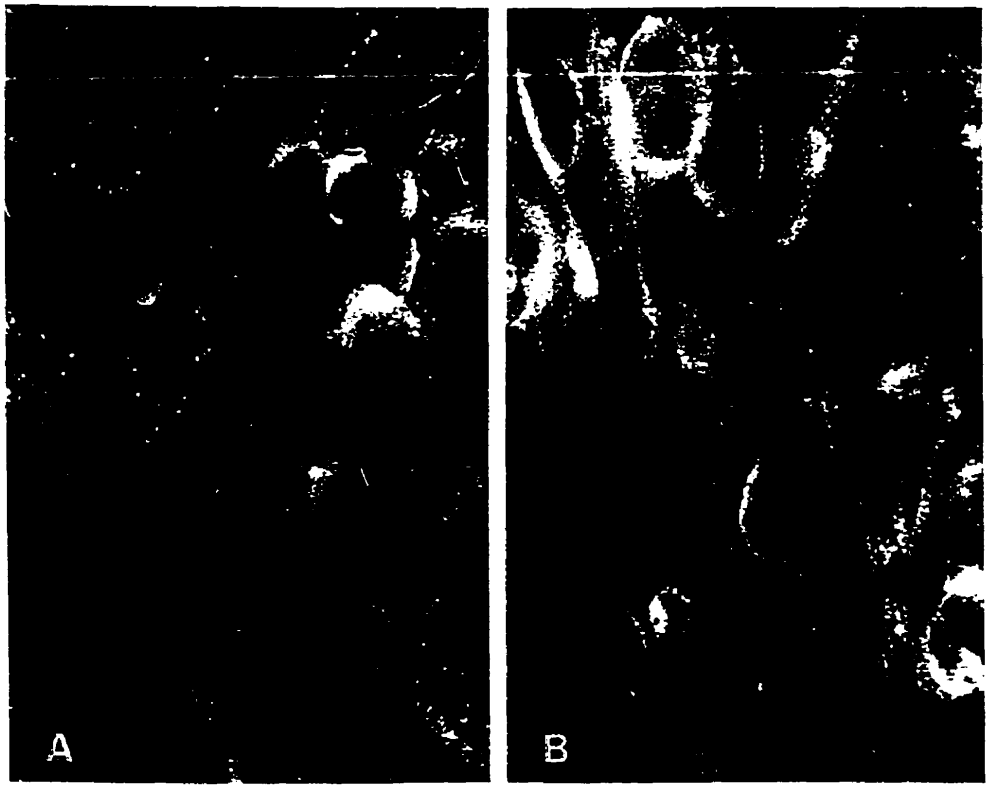
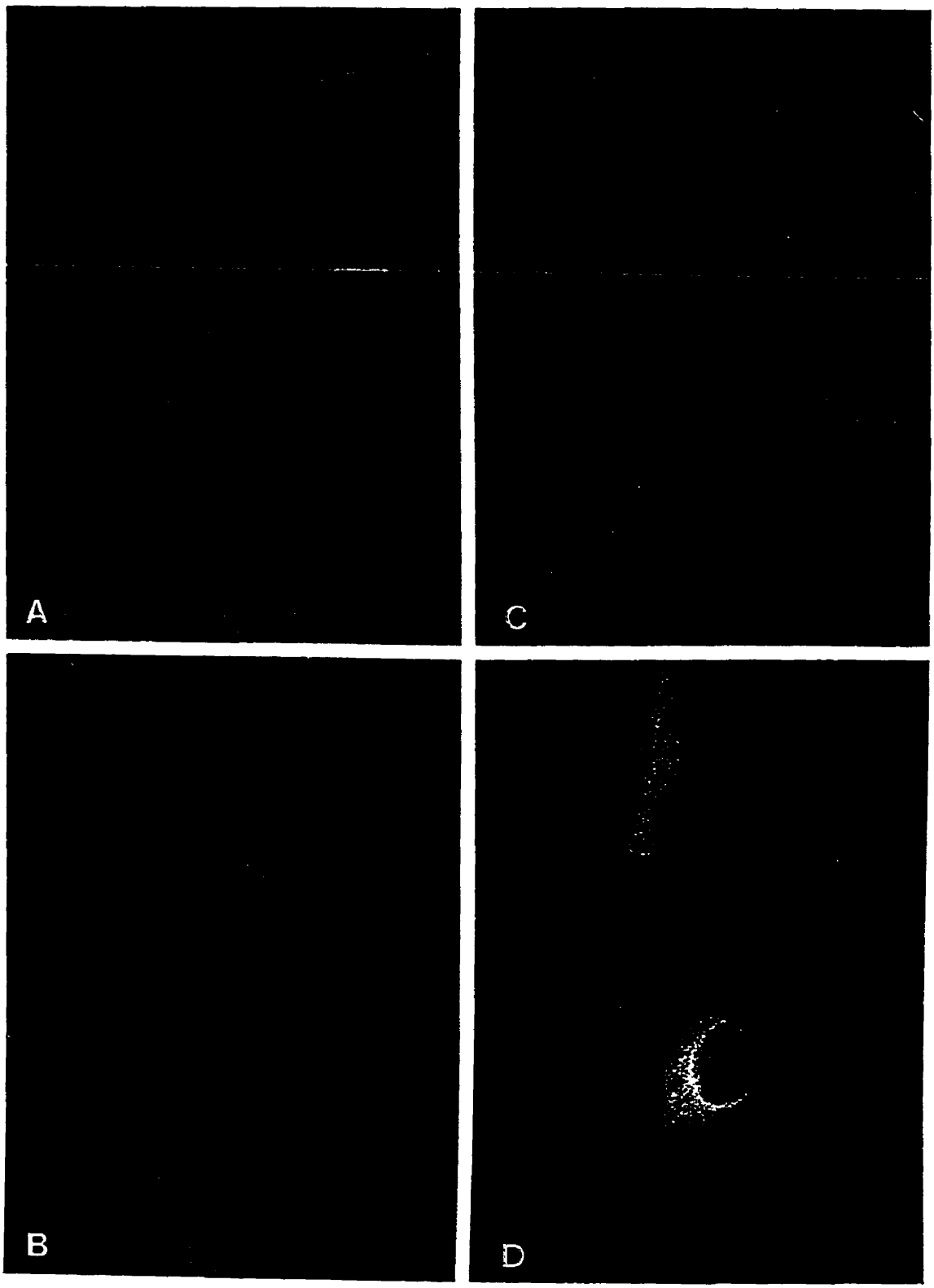

A
B

Figure 4: Effects of dopamine/H_2O_2 (DH) on PC12 cell viability.

PC12 cells are stained with anti-TH antibody (green). Dead cells are identified by nuclear EMA staining (red). Little or no PC12 cell death is present when these cells are grown atop control astrocytes in the absence (A) or presence (C) of DH challenge. PC12 cells grown atop CSH-pretreated astrocytes exhibit minimal death in the absence of DH challenge (B) but considerable death following DH exposure (D).

DH = dopamine (1μM) + H_2O_2 (1μM).


A
C
B
D

(ii) Quantitative analysis

We observed that PC12 cells grown on the surface of CSH-pretreated (iron enriched) astroglia were far more susceptible to dopamine/H_2O_2-related killing than PC12 cells cultured atop control glial substrata (Fig. 5). We then challenged the co-cultures with either 1μM of dopamine alone or 1μM of H_2O_2 alone. Although there was more PC12 cell killing than non-challenged controls ($p<0.05$ for dopamine and $p< 0.01$ for H_2O_2), there was far less killing than when dopamine and H_2O_2 were administered together ($p<0.01$ relative to column 4 (Fig. 5) for both dopamine and H_2O_2 alone). These data suggest that it is the conversion of dopamine into its orthosemiquinone radical (using H_2O_2 as a substrate) which is largely responsible for the PC12 cell killing and not merely the toxic effects of dopamine or H_2O_2 alone.

In order to determine whether the PC12 cell killing in our cultures was due to oxidative stress, we repeated the aforementioned experiments in the presence and absence of potent antioxidants (Fig. 5). When melatonin (100 μM) was administered to the DH-challenged co-cultures, there was a significant attenuation of PC12 cell killing when cultured atop CSH-pretreated astrocytes ($p<0.01$ relative to CSH-pretreated co-cultures challenged with DH alone). Furthermore, the protective effects of melatonin were equivalent when PC12 cells were cultured atop control or CSH-pretreated astrocytes. Co-incubation of DH with ascorbic acid (200 μM) yielded practically identical results as the melatonin experiment; *viz.*, ascorbic acid significantly attenuated DH-related PC12 cell killing in our CSH-pretreated co-cultures ($p<0.01$ relative to CSH-pretreated co-cultures challenged with DH alone). When ascorbic acid was added to DH-challenged co-cultures

there was no significant difference ($p>0.05$) when the PC12 cells were cultured atop control or CSH-pretreated astroglial monolayers. Finally, the grape-derived antioxidant, resveratrol (50 μM) , was also highly effective in protecting PC12 cells grown atop CSH-pretreated astrocytes from DH challenge ($p<0.01$ relative to CSH-pretreated co-cultures challenged with DH alone). These results support our hypothesis that DH-related PC12 cell killing in our co-culture paradigm was due to oxidative stress. In order to ascertain the role of iron in our model, desferoxamine (DFO; 400 μM), an iron chelator, was added to the DH-challenged cultures. Similar to the antioxidant effects, DFO attenuated PC12 cell killing cultured atop CSH-pretreated astroglia when challenged with DH for 24 hours ($p<0.01$). There was, however, a significant difference between PC12 cell killing when the latter were cultured atop control or CSH-pretreated astrocytes and subjected to DH-challenge even in the presence of desferoxamine ($p<0.01$). The protective effects of DFO in our co-culture paradigm attest to the role of iron in the conversion of dopamine into potentially neurotoxic semiquinone radicals (a pseudoperoxidase reaction).

Pretreatment of PC12 cells with nerve growth factor (NGF; 50 μg/ml) for six days was used in order to mimic the dopaminergic phenotype (Fig. 6). Although a greater vulnerability of NGF-pretreated PC12 cells to oxidative challenge was observed when cultured atop CSH pre-treated, as opposed to control astrocytes ($p<0.01$), the extent of PC12 cell death in these co-cultures was significantly less than that observed in non-NGF-exposed PC12 populations (Fig. 7). As in the case of the non-NGF-pretreated PC12 cells, when melatonin (100μM) was co-incubated with DH-challenged co-cultures, melatonin significantly decreased DH-related PC12 cell killing when the latter were cultured atop

CSH-pretreated astrocytes ($p<0.01$). The effects of melatonin were similar in both control and CSH-pretreated astroglial co-cultures challenged with DH. Co-incubation of DH with ascorbic acid (200 μM) diminished PC12 cell killing when cultured atop CSH-pretreated astrocytes ($p<0.01$). In contradistinction to the non-NGF-pretreated PC12 cell co-cultures, there was no significant difference between DH-related PC12 cell death when the latter were cultured atop control or CSH-pretreated astroglial monolayers when ascorbate was added. When resveratrol (50 μM) was co-administered with the DH-challenge, the antioxidant significantly protected against PC12 cell killing ($p<0.01$). However, it differed from non-NGF-pretreated PC12 cells in that there was no significant difference between DH-related PC12 cell killing when the latter were cultured atop control or CSH-pretreated astrocytes in the presence of resveratrol. These results further support our hypothesis that the PC12 cell killing in our co-culture paradigm was due to oxidative stress. These data are in agreement with earlier reports that NGF protects PC12 cells from oxidative challenge (Sampath *et al.*, 1994).

In order to delineate further the role of iron in our cultures, DFO (400 μM) was added to DH-challenged cultures containing NGF-pretreated PC12 cells. DFO significantly attenuated PC12 cell killing atop CSH-pretreated astroglia after challenge of the co-culture with DH for 24 hours ($p<0.01$). There was no significant difference between PC12 cell killing when cultured atop control or CSH-pretreated astrocyte monolayers in the presence of DFO ($p>0.05$). These data further support the role of iron in the non-enzymatic conversion of dopamine into potentially neurotoxic semiquinone radicals.

Figure 5: Effects of DH challenge on PC12/astrocyte co-cultures.

Quantitation of PC12 cell death in co-cultures of (I) PC12 cells atop control astrocytes (□) and (II) PC12 cells atop CSH-pretreated astrocytes (■) under the following conditions (n = 4 for each group). 1,2 = unchallenged; 3,4 = dopamine (1μM) + H_2O_2 (1μM) [DH]; 5,6 = dopamine (1μM); 7,8 = H_2O_2 (1μM); 9,10 = DH + melatonin (100μM); 11,12 = DH + ascorbate (200μM); 13,14 = DH + resveratrol (50μM); 15,16 = DH + deferoxamine (400μM). # = $p<0.05$; ## = $p<0.01$; * = $p<0.001$ relative to column 4; NS = Not Significant. See text for details of treatment protocols and cell death assay.

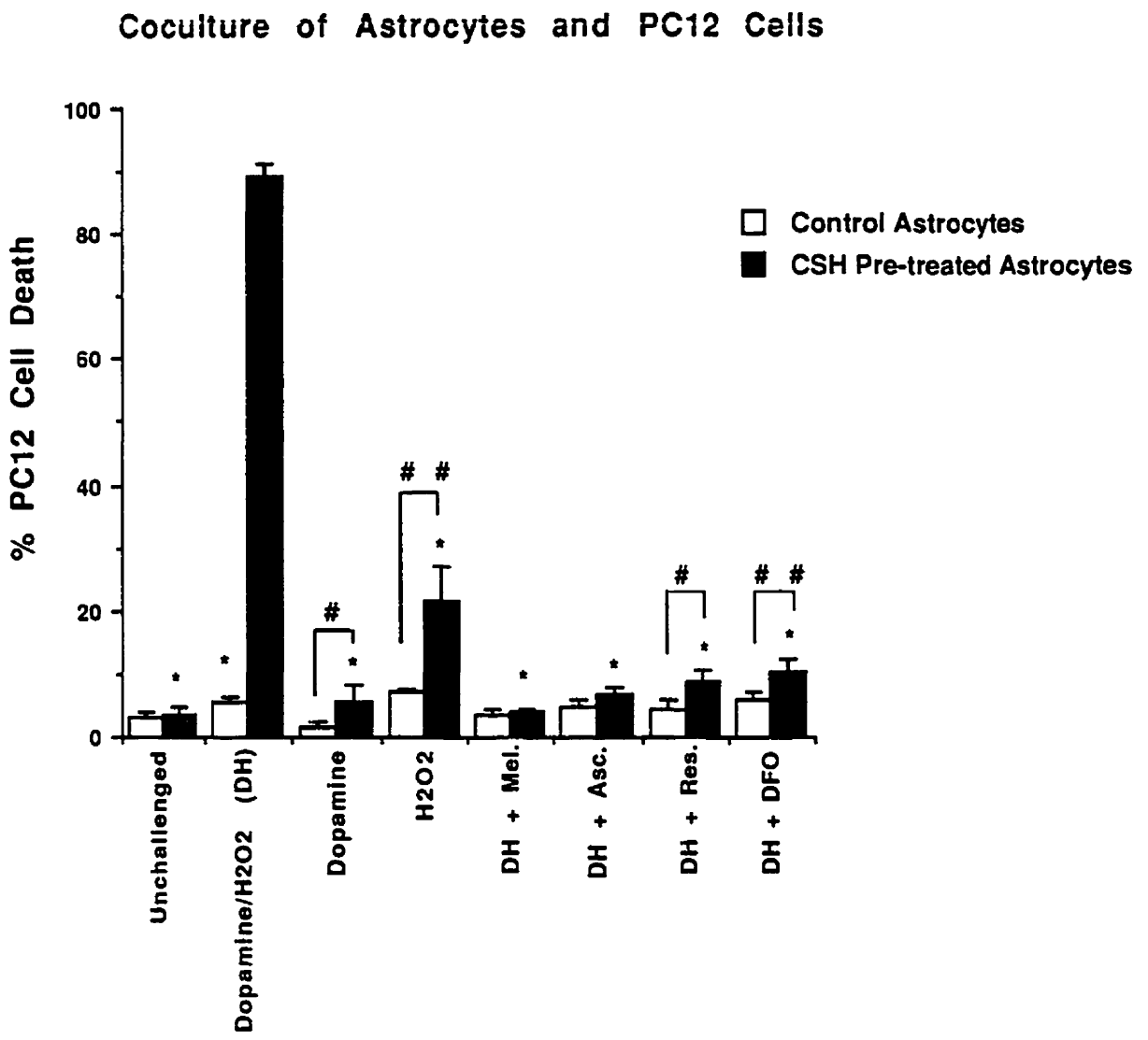

Coculture of Astrocytes and PC12 Cells
% PC12 Cell Death
100
80
60
40
20
0
Control Astrocytes
CSH Pre-treated Astrocytes
Unchallenged
Dopamine/H2O2 (DH)
Dopamine
H2O2
DH + Mel.
DH + Asc.
DH + Res.
DH + DFO

Figure 6: Effects of NGF on PC12 cell morphology in PC12/astrocyte co-cultures.

Under light microscopy, PC12 cells exhibited neuritic extensions within six days of NGF treatment. PC12 cell neuritic outgrowth was indistinguishable between unchallenged control (A) and CSH-pretreated monolayers (B) (unstained)

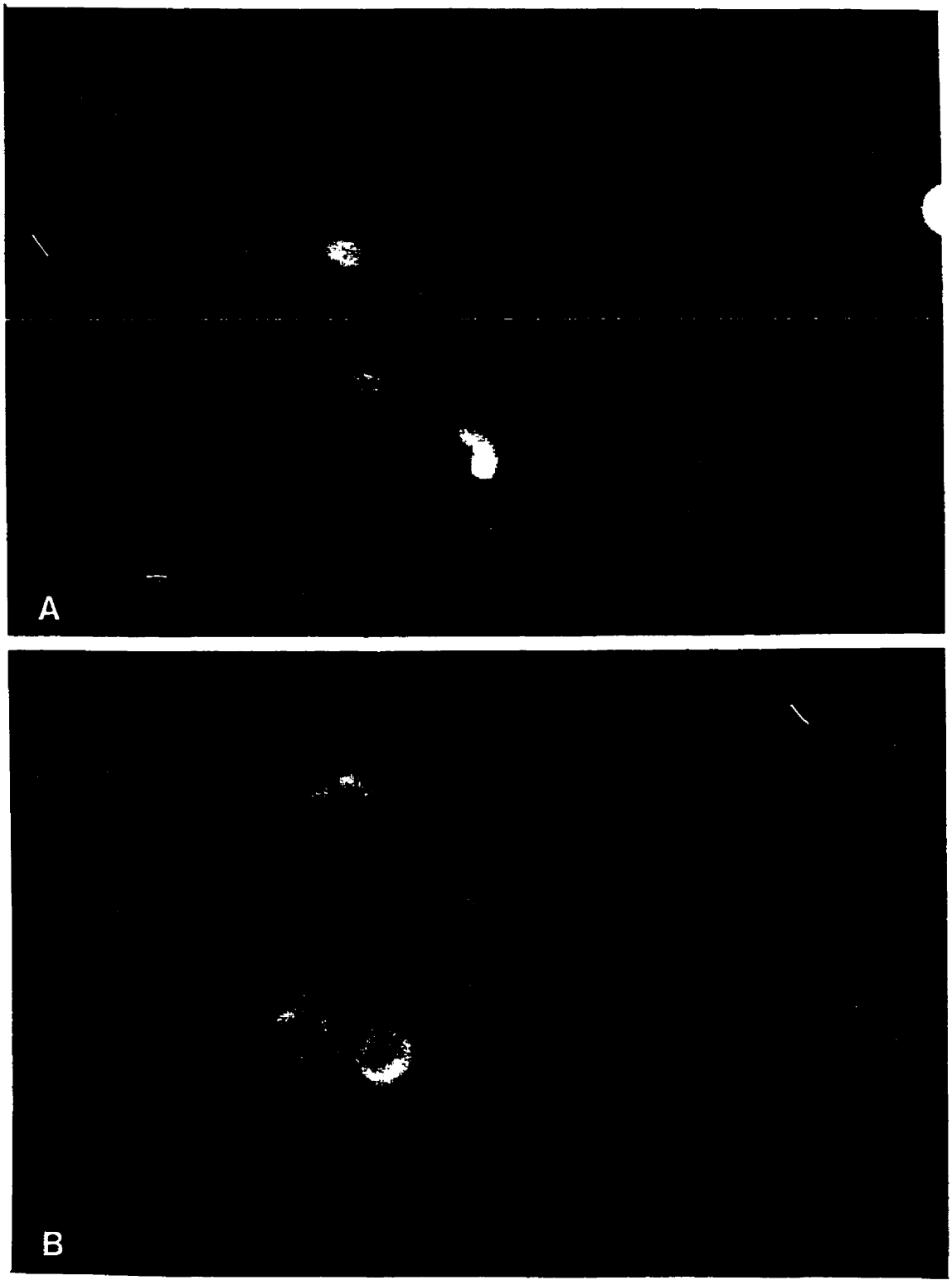
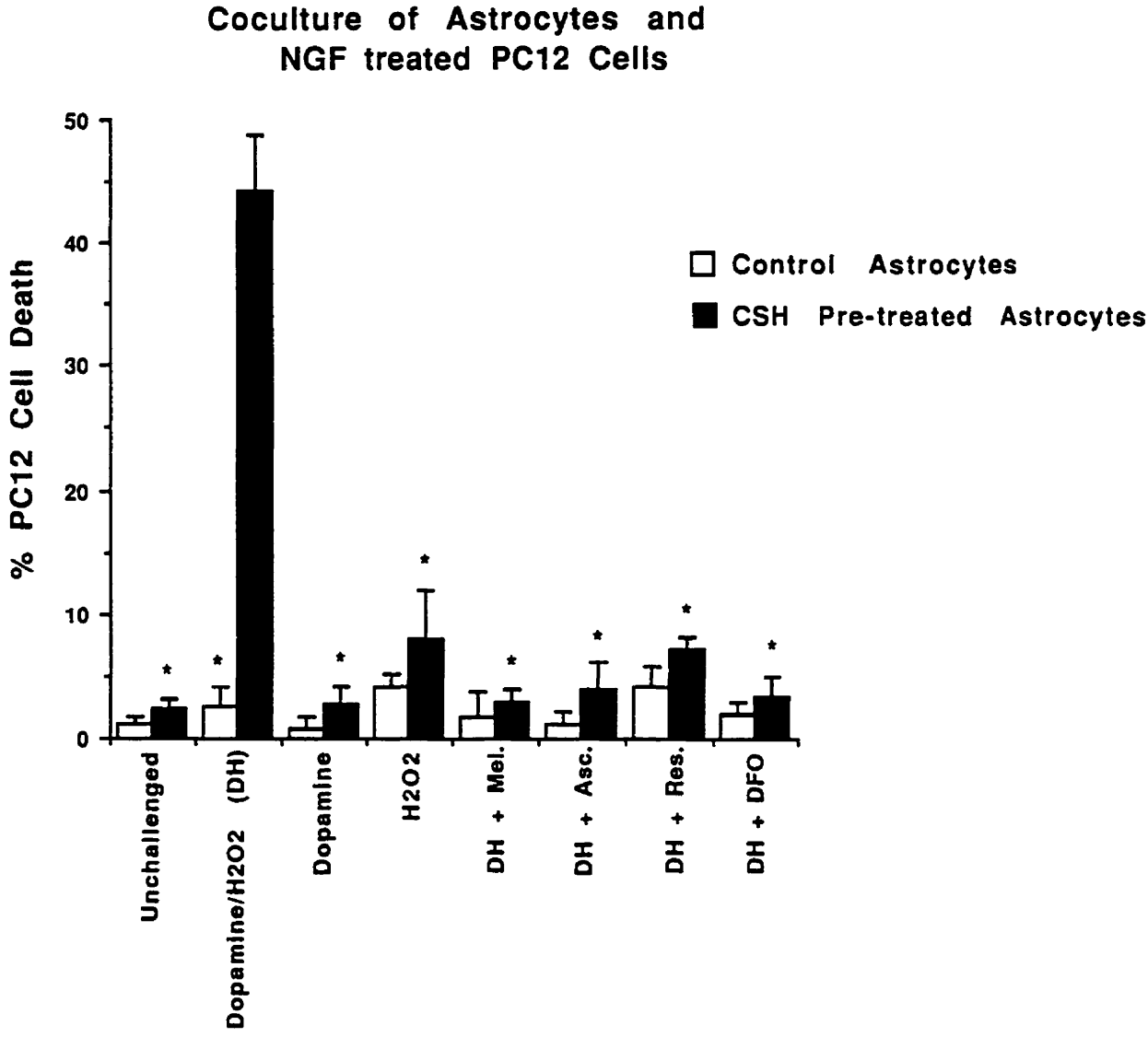

A
B

Figure 7: Effects of DH challenge on death of NGF-pretreated PC12 cells in PC12-astroglial co-cultures.

Quantitation of PC12 cell death in co-cultures of (I) NGF-pretreated PC12 cells atop control astrocytes (□) and (II) NGF-pretreated PC12 cells atop CSH-pretreated astrocytes (■) under the following conditions (n = 4 for each group). 1,2 = unchallenged; 3,4 = dopamine (1μM) + H_2O_2 (1μM) [DH]; 5,6 = dopamine (1μM); 7,8 = H_2O_2 (1μM); 9,10 = DH + melatonin (100μM); 11,12 = DH + ascorbate (200μM); 13,14 = DH + resveratrol (50μM); 15,16 = DH + deferoxamine (400μM). # = $p<0.05$; ## = $p<0.01$; * = $p<0.001$ relative to column 4; NS = Not Significant. See text for details of treatment protocols and cell death assay.

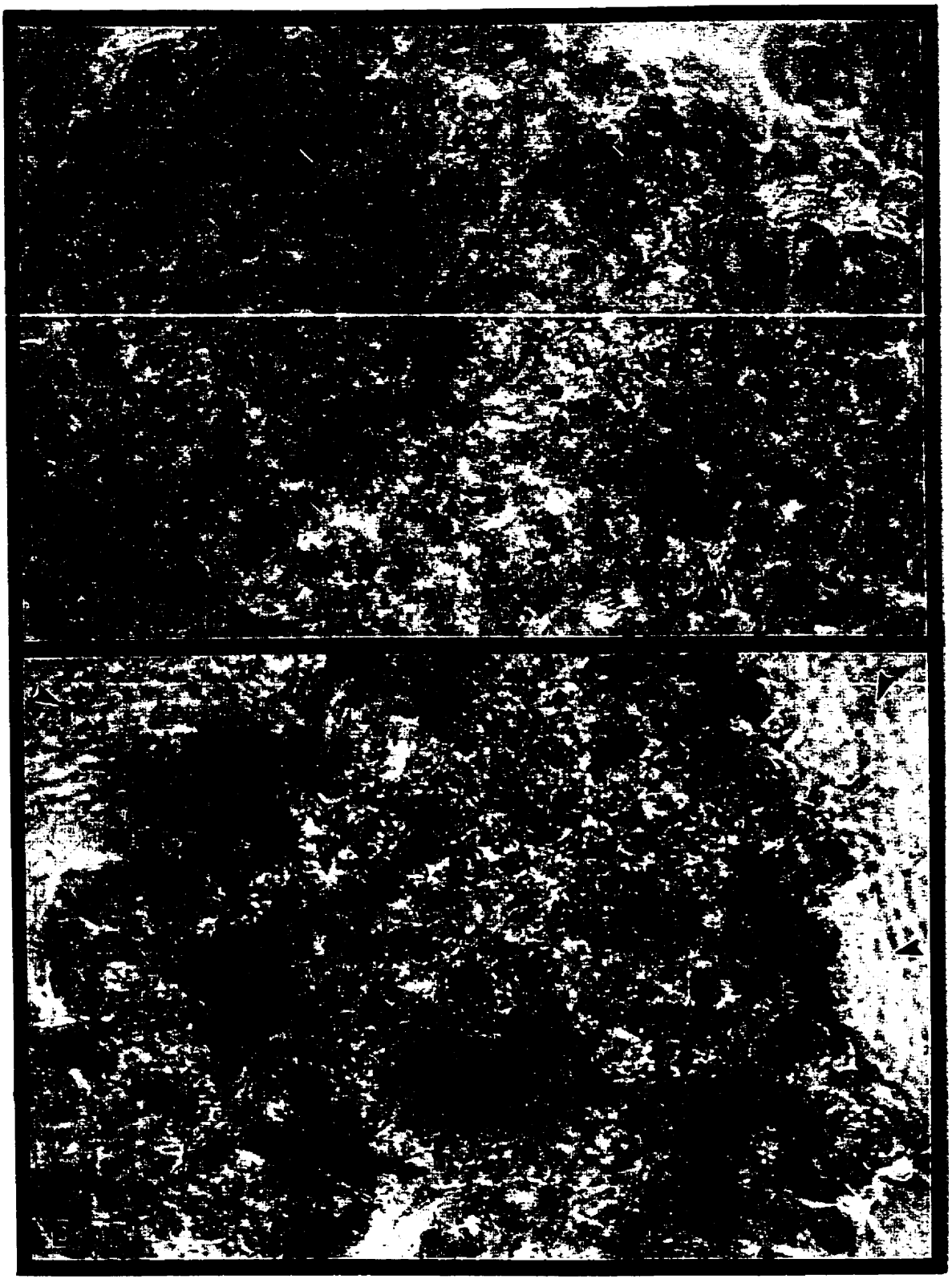

Coculture of Astrocytes and
NGF treated PC12 Cells
% PC12 Cell Death
50
40
30
20
10
0
Control Astrocytes
CSH Pre-treated Astrocytes
Unchallenged
Dopamine/H2O2 (DH)
Dopamine
H2O2
DH + Mel.
DH + Asc.
DH + Res.
DH + DFO

Light Microscopy

In other experiments, trypan blue was used to assess the viability of high-density ("clumped") PC12 cells when cultured atop control and CSH-pretreated astrocytes. The ability of PC12 cells to exclude the vital dye, trypan blue, was greater when these cells were cultured on top of control astrocytes than on CSH-pretreated astrocytes (Fig. 8) In light of the dense PC12 cell clumping, it was impossible to quantitate PC12 cell death and the data were analyzed in a qualitative fashion. Growth characteristics of PC12 cells atop control or CSH-pretreated astrocytes were identical to each other in the absence of DH-challenge. Few PC12 cells retained trypan blue (died) following DH-challenge when the PC12 cells were cultured atop control astroglial monolayers. In contrast, when the PC12 cells were cultured atop CSH-pretreated astrocytes and challenged with DH for 24 hours, large patches of PC12 cells retained the trypan blue dye (died). These data again support our hypothesis that PC12 cells are more vulnerable to DH-challenge when cultured atop CSH-pretreated astrocytes than when cultured atop healthy, young, control astroglia. Furthermore, both the control and CSH-pretreated astroglial monolayers themselves did not retain vital dye in these experiments indicating that the glial monolayers themselves were largely unaffected by the DH-challenge.

Figure 8: Trypan blue retention (cell death) in PC12 cells grown at high density atop confluent monolayers of control (A) and CSH-pretreated (B) astrocytes.

The co-cultures were challenged with dopamine (1μM) and H_2O_2 (1μM) for 24 hours. There are relatively few trypan blue-positive PC12 cells when cultured atop control glial monolayers (Panel A). In contrast, large patches of trypan blue-positive PC12 cells are observed when these cells are cultured atop CSH-pretreated astrocytes (Panel B). Little or no astroglial cell death was observed in either co-culture following DH-challenge (arrows).



DISCUSSION

I - Catecholamine Neurotoxicity

The catecholamine, dopamine, can be readily oxidized to generate reactive oxygen intermediates (Ben-Shachar *et al.*, 1995) and has been shown to be toxic to various mammalian cells in culture (Rosenberg, 1988; Michel *et al.*, 1990). Furthermore, dopamine is thought to act as an endogenous neurotoxin which may be responsible for the striatal neuropathology observed in various disease states including systemic methamphetamine treatment (Schmidt *et al.*, 1985) and brain ischemia (Weinberger *et al.*, 1985; Buisson *et al.*, 1992). Although the mechanism of dopamine action needs to be further elucidated, neuronal cell death resulting from exposure to dopamine is thought to occur through a free radical mechanism of action either spontaneously, in the presence of transition metals (Graham, 1978), or via enzyme-catalyzed reactions (Hastings, 1995). In support of this hypothesis, numerous studies have shown that co-administration of antioxidants, such as ascorbate or glutathione, significantly attenuate dopamine-induced cell death in primary mesencephalic neuronal cultures (Iacovitti *et al.*, 1997) and in several catecholamine-secreting cell lines (Chanvitayapongs *et al.*, 1997). The dopamine precursor, L-dopa, a mainstay of treatment of PD, has also been shown to be toxic to cells *in vitro* (Fahn, 1997) and can participate in the generation of oxidative stress (Spina *et al.*, 1988; Spencer *et al.*, 1994). Free radical intermediates may be derived from direct oxidation of L-dopa or after its metabolism to dopamine (Spina *et al.*, 1988; Spencer *et al.*, 1994; Spencer *et al.*, 1995). In one study, L-dopa exposure was reported to

exacerbate depletion of nigrostriatal neurons in MPTP pretreated mice (Fukuda *et al.*, 1996).

On the other hand, recent evidence suggests that L-dopa may, under certain circumstances, protect neurons cultured atop astroglial monolayers from oxidative insults, an effect that is independent of the conversion of L-dopa to dopamine (Mena *et al.*, 1997). There is evidence that L-dopa may exert its neurotrophic effects on surrounding neurons by augmenting GSH synthesis (Yan et al., 1995). However, it is not yet known to what extent these protective effects are due to i) L-dopa itself (acting as an antioxidant), ii) the upregulation of GSH biosynthesis or iii) stimulation of other cellular antioxidant defences. In view of the contradictory data cited above, it remains highly controversial whether or not L-dopa replacement therapy may accelerate nigrostriatal cell degeneration in PD subjects (Fahn *et al.*, 1998).

In the present study, we observed that combined challenge with dopamine (1 μM) and H_2O_2 (1μM) was more toxic to PC12 cells than when dopamine or H_2O_2 was administered alone. These data suggest that it is the conversion of dopamine into its orthosemiquinone radical (using H_2O_2 as a substrate in a peroxidase-like reaction) that is largely responsible for the PC12 cell killing, and not merely the toxic effects of dopamine or H_2O_2 alone. This observation is in accordance with other studies showing that dopamine and norepinephrine can be oxidized to semiquinones with proven neurotoxic capabilities via peroxidase-mediated reactions (Metodiewa *et al.*, 1989). In the presence of monoamine oxidase-B (MAO-B), dopamine is metabolized to DOPAC and ammonia and the pro-oxidant species, H_2O_2. In the presence of ferrous iron, H_2O_2 can undergo a Fenton

reaction leading to the production of hydroxyl radicals, one of the most damaging free radicals generated in living tissues (Goldfischer, et al., 1966; Graham, 1978; Schipper, et al., 1991). In both the human and rat brain, iron-rich glial inclusions progressively accumulate with advancing age (Schipper, *et al.*, 1981). The glial iron manifests intense, non-enzymatic peroxidase activity and is readily stained with diaminobenzidine (DAB) in the presence of H_2O_2 (Srebro *et al.*, 1971). These redox-active glial granules may therefore play an important role in the generation of free radicals in the course of natural brain aging and in neurodegenerative diseases. It is conceivable that H_2O_2, produced from dopamine oxidation in the presence of MAO-B, may serve as a co-factor for further dopamine oxidation (to neurotoxic semiquinone radicals) by peroxidase-mediated reactions in these senescent astroglia. In addition to dopamine, redox-active glial iron may also facilitate the non-enzymatic oxidation of a) the protoxin MPTP, to the dopaminergic toxin, MPP+, in the presence of MAO inhibitors (DiMonte *et al.*, 1995), and b) the dopamine precursor, DOPA, to 2,4,5-trihydroxyphenylalanine (TOPA) and the non-NMDA excitotoxin, TOPA-quinone (Newcomer *et al.*, 1995).

II - Neuroprotective Effects of Astroglia

Astrocytes perform a wide range of adaptive and supportive functions in the central nervous system. Some astrocytes extend their processes to the surface of the CNS to form a glial membrane that serves as a protective covering for the CNS (Kandel and Schwartz, 1991). Astrocytic "end feet" induce tight junctions between adjacent endothelial cells and constitute the blood brain barrier (Kandel and Schwartz, 1991).

Astrocytes are responsible for the production of proinflammatory and immunomodulatory cytokines, metabolism of various neurotransmitters and they contribute to the maintenance of ion homeostasis (Hertz, 1981; Fedoroff and Vernadakis, 1986a; Wilkin *et al.*, 1990; Plata-Salaman, 1991; Benveniste, 1992). Astrocytes are also thought to be involved in the guidance and migration of neuronal cells and growth cones during embryogenesis (Rakic, 1971; Silver and Shapiro, 1980; Silver et al., 1982) and are thought to be instrumental in the recovery process after CNS injury (Smith et al., 1986). Taken together, these data suggest that astrocytes subserve protective and restorative roles in the injured nervous system.

The results of the present study support earlier reports (Vernadakis, 1988) that healthy, neonatal (control) astrocytes protect PC12 cells from oxidative challenge. As mentioned above, when DH was added to the PC12 cells alone (i.e. in the absence of astroglia) virtually all the PC12 cells were destroyed. On the other hand, when the PC12 cells were cultured atop control, healthy astrocytes and challenged with dopamine and H_2O_2 for 24 hours, minimal PC12 cell death was observed. These observations confirm that young, healthy astroglia confer cytoprotection to co-cultured PC12 cells in the face of DH challenge.

The mechanism by which astrocytes exhibit their cytoprotection is not fully understood but may be due to their antioxidant capabilities, their ability to release growth-promoting substances, or a combination of these factors. Astrocytes have been shown to produce and release neurotrophic and neurite outgrowth-stimulating factors including nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF) and S-100 protein

(Montgomery, 1994). In some cases, glial cells have been shown to mediate the neurotrophic action of fibroblast growth factors which enhances the release of NGF from astrocytes (Engele and Bohn, 1991). In this pathway, NGF is synthesized in the astrocytes, secreted into the extracellular space, taken up by neurons through receptor-mediated mechanisms and retrogradely transported to the cell somas (Levi-Montalcini, 1987; Thoenen *et al.*, 1987). Recent studies have demonstrated increased neuronal cell death when cultured neurons are physically separated from underlying astroglial monolayers; neuronal death in this model was significantly suppressed following treatment with GDNF (Kimura *et al.*, 1998).

GSH is the most important non-enzymatic intracellular antioxidant and serves to quench H_2O_2 produced as an end-product of dopamine oxidation mediated in glial cells by monoamine oxidase-B. Reduced GSH is oxidized to GSSG, and unless there is an excess production of H_2O_2, is immediately reduced back to GSH by glutathione reductase thereby maintaining optimal tissue ratios of GSH and GSSG (Olanow, 1990). Although GSH is mainly found in the cytosolic fraction, about 10% of GSH is localized to the mitochondria (Reed, 1990). With glutathione peroxidase and glutathione reductase present in this organelle as well, a system is in place for the intramitochondrial detoxification of H_2O_2 (Reed, 1990). In addition, mitochondrial GSH plays an important role in maintaining mitochondrial thiols in their reduced states (Sandri, *et al.*, 1990). Both neurons and astrocytes contain high levels of glutathione (Hjelle *et al.*, 1994; Langeveld *et al.*, 1996; Makar *et al.*, 1994), however, neurons are believed to be dependent on astrocytes for the maintenance of their glutathione levels (Sagara *et al.*, 1993) possibly via uptake of the

glutathione precursor, cysteine, from the astrocytes (Kranich *et al.*, 1996; Sagara *et al.*, 1993). Astrocytes can release glutathione into the extracellular milieu and thereby protect surrounding neurons from oxidative stress (Sagara *et al.*, 1996; Yudkoff *et al.*, 1990). Challenging glial-neuronal co-cultures with H_2O_2 resulted in increased neuronal cell death when the latter were cultured atop glutathione-depleted astroglial monolayers (Drukarch *et al.*, 1997). Thus, antecedent exposure of astroglia to oxidative stress may deplete glial GSH and thereby predispose to concomitant or subsequent neuronal injury. A decrease in astrocyte glutathione levels may also disrupt glial glutamate uptake and render neurons increasingly vulnerable to excitotoxicity (Bridges *et al.*, 1991; Murphy *et al.*, 1990).

On the other hand, Desagher and co-workers suggested that the antioxidant, catalase, is primarily responsible for neuronal protection by astrocytes (Desagher *et al.*, 1996). They used aminotriazole (ATZ) to irreversibly inactivate catalase and demonstrated increased H_2O_2-related neuronal cell death when the neurons were cultured on top of catalase-deficient astrocytes. Recent studies corroborating the neuroprotective effects of astroglia have demonstrated that astrocytes protect cultured neurons from toxic insults via a glutathione-dependent mechanism (Dringen *et al.*, 1998). Others have shown that astrocytes diminish the sensitivity of co-cultured neurons to radiation-induced cytotoxicity via the release of an ill-defined soluble protein (Noel *et al.*, 1998). Finally, in addition to the aforementioned *in vitro* studies demonstrating astroglial protection, current studies have reported *in vivo* neuroprotection by astrocytes co-grafted with fetal mesencephalic dopaminergic neurons (Pierret *et al.*, 1998). Taken together, these studies indicate that astroglia confer major antioxidant protection to nearby neurons although the extent to

which GSH, catalase or some other antioxidant system(s) contribute to this cytoprotection remains to be elucidated.

III - Dystrophic Effects of Astroglia

As described in the previous section, there currently exists a large body of literature attesting to the beneficial roles of astrocytes in neuronal survival and neuritic outgrowth. However, virtually all of these effects were demonstrated in experiments using healthy, young astrocytes. Far less is known regarding the abnormal behavior of "stressed" astroglia in the aging and diseased nervous system. For example, as mentioned above, while ATP-dependent uptake of extracellular glutamate by healthy astroglia is an "advantage," failure of this glial mechanism in ischemic brain may represent a serious "disadvantage" predisposing the latter to excitotoxic neuronal injury (Choi and Rothman, 1990). Similarly, progressive mitochondrial damage, sequestration of redox-active transition metals, and augmentation of H_2O_2-generating MAO-B activity in subpopulations of astroglia may render the senescent nervous system particularly prone to oxidative injury while astrocytes in young, healthy brain exhibit properties that are purely adaptive in nature.

Various dystrophic effects exhibited by stressed astroglia include: 1) Following tissue hypoxia, oxidative stress and metal exposure, astrocytes may fail to take up extracellular glutamate and thereby predispose the neuronal compartment to excitotoxic injury (Choi and Rothman, 1990). 2) In response to CNS injury, astrogliosis may lead to the formation of epileptogenic scar tissue. 3) Astrocytes can metabolize protoxins such as,

MPTP, into potent neurotoxins (MPP+) (Albrecht, *et al.*, 1991; Chan *et al.*, 1988; Fedoroff *et al.*, 1986; Langston, 1985). 4) Reactive gliosis is a common pathological feature of the major human neurodegenerative disorders, including Alzheimer's disease (AD), PD, Huntington's disease and amyotrophic lateral sclerosis. Reactive gliosis is characterized by astrocyte hypertrophy, accumulation of glial fibrillary acidic protein (GFAP)-positive intermediate filaments, and possibly hyperplasia (Beach *et al.*, 1989; Duffy *et al.*, 1980; Kushner *et al.*, 1991; Schipper *et al.*, 1993; Tomlinson *et al.*, 1984). Reactive gliosis is accompanied by increases in monoamine oxidase-B (MAO-B) activity in the aging mammalian brain, in AD, and in experimental models of PD. An increase in MAO-B activity can promote the production of excessive H_2O_2 via dopamine oxidation (as described above) promoting further neuronal injury (Saura *et al.*, 1994; Wang *et al.*, 1994). 5) Finally, as in the case of hepatic encephalopathy and certain rare neurodegenerative diseases, astrocytes may exhibit specific cytopathological changes suggesting that they may be the primary targets of the disease process (Norenberg *et al.*, 1974; Seitelberger, 1988).

In the present study, we observed that PC12 cells grown on the surface of iron enriched (senescent-like) astroglia were far more susceptible to dopamine/H_2O_2-related killing than PC12 cells cultured atop control glial substrata. Augmented killing of PC12 cells in the former paradigm was inhibited with potent antioxidants (melatonin, ascorbate, resveratrol) and an iron chelator (DFO) implicating a free radical mechanism of action. These data support the notion that a senescent astroglial phenotype (simulated by CSH-pretreatment) may manifest "dystrophic" properties not observed in their young, healthy

counterparts. In the presence of nerve growth factor we still observed a greater vulnerability of PC12 cells to oxidative challenge when cultured atop CSH pre-treated astrocytes, although the extent of PC12 cell death in these co-cultures was significantly less than that observed in non-NGF-exposed PC12 populations. Although the exact mechanism of growth factor protection needs to be further elucidated, NGF has been shown to increase neuronal catalase activity and glutathione levels which may contribute significantly to enhanced neuronal survival (Jackson *et al.*, 1994; Sampath *et al.*, 1994).

IV - Dopamine Oxidation by Peroxidase-Positive Astrocytes in Primary Culture

Schipper and co-workers used electron spin resonance spectroscopy (ESR) to assess whether the peroxidase activity (mitochondrial iron deposition) in CSH-pretreated cells was capable of oxidizing catecholestrogens and catecholamines to their respective orthosemiquinone radicals (Schipper *et al.*, 1991). Low-amplitude or no o-semiquinone spectra were observed when control astroglial homogenates were incubated with 2-hydroxyestradiol in the presence of H_2O_2 and NADPH co-factors. However, incubation of equimolar concentrations of 2-hydroxyestradiol with CSH-pretreated astroglial homogenates produced high-amplitude o-semiquinone spectra indicative of robust catechol oxidation. In contrast, they observed a marked reduction in signal amplitude in the absence of H_2O_2 substrate attesting to the role of glial peroxidase activity in the oxidative metabolism of catecholestrogens (Schipper *et al.*, 1991).

Analogous to the case of 2-hydroxyestradiol, Schipper *et al*, demonstrated that the peroxidase activity induced by CSH-pretreatment significantly enhances the oxidation of

the catecholamine, dopamine, into its dopamine-o-semiquinone radical in the presence of H_2O_2 (Schipper *et al.*, 1991). This latter observation is in accordance with others who have shown that dopamine and norepinephrine can be oxidized to semiquinones with proven neurotoxic capabilities via peroxidase-mediated reactions (Metodiewa *et al.*, 1989).

In the present study, dopamine and H_2O_2 (DH) were used to challenge the PC12 cell/astrocyte co-cultures. PC12 cells cultured atop CSH-pretreated (peroxidase-positive) astrocytes were more vulnerable to DH-challenge than when cultured atop healthy, control astroglial monolayers. This study is in agreement with the previously mentioned ESR data and it is therefore conceivable that the oxidation of dopamine by the peroxidase positive astrocytes (using H_2O_2 as a substrate) into neurotoxic semiquinone radicals was responsible for the PC12 cell killing in our co-culture paradigm. In support of the latter hypothesis, when dopamine alone or H_2O_2 alone were used to challenge the co-cultures, we observed significantly less PC12 cell killing than when the two compounds were administered together. These data suggest that it is the conversion of dopamine into its o-semiquinone radical, using H_2O_2 as a substrate, by the peroxidase-positive (iron-rich) astrocytes that is responsible for the PC12 cell killing, and not simply the effects of dopamine or H_2O_2 alone.

In support of a free radical mechanism of DH action, we demonstrated significant attenuation of DH-related PC12 cell killing in our co-cultures following co-administration of the potent antioxidants, ascorbate, melatonin or resveratrol. Ascorbic acid (vitamin C) has previously been postulated to serve as an antioxidant in human neurodegenerative

disorders such as amyotrophic lateral sclerosis (Kok, 1997). The role of ascorbate as an important endogenous antioxidant in humans is supported by 1) a correlation between advancing age and decreased ascorbate plasma levels, 2) the ability of ascorbate to prevent the formation of peroxynitrite radicals, and 3) a close correlation between CNS ascorbate levels and susceptibility to neural injury (Kok, 1997). In contrast, ascorbic acid may at times behave as a pro-oxidant by increasing intracellular H_2O_2 generation and promoting the redox cycling of ferric iron to the more reactive ferrous state (Mendiratta *et al.*, 1998). Others have shown that vitamin supplementation of 500 mg of vitamin C caused a significant increase in 8-oxo-adenine levels, a marker of oxidative nucleic acid damage (Podmore *et al.*, 1998). Whether ascorbate behaves as a prooxidant or an antioxidant may be critically dependent on cellular concentrations of the vitamin. In the present study, 200 µM of ascorbic acid significantly inhibited DH-related PC12 cell death when the latter were cultured atop CSH-pretreated astroglial monolayers. Thus, ascorbate clearly behaved as an antioxidant in the dosage utilized in the current study.

Melatonin, a hormone secreted by the pineal gland, possesses antioxidant capabilities and has previously been shown to scavenge reactive oxygen species such as hydroxyl radicals (Tan *et al.*, 1993; Pierrefiche *et al.*, 1993), peroxyl radicals (Pieri *et al.*, 1994), and superoxide radicals (Pierrefiche *et al.*, 1993). Miller and co-workers reported that melatonin also functioned in scavenging free radicals produced by catecholamine autoxidation (Miller *et al.*, 1996). Because of the lipid solubility of melatonin, this antioxidant can easily traverse the plasma membrane and participate in the intracellular scavenging of superoxide and hydroxyl radicals derived from the electron transport chain

or xenobiotic metabolism. Roth and co-workers found that high concentrations of melatonin (100 μM) attenuated PC12 cell killing in response to oxidative challenge which is in agreement with the results of the present study.

Another novel antioxidant used in our experiments was the grape-derived phytoalexin, resveratrol. Resveratrol, present in significant concentrations in red wine, is thought to protect arterial walls from oxidative damage and may, in part, contribute to the low incidence of atherosclerosis in France (Renaud *et al.*, 1992). The antioxidant capabilities of resveratrol are supported by a number of studies showing resveratrol-related: i) inhibition of LDL oxidation (Frankel *et al.*, 1993), ii) cytoprotection against lipid peroxidation (Arichi *et al.*, 1982), and iii) anti-cancer effects (Jang *et al.*, 1997). More germane to the present study, resveratrol has recently been shown to protect PC12 cells from oxidative damage (Chanvitayapongs *et al.*, 1997). As in the case of ascorbate and melatonin, resveratrol administration significantly attenuated DH-related PC12 cell killing when the latter were grown atop CSH-pretreated (iron-rich) astroglia.

Deferoxamine, an iron chelator, has been used in a variety of clinical settings including tumor therapy (Donfransesco et al., 1996), acute iron poisoning (Howland, 1996), heart disease (Kontoghiorghes, 1995) and in animal models of MPTP neurotoxicity (Lan and Jiang, 1997). In these conditions, DFO inhibited iron accumulation thereby ameliorating free radical-related tissue damage. In the present study, DFO was used in an attempt to more directly implicate cellular iron in DH-related PC12 cell toxicity. Similar to the antioxidant effects, DFO significantly attenuated DH-related PC12 cell killing when the PC12 cells were cultured atop CSH-pretreated (iron-enriched) astroglia. The

protective effects of DFO in our co-culture paradigm supports the role of iron in the bioactivation of dopamine into neurotoxic semiquinone radicals. This finding is in accord with our laboratory's central hypothesis that iron-mediated pseudoperoxidase activity in senescent astroglia (simulated by CSH-pretreatment) enhances the vulnerability of aging neural tissues to oxidative insults.

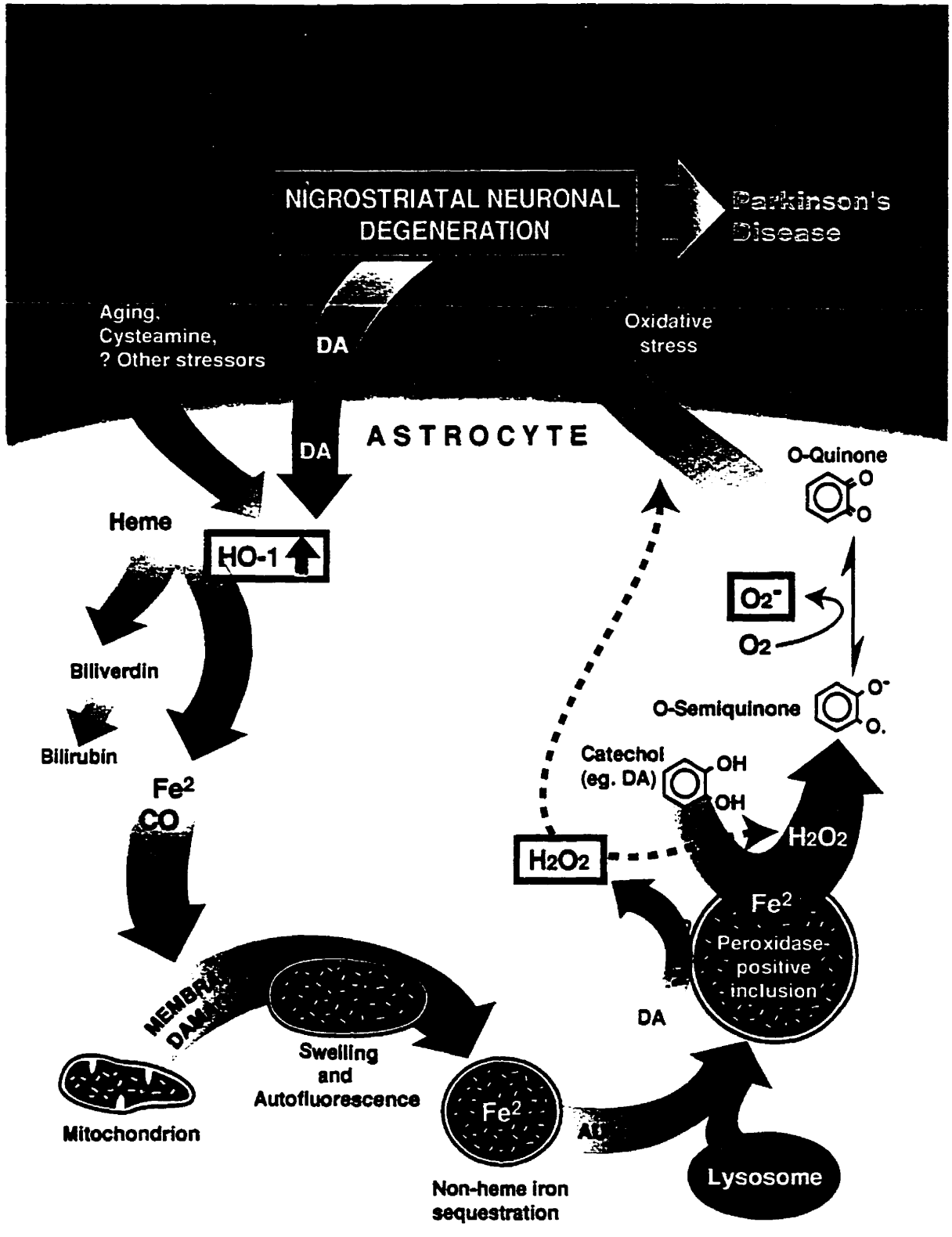
V - Model for Pathological Glial-Neuronal Interaction in Parkinson's Disease

Taken together, the observations on CSH- and dopamine-stressed astroglia have led Dr. Schipper to put forth a comprehensive model for the relationship between glial inclusion formation, iron sequestration, and the perpetuation of oxidative neuronal injury in the aging and degenerating nervous system (Fig. 9; Schipper et al., in press): (a) In response to oxidative stress, various HSP's and HO-1 are up-regulated in subpopulations of astroglia within the senescent basal ganglia and other subcortical brain regions. Induction of HO-1 in response to oxidative stress may lead to HO-mediated release of CO and free ferrous iron culminating in mitochondrial injury. (b) Damage to the mitochondrial compartment renders this organelle permeable to non-transferrin-derived, low molecular weight iron that is maintained in the redox-active ferrous state by abundant reducing equivalents of the electron transport chain. (c) The redox-active mitochondrial iron may cause further oxidative stress and thereby participate in a vicious cycle of pathological events long after the initial insult has dissipated. This model raises the possibility that exacerbation of the stress-related trapping of iron in the mitochondrial compartment of astroglial cultures may be an important mechanism responsible for the accumulation of

mitochondrial iron in the basal ganglia of PD subjects. This iron may originate from degenerating neurons, glia or the cerebrospinal fluid (CSF). Small amounts of iron are present in the CSF of healthy control patients and this metal has been shown to increase in the CSF of patients with neurodegenerative disorders (Gutteridge, 1992). In agreement with this model are reports that excess brain iron and mitochondrial electron transport chain deficits are present in the brains of PD subjects (Connor *et al.*, 1990; Jellinger *et al.*, 1990; Olanow, 1992). Most importantly, by oxidizing dopamine and environmentally-derived xenobiotics to neurotoxic intermediates, redox-active glial iron could serve as a "final common pathway" perpetuating nigrostriatal degeneration initiated by as yet undetermined genetic and epigenetic factors in patients with PD.

Figure 9: A model for pathological glial-neuronal interaction.

(From Schipper, H.M. Glial iron sequestration and oxidative injury in the aging and degenerating nervous system. Wang, E. and Snyder, D.S, eds. Handbook of the Aging Brain, Academic Press, San Diego, p.243-257).


NIGROSTRIATAL NEURONAL DEGENERATION
Parkinson's Disease
Aging,
Cysteamine,
? Other stressors
DA
Oxidative stress
DA
ASTROCYTE
O-Quinone
Heme
HO-1
O_2^-
O_2
Biliverdin
O-Semiquinone
Bilirubin
Catechol (eg. DA)
OH
OH
Fe^2
CO
H_2O_2
H_2O_2
Fe^2
Peroxidase-positive inclusion
DA
Swelling and Autofluorescence
Mitochondrion
Fe^2
Non-heme iron sequestration
Lysosome

VI - CONCLUSION

Although, the mechanisms responsible for the accumulation of redox-active brain iron in PD remain poorly understood, there is considerable evidence implicating excessive basal ganglia iron and catechol-derived free radicals in the pathogenesis of this disease. The progressive accumulation of autofluorescent, peroxidase-positive astrocytic granules represents a fundamental and highly consistent bio-marker of aging in the vertebrate CNS. It is only in recent years that the subcellular origin of these inclusions, the mechanism(s) underlying their biogenesis, and their potential role in brain aging and neurodegeneration have begun to be understood. These gliosomes are thought to be "stress granules" which are ultimately derived from metal-laden mitochondria engaged in a complex autophagic process. The fact that oxidative (or sulfhydryl) stress appears to initiate the formation of these granules, which are themselves redox-active, may have several important implications. First, we may be able to "map" regions of the CNS at increased risk of oxidative injury in normal aging and under neuropathological conditions by delineating the topography of these inclusions. Second, our ability to mimic this senescent glial phenotype in primary cultures (by CSH exposure) provides us with a model to investigate i) the role of HO-1 and other heat shock proteins in the biogenesis of deleterious neural inclusions, ii) stress-related (dys)regulation of MnSOD and other antioxidant enzymes in aging and degenerating neural tissues and, iii) mechanisms responsible for the sequestration of brain iron in Parkinson's disease and other neurodegenerative conditions. The current project was undertaken to determine whether the non-enzymatic peroxidase activity characteristic of senescent astroglia promotes the bio-activation of endogenous catechols to potential

neurotoxins. To begin testing this hypothesis, we set out to determine whether catecholamine-secreting PC12 cells grown atop confluent monolayers of CSH-pretreated (iron-rich) astrocytes, a senescent glial phenotype, are more vulnerable to dopamine/H_2O_2-related killing than PC12 cells co-cultured with young, control astroglia. The results of this study clearly demonstrate that PC12 cells grown on the surface of CSH-pretreated (iron-enriched) astroglia are more susceptible to dopamine/H_2O_2-related killing than DH-exposed PC12 cells cultured atop control (young, healthy) glial substrata. These data support Dr. Schipper's earlier ESR findings that the peroxidase activity in CSH-pretreated astrocytes is capable of converting dopamine into potentially neurotoxic o-semiquinone derivatives. The effects of DH-challenge were abrogated in the presence of potent antioxidants and an iron chelator further supporting our contention that oxidative stress was responsible for the killing of PC12 cells in our co-culture model. The results of the present study indicate that the aging-dependent increases in numbers of peroxidase-positive astrocytes which have been documented in rodent and human brain may render the senescent nervous system particularly prone to parkinsonism and other free radical-related neurodegenerative diseases.

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APPENDIX

The Role of HO-1 in the up-regulation of the MnSOD Gene in Cysteamine-and Dopamine-challenged Astroglia

Introduction

A unique subpopulation of granule-laden neuroglia initially referred to as Gomori-positive astrocytes on the basis of their affinity for chrom-alum hematoxylin and aldehyde fuchsin (Gomori stains), resides in limbic and periventricular brain regions of all vertebrates studied to date including humans (Srebro *et al.*, 1971; Srebro, *et al.*, 1975; Wislocki *et al.*, 1954; Schipper *et al.*, 1991; Schipper *et al.*, 1995). These astrocytic inclusions possess a high sulfur content (Srebro *et al.*, 1971), emit orange-red autofluorescence (Goldgefter *et al.*, 1980) and stain intensely with diaminobenzidine (DAB), a marker for endogenous peroxidase activity (Srebro *et al.*, 1971). Iron-catalyzed peroxidation has been implicated as the mechanism for non-enzymatic H_2O_2 reduction in these cells (Goldfischer, *et al.*, 1966). In both the human and rat, peroxidase-positive glial inclusions progressively accumulate with advancing age (Schipper, *et al.*, 1981). Dr. Schipper has developed a model wherein the aging-related accumulation of peroxidase-positive astroglia can be greatly accelerated by treating neonatal rat astroglia with the sulfhydryl compound, cysteamine (CSH; Schipper *et al.*, 1990). The ability to generate these primary cell cultures has considerably enhanced our understanding concerning the origin of these peroxidase-positive inclusions, the mechanism(s) responsible for their biogenesis, and the role these cells may play in brain aging and neurodegenerative diseases.

CSH (880μM in culture medium administered twice weekly from *in vitro* day 6-18) induces a massive accumulation of astrocytic inclusions in the context of a cellular stress (heat shock) response (Mydlarski *et al.*, 1993; Schipper *et al.*, 1990) These CSH-induced inclusions are structurally and histochemically identical to the Gomori-positive glial granules that progressively accumulate in the aging subcortical brain. Elemental iron is detected in the inclusions by electron microprobe analysis, and the presence and concentration of the metal correlates closely with the presence and intensity of DAB (peroxidase) staining (McLaren *et al.*, 1992). Many astroglial mitochondria exhibit progressive swelling, rearrangement of cristae, sequestration of redox-active iron, and, in some cases, fusion with the lysosomes after 24-72 hours of CSH exposure (Brawer *et al.*, 1994a). As in the case of the CSH-pretreated cultures, peroxidase-positive glial inclusions in the intact rat and human brain invariably exhibit mitochondrial epitopes (Brawer *et al.*, 1994b; Schipper *et al.*, 1995). Taken together, these observations indicate that a) the iron-laden astrocyte granules are derived from abnormal mitochondria engaged in a complex autophagic process and b) CSH accelerates the appearance of a senescent phenotype in these cells.

HO-1 is a 32-kd member of the stress protein superfamily that catalyzes the rate limiting step in heme degradation (Ewing *et al.*, 1991; Willis *et al.*, 1996). The HO-1 gene has a heat shock element in its promoter region and is rapidly induced upon exposure to heme as well as metal ions, ultraviolet light, sulfhydryl compounds and various pro-oxidants (Dwyer *et al.*, 1992; Applegate *et al.*, 1991; Keyse *et al.*, 1989). Induction of

HO-1 in response to oxidative stress may be a fundamental component of the cellular anti-oxidant defense system (Applegate *et al.*, 1991; Abraham *et al.*, 1995). Stocker suggested that augmentation of HO-1 activity in oxidatively challenged cells may serve to normalize the redox microenvironment by converting pro-oxidant heme to anti-oxidant bilirubin (Stocker 1990). The formation of astrocytic inclusions in senescent and CSH-treated astroglia may be due, in part, to the up-regulation of HO-1. After six hours of exposure to CSH, cultured astroglia exhibit a 4-10 fold increase in HO-1 mRNA and protein levels, as well as a three fold increase in HO enzymatic activity compared to controls (Chopra *et al.*, 1995; Manganaro *et al.*, 1995; Mydlarski *et al.*, 1995). Conceivably, induction of HO-1 in CSH-pretreated astroglia may promote oxidative injury to mitochondrial membranes via the liberation of free ferrous iron and CO (Zhang and Piantadosi, 1992), and thereby facilitate the transformation of normal astrocytic mitochondria to peroxidase-positive cytoplasmic inclusions.

Two categories of antioxidant defense mechanisms have evolved to protect cells from cytotoxic redox reactions: 1) The enzymatic defenses which include: superoxide dismutases (SOD's), glutathione (GSH) peroxidase, glutathione reductase and catalase, and 2) the non-enzymatic defenses such as: ascorbic acid (vitamin C), α-tocopherol (vitamin E), and GSH. In neural and non-neural cells, SOD catalyzes the dismutation of superoxide radicals to H_2O_2. There are three forms of SOD: a copper-zinc SOD (CuZnSOD) which is specific to the cytosol, a manganese SOD (MnSOD) in the mitochondrial compartment and an iron SOD found in bacteria (Fridovitch, 1976). Free radical scavenging enzymes such as catalase and reducing substances such as reduced

glutathione are reportedly deficient in the basal ganglia of PD subjects (Ambani *et al.*, 1975). In contrast, manganese superoxide dismutase (MnSOD) is elevated in PD which has been interpreted as a compensatory response to oxidative stress (Karla *et al.*, 1992). Dr. Schipper has shown that after 3-6 days of CSH exposure, astroglia exhibit augmented MnSOD mRNA levels suggesting that MnSOD may play a vital role in protecting astroglial mitochondria from oxidative damage (Manganaro *et al.*, 1995). In the present study, we set out to determine whether the late induction of MnSOD in CSH- or dopamine (DA)-pretreated astrocytes is contingent upon, or entirely independent of, antecedent up-regulation of HO-1.

Materials and Methods

Materials

Neonatal Sprague-Dawley neonatal rats were obtained from Charles River Canada. Ham's F-12, high glucose Dulbecco's modified Eagle's medium (DMEM) and Minimum essential media, lacking glycine (MEM) were purchased from GIBCO BRL (Life Technologies, Burlington, Ontario). Horse serum and fetal bovine serum were purchased from WISENT (St-Bruno, Quebec). Cysteamine (CSH), dopamine and penicillin-streptomycin were obtained from Sigma Chemical Co. (St. Louis, MO.). HEPES, melatonin and research grade guanidine thiocyanate were obtained from ICN Biomedicals (Aurora, Ohio). Tissue culture flasks (75 cm^2) and ascorbic acid were obtained from Fisher Scientific Ltd. (Montreal, Qc, Canada). dCTP (alpha-32P)(3000 Ci/mmoles) was obtained from Dupont (Boston, MA). Oligolabelling kit, was purchased from Pharmacia LKB (Pharmacia, Canada). Hybond nylon membranes were obtained from Amersham Corp. (Arlington Heights, IL, USA). Vacuum Blotter was obtained from Bio-Rad Laboratories (Richmond, CA, USA). Hybridization bottles, nylon mesh and hybridization oven were purchased from Bell Co Glass Co. QIAGEN PLASMID kits were obtained from QIAGEN Inc. (Chatsworth, CA). Human heme oxygenase-1 (Xho1-EcoR1) (1 Kb) in pBluescript SKII+) was obtained from Dr. S. Shibahara *et al.*, (Freidrich-Miescher Institute, Basel). Rat MnSOD cDNA (EcoR1 cDNA fragment (~ 1.4 Kb) in pSP65) was sequenced by Dr. Y-S Ho of Duke University and obtained from Dr. B.T. Mossman (University of Vermont). pBK-CMV vector was obtained from Stratagene. X-ray film was obtained from Eastman Kodak Co. (Rochester, NY). Millipore membranes (0.22 μm)

were purchased from Millipore Corporation (Bedford, MA). HO-1 rabbit anti-rat antibody was purchased from StressGen (Victoria, BC., Canada). MnSOD sheep anti-human antibody was purchased from Research Plus (Bayonne, NJ. USA). Tin-mesoporphyrin (SnMP) was obtained from Porphyrin products (Logan, Ut). Resveratrol was a gift from Pharmascience Inc. (Montreal, Qc. Canada)

Brain cell cultures

Primary neural cell cultures were prepared by mechanoenzymatic dissociation of cerebral tissue as previously described (Chopra *et al.*, 1995). Cells were grown in Ham's F12 and high glucose DMEM (50:50 v/v) supplemented with 10 mM HEPES, 5% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively). The cells were plated in 75 cm^2 tissue culture flasks at a density of $1X10^6$ cells/mL. Cultures were incubated at 37°C in humidified 95% air-5% CO_2 for 6 hours at which time they were vigorously shaken 20-30 times with replacement of fresh media to remove adherent oligodendroglia and microglia from the astrocytic monolayers (LeBlanc *et al.*, 1991). The cultures were incubated in the above-mentioned conditions for 6 days at which point more than 98% of the cells comprising the monolayer are astroglia as determined by immunohistochemical labeling for the astrocyte-specific marker, glial fibrillary acidic protein (GFAP) (Chopra *et al.*, 1995; Schipper and Mateescu-Cantuniari, 1991).

Immunofluorescence

The monolayers were fixed with 4% paraformaldehyde for 20 minutes at room temperature. After fixation, the cultures were washed in washing buffer (Sodium Phosphate 10mM, NaCl 0.5M, 0.2% Triton X and 0.5% BSA) for 10 minutes, rinsed with PBS, and incubated with sheep anti-human MnSOD (diluted 1/100) overnight. After rinsing with PBS, the specimens were incubated with FITC-conjugated anti-sheep antibody (diluted 1/50) in the dark at room temperature for one hour. The monolayers were then incubated with rabbit anti rat HO-1 (1/100 dilution) overnight at $4^{o}C$ followed by incubation with rhodamine-conjugated anti-rabbit antisera (1/50 dilution) for one hour at room temperature. The chambers were removed from the chamber slide and the glass slides were mounted with 50% glycerol/PBS.

Laser scanning confocal microscopy

The rhodamine- and FITC-labeled slides were scanned using a Bio-Rad MRC-600 laser scanning confocal microscope. This system is equipped with a 15 mW krypton/argon laser that excites samples with lines at 488,568, and 647 nm. The excitation filter wheel was used in the dual excitator position which allows only the 488 and 568 nm lines of the laser to reach the sample. The emission wavelengths were detected from the samples using the K1 and K2 filter block set. The latter allows the detection of wavelengths between 522 and 554 nm on the green channel and 585 nm or greater on the red channel. Both channels were 70% open. Images scanned on the two channels (green and red) were

merged to produce a single profile. After scanning, the images were collected using COMOS software package and stored on optical disk.

Drug treatments:

To assess the effects of cysteamine and catecholamines on MnSOD gene expression, astroglial cultures were left untreated (control) or received dopamine (DA;1 uM) or cysteamine (CSH; 800 uM) in DMEM for 3 or 6 days. Cultures were either left untreated (control) or received 880μM cysteamine or 1μM dopamine (DA) with each change of culture medium (twice weekly) after 6 days *in vitro* (DIV). Stock solutions were freshly prepared by dissolving the CSH in DMEM and sterilized by filtering with a 0.22 μm Millipore membrane. The pH was adjusted between 7.2 and 7.4. This CSH regimen induces the accumulation of peroxidase-positive (iron-rich) cytoplasmic inclusions in cultured astroglia as previously described (Schipper and Mateescu-Cantuniari, 1991). As in the case of CSH, physiologically- and pharmacologically-relevant concentrations of dopamine (1-25 μM) rapidly (within 3-6 hours) augmented HO-1 mRNA levels in primary cultures of neonatal rat astroglia (unpublished data).

To determine the role of HO-1 in dopamine or cysteamine-related MnSOD expression, DA (1 uM) or CSH (800 uM) were administred to the astroglial cultures in the presence or absence of the HO-1 competitive inhibitor, tin-mesoporphyrin (SnMP; 1 uM) for 3 or 6 days. This concentration of SnMP was previously shown to completely block heme oxygenase activity in our glial cultures (Schipper *et al.*, submitted). Cultures receiving SnMP were light-shielded to prevent metalloporphyrin photoactivation.

RNA Isolation and Northern Analysis

Cultured astrocytes were harvested using a rubber policeman and cytoplasmic RNA was isolated using an acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski, *et al.*, 1987). Ten micrograms of RNA was denatured and size-separated by electrophoresis on 1% agarose/formaldehyde gels. RNA integrity was confirmed by ethidium bromide staining. The RNA was transferred onto Hybond-N filter paper and covalently cross-linked to the membrane by UV light for two minutes. The hybridization probe (HO-1; 1.0 Kb and MnSOD 1.4 Kb) was prepared by random primer-generated ds DNA probes using the Random Primer DNA Labelling System (Feinberg *et al.*, 1984). Prehybridization was performed for 12 hours at 42°C in a buffer containing formamide deionized, 5x Denhardt's reagent, 6x SSPE and 0.5% SDS. The hybridization buffer consisted of the prehybridization buffer without 5 x Denhardt's reagent, and ^{32}P-labelled denatured DNA probe (Noonberg *et al.*, 1994). Equal loading of RNA was confirmed by hybridization with a cDNA for the (housekeeping) gene, GAPDH. All washes were performed under stringent conditions (1x SSC and 0.2% SDS for 45 minutes at room temperature, 0.4 x SSC and 0.2% SDS for 15 minutes at 65°C, 0.1 x SSC and 0.2 % SDS for 15 minutes at 65°C). The RNA hybridizing with cDNA probes was visualized by autoradiography using an intensifying screen at -80°C (Church *et al.*, 1984). Resulting bands on the autoradiograph were analysed using a Phospho Imager S1 densitometer. Densitometry data were normalized by calculating the ratios of the HO-1 mRNA signals to the GAPDH signals.

Transfection of Human HO-1 cDNA into Rat Astroglia.

HO-1 cDNA (1.0 Kb) cloned in a pBLuescript vector was cut out by digestion with Xba I and Xho I and inserted into the Xba I and Xho I site of the pBK-CMV vector (Stratagene, 4512 bp). The same plasmid without the HO-1 cDNA was used as a control. For transient transfection, cells were transfected with HO-1 DNA-Lipofectamine complex using the lipofectamine method and selected with neomycin. The cells were incubated at 37°C in a CO_2 incubator until 50 % confluence at which point the growth medium was changed to serum-free DMEM. For each transfection, 15 µg of HO-1 cDNA and 50 µl of cationic lipid reagent were diluted respectively into 800 µl serum-free medium. The two solutions were combined and incubated at room temperature for 45 minutes to allow DNA-lipid complexes to form. For each transfection, DNA-lipid complexes were diluted in 6.5 ml of serum-free medium and added to the cells. Following incubation for 12 h at 37°C, the transfection mixture was removed and replaced with complete medium. The transfection efficiency was approximately 31% as determined using the β-galactosidase/X-gal method (An *et al.*, 1982). To determine the role of HO-1 in the induction of the MnSOD gene, MnSOD mRNA levels were measured in HO-1-transfected astrocytes in the presence or absence of SnMP(1µM). To assess the role of oxidative stress in the induction of MnSOD in HO-1-transfected astrocytes, MnSOD mRNA levels were measured in HO-1-transfected astrocytes exposed to, acid (200µM), melatonin (100µM) or resveratrol (100µM) administered in culture media from the time of transfection until mRNA harvesting.. MnSOD mRNA levels were determined on post-transfection days 1, 3 and 6 as described above.

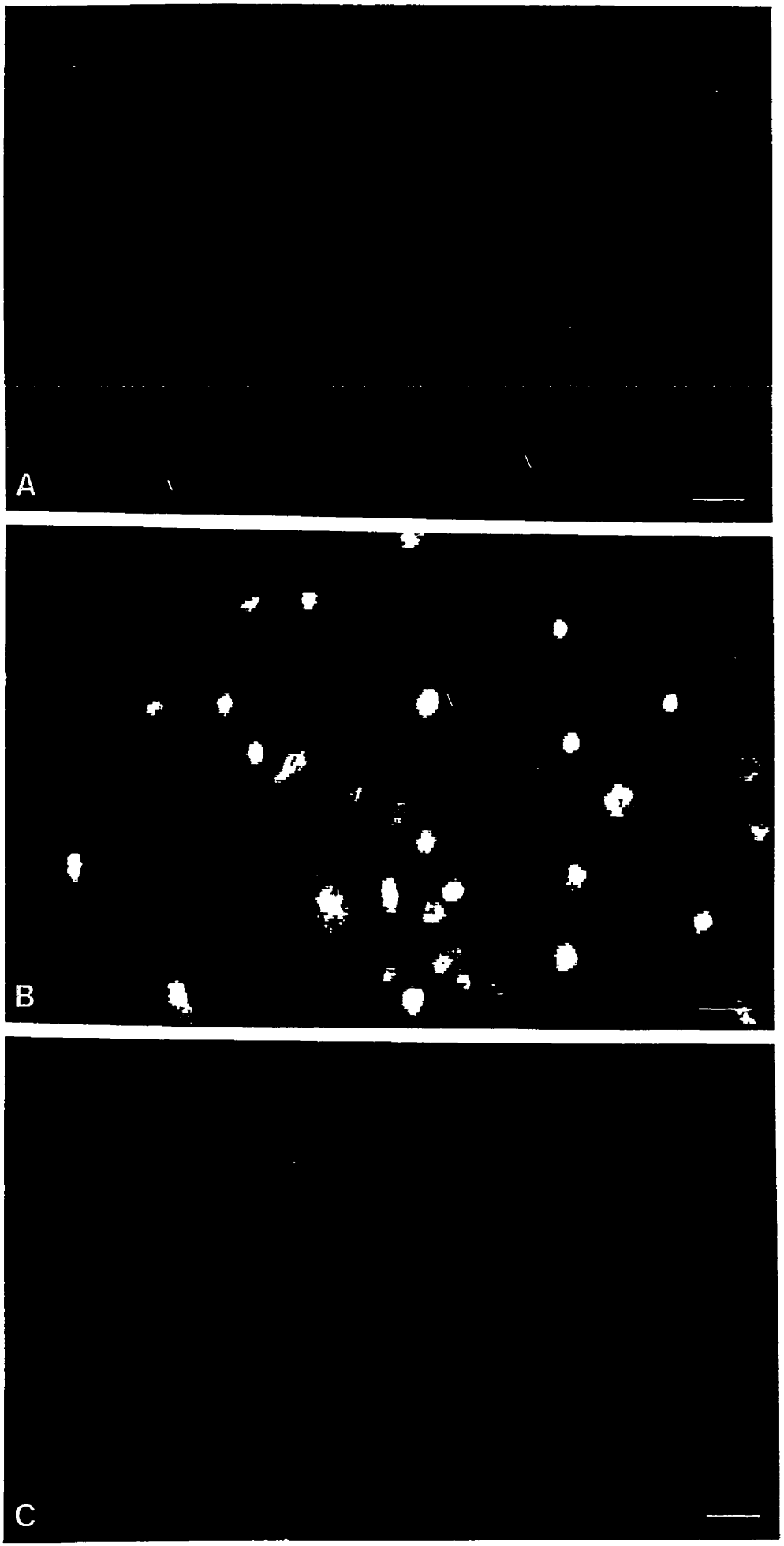
Results

HO-1 and MnSOD Immunofluorescence in CSH-treated astrocytes.

Using confocal microscopy, we observed no HO-1 or MnSOD immunofluorescence in control (untreated) astroglia after 3 days in culture. After 6 days in culture, control astrocyte monolayers exhibited immunostaining for HO-1 but not for MnSOD. In CSH-treated astrocytes, intense HO-1 staining was observed after 3 days of treatment. However, like the control astrocytes, no MnSOD immunofluorescence was observed at this time point. In contrast, after 6 days of CSH exposure, the astrocytes stained intensely for both HO-1 and MnSOD (Fig. 1a). To delineate further the role of HO-1 in the late expression of MnSOD, we administered CSH to the astroglia along with the competitive inhibitor of HO-1, SnMP (1μM). After 3 days of treatment, there was enhanced HO-1 immunofluorescence and no detectable MnSOD expression (Fig. 1b), as previously noted in the absence of SnMP (Fig. 1a). However, after 6 days of treatment, HO-1 immunostaining remained intense, but MnSOD expression was abrogated by the addition of SnMP (Fig. 1c).

Figure 1: Laser scanning confocal images of astroglial monolayers immunostained for HO-1 and MnSOD.

Control astroglial cultures exhibit HO-1 staining (red) after 6 days in culture but no MnSOD immunofluorescence (A). Treatment with CSH (880μM) promotes the up-regulation of HO-1 (red) and late expression of MnSOD (green) resulting in intense yellow fluorescence in this merged confocal image (B). In the presence of the HO-1 competitive inhibitor, SnMP (1μM), CSH treatment no longer elicits MnSOD expression and only HO-1 immunofluorescence (red) is observed (C). Bars = 25 μM


A
B
C

Northern Analysis

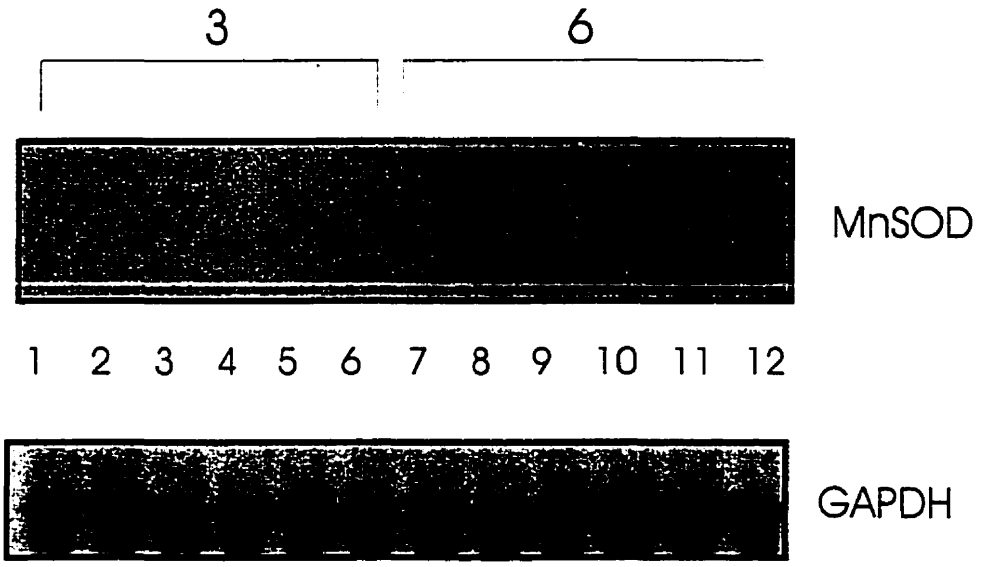
Effects of CSH and DA on glial MnSOD mRNA Levels.

As previously noted (Manganaro *et al.*, 1995; Schipper *et al.*, 1997 (abst.)), both CSH (880 μM) and DA (1 μM) significantly augmented glial HO-1 mRNA levels within 3-6 hours of treatment (data not shown). MnSOD mRNA levels were faint or undetectablein control, CSH-exposed and DA-exposed astroglia on post-treatment days 1 and 3. On post-treatment day 6, robust MnSOD mRNA bands corresponding to the 5 transcripts arising from differential polyadenylation of MnSOD mRNA (???) were in glial cultures exposed to CSH (880 μM) or DA (1 μM), but remained undetectable in the untreated control cultures (Fig. 2). To evaluate the role of antecedent HO-1 induction in the late up-regulation of MnSOD, MnSOD mRNA levels were assessed on post-treatment day 6 in additional cultures exposed to CSH (880 μM) or DA (1 μM) in the presence of the HO-1 inhibitor, SnMP (1 μM). As depicted in Fig. 2, CSH- and DA-related induction of the MnSOD gene was completely abrogated by SnMP treatment.

Figure 2: Effects of CSH and DA on glial MnSOD mRNA levels in the presence and absence of SnMP.

Cell monolayers were either left untreated (controls) (lanes 1,7), received 880 μM CSH (lanes 2,8), or received 1 μM of dopamine (lanes 3,9). To assess the role of HO-1 in subsequent MnSOD expression, CSH (lanes 4,10) or DA (lanes 5,11) was administered in the presence of 1μM SnMP. In control experiments, SnMP was administered alone (lanes 6,12). MnSOD mRNA levels were measured in all groups after 6 days of drug treatment.

Days of treatment

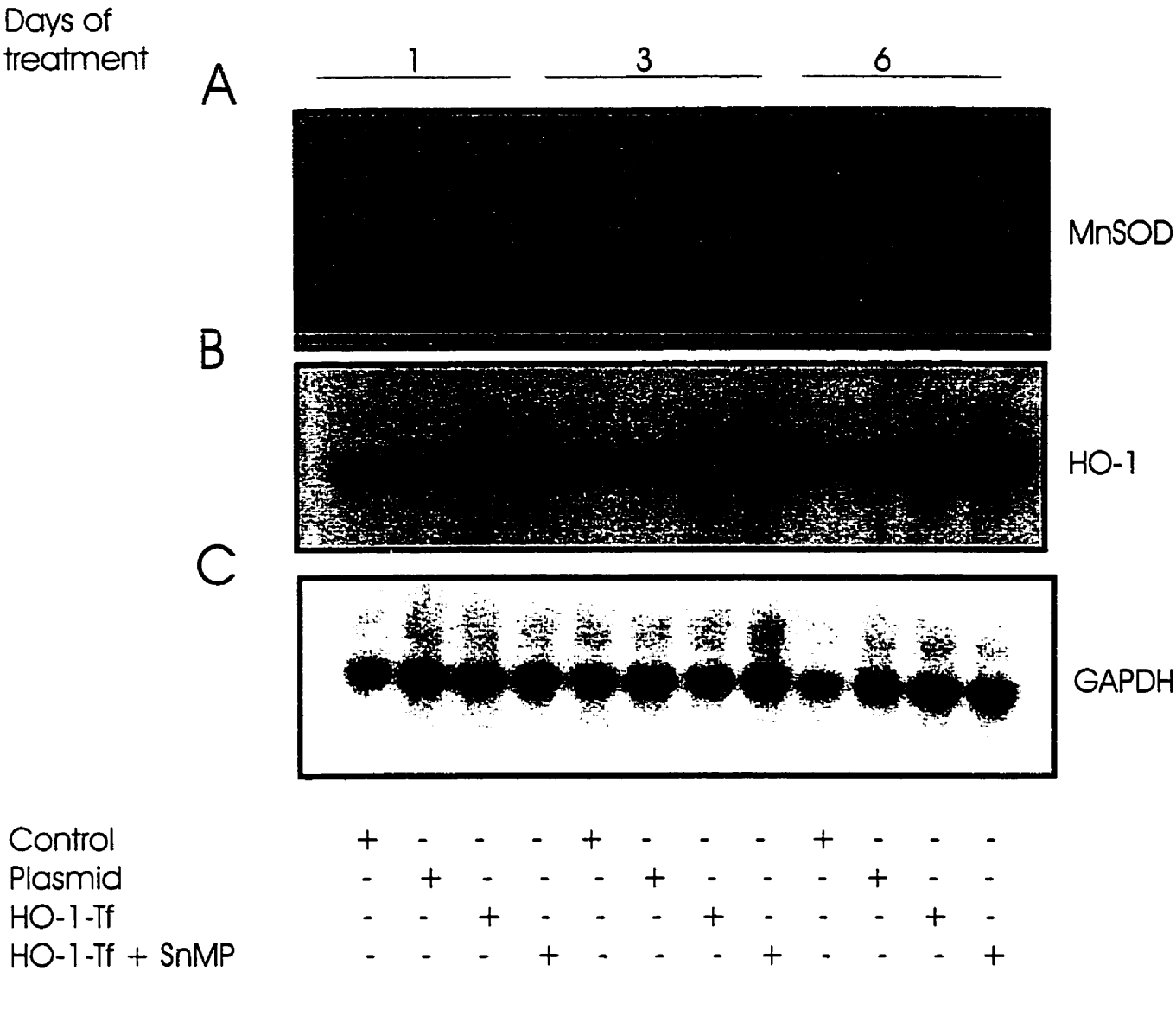


HO-1 mRNA and MnSOD mRNA Levels in HO-1-Transfected Astroglia

To determine further the role of HO-1 in the up-regulation of MnSOD, rat astrocyte cultures were transiently transfected with human HO-1 cDNA using LIPOFECTAMINE method as described above. Plasmids lacking HO-1 cDNA insert (sham-transfected cells) and non-transfected cells served as controls. As seen in Fig. 3, HO-1 mRNA levels were significantly augmented in HO-1 transfected cells on post-transfection days 1, 3 and 6 relative to sham- and non-transfected controls. The potential stress of sham transfection did not increase endogenous HO-1 mRNA levels above non-transfected control values (Fig. 3). MnSOD mRNA levels were clearly augmented in the HO-1-transfected cells on post-transfection days 3 and 6 relative to control preparations (Fig. 3). Addition of SnMP (1 μM) to the HO-1-transfected cultures had no effect on HO-1 mRNA levels (as expected) but completely inhibited MnSOD induction on post-transfection days 3 and 6 (Fig. 3).

Figure 3: Effects of HO-1 transfection on glial MnSOD mRNA levels in the presence and absence of SnMP.

HO-1 transfected and control astrocytes were assessed at 1, 3 or 6 days for MnSOD mRNA levels (A) and HO-1 mRNA levels (B) and GAPDH (C). The astroglial monolayers were either left untreated (controls) (lanes 1,5,9), received the plasmid vector only (lanes 2,6,10), were transfected with the human HO-1 gene (lanes 3,7,11), or received 1 μM SnMP after HO-1 transfection (lanes 4,8,12).

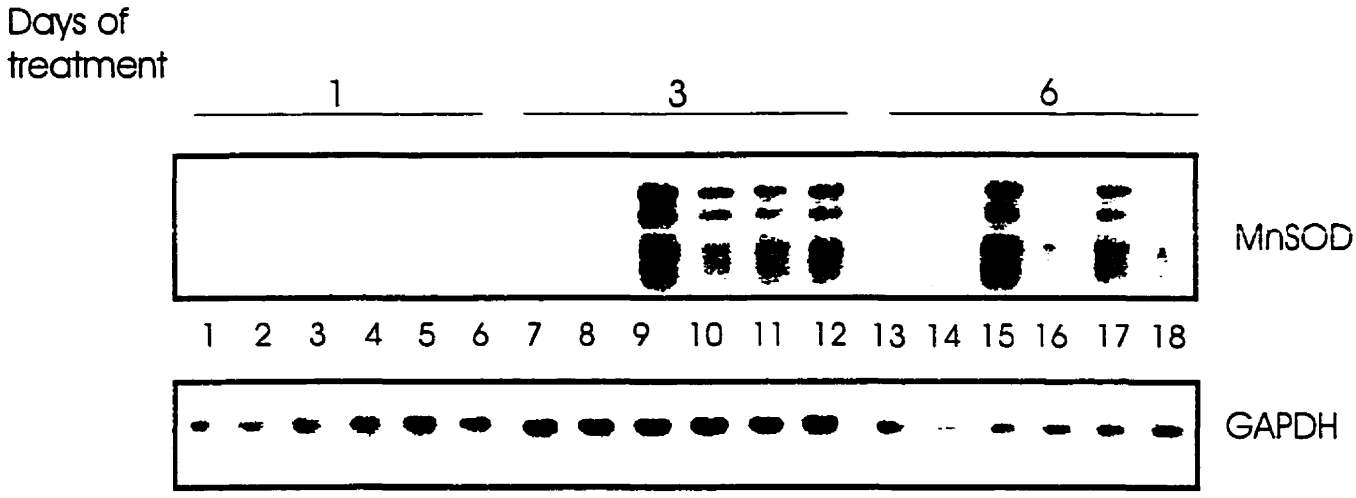

Days of treatment
1
3
6
A
MnSOD
B
HO-1
C
GAPDH
Control + - - - + - - - + - - -
Plasmid - + - - - + - - - + - -
HO-1-Tf - - + - - - + - - - + -
HO-1-Tf + SnMP - - - + - - - + - - - +

Effect of Anti-oxidants on MnSOD mRNA levels in HO-1-transfected astrocytes

To determine whether free radicals mediate MnSOD induction in HO-1-transfected astrocytes, MnSOD mRNA levels were measured in additional cultures on post-transfection day 6 following exposure to ascorbic acid (200 μM), melatonin (100 μM) or resveratrol (100 μM). As shown in Figure 4, each antioxidant effectively suppressed MnSOD gene expression in the HO-1-transfected glia indicating that free radical generation may signal MnSOD up-regulation in HO-1-transfected astroglia.

Figure 4: Effects of HO-1 transfection on glial MnSOD expression in the presence and absence of antioxidants.

MnSOD (A) and GAPDH (B) mRNA levels in HO-1-transfected astroglia. Cell monolayers were either left untreated (controls) (lanes 1,7,13), received plasmid vector only (lanes 2,8,14), or were transfected with the human HO-1 gene (lanes 3,9,15). To assess the role of HO-1 on subsequent MnSOD expression, MnSOD mRNA levels were measured in HO-1-transfected astroglia exposed to 200 μM ascorbic acid (lanes 4,10,16), 100 μM melatonin (lanes 5,11,17) or 100 μM resveratrol (lanes 6,12,18).


Days of treatment
1
3
6
MnSOD
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
GAPDH

Discussion

Dr. Schipper's laboratory has demonstrated that treatment of neonatal rat astroglial cultures with the sulfhydryl agent, CSH, induces a massive accumulation of astrocytic inclusions in the context of a cellular stress (heat shock) response (Mydlarski *et al.*, 1993; Schipper *et al.*, 1990). These CSH-induced inclusions are identical to glial granules that progressively accumulate in the aging subcortical brain. These inclusions exhibit orange-red autofluorescence and non-enzymatic peroxidase activity (Schipper *et al.*, 1991; Schipper *et al.*, 1990). The gliosomes are membrane-bound, variable in size and shape, and exhibit an intensely electron-dense granular matrix (Brawer *et al.*, 1994a; McLaren *et al.*, 1992). Elemental iron is detected in the inclusions by electron microprobe analysis, and the presence and concentration of the metal correlates closely with the presence and intensity of DAB (peroxidase) staining (McLaren *et al.*, 1992). Many astroglial mitochondria exhibit progressive swelling, rearrangement of cristae, sequestration of redox-active iron, and, in some cases, fusion with the lysosome after 24-72 hours of CSH exposure (Brawer *et al.*, 1994a). Subcutaneous injections of CSH (150-300 mg/kg biweekly for three weeks) in young adult rats induces a 2-3 fold increase in the number of peroxidase-positive astrocyte inclusions in many brain regions including the basal ganglia and hippocampus (Schipper *et al.*, 1993). As in the case of CSH-pretreated cultures, peroxidase-positive glial inclusions in the intact rat and human brain invariably display mitochondrial markers (Brawer *et al.*, 1994b; Schipper *et al.*, 1995). Taken together, these observations indicate that a) the iron-laden astrocyte granules are derived

from abnormal mitochondria engaged in a complex autophagic process and b) CSH accelerates the appearance of a senescent phenotype in these cells.

A considerable body of evidence suggests that intracellular oxidative stress may be the "final common pathway" responsible for the transformation of normal astrocyte mitochondria to peroxidase-positive inclusions both *in vitro* and in the intact brain: 1) Prior to inducing astrocyte granulation, CSH up-regulates stress proteins which typically respond to oxidative stress (e.g.: heat shock protein (HSP) 27, 90) but have little or no effect on redox-insensitive proteins such as glucose-regulated protein (GRP) 94 (Mydlarski *et al.*, 1995; Mydlarski *et al.*, 1993). 2) In the presence of transition metals, CSH undergoes redox cycling with the generation of pro-oxidant species including thiyl radicals, superoxide, H_2O_2 and the hydroxyl radical (Munday, 1989). H_2O_2 induces HSP and heme oxygenase-1 (HO-1) expression in rat astrocytes and stimulates the accumulation of peroxidase-positive astrocyte granules in primary culture following prolonged treatment (Mydlarski *et al.*, 1995). 3) Ionizing radiation, a known generator of intracellular pro-oxidant intermediates, increases numbers of peroxidase-positive glial granules in the rat hypothalamus in a dose-dependent manner (Srebro, 1971). If a causal relationship to intracellular oxidative stress is confirmed, determination of the topography and intensity of endogenous glial peroxidase activity may permit accurate "mapping" of CNS regions particularly susceptible to chronic oxidative stress during normal aging and under pathological conditions.

In the present study, we confirmed earlier findings from Dr. Schipper's lab that after 3-6 days of CSH exposure, astroglia exhibit augmented MnSOD mRNA levels

suggesting that MnSOD may play an important role in protecting astroglial mitochondria from CSH-related oxidative stress (Manganaro *et al.*, 1995). In addition, we provided evidence that, like CSH, DA stimulates early HO-1 up-regulation and a late, possibly compensatory, induction of the MnSOD gene. In a separate study recently completed in Dr. Schipper's lab, it was shown that co-administration of antioxidants inhibits DA-related HO-1 induction in cultured astroglia implicating a free radical mechanism of DA action in these cells (unpublished data).

There is currently a controversy in the literature as to whether HO-1 activity confers antioxidant protection to cells or, conversely, whether HO-1 may exacerbate or amplify intracellular oxidative stress. Stocker suggested that augmentation of HO-1 activity in oxidatively challenged cells may serve to normalize the redox microenvironment by converting pro-oxidant heme to anti-oxidant bilirubin (Stocker 1990). On the other hand, Zhang and Piantadosi (1992) have demonstrated that free ferrous iron and carbon monoxide (CO) derived from heme oxygenase-catalyzed breakdown of heme may specifically augment free radical generation within the mitochondrial compartment. Based on the latter, Dr. Schipper has conjectured that the mitochondria-derived astrocytic inclusions observed in senescent and CSH-pretreated astroglia may arise, in part, as a consequence of augmented HO-1 activity. After six hours of exposure to CSH, cultured astroglia exhibit a 4-10 fold increase in HO-1 mRNA and protein levels, as well as a three fold increase in HO enzymatic activity compared to controls (Chopra *et al.*, 1995; Manganaro *et al.*, 1995; Mydlarski *et al.*, 1995). Conceivably, induction of HO-1 in CSH-pretreated astroglia may promote oxidative injury to mitochondrial membranes via the

liberation of free ferrous iron and CO, and thereby facilitate the transformation of normal astrocytic mitochondria to peroxidase-positive cytoplasmic inclusions.

In the present study we determined that HO-1 is necessary and sufficient for the "late" induction of MnSOD in CSH-treated, DA-treated and HO-1-transfected astrocyte cultures. Others have shown that MnSOD is up-regulated in PC12 cells and confers cytoprotection to these cells following exposure to nitric oxide (NO) (Gonzalez-Zulueta *et al.*, 1998). Since CO and NO share many similar biophysical properties (Verma *et al.*, 1993), it is possible that CO, liberated during HO-1 catalyzed heme degradation, may act in a fashion akin to that of NO and stimulate induction of the MnSOD gene. Furthermore, Dr. Schipper has recently demonstrated that, like CSH, DA can elicit the selective trapping of non-heme iron by the mitochondrial compartment, and the antecedent up-regulation of HO-1 appears to be necessary for mitochondrial iron sequestration in these cells (unpublished data). Taken together, these data suggest that HO-1 may amplify and prolong oxidative stress in astrocytes long after initiating insults (e.g. CSH, DA) have dissipated. The latter hypothesis supports the notion that HO-1 may be acting as an intracellular pro-oxidant, rather than as an anti-oxidant, in "stressed" astroglia. It is not yet known whether HO-1 mediates similar dystrophic effects in other cell types although it is interesting to note that inhibition of HO-1 activity significantly attenuates menadione-related (oxidative) DNA damage in human MCF-7 cells (Nutter *et al.*, 1994).

The data reported herein may be germane to the pathogenesis of Parkinson's disease (PD). PD is a neurodegenerative disorder of the aging brain which is characterized by the accelerated loss of dopaminergic (DA) neurons in the pars compacta of the

substantia nigra. There is currently a broad consensus implicating oxidative stress as a major factor in the pathogenesis of this disease (Cohen *et al.*, 1994; Fahn *et al.*, 1992). This hypothesis is supported by recent observations that: a) excessive concentrations of hydrogen peroxide (H_2O_2) are produced by monoamine oxidase B (MAO-B)-mediated deamination of DA in both idiopathic PD and experimental parkinsonism, b) the neurotoxins 6-hydroxydopamine, manganese and MPTP, induce parkisonism in animals, at least in part, via the generation of free radicals (Barbeau *et al.*, 1984; Cadet *et al.*, 1989). c) Lipid peroxidation in the substantia nigra is augmented in post mortem Parkinsonian brain relative to aged matched controls (Dexter et al., 1989), and d) free radical scavenging enzymes such as catalase, and reducing substances such as reduced glutathione are reported to be deficient in the basal ganglia of PD subjects (Ambani et al., 1975; Perry et al., 1982). In contrast, manganese superoxide dismutase (MnSOD) is **elevated** in PD which has been interpreted as a response to oxidative stress (Karla *et al.*, 1992; Riederer *et al.*, 1989).

Recently, HO-1 has also been shown to be overexpressed in neurodegenerative diseases, possibly in response to chronic oxidative stress. Recent studies have confirmed that, in comparison to normal senescent brains matched for age and post-mortem interval, AD patients demonstrate a marked overexpression of HO-1 in hippocampal neurons and astrocytes (Schipper *et al.*, 1995). As well, there is a greater percentage of GFAP-positive astrocytes in the PD substantia nigra expressing HO-1 compared to age-matched controls (Schipper *et al.*, 1998). Although HO-1 up-regulation in these conditions may confer some cytoprotection by degrading pro-oxidant heme to the antioxidant bile pigment,

bilirubin, our findings suggest that heme-derived ferrous iron and CO may contribute to the development of mitochondrial electron transport chain deficiencies and excess mitochondrial DNA mutations reported in the brains of Alzheimer and PD subjects (Beal, 1995; Reichmann *et al.*, 1994).

Taken together, the observations on CSH- and dopamine-stressed astroglia have led Dr. Schipper to put forth a comprehensive model for the relationship between glial inclusion formation, iron sequestration, and the perpetuation of oxidative neuronal injury in the aging and degenerating nervous system (a) In response to oxidative stress, various HSP's and HO-1 are up-regulated in subpopulations of astroglia within the senescent basal ganglia and other subcortical brain regions. Induction of HO-1 in response to oxidative stress may lead to HO-mediated release of CO and free ferrous iron culminating in mitochondrial injury. (b) Damage to the mitochondrial compartment renders this organelle permeable to non-transferrin-derived, low molecular weight iron that is maintained in the redox-active ferrous state by abundant reducing equivalents of the electron transport chain. (c) The redox-active mitochondrial iron may cause further oxidative stress and thereby participate in a vicious cycle of pathological events long after the initial insult has dissipated. Finally, (d) the up-regulation of MnSOD observed in Parkinsonian subjects may be a compensatory response to oxidative mitochondrial damage, and intracellular ferrous iron and/or CO accruing from the antecedent induction of HO-1 may be responsible for signaling the compensatory rise in MnSOD. In agreement with this model are reports that augmented neural HO-1 and MnSOD expression, leads to excess brain iron and mitochondrial electron transport chain deficits characteristic of PD subjects

(Connor *et al.*, 1990; Jellinger *et al.*, 1990; Olanow, 1992; Schipper *et al.*, 1998). By oxidizing dopamine and environmentally-derived xenobiotics to neurotoxic intermediates, the redox-active mitochondrial iron could serve as a "final common pathway" perpetuating nigrostriatal degeneration initiated by as yet undetermined genetic and epigenetic factors in patients with PD.

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