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Differential Effects of Cysteamine on Heat Shock Protein Induction and Cytoplasmic Granulation in Astrocytes and Glioma Cells

by

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Abstract

A subpopulation of astrocytes in the limbic and periventricular brain regions of many vertebrates, including humans, progressively accumulates cytoplasmic inclusions with advancing age which are morphologically and histochemically distinct from the aging pigment, lipofuscin. These inclusions exhibit orange-red autofluorescence consistent with porphyrins or oxidized flavoproteins and non-enzymatic peroxidase activity mediated by ferrous iron and perhaps other transition metals. The sulfhydryl compound, cysteamine (CSH), promotes the accumulation of identical autofluorescent, peroxidase-positive granules in primary astrocyte cultures. In CSH-treated cultures and in the aging periventricular brain, these glial inclusions appear to originate from swollen, iron-laden mitochondria engaged in a macroautophagic process. In the present study, we demonstrated that within 6 hours of CSH-treatment, long before cytoplasmic granulation occurs, cultured astroglia exhibit increased levels of HSP 27, HSP 90 and heme oxygenase (HO-1) protein and mRNA, relative to control (untreated) cultures. In contrast to primary astroglia, the astrocyte-derived C6 glioma cell line constitutively expresses HSP 27, HSP 90, and HO-1 at low levels. Moreover, CSH treatment elicited relatively minor increases in HO-1 mRNA and had no effect on HSP 27 and HSP 90 mRNA and protein in the glioma cells. Untreated glioma cells exhibited yellow autofluorescent cytoplasmic granules consistent with lipofuscin which was not affected by CSH exposure. Moreover, CSH treatment failed to induce the accumulation of red autofluorescent, peroxidase-positive inclusions in these cells. Taken together, these findings support the notion that the biogenesis of peroxidase-positive astrocytic granules in CSH-treated cultures and in the aging periventricular brain is dependent on an antecedent stress (heat shock) response. Evidence is reviewed implicating intracellular oxidative stress as the "final common pathway" leading to the biogenesis of these glial inclusions in CSH-treated cultures and in the aging periventricular brain. In further

support of this contention, we demonstrated that CSH treatment up-modulates the expression of the mitochondrial antioxidant MnSOD in cultured astroglia. Augmented levels of MnSOD mRNA and protein were only noted after 5 days CSH exposure. The relatively late induction of MnSOD suggests that it is not part of generalized cellular stress response. It may serve as a late cytoprotective mechanism in astrocytes against secondary superoxide injury resulting from mitochondrial damage rather than due to the direct effects of CSH. CSH exposure appears to accelerate a spectrum of pathophysiological processes in astroglia germane to normal brain aging and various senescence-related neurodegenerative disorders including: 1) the transformation of astroglial mitochondria to Gomori-positive inclusions, 2) stress-related sequestration of redox-active glial iron, 3) dysregulation of MnSOD and other antioxidant enzymes, and 4) the establishment of reactive gliosis.

RÉSUMÉ

Une souspopulation d'astrocytes dans les systèmes limbiques et périventriculaires du cerveau de plusieurs vertébrés, y compris l'humain, accumulent des inclusions cytoplasmiques qui sont morphologiquement et histochimiquement distinctes du pigment de veillissement, la lipofuscine. Ces inclusions démontrent une autofluorecence rouge-orange qui peut être attribué aux porphyrines ou aux flavoprotéines oxidées et à une activité de péroxidase non-enzymatique provoquée par les ions ferreux ainsi que d'autres métaux de transition. La cystéamine (CSH), un composé sulfhydryle, promeut l'accumulation d'identiques granules autofluorescents et péroxidase positifs dans les cultures primaires d'astrocytes. Dans les cultures traitées à la CSH et dans la région périventriculaire du cerveau veillissant, ces inclusions gliales semblent provenir de mitochondries, gonflées et chargées de fer, qui sont engagées dans un processus de macroautophagie. Dans l'étude présente, nous démontrons qu'aprés 6 heures de traitement à la CSH, bien avant que la granulation cytoplasmique ne surveiment, les astroglies cultivées montrent des niveux élevés de protéines et d'ARNm pour l'HSP 27, l'HSP 90 et l'oxygénase d'hème (HO-1), par rapport aux cultures contrôles. Par opposition aux astrocytes primaires, la lignée cellulaires de gliome C6 (dérivée d'astrocyte) exprime constitutivement l'HSP 27, L'HSP 90, et HO-1, a un niveau peu élevé. De plus, le traitement à la CSH de la lignée C6 ne provoque que des augmentations mineures de l'ARNm pour l'HO-1 et n'a pas d'effet sur le niveau d'ARNm et de protéines pour les HSP 27 et 90. Les cellules de gliome non-traitées ont des granules cytoplasmiques ayant une autofluorescence jaune conforme à la lipofuscine, qui n'est pas affectées par le traitement à la CSH. Additionellement, ce traitement n'induit pas l'accumulation d'inclusions peroxidase positives et autofluorescentes rouge dans cette lignée cellulaire. Ces données renforcent donc la notion que la biogenèse des granules peroxidase positifs dans les cultures d'astrocytes traitées à la CSH et dans la région périventriculaire du

cerveau viellissant dépend d'une réponse à un stress (choque thermique) antécédent. Nous révisons les faits impliquant le stress oxydatif intracellulaire comme étant la dernière étape commune menant à la biogenèse de ces inclusions gliales dans les cultures traitées à la CSH et dans le région périventriculaire du cerveau viellissant. Pour renforcer d'avantage cette affirmation, nous démontrons que le traitement à la CSH augmente l'expression de l'antioxidant mitochondrial MnSOD dans les astroglies culitvées. Cette augmentation des niveaux d'ARNm et protéine MnSOD n'est constatée qu'après 5 jours de traitement. L'induction tardive de l'MnSOD insinue que cette enzyme ne fait pas partie d'une réponse généralisée au stress cellulaire. Cette induction tardive dans les astrocytes sert peut être de mécanisme cytoprotecteur contre les dommages secondaires dûs aux superoxides générés par les mitochondries endommagées plutôt que par les effets directs de la CSH. Le traitement à la CSH semble accélérer un large spectre de procédés pathophysiologiques dans les astroglies, semblables au viellissement normal du cerveau et à une variété de conditions neurodégénératrices inclusant 1) la transformation des mitochondries astrogliales en inclusions Gomori-positives, 2) la séquestration de fer réduit et actif induite par le stress, 3) la régulation abérrante de l'MnSOD ainsi que d'autres enzymes antioxydantes, et 4) la gliose réactive.

To my parents and brother, for their
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Contributions to Original Knowledge

1) Previous results have shown that cysteamine (CSH) induces the formation of autofluorescent, peroxidase-positive cytoplasmic inclusions in primary astroglial cultures. The present results demonstrate that CSH is incapable of inducing similar inclusion formation in astrocyte-derived C6 glioma cells. C6 glioma cells exhibited yellow autofluorescent cytoplasmic granules consistent with lipofuscin which was not affected by CSH exposure.

2) CSH rapidly elevates heat shock protein (HSP) 27, HSP 90 and heme oxygenase-1 (HO-1) mRNA levels in primary astroglial cultures but only HO-1 mRNA in C6 glioma cells. Furthermore, C6 glioma cells constitutively express HSP 27, HSP 90, HO-1 at low levels. Taken together, the results support the notion that the biogenesis of peroxidase-positive cytoplasmic granules in CSH-treated astroglial cultures is dependent on an antecedent cellular stress (heat shock) response.

3) CSH-treatment results in a relatively late upregulation of manganese superoxide dismutase (MnSOD) mRNA and protein levels in primary astrocyte cultures. Induction of MnSOD is not part of a general stress response but may act as a late cytoprotective mechanism in astrocytes, possibly against superoxide injury. The latter may be a result of mitochondrial damage incurred by CSH rather than due to the direct effects of this sulfhydryl agent.

Preface

The author was responsible for all the data presented in this thesis. The data has been published in the following manuscript:

Chopra, V.S., Chalifour,L.E., and Schipper,H.M. Differential effects of cysteamine on heat shock protein induction and cytoplasmic granulation in astrocytes and glioma cells. Molecular Brain Research. 31 (1995) 175-184.

In addition, the candidate was also responsible for the data on MnSOD presented in Fig. 2-5 of the following published manuscript:

Manganaro,F., Chopra,V.S., Mydlarski,M.B., Bernatchez,G., and Schipper,H.M. Redox perturbations in cysteamine-stressed astroglia: implications for inclusion formation and gliosis in the aging brain. Free Radical Biology and Medecine. (1995) in press.

Table of Contents

page

List of Figures

Page

Introduction

The central nervous system (CNS) tissue consists of a multitude of cell types which interact with each other in a highly complex and precise manner. The cell types consist of neurons, neuroglia, endothelia, specialized epithelial cells (e.g. choroid plexus) and mesenchymal cells (Fedoroff and Vernadakis, 1986b). The majority of cells within the CNS are the neuroglia which comprise approximately 50% of the total volume of the brain. The neuroglia are subdivided into two major groups: 1) microglia and 2) macroglia (Carpenter, 1983).

Microglia

Microglia are believed to be derived from the mesodermal cell layer of the developing embryo. The microglia enter the CNS as perivascular mesenchymal cells (Carpenter, 1983). However, these findings are still somewhat controversial and some investigators have implicated a neuroectodermal origin for these cells (DeGroot et al., 1990).

At the fine structural level, microglia are characterized by their small nuclei, electron-dense cytoplasm containing dense bodies, very short strands of endoplasmic reticulum, and absence of intermediate filaments (Mugnaini and Walberg, 1964; Mori and LeBlond, 1969; Peters et al., 1976; Murabe and Sano, 1981). In response to injury or disease, microglia undergo mitotic division and migrate to the site of damage where they engage in phagocytosis of degenerating neuropil constituents (Graeber et al., 1988; Kandel and Schwartz, 1991). Microglia are highly responsive to cytokines such as interferon-γ (IFN-γ; Graeber et al., 1988). IFN-γ induces the expression of major histocompatability complex (MHC) class II on microglia (Sethna et al., 1991). It has also been shown using immunohistochemical techniques that microglia have immunoglobulin Fc and 3 complement receptors on their surface membranes (Perry et

al., 1985). These receptors strongly suggest that microglia play a role in the immune response. Microglia aid in the removal of cellular debris and noxious substances and may be involved in facilitating repair of damaged tissue within the CNS (Benveniste, 1992). Microglia have been implicated in a number of inflammatory and degenerative neurological disorders including multiple sclerosis, AIDS dementia complex, Parkinson's disease, and Alzheimer's disease. (Banati et al., 1993).

Macroglia

The macroglia are composed of two major cell types, the oligodendrocytes and astrocytes. Like neurons, the macroglia are derived from the ectodermal layer of the developing embryo and commitment to the neuroglial cell lineage occurs before the neural tube is formed (Fedoroff and Vernadakis, 1986a). At this time it is unclear if the neural and macroglial cells arise from a multipotential cell or from two or more populations of cells which exist in the ectodermal layer of the developing embryo (Fedoroff and Vernadakis, 1986a). Multipotential cells may potentially give rise to macroglial or neuronal cells at later stages of embryonic development and even in early postnatal development (Raff and Muller, 1984; Williams et al., 1985; Goldman et al., 1986; Fedoroff and Vernadakis, 1986a). Astrocytes and oligodendroglia are thought to arise from primitive stem cells according to the following development scheme (fig.1). The stem cells are multipotential cells. Glioblasts are cells commited to the macroglial cell lineage. Astoblasts are cells commited to the astroglial cell lineage. Astrocytes are terminally differentiated cells (Fedoroff and Vernadakis, 1986a).

Oligodendrocytes

Oligodendrocytes are generally smaller than astrocytes and contain relatively few processes, which radiate short distances from their pear or spherical-shaped cell

bodies (Carpenter, 1983). Ultrastructurally, oligodendrocytes are characterized by a cytoplasm which varies in electron density from pale to very dense, is rich in ribosomes and microtubules, contains no filaments, and has well developed cisternae of rough endoplasmic reticulum (rER; Mori and LeBlond, 1970). In the human brain three main types of oligodendrocytes can be identified on the basis of their location and presence during development: 1) Perineuronal stellate cells which are found apposed to the neuronal perikarya or dendrites. This cell type is commonly observed in the adult CNS. 2) Interfascicular cells which are found in abundance in the white matter of the fetus or newborn after which time their numbers decrease dramatically. After the myelin sheath is formed, only the nuclei of these cells remain conspicuous and very few processes are observed. 3) Perivascular oligodendrocytes which establish contacts with adjacent blood vessels (Carpenter, 1983). As previously mentioned, the main function of oligodendrocytes is to form the myelin sheaths which encapsulate the neuronal axons, thereby promoting saltatory conduction of electrical signals (Bunge et al., 1968; Peters and Vaughn, 1970; Kandel and Schwartz, 1991). Oligodendrocytes can be readily identified by the presence of the protein, galactocerebroside (GC), the major glycolipid component of myelin (Fedoroff and Vernadakis, 1986a).

Astrocytes

Astrocytes are the most numerous and diversified class of neuroglia in the fetal and mature CNS (Schipper, 1991). Astrocytes can be identified by their stellate perikarya and numerous processes which extend into the surrounding neuropil (Carpenter, 1983). The processes of some astrocytes extend to the surface of the CNS and form the glial membrane or *glia limitans*. The *glia limitans* may serve as a protective ensheathment for the CNS (Kandel and Schwartz, 1991). Astrocyte processes referred to as "end-feet" abut on blood vessels and induce the formation of tight junctions between adjacent endothelial cells. Endothelial tight junctions

constitute the blood brain barrier which effectively limits the entry of many blood-borne substances into the CNS (Kandel and Schwartz, 1991).

Ultrastructurally, astrocytes exhibit euchromatic nuclei and an electron-lucent cytoplasm containing relatively few mitochondria, ribosomes and some lipid droplets. Astrocytes also contain some smooth endoplasmic reticulum (ER), Golgi bodies, multivesicular bodies, and some lysosomal dense bodies (Mugnaini and Walberg, 1964; Braak, 1975; Montgomery, 1994). These cells can be readily identified in fetal and mature CNS by the presence of cytoskeletal components such as glial fibrillary acidic protein (GFAP), or vimentin (Fedoroff and Vernadakis, 1986a, Schipper, 1991). GFAP, which is found exclusively in astroglial cells and tanycytic processes of the adult CNS, is an intermediate filament protein which was initially isolated by Eng et al. (1971) from multiple sclerosis plaques. S-100 protein is another marker for astroglial cells and was initially discovered by Moore et al. (1965). The physiological function of S-100 remains unknown (Takahashi, 1992; Fedoroff and Vernadakis, 1986a). Recent findings by Selinfreund et al. (1991) have demonstrated that nanomolar concentrations of S-100 beta are capable of inducing proliferation of primary astrocyte cultures suggesting that S-100 beta has neurotrophic properties. Antibodies generated against S-100 are often used to detect astrocytes (Fedoroff and Vernadakis, 1986a) and astrocyte-related cells such as ependymal cells (Cocchia, 1981) and retinal Muller cells (Cocchia et al., 1982, 1983; Terenghi et al., 1983; Kondo et al., 1983, 1984).

Fibrous and Protoplasmic Astrocyte Sub-Types

Pioneering work by Andriezen (1893), Retzius (1894), Azoulay (1894), Kollicker (1896) and Cajal (1909) indicated that astrocytes can be subdivided into protoplasmic and fibrous types based on their morphology and location within the CNS (Fedoroff and Vernadakis, 1986a; Miller et al., 1989). Fibrous astrocytes are

found mainly in the white matter, have a stellate appearance and exhibit numerous vascular "end feet". Some of the processes emanating from fibrous astrocytes extend into the nearby grey matter. Their cytoplasm is replete with 10nm intermediate filaments associated with GFAP. Protoplasmic astrocytes are found in the grey matter. These cells have short, sheet - like processes which are often ramified (Fedoroff and Vernadakis, 1986a). The cytoplasm of protoplasmic astrocytes contains relatively few bundles of intermediate filaments (Fedoroff and Vernadakis, 1986a). These two main types of astrocytes can be further subdivided based on their morphology, location, and relation to blood vessels, pia etc. (Miller et al., 1989).

A2B5 Positive and A2B5 Negative Astrocytes

The development of the A2B5 antibody by Raff et al. (1983a) has led to another classification scheme for astrocyte sub-types. In primary glial cultures, A2B5-positive astroglia are known as type 2 astrocytes whereas those that do not exhibit the epitope are referred to as type 1 astrocytes (Raff et al., 1983a). On the basis of their location (white vs grey matter) Raff postulated that type 1 and type 2 astrocytes were protoplasmic and fibrous astrocytes, respectively. However, Raff et al. (1983b) found that A2B5 positive cells were not exclusively type 2 astrocytes but could also be oligodendrocytes. They found that cultured A2B5 positive cells develop into oligodendrocytes if no serum is added to the culture media. However, if serum is present in the culture media (10% fetal calf serum; FCS), the cells develop into type 2 astrocytes. Thus, the A2B5 positive cells were subsequently referred to as oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells (Raff et al., 1983b). These results suggest that during development O-2A cells require a signal (presumably also found in FCS) to differentiate into fibrous astrocytes and if these signals are not present oligodendrocytes develop by a default mechanism. Stockli et al. (1991) have demonstrated that ciliary neurotrophic factor (CNTF) participates in the induction of

type 2 astrocyte differentiation *in vitro*. Using immunohistochemical analyses they have also demonstrated that the expression of CNTF is confined to a subpopulation of type 1 astrocytes. In addition, it has been shown that platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs) and neurotrophin-3 are survival and proliferation factors for newly formed oligodendrocytes and their precursors in culture (Barres et al., 1992, 1994b). However, Hart et al. (1992) have shown that PDGF is incapable of inducing mitosis in differentiated oligodendrocytes. They have suggested that this lack of responsiveness is due to an interruption or alteration in the intracellular signalling pathway(s) within the differentiated oligodendrocytes. Barres et al. (1994a) have recently demonstrated that thyroid hormone, glucocorticoids, and retinoic acid are involved in the timing of oligodendrocyte development. Their presence is not required for differentiation of precursor cells into oligodendrocytes but rather help to coordinate the timing of differentiation to specific periods during ontogenesis.

With the application of immunolabeling techniques, a classification scheme has evolved based on the differential expression of surface antigens by a host of progenitor and mature cells of astroglial lineage (Malhotra, 1988).

Family of Astrocyte Cell Types

Classification schemes for astrocytes based on whether they are protoplasmic VS. fibrous, or type 1 or type 2 are incomplete because they do not address the wide variety of specialized astroglial found in the CNS. Astrocytes constitute a diverse "family" of cell types which includes radial glia, fibrous astrocytes, protoplasmic astrocytes, ependymal cells, tanycytes, Muller cells, choroid plexus epithelium, Golgi-Bergmann cells, pinealocytes, and Raff's type 1 and type 2 astrocytes (Fedoroff and Vernadakis, 1986a). A full discussion of each cell type is beyond the scope of this discussion. Briefly, ependymal cells, tanycytes, and choroid plexus epithelial cells line

the ventricular system. Golgi-Bergmann and Muller cells are found in the cerebellum and the retina, respectively, and pinealocytes and pituicytes are found in the pineal gland and neurohypophysis, respectively.

Common Functions of Astrocytes in the CNS

A large body of evidence has clearly demonstrated that astrocytes perform a wide variety of functions within the developing and adult CNS. Astrocytes are thought to be involved in the guidance of migrating neuronal cell bodies (Rakic, 1971) and growth cones (Silver and Shapiro, 1980; Silver et al., 1982) during embryogenesis. Astrocytes are involved in the induction of formation and maintenance of the blood-brain barrier (Janzer and Raff, 1987), the maintenance of ion homeostasis (Hertz , 1981), sequestration and metabolism of various neurotransmitters (Fedoroff and Vernadakis, 1986a; Wilkin et al., 1990), and production of proinflammatory and immunomodulatory cytokines (Plata-Salaman, 1991; Benveniste, 1992). In fact, it has been demonstrated by Benveniste et al. (1989) that the cytokine IFN-γ can induce MHC class II gene expression in primary astrocyte cultures. This suggests that astrocytes can act as antigen presenting cells to various immunocytes such as T cells and monocytes/macrophages and possibly microglia. Evidence is accumulating which suggests that astrocytes may also play a role in facilitating recovery after CNS injury. For example, local implantation of immature astroglia has been shown to promote axonal regeneration following transection of the corpus callosum (Smith et al., 1986). Secretion of neurotrophic substances such as nerve growth factor (NGF) or glial derived neurotrophic factor (GDNF) by astrocytes may play an important role in neuronal regeneration following CNS injury or disease (Lindsay, 1979).

Pathological Effects of Astrocytes in the CNS

While it is clear that astrocytes play an important role in normal functioning of the CNS, under certain circumstances these cells may cause harmful changes which ultimately lead to a decline in neurologic function. Astrocyte-derived tumours are among the most common forms of neoplastic transformation in the human CNS (Carpenter, 1983). Under the conditions of hypoxia, oxidative stress, and metal exposure, astrocytes may play a role in excitotoxic brain damage (Fedoroff and Vernadakis, 1986b). For example, Volterra et al. (1994) demonstrated that reactive oxygen species (ROS) inhibit glutamate uptake in cortical astrocytes. The inhibition of glutamate uptake leads to a progressive increase in glutamate concentrations within the synaptic cleft or perisynaptic space, which in turn results in the robust activation of glutamate receptors in postsynaptic cell membranes. Activation of glutamate receptors, in turn, can lead a cascade of intracellular biochemical changes culminating in cell death (for review see Wood et al., 1990).

Under conditions of ischemia, trauma, hypoglycemia, epileptic seizures, hypotonicity, and fulminant hepatic failure, astrocytes often exhibit considerable swelling. Astrocyte swelling is caused by an excess in intracellular osmoles with secondary influx of water. The astrocyte swelling may lead to increased intracranial pressure resulting in mechanical and ischemic CNS injury (Norenberg, 1994).

Astrocytes are capable of metabolizing protoxins into potent neurotoxins. For example, Ransom et al. (1987) and Brooks et al. (1989) have shown that astrocytes can convert the protoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into the dopaminergic neurotoxin MPP+ via the enzyme monoamine oxidase B (MAO-B).

In response to the cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), and the endotoxin lipopolysaccharide (LPS), astrocytes have been shown to produce toxic concentrations of nitric oxide (NO·) and peroxynitrites (ONOO) which are capable of damaging surrounding neurons (Skaper

et al., 1995). This increase in NO· and ONOO is due to an increase in the activity of the enzyme, NADPH diaphorase or NO· synthase. The neurotoxicity associated with the cytokines and LPS can often be attenuated by the addition of NO· synthase inhibitors, N-omega-nitro-L-arginine and N-omega-nitro-D-arginine methyl ester, as well as oxyhemoglobin which inactivates NO· (Skaper et al., 1995). In addition, Balanos et al. (1994) have shown that administration of LPS and interferon-gamma (INF-γ) to astrocyte cultures leads to both an increase NO· synthase activity and an inhibition of the respiratory chain enzymes: cytochrome c oxidase, NADH-ubiquinone-1 reductase, and succinate-cytochrome c reductase. They found that the NO· synthase inhibitor, NG-monomethyl-L-arginine, prevented inhibition of the respiratory chain complexes. These results suggest that inhibition of the mitochondrial respiratory chain after induction of NO· synthase activity may represent a mechanism of NO· mediated neurotoxicity.

In response to injury or disease of the CNS, astrocytes exhibit a very rapid and characteristic response known as reactive astrogliosis. In this response, astrocytes increase both in size and number. These astrocytes also show an increase in glycogen content, Golgi-membranes, ER, various enzymes activities and numbers of mitochondria. The astrocytes also elaborate more extensive processes and significantly increase their content of GFAP (Miyake et al., 1988; Yong et al., 1991). Reactive gliosis results in the formation of a gliotic scar at the site of injury. These scars are filled with GFAP filaments and encapsulated by the *glia limitans* (Maxwell et al., 1990; Reir et al., 1983). The glial scars may deter successful axonal regeneration (Rudge et al., 1990; Reir et al., 1990) and serve as epileptogenic foci (Fedoroff and Vernadakis, 1986b). Reactive astrogliosis is a characteristic feature of many neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease, and occurs to a lesser extent, in the course of normal brain aging. Increases in MAO-B activity accompany reactive

gliosis in the aging mammalian brain and in experimental models of Parkinson's disease (Beach et al., 1989; Duffy et al., 1980; Kushner et al., 1991; Schipper et al., 1993). Under these conditions, neuronal injury may be precipitated by excessive H_2O_2 derived from the accelerated deamination of dopamine and other monoamines. The astrocytic compartment may also contribute substantially to the pathogenesis of Alzheimer's disease by serving as an important site for cathepsin mediated proteolysis, amyloid metabolism, cytoskeletal protein hyperphosphorylation and the expression of apolipoprotein E (Oropeza, R. et al. 1987; Nakamura et al., 1991; Eddelston,M., et al., 1993; Nieto-Sampedro and Mora, 1994). As described in the following actions, the unique histochemical properties of Gomori-positive astrocytes suggest that those cells may play an important role in brain aging and neurodegeneration.

Gomori-Positive Astrocytes

Gomori-positive astrocytes are a unique subpopulation of granule-laden neuroglia found in the hippocampus, striatum, and periventricular brain regions of all vertebrate species examined to date, including humans (Schipper, 1991). These astrocytes contain granules that are homogeneously and intensely osmiophilic, variable in size, and round to angular in shape. These granules have no membrane or may be surrounded by a single limiting membrane. The granules may be contiguous with ER that is filled with an electron dense material similar to that which characterizes the granules (Brawer and Sonnenshein, 1975; Srebro and Lach, 1987).

Histochemically, these cytoplasmic inclusions can be identified by their affinity for Gomori stains (chrome alum hematoxylin and aldehyde fuchsin) and metachromasia in toluidine blue-stained sections (Creswell et al., 1964; Schipper et al., 1982). A high sulfur content content may underlie the inclusions' affinity for Gomori stains (Srebro and Cichocki, 1971; Wagner and Pilgrim, 1978). Aldehyde fuchsin is known to stain other structures in the CNS such as corpora amylacea,

oxidized neuronal lipofuscin, neuromelanin (Barden, 1978), neuronal dense bodies and neurosecretory material (Creswell et al., 1964). Chrome alum hematoxylin stains basically the same structures as aldehyde fuchsin with the exception of neuronal perikarya (Creswell et al., 1964; Noda, 1959; Wislocki and Leduc, 1954). Based on their tinctorial characteristics, the Gomori-positive inclusions were believed to be a form of lipofuscin or a phagocytosed neurosecretion (Lofgreen, 1961; Noda, 1959, Schipper, 1991). Indeed, the numbers of Gomori-granules and lipofuscin both increase progressively with age. However, the atypical autofluorescence pattern and the absence of neutral lipid (negative Sudan Black and Sudan III reactions) and visible pigment under light microscopy exclude lipofuscin as a component of these astrocytic granules (Schipper, 1991).

The astrocytic inclusions emit an orange-red autofluoresence, consistent with the presence of porphyrin or oxidized flavoprotein (Goldgefter et al., 1980; Wang et al., 1995), and stain intensely with diaminobenzidine (DAB), a marker for endogenous peroxidase activity (Srebro, 1971). Schipper et al. (1990a) has previously demonstrated full DAB staining of the astrocytic granules in the presence of aminotriazole, a catalase inhibitor, thereby ruling out catalase as the source of the peroxidatic reaction. Furthermore, the heat stable and pH-resistant nature of the DAB oxidation, occurring over a pH range of 4 to 10.5 and at a temperature of 95 - 100°C, indicates that the peroxidase activity in these astrocyte is not likely enzyme-mediated. These histochemical data favour iron-catalyzed "pseudoperoxidation" as the source for H_2O_2 reduction in these cells. Indeed, the presence of iron in these inclusions has been demonstrated using Perl's ferric ferrocyanide method (Srebro et al., 1972) and by X-ray microprobe analysis (McLaren et al., 1992; Brawer et al., 1994a)..

As stated above, the numbers of Gomori-positive astrocytes and their granule content progressively increase with advancing age in both rodents and humans (Schipper et al., 1981; Schipper, 1991). In the rodent hypothalamus , X-irradiation

(Srebro, 1971) and chronic estrogenization (Brawer et al, 1980; Brawer et al., 1983) induce the accumulation of these astrocytic inclusions. As discussed below, this estrogenic effect on Gomori-positive hypothalamic astrocytes may play a critical role in the development of reproductive failure in rodents (Schipper, 1993).

Topography of Gomori Positive Astrocytes

Gomori-positive granules are found consistently in subependymal brain regions and circumventricular organs (Creswell et al., 1964; Goldgefter, 1976; Keefer and Christ, 1976; and Srebro and Cichoki, 1971). Much of the detailed work on the location of Gomori-positive astrocytes within the CNS has been performed on the rat. In the rat telencephalon, the Gomori-positive granules are found in the organum subfornicale and organum vasculosum of the lamina terminalis, throughout the olfactory bulb, caudate nucleus (adjacent to the lateral ventrical), and in specific regions within the hippocampal formation (Schipper, 1991; Schipper and Mateescu-Cantuniari, 1991). Within the dorsal hippocampus, Gomori-positive astrocytes are found in the hilus of the dentate gyrus, and in the lacunosum molecular layer and stratum oriens of regions CA1 and CA2 adjacent to the corpus callosum (IBID). In the rat diencephalon, these astrocytes are found prominently throughout the entire rostro-caudal region of the arcuate nucleus and premammillary area. The subependymal third ventricular region and the suprachiasmatic nuclei also contain moderate numbers of these granulated astroglia (IBID). Within the rat mesencephalon, Gomori-positive astrocytes are found in the superficial layers of the superior colliculi, the periaqueductal grey, and dorsal to the raphe nuclear complex (IBID). In the rhombencephalon, the Gomori-positive astrocytes are most abundant in the area postrema. Some of these cells are also found in the nucleus gracilis, dorsal motor nucleus of the vagus, locus coeruleus, and scattered throughout the olivary complex and lateral cerebellum. (Keefer and Christ, 1976; Schipper, 1991).

Gomori-positive astrocytes are also present in periventricular and limbic regions of the aging human brain (Schipper, 1991; Schipper and Cisse, 1995). As in rodents, these cells contain cytoplasmic inclusions which exhibit an affinity for chrome alum hematoxylin, emit orange-red autofluorescence and stain intensely for (non-enzymatic) peroxidase activity.

Gomori-Positive Astrocytes in Primary Culture

Srebro and Mackinska (1972) first detected the presence of Gomori-positive brain cells in fetal rat and human explants. They postulated that these cells were glial in origin because their tinctorial and fluorescent properties were similar to those of periventricular astrocytes *in situ*. They did not however, perform more definitive ultrastructural and immunohistochemical analyses on these cells. In dissociated embryonic day 17 and neonatal day 2 rat brain cultures, Schipper et al. (1990b) demonstrated the co-localization of peroxidase-positive cytoplasmic granules to astrocytes by combined DAB histochemistry / anti-GFAP immunostaining. These inclusions were shown to be Gomori-positive by chrom alum hematoxylin staining. The numbers of Gomori glia and their granule content increased between day 10 and 50 *in vitro*. In the older cultures large numbers of DAB-positive inclusions were present in isolated flat and stellate astrocytes and in confluent astrocytes monolayers. The DAB oxidation reaction in the cultured Gomori astrocytes occurred over a pH range of 3-11 and could not be inactivated by aminotriazole (catalase inhibitor), or by tissue preheating at 95°C. As in the *in situ* experiments, these results suggest a non-enzymatic, iron-catalyzed (pseudo-) peroxidation as the source of the DAB oxidation in these cells. Furthermore, the Gomori-granules emit an orange-red autofluorescence identical to those observed *in situ* consistent with the presence of porphyrin or oxidized flavoprotein. As *in situ*, ferrous iron appears to mediate the peroxidase reaction in these granules (McLaren et al., 1992).

Effects of Cysteamine

Schipper et al. (1990b) have demonstrated that the sulfhydryl agent, cysteamine (CSH) and its disulfide, cystamine, induce a massive accumulation of DAB / Gomori-positive astrocytes sampled on 18 days *in vitro*. However, equimolar concentrations of L-cysteine or ethanolamine, which differ from cysteamine by single functional group modifications, do not stimulate a similar accumulation of Gomori-positive granules in cultured astroglia. More recently, Schipper et al. (1993) have shown that subcutaneous injections of CSH into young adult rats resulted in a 2-3 fold increase in numbers of peroxidase-positive astrocyte granules in striatum, hippocampus and other brain regions, suggesting that CSH accelerates an aging process in these cells.

Ultastructurally, the CSH-induced Gomori-granules are very similar to those found in periventricular astrocytes *in situ*. The CSH-induced inclusions are round/ovoid in shape, variable in size, membrane bound, and contain an intensely electron-dense granular matrix (McLaren et al., 1992). Using energy dispersive X-ray microanalysis, McLaren et al. (1992) have shown that elemental iron is found subcompartmentalized within the Gomori-granules. The presence and intensity of the iron correlates with that of DAB stain suggesting that the pseudoperoxidase activity of these cells is due to the presence of the redox-active iron. Recently, Brawer et al. (1994) have demonstrated that the CSH-induced glial inclusions originate from swollen, iron-laden mitochondria engaged in a macroautophagic process. Within 24-72 hours of CSH exposure, many astrocytes contained mitochondria which were swollen, devoid of cristae, filled with redox-active iron, and which occasionally appeared to be incorporated within acid phosphatase-positive lysosomes (Brawer et al., 1994a). Using a number of FITC-labelled antibodies against organelle-specific proteins and confocal microscopy, Schipper et al. (1993) have shown that the Gomori-

granules partially colocalize to lysosomes and, to a lesser extent, rough endoplasmic reticulum (rER) and early endosomes.

Role of Gomori-Positive Astrocytes in Aging of the Reproductive Hypothalamus

For many years it has been known that gonadal steroids play an important role in the differentiation, development, and maturation of the hypothalamus. Steroids also provide signals to the hypothalamus and pituitary that are crucial for the regulation of the ovulatory cycle (Brawer et al., 1993). However, under certain conditions, steroids can mediate changes which alter the ability of the CNS to regulate the ovulatory cycle; one such example is persistent estrus. Persistent estrus (PE) is characterized by the loss of the pre-ovulatory gonadotrophin (LH) surge and anovulatory sterility. The tonic plasma LH levels result in the formation of polycystic ovaries devoid of fresh corpora lutea, and vaginal cornification (Schipper, 1993). The vaginal cytologies suggest that the reproductive tracts are under chronic estrogen stimulation (Finch et al., 1984). The mechanism by which the cessation of the ovulatory cycle occurs can vary depending on the species in question. The cessation of mentrual cycles in women arises from a decline in gonadal function, although the hypothalamus may also be involved (Meites et al., 1987). However, in female rats the decline in the reproductive system seems to be due to a decrease in the ability of the hypothalamus to release gonadotrophin releasing hormone (Meites et al., 1987; Finch, 1978). The onset of PE can occur as a consequence of natural aging (Ascheim, 1976; Schipper et al., 1981) or as a result of external influences such as exposure to continuous illumination or a single intramuscular dose of 2 mg estradiol valerate (EV; Brawer et al., 1978; Brawer et al., 1980). Administration of EV to young adult female rats results in axodendritic damage in the arcuate nucleus of the hypothalamus associated with the formation of myelin figures, synaptic loss and remodelling, increased numbers of reactive microglial cells containing phagocytosed material, and an accumulation of astrocytes

exhibiting a massive proliferation of Gomori-granules. Recently it has also been shown that EV-induced PE results in a selective loss of β-endorphin containing neurons with the arcuate nucleus of the hypothalamus (Desjardins et al., 1993). Brawer et al. (1980) have demonstrated that ovariectomy prior to EV treatment or continuous illumination prevented the development of hypothalamic pathology in adult female rats, suggesting that an ovarian product is required for the condition to occur. Conversely, chronic exposure to high-physiological concentrations of unconjugated estradiol in gonadectomized male and female rats results in similar senescence-like pathological changes within the arcuate nucleus (Schipper et al., 1982; Brawer et al., 1983). These results implicate estradiol as the ovarian product responsible for promoting the development of pathological changes in the aging rodent hypothalamus. Schipper has suggested that peroxidase reactions within astrocytes of the arcuate nucleus may play a pivital role in the development estradiol-mediated neural injury (Schipper et al., 1990a; Schipper, 1993). Several lines of evidence have shown that in the hypothalamus, 2-hydroxylases and peroxidases can convert estradiol into catecholestrogen (Ball and Knuppen 1978; MacClusky et al., 1981; Mondschein et al., 1986). The catecholestrogens may in turn be converted into reactive semiquinones by a peroxidase/H_2O_2 mediated reaction or by spontaneous autoxidation. During the formation of semiquinones, neurotoxic superoxide anion and H_2O_2 are also generated (Kalyanaraman et al., 1984, 1985; Schipper, 1993). In support of this free radical hypothesis of hypothalamic damage, it has been shown that dietary supplementation with the potent antioxidants α-tocopherol (Desjardins et al., 1992) or 21-aminosteroids (Schipper et al., 1994) reduces EV-induced damage to the arcuate nucleus. As in the case of the peripheral sex steroid target tissues, many periventricular glia contain estrogen receptors (Lee, 1982; Stopa et al., 1989; Langub and Watson, 1992). Thus, chronic estrogenization may directly induce cytoplasmic granulation in these cells. The intense peroxidase activity manifest within Gomori-

positive glia may oxidize catecholestrogens to ortho-semiquinones and other reactive oxygen species which, in turn, could promote axo-dendritic damage within the neuroendocrine hypothalamus with ensuing reproductive senescence (Schipper et al. 1990a). In the following section, direct evidence implicating glial peroxidase activity in the generation of pro-oxidant intermediates is discussed.

Redox Activity of Peroxidase-Positive Astrocytes and its Possible Role in Neurodegenerative Disorders

The non-enzymatic peroxidase activity of Gomori astrocytes may be of great significance with respect to normal brain senescence as well as the pathogenesis of aging-related neurologic and neuroendocrine disorders. Schipper and co-workers (1993) used electron spin resonance spectroscopy with magnesium spin stabilization to determine whether Gomori-positive astrocytes are capable of converting catecholestrogens and catecholamines to free radical intermediates. They determined that in the presence H_2O_2, the CSH-induced peroxidase activity in cultured astrocytes promotes the robust oxidation of 2-hydroxyestradiol (catecholestrogen) to its respective ortho-semiquinone radical. Exposure of control, untreated astrocyte cultures to catecholestrogens resulted in faint or no o-semiquinone spectra. These observations support the hypothesis that these redox-active glial granules convert neutral catecholestrogens into neurotoxic free radical intermediates which may be toxic to neuropil constituents within the arcuate nucleus. This view is supported by the fact that antioxidants such as α-tocopherol and 21-aminosteroids attenuate estradiol-induced depletion of hypothalamic β endorphin in this brain region (Desjardins et al., 1992).

As in the case of 2-hydroxyestradiol, electron spin resonance spectroscopy has demonstrated that the CSH-induced peroxidase activity in cultured astrocytes are capable of converting dopamine (DA) into its dopamine-o-semiquinone derivative

(Schipper et al., 1991). These results are consistent with those of Metodiewa et al. (1989) who showed that dopamine and norepinephrine are readily oxidized to semiquinones with proven neurotoxic activity *in vitro* via peroxidase mediated reactions. The main catabolic pathway for DA turnover is oxidative deamination by the mitochondrial enzyme, monoamine oxidase-B (MAO-B), which leads to the formation of H_2O_2 (Cohen, 1987). H_2O_2 may cause oxidative damage directly (Scott,1988) or it may undergo further metabolism to more reactive oxyradicals such as ·OH. (Gutteridge et al., 1985; Southorn et al., 1988). In addition to oxidative deamination, DA can undergo spontaneous autoxidation and peroxidase / H_2O_2 mediated oxidation to form neurotoxic quinones, ortho-semiquinones and the oxygen radicals O_2· and H_2O_2 (Graham, 1978). The oxidation of dopamine and other catechols to semiquinones and oxyradicals by peroxidase-positive astrocytes may play a role in the pathogenesis of Parkinson's disease in accord with the prevailing Free Radical Hypothesis of this condition. Several lines of evidence have confirmed that free radicals play an important role in the pathogenesis of Parkinson's disease (Cohen and Werner, 1994; Fahn and Cohen, 1992; Jenner, 1992). For example, Dexter et al. (1989) have demonstrated that there is more lipid peroxidation in the substantia nigra of post-mortem human PD brain than in non-parkinsonian controls matched for age and post-mortem interval. Subjects with Parkinson's disease (PD) have high levels of tissue iron within the substantia nigra and basal ganglia (Youdim et al., 1993). This excessive iron may accelerate the reduction of H_2O_2 to HO· and thereby promote neuronal degeneration (Cohen, 1987). As described above, ferrous iron may also behave as a pseudo-peroxidase activity capable of oxidizing catecholamines, such as dopamine, to neurotoxic ortho-semiquinone radicals (Goldfischer et al., 1966; Schipper, 1991). Since astrocytes may contain both MAO-B enzyme activity and non-enzymatic peroxidase activity, it may possible that H_2O_2 produced by MAO-B oxidation of dopamine acts as a co-factor for further dopamine oxidation (to

potentially neurotoxic ortho-semiquinones) by peroxidase-mediated reactions. Thus, the aging-related accumulation of peroxidase-positive astrocyte granules in rat and human striatum (Schipper, 1990b; Schipper et al., 1991) may predispose this region to oxidative stress and thereby render the senescent CNS prone to Parkinson's disease and other free radical-related neurodenerative conditions.

Possible Mechanisms of CSH Action

The exact mechanism by which CSH induces astrocyte granulation remains unclear. Petersen et al. (1989) have demonstrated that CSH induces heme oxygenase (HO) activity within rat hepatocytes. HO is the rate limiting enzyme in the catabolism of heme (Shibahara et al., 1987). HO in the presence of molecular oxygen and nicotinamide-adenine dinucleotide phosphate, coverts heme to biliverdin, carbon monoxide, and ferric iron. HO consists of 2 isozymes referred to as HO-1 and HO-2 (Schacter, 1988). HO-1 can be induced by a variety of different stimuli while HO-2 is non-inducible and constitutively expressed. HO-1 can be upregulated by metal ions, intermediates of heme metabolism, hormones, thiol reactive substances, toxins, organic solvents, heat shock, free radical insults (including anti-oxidant depletion and X-irradiation) and neoplastic states (for a review see Stocker, 1988). HO-1 is a stress protein and the promoter region of the HO-1 gene contains a heat shock element (HSE) consensus sequence (Shibahara et al., 1988). Stress proteins are a superfamily of highly conserved polypeptides which are found in all eukaryotic cells. Cellular stressors such as sublethal exposure to heat, H_2O_2, metal ions, and sulfhydryl agents are known to induce the accumulation of these stress proteins. Members of the stress protein superfamily include HSP90, HSP72, HSP27 and a group of glucose-regulated proteins (e.g. GRP94; Kochevar et al, 1991; Pelham ,1986). Unlike most members of the stress protein superfamily, GRP94 appears to respond to a more restricted range of stimuli such as glucose deprivation and calcium ionophores but not to generalized

intracellular oxidative stress (Lindquist and Craig, 1988). Also, ubiquitin and some α-crystallins contain HSE consensus sequences in their promoter regions and can be up-regulated in response to cellular stressors in a manner similar to HO-1 and other HSPs (Applegate et al., 1991; Hightower, 1991). Heat shock proteins (HSPs) subserve a diverse range of cellular functions. Under normal conditions, it is thought that HSPs are involved in protein folding, unfolding, oligomerization, translocation, and activation of transcription factors (Poola and Kiang, 1994). HSPs are thought to protect cells undergoing stress by preventing damage to the translational apparatus, maintenance of lipid membrane integrity, accelerating degradation of denatured or abnormal proteins and protecting against deleterious protein aggregation by binding to exposed hydrophobic surfaces (Burdon et al, 1987). HO-1 may protect cells undergoing oxidative stress by degrading pro-oxidant metalloporphyrins (heme) to biliverdin and bilirubin which are known to have free-radical scavenging properties (Applegate et al., 1991).

Recent evidence from Dr. Schipper's laboratory suggests that chronic activation of the cellular stress response may be an important mechanism leading to the formation of Gomori-granules in CSH-treated astroglial cultures and in astrocytes of the senescent periventricular brain (Mydlarski et al., 1993, Mydlarski and Schipper, 1993). They demonstrated that 6 hour CSH-treatment induces the accumulation of HO-1, HSP90, 72, and 27, and ubiquitin in cultured astroglia (Mydlarski et al., 1993). In addition, the CSH-pre-treated astroglia were found to be more resistant to H_2O_2 toxicity and mechanoenzymatic trauma (trypsinization) consistent with the fact that the astrocytes have undergone a heat shock response (Mydlarski et al., 1993). Furthermore, subcutaneous CSH administration to young adult male rats induces significant increases in concentrations of DAB-positive astrocytic granules and numbers of astroglia expressing HSP27, 72, 90 and GRP94 in hippocampus, striatum and other brain regions (Schipper et al., 1993b). In another set of experiments, using

confocal microscopy, it was shown that HSP27, GRP94, ubiquitin and to a lesser extent HSP72 (but not HSP90 or α-B-crystallin) co-localized to the autofluorescent granules in CSH-treated astroglial cultures and in astrocytes of the third ventricular subependymal zone in young adult rats (Mydlarski et al., 1993). Interestingly, estrogen-related astroglial granulation in the arcuate nucleus and the third ventricular subependymal zone also appears to be contingent on antecedent HSP expression in these cells (Mydlarski et al., 1995). The Gomori granules may be similar to the "stress" granules previously reported by Nover et al. (1989) in heat shocked tomato cell cultures. They have suggested that heat shock granules may protect various mRNAs and critical proteins from stress-related damage and degradation.

Recent developments suggest that CSH not only influences the heat shock response but may also perturb various enyzmatic antioxidant defense mechanisms in cultured astroglia such as gluthathione reductase, glutathione peroxidase, catalase and superoxide dismutase (SOD). Gutathione reductase reconstitutes reduced glutathione from the oxidized form of this important cellular antioxidant. Glutathione peroxidase coverts toxic H_2O_2 into H_2O and organic peroxides into nontoxic alcohol compounds. Catalase converts H_2O_2 into H_2O, and H_2O_2 in the presence of alcohol into H_2O and acetaldehyde. SOD catalyzes the dismutation of superoxide radicals ($O_2\cdot$) into H_2O_2. Two forms of SOD are found in eukaryotes: CuZnSod in the cytosol and MnSOD in the mitochondria (Cohen and Werner, 1994). Schipper et al. have shown that CSH can increase the activity of MnSOD (but not of CuZnSOD) and can supress the activities of glutathione peroxidase, glutathione reductase, and catalase in cultured astrocytes (Manganaro et al., 1995). Futhermore, they have shown that CSH induces a transient increase in the levels of reduced glutathione. Similarly, subcutaneous administration of CSH results in increased MnSOD, but decreased catalase and glutathione peroxidase, activity in adult rat diencephalon (Manganaro et al., 1995). In addition to the aforementioned heat shock response, upregulation of MnSOD activity may play an

important role in protecting astroglial mitochondria from the oxidative damage induced by CSH and other free radical generating substances.

It is possible that CSH is mediating its effects after being metabolized into some other product. For example, the oxidation of CSH in the presence of transition metals generates several pro-oxidant species including thiyl radicals, superoxide, H_2O_2 and HO· (Munday, 1989). In support of this hypothesis, Manganaro et al. (1995) have shown that CSH causes lipid peroxidation in isolated mitochondria and that this CSH effect can be completely inhibited by addition of catalase. This suggests the autoxidation of CSH results in the formation of H_2O_2 which in turn causes lipid peroxidation within the mitochondrial compartment. Furthermore, H_2O_2, a potent oxidant and inducer of HO-1 in rat astrocytes, is also capable of inducing granulogenesis in astrocyte cultures in a manner similar to CSH (Mydlarski et al., 1993). Taken together, these results suggest that chronic intracellular oxidative stress may be the "final common pathway" responsible for the up-regulation of HO-1 and other HSPs which, in turn, participate in the biogenesis of mitochondria-derived astrocytic inclusions *in vitro* and in the aging brain. This hypothesis is consistent with previous work by Srebro (1971) who showed that X-irradiation, a known generator of intracellular prooxidant intermediates, increases the numbers of peroxidase-positive inclusions in hypothalamic astrocytes in a dose dependent manner. In contrast to primary astrocytes, preliminary data indicate that the astrocyte-derived C6 glioma cell line does not accumulate peroxidase-positive inclusions in response to CSH exposure and therefore provides an important control tissue for delineation of mechanisms responsible for cytoplasmic granulation in astrocytes.

C6 Glioma Cell Line

The C6 glioma cell line was first cloned from a rat glial tumor induced by exposure to N-nitrosomethylurea (Benda et al., 1968). C6 glioma cells express the

astrocyte-specific protein, S-100. In addition, Roser et al. (1991) have shown that C6 cells consist of a mixed population of cells which either express vimentin or lack any cytoplasmic intermediate filaments. The exact mechanism by which transformation of astrocytes into C6 glioma occurred remains unclear. However, recent studies suggest that the absence of GFAP in C6 cells may play a role in tumor formation. Toda et al. (1994) generated stably transfected C6 cells with a cDNA for murine GFAP. The expression of GFAP in the C6 cells resulted in altered cell morphology and marked suppression of cell growth, suggesting that GFAP may act as a tumor suppressor in gliomas. Like primary astrocytes, C6 glioma are also responsive to various cytokines and trophic factors. It has been demonstrated that nerve growth factor (NGF) and retinoic acid (RA) both inhibit the proliferation of C6 cells *in vivo* (Rodts and Black, 1994). Conversely, estradiol, PDGF and S-100 beta seem to augment the viability and the proliferative status of C6 glioma (Bishop and Simpkins, 1994; Strawn et al., 1994; Selinfreund et al., 1991). In response to β-adrenergic receptor agonists, C6 cells have been shown to synthesize and secrete of NGF. Recently, it has been shown that simultaneous administration of TNF-α and interferon-gamma (IFN-γ) followed by incubation with LPS results in the induction of NO· synthase activity in C6 glioma cells (Feinstein et al., 1994).

The effects of CSH on C6 glioma cells is currently under investigation in Dr. Schipper's laboratory. Preliminary results indicate that CSH is incapable of engendering the appearance of peroxidase-positive granules in C6 glioma cells. Furthermore, untreated C6 exhibit have a higher baseline levels of glutathione, glutathione reductase, and catalase in comparison with primary astrocytes cultured for equivalent lengths of time. These findings raise the possibility that bolstered antioxidant defenses protect C6 cells against CSH-mediated oxidative mitochondrial injury and subsequent granulation. The goal of the present thesis is to futher explore the notion that CSH-related cytoplasmic granulation in astroglia (a phenomenon

simulating normal senescent changes in periventricular brain regions *in situ*) is contingent upon an antecedent induction of a cellular stress (heat shock) response. Furthermore, since CSH-mediated oxidative stress in astroglia appears to selectively target the mitochondrial compartment and enhances the activity of the mitochondrial enzyme, MnSOD, we set out to determine whether CSH would up-regulate MnSOD at the transcriptional level in cultured astroglia.

Aims of the Present Thesis

The aims of the thesis were to test the following hypotheses:

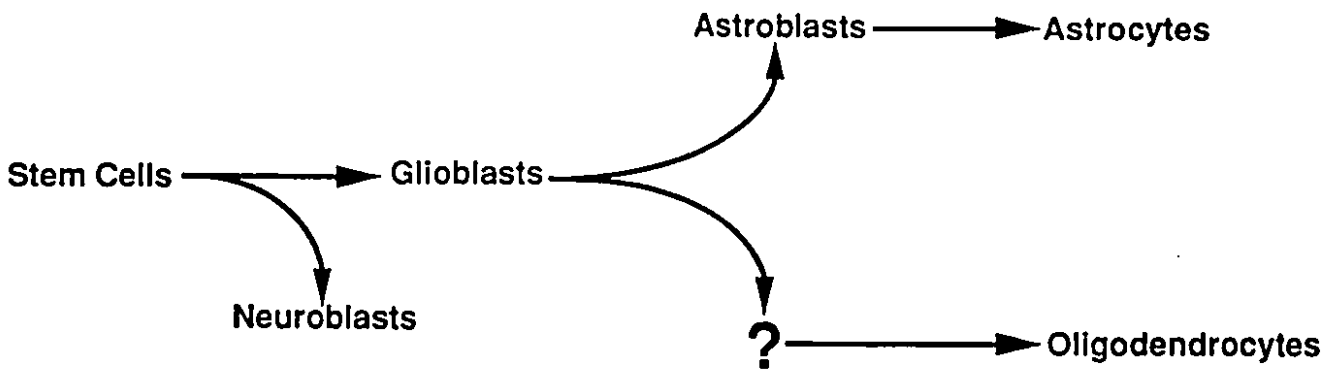
Hypothesis 1: Cysteamine or its metabolites induces the biogenesis of peroxidase-positive granules in purified astrocyte cultures but not in the astrocyte-derived C6 glioma cell line.

Hypothesis 2: Cysteamine or its metabolite induces a cellular stress response leading to the rapid accumulation of heat shock proteins and heme oxygenase-1 in astrocytes but not in C6 glioma cells.

Hypothesis 3: Cysteamine exposure elevates heat shock protein and heme oxygenase-1 mRNA levels prior to stress protein accumulation and granulation in astrocytes but not in C6 glioma cells.

Hypothesis 4: CSH up-regulates MnSOD mRNA and protein levels as a putative cytoprotective mechanism in cultured astroglia.

Figure 1: Classical scheme for the development of macroglia. The stem cells are multipotential cells. Glioblasts are cells committed to the astroglial cell lineage. Astrocytes and oligodendrocytes are terminally differentiated cells.


Astroblasts
Astrocytes
Stem Cells
Glioblasts
Neuroblasts
?
Oligodendrocytes

Materials and Methods

Materials

Superscript cDNA Synthesis Kit and the Random Primer DNA Labelling System were purchased from GIBCO/BRL. Gene Screen Plus hybridization filter membranes were obtained from NEN/Dupont. Hybond hybridization filter membranes were obtained from Amersham. Vacuum Blotter was purchased from BioRad. Slot-Blot manifolds were purchased from Tyler Research Instruments, Edmonton, Canada. Hybridization bottles, nylon mesh and hybridization oven were purchased from BellCo Glass Co. Sprague Dawley neonate rats were obtained from Charles River Inc. Quebec. The rat C6 glioma cell line (ATCC CCL 107) was purchased from ATCC. Ham's F12, high glucose Dulbecco's Modified Eagle Medium, heat-inactivated horse serum and fetal calf serum were purchased from GIBCO. All other chemicals were purchased from Sigma.

Plasmids

Plasmids containing human heme oxygenase cDNA (HH01) (Dr. S. Shibahara, Freidrich-Miescher-Institut, Basel), rat MnSOD cDNA (Dr. B. T. Mossman, U. Vermont) and human pGAPDH cDNA (Dr. D. Ann, U. Minnesota) were gifts. Heat shock protein 27 (clone SPD-910), and heat shock protein 90 (clone SPD-930) were purchased from StressGen, Victoria, Canada. Plasmid DNAs were isolated using standard techniques and purified by centrifugation through an ethidium bromide / cesium chloride gradient (Ausubel et al., 1993). The concentration and purity of each plasmid solution was determined by spectrophotometry.

Antibodies

Rabbit polyclonal antisera against heme oxygenase-1 (SPA-895) and monoclonal antibodies specific for HSP 27 (SPA-800) and HSP90 (SPA-830) were purchased from StressGen, Victoria, Canada. Secondary antibodies complexed to horseradish peroxidase

were purchased from Sigma. Sheep anti-human MnSOD was purchased from Research Plus (Bayonne,NJ).

Primary astrocyte and C6 glioma cultures

Primary neuroglial cultures were prepared by mechanoenzymatic dissociation of cerebral tissue obtained from 2 day old Sprague Dawley rat pups, as previously described (Schipper et Mateescu-Cantuniari, 1991). Rat pups were sacrificed by carbon dioxide inhalation, and sterilized by dips in 70% ethanol. The heads of the pups were decapitated using sterile operating scissors. The skin from the base of the head to the mid-eye area was cut using sterile microdissecting scissors. The skin was then folded to the sides 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 a sterile, curved forceps and placed in 1X Hank's buffered salt solution where the cerebellum and the meninges were removed using a sterile microdissecting forceps. The remaining brain material was cut into small pieces using a scalpel, and passaged successively through a 10 ml, 5 ml, and fire-polished pasteur pipettes. The partially homogenized brain tissue was then incubated for 30 minutes in an equal volume of papain enzyme solution (7,4mM $MgCl_2 \cdot 6H_2O$, 0.1% Papain, 0.01% deoxyribonuclease, 0.1% dispase II). The material was then passed 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 1000rpm 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. The brain cells were resuspended in Ham's F12 and Dulbecco's Modified Eagle Media (DMEM; 50:50 V/V) supplemented with 10mM HEPES, 5% heat-inactivated horse serum, 5% fetal calf serum and penicillin-streptomycin (50U/ml and 50µg/ml, respectively). The cells were seeded onto 75cc flasks precoated with 0.01% poly-D-lysine at a density of 10^7/ flask. The cultures were incubated at 37°C in

humidified 95% air-5% CO_2 for 6 days. Approximately 90% of cells in these cultures are astrocytes as determined by GFAP immunolabeling (Goldman et al., 1986; Schipper et Mateescu-Cantuniari, 1991). After 6 days, the cells were either left untreated (control) or received 880μM cysteamine (CSH) in culture media for 3hr and 6hr in the short-term studies (immunoblots, slot-blots, and Northern blots) or for 12 days in long-term studies (autofluorescence and DAB histochemistry). In the latter, CSH was administered with each change of culture medium (twice weekly).

In order to further enrich the cultures for astroglia, a modification to the protocol described above was performed: after cells had been seeded onto the flasks, the cultures were left in the incubator at 37°C for 6 hours. The flasks were then shaken vigorously by hand 0, 15, 20 or 30 times. The culture media was then removed and replaced with fresh media. After 6 DIV the cultures were treated as above The purity of the cultures was determined using GFAP immunostaining. It was found that after 30 shakes, the cultures were comprised of >98% astrocytes. The Western and Northern blots for MnSOD were performed using cultures prepared by this modified protocol (using flasks shaken 30 times). For the Westerns, the cultures were either left untreated (controls) or were exposed to 880μM CSH for 3 or 5 days. The cultures used for the Northern blots were either left untreated (controls) or exposed to 880μM CSH for for 30 min, 1hr, 3hr, 6hr, 9hr, 12hr, 1 day, 3 days, 5 days, 6 days, or 12 days.

Rat C6 glioma cells (ATCC CCL 107, 38th passage) were grown in Ham's F12 and DMEM (50:50 V/V) supplemented with 10mM HEPES, 10% fetal calf serum and penicillin-streptomycin (50U/ml and 50μg/ml, respectively). The cultures were incubated as above. The C6 glioma cells were left untreated (control) or received 800μM or 1.76mM CSH in the short term studies (3 and 6hr). In the long-term studies (12 days), the cultures received 880μM or 1.76mM CSH with each change of culture media (twice weekly).

Autofluorescence and histochemistry

Autofluorecence: Primary astrocytes and C6 glioma cells (control and 12 days treated) were transferred to slides and fixed by incubation in ice-cold methanol:acetone (50:50 v/v) at 20°C for 10 minutes. The slides were then mounted in aqueous media and examined using a Bio-Rad MRC-600 Laser Scanning Imaging system equipped with a 15 mW krypton / argon laser which excites the samples at 488, 568, and 647 nm (Cisse and Schipper, 1993).

Histochemistry: The primary astrocyte and C6 glioma cells were transferred onto glass slides and fixed with 4% paraformaldehyde for 10 min. at 4°C. The slides were incubated in Karnovsky medium containing 0.05% 3,3' diaminobenzidine (DAB) and 0.002% H_2O_2 in 0.1M phosphate buffered saline (PBS) for the demonstration of non-enzymatic (iron-mediated) peroxidase activity as previously described (Schipper, 1991; Schipper et al., 1990a,b). The slides were incubated for 30 minutes during which time they were kept in the dark and gently rocked. The slides were washed under tap water for 3 minutes and counterstained with methyl green. The slides were then dehydrated using 70%, 80%, 90%, 100% ethanol. The slides were then cleared, mounted with Permount, and coverslipped. To control for enzymatic peroxidase activity, some slides were i)heated at 95°C for 15 min. prior to incubation, or ii) pre-incubated for 30 min. in 10mM of the catalase inhibitor, 3-amino-1,2,3-triazole (AT, Sigma) followed by incubation in DAB-hydrogen peroxide and 0.1M AT (Schipper et al., 1990b).

Gel electrophoresis and immunoblotting

Control and treated (3hr, 6hr, 3 day, and 5 day CSH exposure) primary astrocyte and (3hr and 6hr) C6 glioma monolayers were washed with ice-cold PBS and gently scraped. Cells were collected by gentle centrifugation (112g for 5 minutes) and solubilized in 5 volumes of 4% SDS sample buffer by passage through 18 and 26 gauge needles. Samples were heated to 100°C for 10 minutes and centrifuged (6720g for 10

min.). SDS/PAGE was performed using the method of Laemmli (1970). Protein concentration was determined using the method of Lowry et al. (1951). 50µg of total cellular protein was loaded onto each lane and electrophoresed at 20mA. Proteins were stained with Coomassie brilliant blue or were electophoretically transferred to nitrocellulose membranes (Towbin et al., 1979).. Western blots were performed using a standard method (Ausubel et al., 1991). Nitrocellulose membranes were blocked with 0.5% Tween-20 in Tris buffered saline (TBS; 0.02 M Tris/HCl, pH 7.2, 0.5 M NaCl) at room temperature for 1 hour and incubated overnight at 4^0C with anti-sera specific for HSPs 27, 90, and heme oxygenase-1 (1:1000 dilution). After several washes, the nitrocellulose membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for one hour at room temp. The revelation media consisted of 50mg% 4-chloro-1-naphthol and 5.3mM hydrogen peroxide in TBS.

Extraction of RNA

RNA was prepared from untreated and CSH-exposed (30 min, 1hr, 3 hr, 6 hr, 9hr, 12hr, 1 day, 3 day, 12 day) primary astroglial and C6 glioma cell cultures (3hr and 6hr) using the single-step method for RNA isolation (Chomczynski and Sacchi, 1987). Following treatment, the RNA was isolated from the cell cultures (control, 3hrs and 6hrs CSH). The culture media was removed from the cells and replaced with 1ml (per dish) of denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate pH 7.0, 0.1M 2-mercaptoethanol, 0.5% sarkosyl). The cells were dislodged from the dish by gently swirling the denaturing solution. The cell suspension was then placed in sterile polypropylene 15ml conical tubes and stored at -80^{o}C until further processing. Frozen 1ml cell suspensions were thawed at 42^{o}C and passed through a pipette 10 times to ensure optimal lysis. Following lysis, 0.1ml of 2M sodium acetate (pH 4.0) and 1.0ml of ice-cold water-saturated phenol was added and thoroughly mixed. After adding 0.2ml of 49:1 chloroform isoamyl alcohol, the solution was incubated for 20

minutes on ice. The solution was then spun at 3000rpm at 4°C for 20 minutes in a swinging bucket IEC centrifuge. The upper aqueous phase was transferred to a sterile, RNase-free 15ml corex tube.and an equal volume of 100% isopropanol was added to the tube. This solution was then incubated at -20°C for 30 minutes and then centrifuged at 10 000rpm for 20 minutes at 4°C. The supernatant was discarded and the RNA pellet was resuspended in 0.3mls of denaturing solution. 0.3mls of 100% isopropanol was added to this solution which was incubated for 30 minutes at -20°C. The sample was spun at 13 000rpm for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet was washed with 0.5mls of 70% ethanol. The solution was incubated for 15 minutes at room temperature and then spun at 13 000rpm for 5 minutes at room temperature. The RNA pellet was then dried under vacuum for 10 minutes and resuspended in 150µl of diethylpyrocarbonate treated H_2O. To remove any traces of genomic DNA, the RNA solution was treated with 10 units of RQ1RNase-free DNAse for 30min., reacted with proteinase K, phenol extracted and re-ethanol precipitated. The RNA pellet was then resuspended in 150µl of DEPC-treated H_2O. The concentration and purity of the RNA solution was determined by spectrophotometry. An aliquot of the RNA solution was electrophoresed through a 1% agarose gel containing ethidium bromide and examined for intact 28S and 18S rRNA bands.

Slot Bot hybridization

mRNA levels were assessed using a novel, slot-blot hybridization assay developed by Chalifour et al (1994). This method involved 5 steps: 1) generation of the first strand cDNA. 2) Construction of the random primer-generated dsDNA probes. 3)Preparation of the slot-blot membrane. 4) Prehybridization and hybridization of the primer-generated dsDNA probes to the cDNAs on the slot-blot membrane. First strand cDNA was made from RNA extracted from primary astrocyte and C6 glioma cultures using the GIBCO/BRL Superscript cDNA Synthesis Kit. In brief, 10µg of total RNA

were added to 0.5µg (100nM) oligo dT in a total volume of 11µl and heated at 70°C for 10 minutes and chilled on ice for 5 minutes. The following were added: 4µl of reverse transcriptase buffer, 2µl of 0.1M dithiothreitol, 1µl of [^{32}P]-dCTP (ICN 3000Ci/mM specific activity). After incubation for 2 minutes at 37°C, 1.0µl of reverse transcriptase was added and incubated for 2 hours. To verify the presence of the first strand cDNA and to determine the length of the cDNA fragment, 2µl of the reaction mixture were elctrophoresed through a 1% agarose gel and exposed to X-OMAT film for 2-3 hours. If the labelled cDNA was between 0.5 and 2Kb in length the experiment was continued.

The first strand cDNA was used as a template to produce random primer-generated dsDNA probes using the Random Primer DNA Labelling System from GIBCO/BRL. The first strand cDNA reaction mixture was heated for 5 minutes in a boiling water bath and quickly chilled on ice. The following were then added: 15µl random primers buffer, 2.5µl of 0.5mM dNTPs (dGTP, dATP, dTTP), 5ml [^{32}P]-dCTP, and distilled water to a volume of 49µl. After the addition of 1µl of Klenow fragment (3units/ml),the random primers reaction was allowed to proceed for 3 hours at 25°C. The reaction was terminated with 5µl of 0.2M EDTA (pH7.5). A Sephadex G50 column was used to seperate incorporated from unincorporated radioactive nucleotide (Ausubel et al., 1992).

The plasmid cDNAs, HSP 27, HSP 90 and HO-1, as well as positive control plasmid, GAPDH, and negative control plasmid, pBR322, were crosslinked to Gene Screen Plus nylon membrane using the Dot Blot procedure suggested by the manufacturer at concentrations ranging from 25ng to 250ng per slot. In brief, the plasmids were denatured in 0.25 N NaOH for 10 minutes, diluted to 250ng and 25ng per 20µl with 0.125 N NaOH / 0.125 X SSC (0.01875 M NaCl; 0.001875 M sodium citrate) and the denatured / diluted DNA was applied to the membrane using light suction. The membrane was removed from the manifold, allowed to air dry and baked at 80°C under a vacuum for 1.5 hours. The filters were then wrapped in Saran wrap and stored in batches

at room temperature.

The prehybridization, hybridization and washes were performed according to the Gene Screen Plus manufacturer's instructions. In brief, the membranes containing the cDNAs was soaked in 2 X SSC (0.3M NaCl / 0.03M sodium citrate) , layered on top of a nylon mesh and placed in a roller bottle. The membrane was prehybridized in 10mls of prehybridization solution (prehyb; 50% dextran sulphate, 10% SDS) for 2 hours at 63^0C followed by incubation in 5mls of hybridization solution (hyb) consisting of fresh prehyb, denatured DNA probe ($2x10^6$ cpm/ ml of prehyb), and denatured, sheared herring sperm DNA (100mg/ ml of hyb). The filter was incubated with the hyb for 16 hours at 63^0C. After the 16 hours, the hyb was removed and the membrane was washed twice with 15mls of 2 X SSC at room temperature, twice with 15mls 2 X SSC / 1% SDS for 30 minutes at 63^0C, and twice with 15mls 0.1 X SSC at room temperature. The filter was placed on a 7.0 X 7.5cm sheet of Whatman filter paper and wrapped in Saran wrap. The filter was exposed to X-OMAT film for a period of 5 days at -80^0C.

Quantitation

Densitometry was performed using a LKB 222-020 Ultrascan XL laser densitometer and the area under each peak was recorded. The corrected band intensities were determined by subtracting the band intensities of PBR322 from those of the cDNAs of interest. These values were then divided by the corrected band intensity of GAPDH. The data were analyzed using the Student's t-test with $p<0.05$ indicating statistical significance.

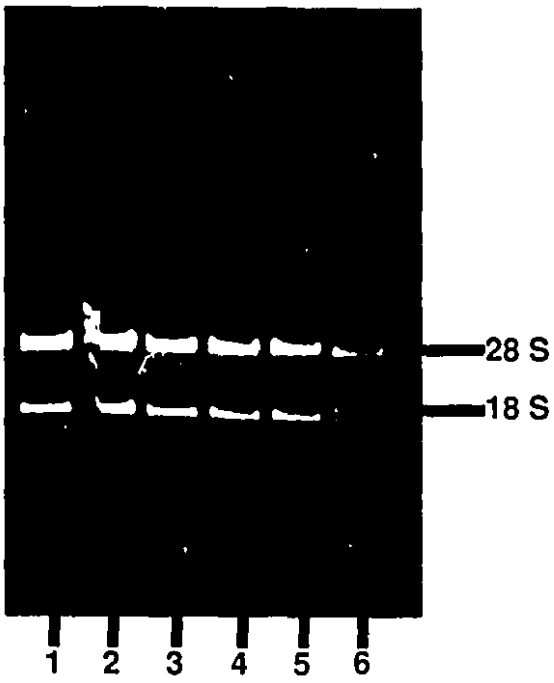
Northern Blot

Conventional Northern analyses were performed to validate our slot blot hybridization assays (Ausubel et al, 1993). Total RNA was isolated from control and treated astrocytes and glioma cells. 15ug of each RNA sample was electrophoresed in a

1% formaldehyde-agarose gel and transferred onto Hybond nylon membrane by Vacuum blotter and cross-linked to the membrane by UV light.. Prehybridization was done for 2hrs at 42^0C in a buffer containing 50% formamide, 5X Dendhardt's reagent, 5X SSPE, and 0.5% SDS. The hybridization buffer consisted of the prehybridization buffer, 0.1mg/ml denatured herring sperm DNA and $5X10^6$ CPM/ml ^{32}P labelled, denatured DNA probe. The HSP27 cDNA probe was prepared from a 400bp restriction fragment (Pst-1), the HSP90 cDNA probe from a 1300bp fragment (Pst-1), the HO-1 cDNA probe from a 1000bp restriction fragment (Xho-1 - EcoR1), and the GAPDH cDNA probe from a 474bp restriction fragment (Xba-1 and Hind III). These fragments were used as templates to produce random primer-generated dsDNA probes using the Random Primer DNA labelling System from GIBCO/BRL in a manner similar to the probes generated for the slot-blot hybridization.. The hybridization was performed overnight at 42^0C. The blots were successively washed with 2XSSC, 0.1% SDS for 20 min at room temperature, 0.1X SSC, 0.1% SDS for 20 min at 60^oC and exposed to the X-ray film. The northern hybridization of MnSOD was performed with some modifications to the method performed above. The 1400bp MnSOD cDNA probe (EcoR1) was prepared as mentioned above. Prehybridization was performed for 4 hours at 65^oC in a buffer containing 5X Dendhardt's reagent, 5X SSPE, 1.0% SDS and 0.1mg/ml denatured salmon sperm DNA. The hybridization buffer consisted of the prehybridization buffer and ^{32}P labelled (10^6 cpm/ml), denatured DNA probe. The hybridization was performed overnight at 65^oC. The blots were sucessively washed with 2.0X SSC, O.1% SDS for 20 min. at room temperature, 1.0X SSC for 20 min. at 65^oC, 0.1X SSC, 0.1% SDS for 20 min. at 65^oC and exposed to X-ray film at -80^oC. The autoradiographs were quantitated by densitometry (Molecular Dynamics) and the data were expressed in arbitrary units as the ratio between the mean of the 5 MnSOD transcripts and 18s ribosomal RNA. The HO-1 transcript was quantitated in the same manner as MnSOD. Berfore reprobing with another probe, the blots were stripped by boiling at 100^oC for 2 min. Blots were re-

analyzed with (^{32}P) ribosomal probe as performed by Lehoux et al. (1987) with the following modification: the 18s ribosomal RNA band was recovered by electrophoresis and precipitated with 1/10 volumes 3M sodium acetate and 2 volumes of 100% ethanol.

Figure 2: Example of RNA quality: Lanes 1, 2, 3 denote untreated astrocytes, 3 hr CSH-treated (880μM) and 6 hr CSH-treated (880μM) astrocytes, respectively. Lanes 4, 5, 6 denote untreated C6 glioma, 3 hr CSH-treated (880μM) glioma, and 6 hr CSH-treated (880μM) glioma, respectively.


28 S
18 S
1
2
3
4
5
6

Results

Autofluorescence and histochemistry

The astroglial and C6 glioma cultures exhibited differences in autofluorescence upon exposure to 880μM CSH. As previously reported (Schipper et al., 1990b), primary astrocyte cultures treated with CSH demonstrate an increase in orange-red autofluorescence consistent with the presence of porphyrin or oxidized flavoproteins (Goldgefter et al., 1980) relative to control (untreated) astrocyte cultures (Figure 3a and b). C6 glioma demonstrated a predominantly yellow autofluorescence. The extent of yellow autofluorescence did not vary significantly between the untreated (control) and treated (880μM - 1.76mM) C6 glioma cultures (Figure 3c and d).

Long term exposure of astroglial cultures to CSH resulted in a marked accumulation of DAB-positive inclusions dispersed throughout the cytoplasm (Figure 4a and b) as previously observed (Schipper and Mateescu-Cantuniari, 1991). In contrast, no DAB-positive cytoplasmic inclusions were observed in either control or CSH-treated C6 glioma cultures. Glioma cells occasionally exhibited faint, diffuse DAB staining of their cytoplasm which was unaffected by CSH exposure (Figure 4c-d).

Heat pretreatment of control or CSH-treated astrocyte or C6 glioma cells did not abolish DAB staining. Pre-incubation of the astrocyte and C6 glioma cells with the catalase inhibitor, AT, similarly, did not influence peroxidase staining. Taken together, these results indicate that the peroxidase activity in these cells is non-enzymatic in nature and likely mediated by ferrous iron (Goldfischer et al., 1966).

Figure 3: Laser scanning confocal images of the autofluorescent granules in Primary astroglial cultures vs C6 glioma cell lines: a and b denote control (untreated) and CSH-treated (880μM X 12 days) astroglial cultures, respectively. c and d denote Control and CSH-treated (1.76 mM X 12 days) C6 glioma cell cultures, respectively. Bar=50um

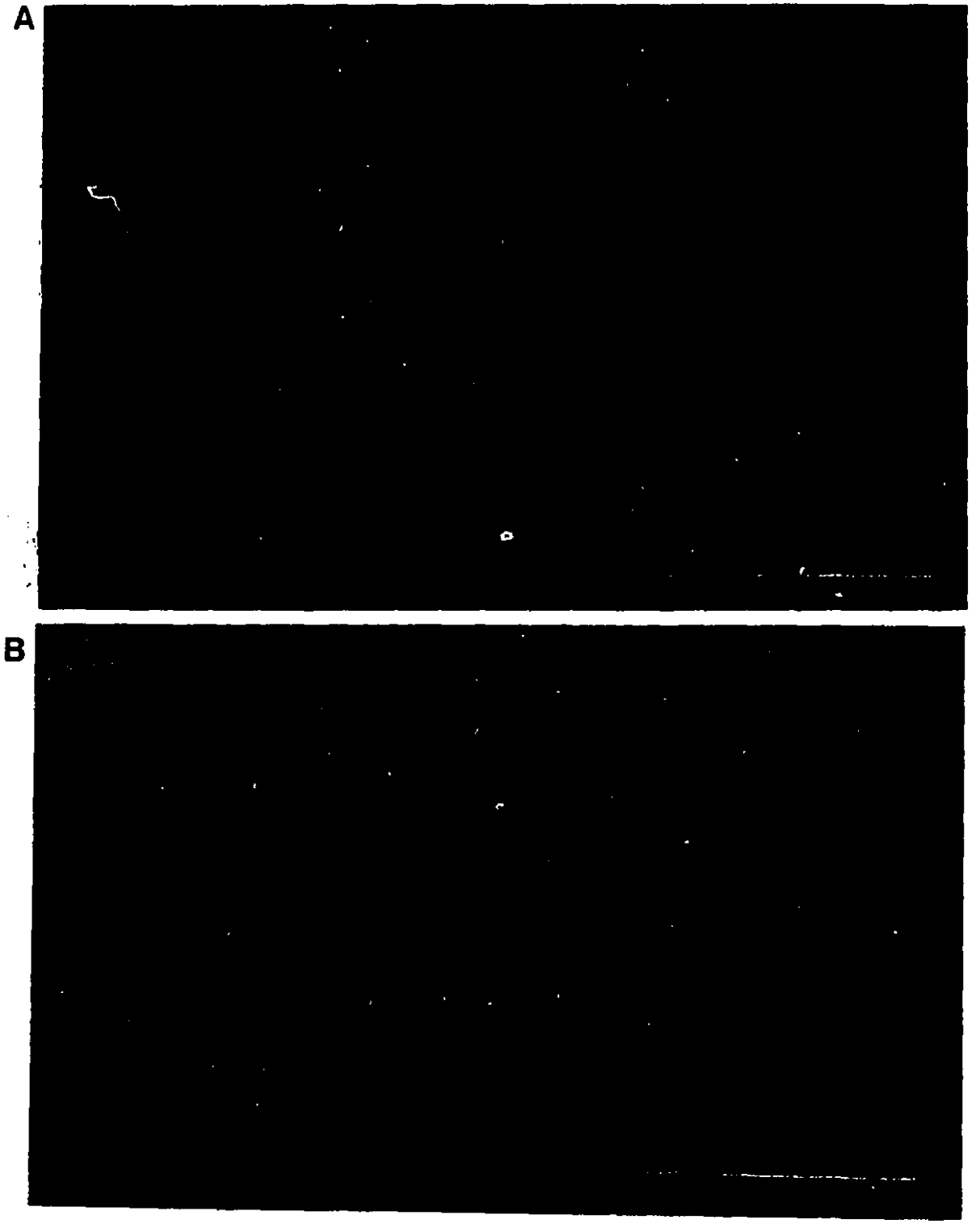

A
B

Figure 3:(continued)

C

D

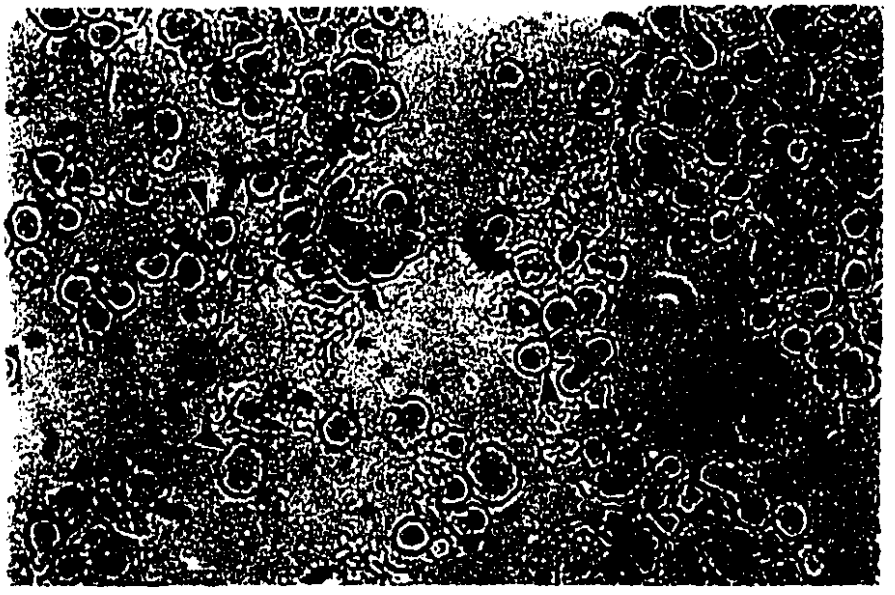
Figure 4: DAB staining, for endogenous peroxidase activity, with a methyl green counterstain. a and b denote Control (untreated) and CSH-treated astroglial cell cultures, respectively. c and d denote Control and CSH-treated (1.76mM X 12 days) C6 glioma cell cultures, respectively. X372.

A

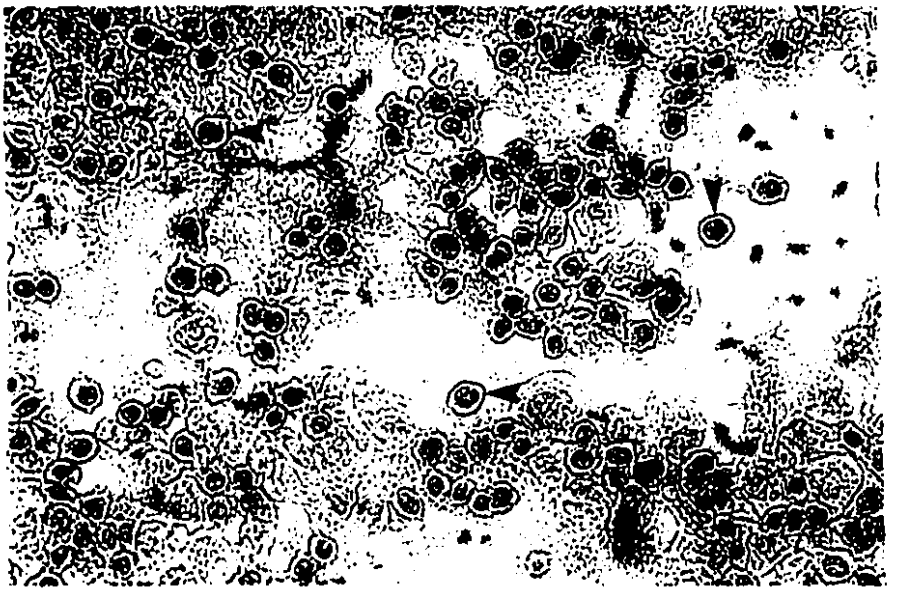
B

Figure 4: (continued)

C



D



Gel Electrophoresis and Western Blots For HSPs and HO-1

Coomasie blue-stained gels demonstrated that CSH exposure induced the appearance of a number of novel protein bands in astroglial cultures (Figure 5, lanes 1-3). Within 3 hours the total protein profile of treated cells demonstrated an increased intensity of protein bands at molecular weights of 27 kDa, and 90kDa (lane 2). Protein bands at 32 kDa (HO-1) were also observed, although the difference in intensity between control and treated astrocyte homogenates was minimal. After 6 hours of CSH exposure, protein preparations from the treated cells demonstrated more intense bands at molecular weights 27 kDa and 90 kDa (lane 3) than was found in protein homogenates prepared from the corresponding untreated cells (lane 1). Also, many additional bands found in control samples disappeared or decreased in intensity in treated cultures. Markedly different results were obtained for the control and treated C6 glioma culture preparations (figure 5, lanes 4-6). Protein bands at 27 kDa and 90 kDa were found in both control and treated (lanes 5 and 6) C6 glioma cultures, indicating that these proteins are constitutively present in C6 glioma. Again, protein bands at 32 kDa were observed but there was no apparent differences between control and treated cultures.

Western blots of control astroglial cultures demonstrated faint staining for HO-1, HSP 27 and HSP 90 (Figure 6, panel A). CSH-treated astroglial cultures (3 hours) displayed modest staining for each of these stress proteins (lanes 2, 5 and 8). Astroglial cultures treated with CSH for 6 hours exhibited intense staining for the various HSPs (lanes 3, 6 and 9). Western blots of HSP 90 demonstrated additional bands at 37 and 27 kDa. As reported by the manufacturer, the SPA-830 monoclonal antibody, while highly specific for HSP 90, shows some cross-reactivity with 35-40 kDa protein components of pre-mRNA particles. Western blots of control and treated C6 glioma cultures displayed identical staining

intensity for HSP 27 and HSP 90 (figure 6, panel B, lanes 4-9). Again, Western blots of HSP 90 demonstrate bands at 47 and 37 kDA. In contrast to the other stress proteins, HO-1 immunoreactivity in the glioma preparations was substantially increased after 6 hr CSH exposure (panel B, lane 2 and 3) relative to the untreated controls (panel B, lane 1).

Figure 5: Coomassie brilliant blue stained SDS-PAGE (to verify consistency of protein laoding per lane) prepared from control and CSH-treated (880μM X 3 hr or X 6 hr) primary astroglial (lanes 1-3) and C6 glioma (Lanes 4-6) cultures. Lane 1: untreated astrocytes. Lane 2: 3 hr CSH treatment. Lane 3: 6 hr CSH treatment. Lane 4 untreated glioma. Lane 5 and lane 6: 3 hr and 6 hr CSH exposure, respectively.

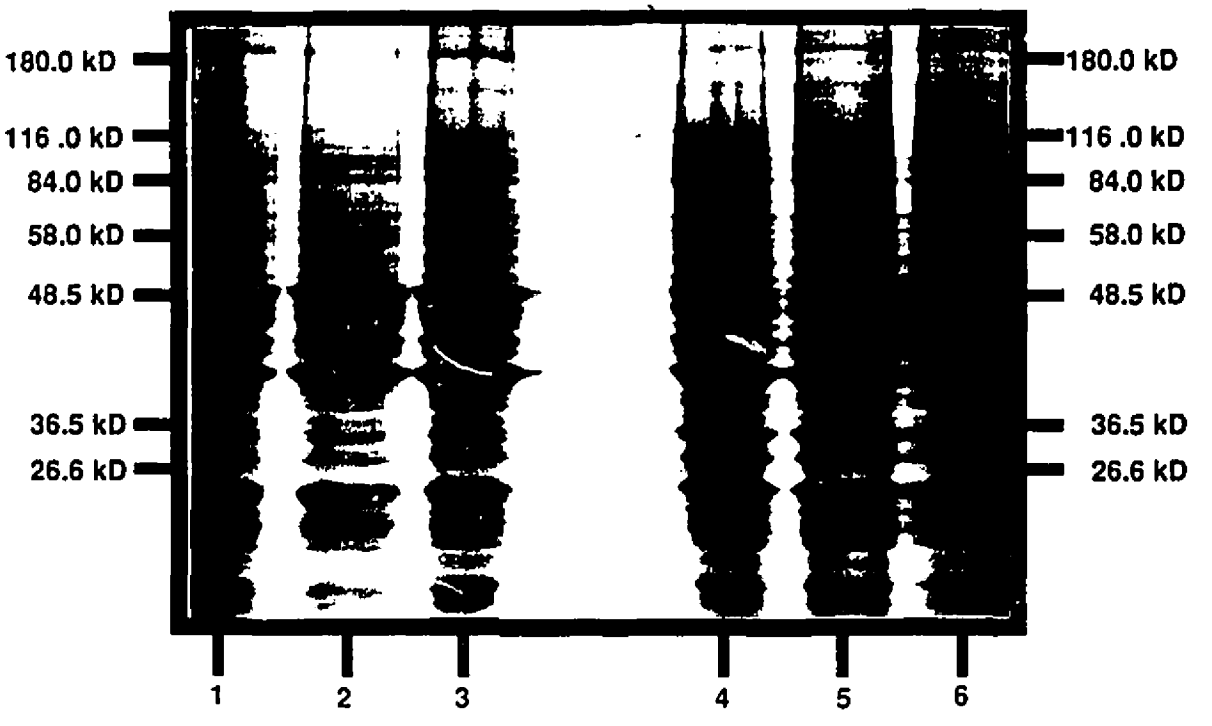
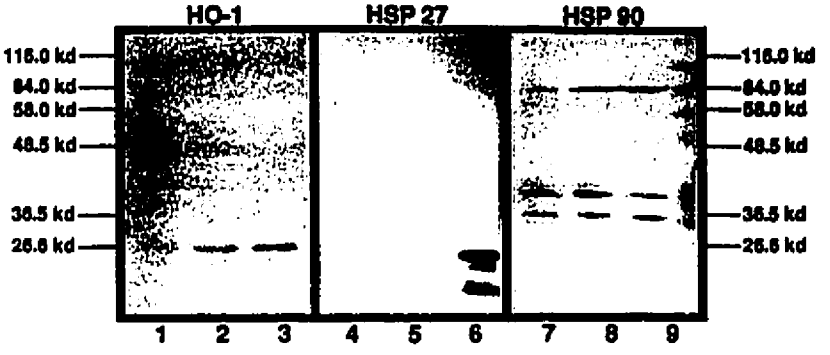

180.0 kD
116 .0 kD
84.0 kD
58.0 kD
48.5 kD
36.5 kD
26.6 kD
180.0 kD
116 .0 kD
84.0 kD
58.0 kD
48.5 kD
36.5 kD
26.6 kD
1
2
3
4
5
6

Figure 6: Western blots of CSH-treated and untreated astrocyte and glioma cultures.

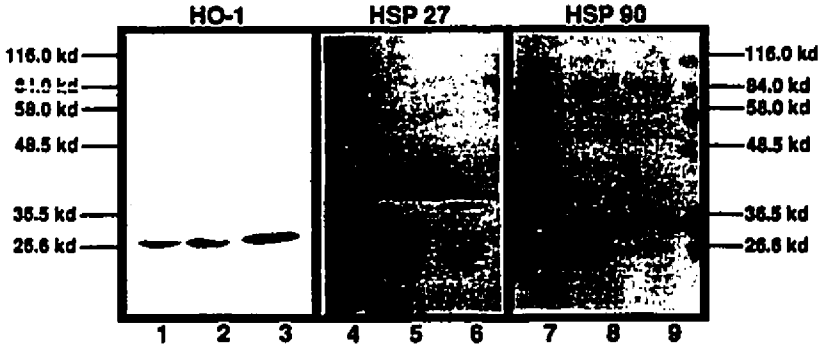
Panel A) Astrocytes. 50µg of total cellular extract were loaded per lane, electrophoresed through an SDS-PAGE gel and transferred to nitrocellulose. Samples in lanes 1-3 were incubated with rabbit anti-HO-1. Lane 1: untreated astrocytes. Lanes 2 and 3: 3 hr and 6 hr CSH-treated (880µM) astrocytes, respectively. Lanes 4-6 were incubated with mouse anti-HSP27. lane 4: untreated astrocytes. Lanes 5 and 6: 3 hr and 6 hr CSH-treated (880µM) astrocytes, respectively. Lanes 7-9 were incubated with mouse anti-HSP 90. Lanes 7: untreated astrocytes. Lanes 8 and 9: 3 hr and 6 hr CSH-treated (880µM) astrocytes, respectively. The mobility of pre-stained protein markers is indicated on the left and right of the gel.

Panel B) C6 glioma preparations. 50µg of total cellular extract were loaded per lane, electrophoresed through an SDS-PAGE gel and transferred to nitrocellulose. Samples in lanes 1-3 were incubated with rabbit anti-HO-1 antibodies. Lane 1: untreated glioma cells. Lanes 2 and 3: 3 hr and 6 hr (880µM) CSH-treated glioma cells, respectively. Lanes 4-6 were incubated with mouse anti-HSP 27 antibodies. Lane 4: untreated gliomas. Lanes 5 and 6: 3 hr and 6 hr CSH-treated (880µM) gliomas, respectively. Lanes 7-9 were incubated with mouse anti-HSP 90 antibodies. Lane 7 untreated glioma. Lanes 8 and 9: 3 hr and 6 hr CSH-treated (880µM) gliomas, respectively. The mobility of pre-stained protein markers is indicated on the left and right of the gel.

A Astrocyte



B C6 Glioma



mRNA Determination for HSPs and HO-1

CSH-treated astroglial cultures exhibited higher levels of HSP mRNAs than the control cultures as determined by the slot-blot hybridization assay (Figure 7 and 8). The RNA obtained after 3 hours of CSH treatment demonstrated an increase in HSP mRNA levels relative to RNA obtained from control cultures. Furthermore, RNA prepared after 6 hours of CSH exposure showed 3 to 10-fold increases in the mRNA levels of various HSPs relative to the control cultures: HO-1, HSP 27 and HSP 90 mRNAs were increased 8-, 3- and 10-fold, respectively, after 6 hours CSH exposure relative to the untreated controls ($p<0.05$ for each comparison; fig 7). In contrast, HO-1, HSP 27 and HSP 90 mRNA levels in CSH-treated glioma cultures remained unchanged relative to those in the untreated glioma preparations ($p>0.05$ for each comparison; fig 8).

The results of Northern hybridizations for stress protein mRNAs were similar to those obtained using slot blot hybridization method with the exception that the C6 glioma cells consistently displayed an increase in HO-1 mRNA levels after 3 and 6hr CSH treatment (Figure 9; lanes 3-4). As expected, bands for HO-1, HSP 27, and HSP 90 were 1.8 kb, 0.95 kb, and 2.7 kb in size, respectively. As depicted in the panel for HSP 27, the probe binds non-specifically to 18s ribosomal RNA as well as to an unknown RNA species at 1.8 kb. Non-specific binding to a 1.8 kb has been previously observed in human breast cancer cell lines by Faqua et al. (1989). They have suggested that the 1.8 kb band may represent an unspliced, precursor RNA or a transcript from a related fragment. Slot-blot and Northern analyses of HO-1 mRNA levels in CSH-treated and control glioma cells were repeated four times using material from four separate culture preparations. On each occasion, results identical to those described above were obtained.

In the short term glioma study, no significant differences between the 880μM and the 1.76mM CSH doses were observed with respect to the pattern or magnitude of mRNA and protein expression as determined by Slot-blot, Northern and Western analyses.

Figure 7: Slot blot hybridizations demonstrating heat shock protein mRNA levels for control and CSH-treated (880uM X 3h or X 6h) primary astrocytes. Band intensities were measured by densitometry. Columns represent the corrected intensities (see Materials and Methods) for the HSP 27, HSP 90 or HO-1 bands. The vertical bars represent means and standard errors of 3 experiments

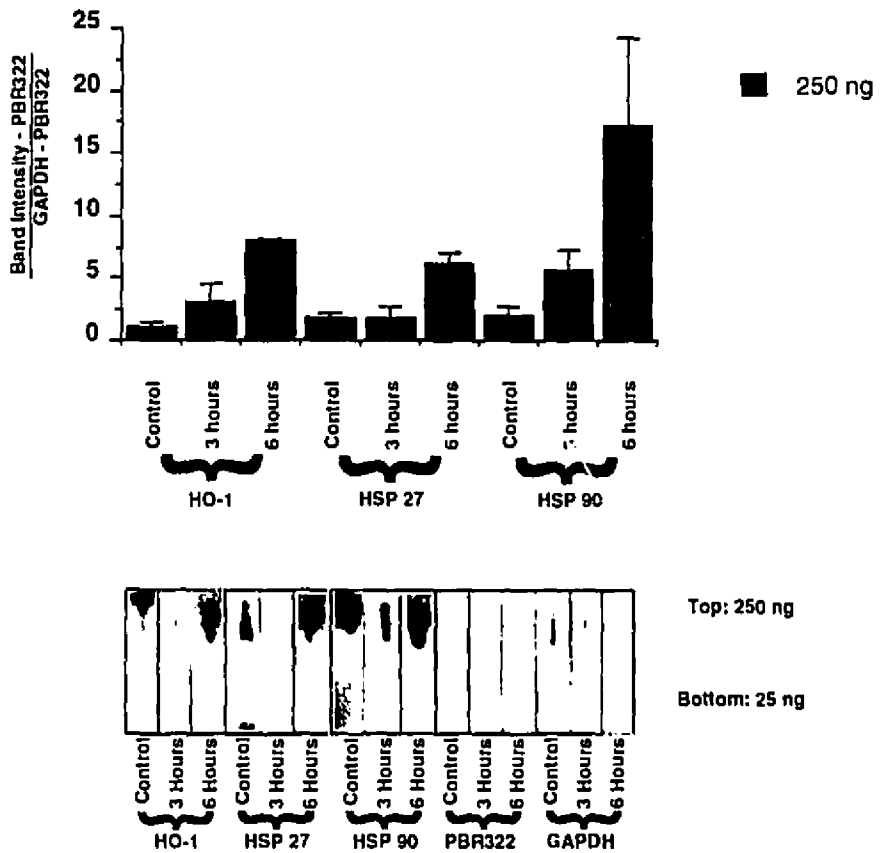
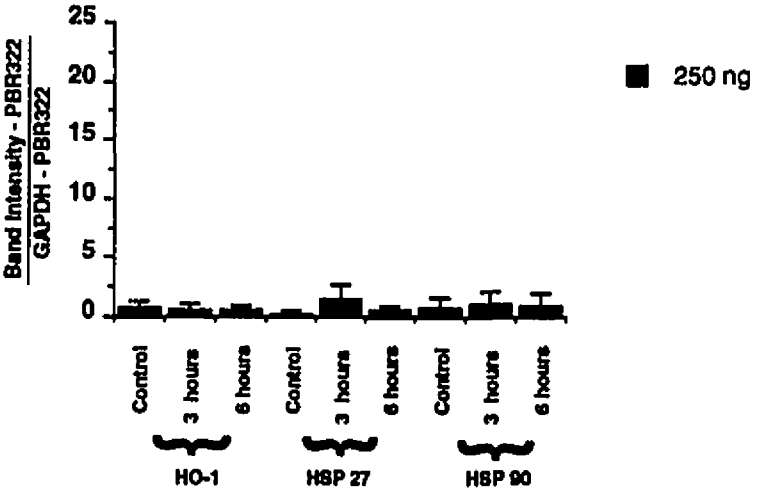

Band Intensity - PBR322
GAPDH - PBR322
25
20
15
10
5
0
250 ng
Control
3 hours
6 hours
HO-1
HSP 27
HSP 90
Top: 250 ng
Bottom: 25 ng
PBR322
GAPDH

Figure 8: Slot blot hybridizations demonstrating heat shock protein mRNA levels for control and CSH-treated (880μM X 3h or X 6h) C6 glioma cells. Band intensities were measured by densitometry. Columns represent the corrected intensities (see Materials and Methods) for the HSP 27, HSP 90 or HO-1 bands. The vertical bars represent means and standard errors of 3 experiments


Band Intensity - PBR322
GAPDH - PBR322
25
20
15
10
5
0
250 ng
Control
3 hours
6 hours
HO-1
HSP 27
HSP 90

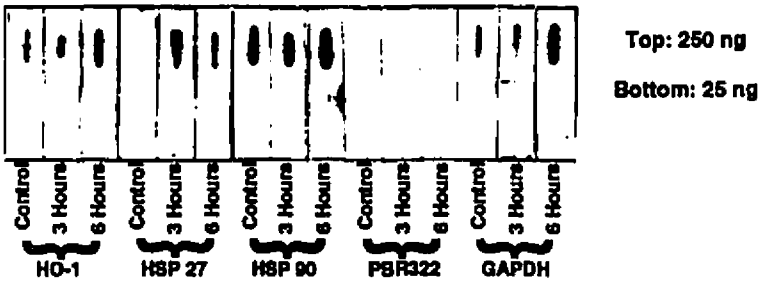
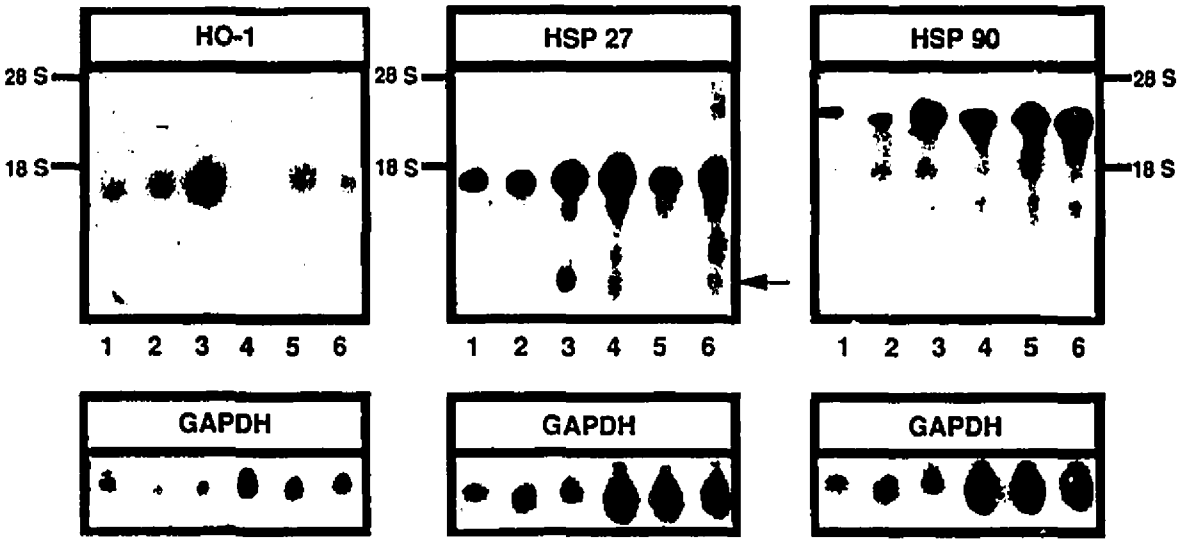

Top: 250 ng
Bottom: 25 ng
Control
3 Hours
6 Hours
HO-1
HSP 27
HSP 90
PBR322
GAPDH

Figure 9: Northern blot analysis demonstrating heat shock protein mRNA levels for control and CSH-treated astrocytes and C6 glioma cells.

Lanes 1, 2, 3 denote untreated astrocytes, 3 hr CSH-treated (880μM) astrocytes, and 6 hr CSH-treated (880μM) astrocytes, respectively. Lanes 3, 4 , 5 denote untreated C6 glioma, 3 hr CSH-treated (880μM) glioma, and 6 hr CSH-treated (880μM) glioma, respectively.


HO-1
HSP 27
HSP 90
28 S
18 S
1 2 3 4 5 6
GAPDH

Shaking Method for the Purification of Primary Astrocyte Cultures

In order to increase the purity of the cultures for astroglia, the flasks were shaken various number of times after the initial 6 hour incubation at 37°C. This facilitates the removal of oligodendroglia and superoxide-producing microglia which may be present in the cultures. Flasks shaken 15, 20, or 30 times were found to contain 93.5%, 96%, and 98% GFAP-positive astrocytes, respectively (figure 10). The unshaken flasks were found to have 91.5% GFAP-positive astrocytes. Since the flasks shaken 30 times exhibited greater astrocyte enrichment than the unshaken flasks, they were used to perform the Western and Northern blots for MnSOD.

Western Blots for MnSOD

Western blots using anti-MnSOD antisera revealed discrete protein bands at 22 kDa (figure 11). Immunoreactive MnSoD was considerably more abundant in the CSH-treated cells after 3 and 5 days of exposure than in the non-treated cells (where immunoreactivity was consistently faint or absent). Unlike the case of HSPs and HO-1, there was no induction of immunoreactive MnSOD at earlier time points (3 hr, 6 hr, 1 day).

Northern Blots for MnSOD

Northern blots for MnSOD demonstrated that all five transcripts were expressed in the primary astrocyte cultures. Each of these mRNA transcripts has been shown to give rise to mature, functional protein (Hurt et al., 1992). MnSOD mRNA levels (figure 12A) in both control and CSH-treated astrocytes increased in parallel between 0 and 12 hours following initiation of the treatment. However, after 6 days of CSH-treatment, MnSOD mRNA levels were approximately twice those observed in controls (figure 13A). HO-1 mRNA levels were rapidly

augmented in response to CSH exposure (figure 12B-13B) confirming that CSH induced a cellular stress response in these cells as previously reported by Mydlarski et al. (1993). The results of the northern analysis were quatitiated by densitometry relative to 18s ribosomal RNA levels (figure 14). The effects of CSH on MnSOD mRNA levels were repeated 4 times using separate culture preparations. On each occasion, results similar to those described above were obtained.

Figure 10: Percentage of GFAP-positive astrocytes vs. number of shakes. Percentage purity was determined by GFAP immunohistochemistry (see methods). Vertical bars represent the standard error of 3 observations.

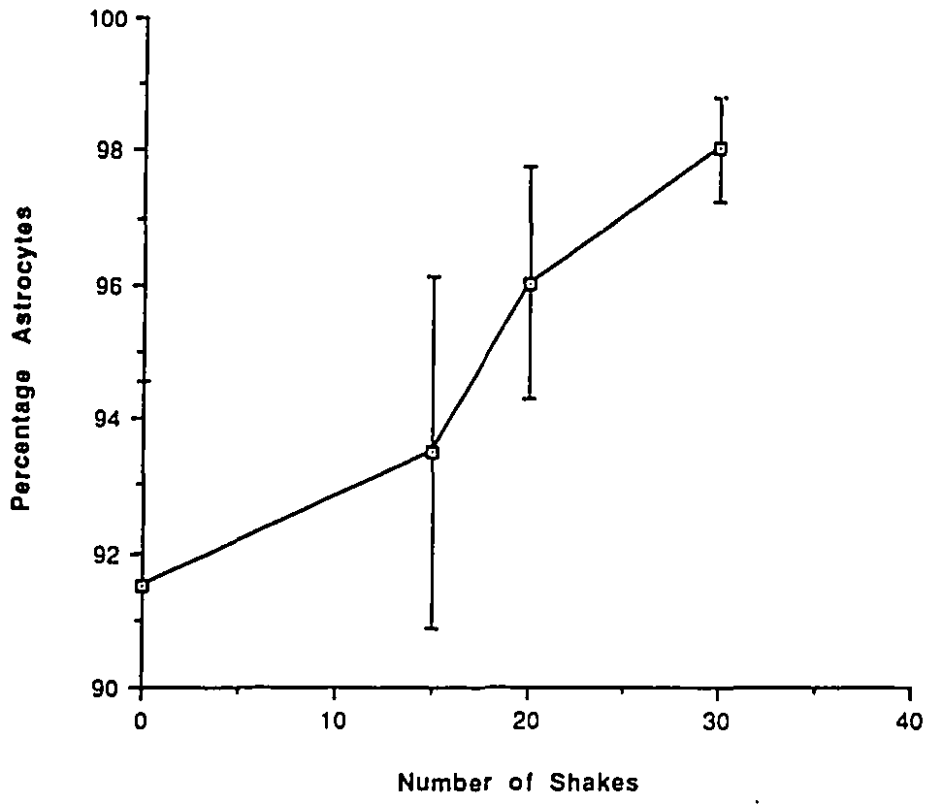

100
98
96
94
92
90
Percentage Astrocytes
0
10
20
30
40
Number of Shakes

Fig. 11: Western blots of CSH-treated and untreated astrocytes. Lanes a and b denote control astrocytes and lanes c and d indicate CSH-treated astrocytes. Lanes a and c: 3 days, lanes b and d: 5 days. The blot was incubated with sheep anti-human MnSOD. The mobility of pre-stained protein markers is indicated on the left of the figure.

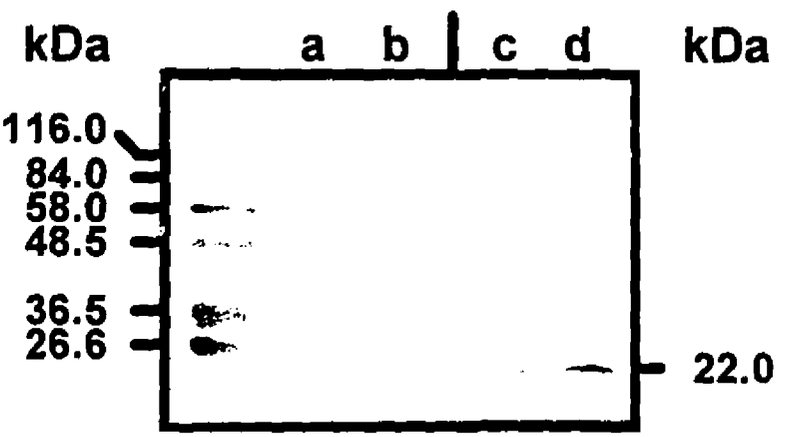

kDa
a
b
c
d
kDa
116.0
84.0
58.0
48.5
36.5
26.6
22.0

Fig. 12: Northern analysis of the short term effects of CSH on MnSOD and HO-1 mRNA levels in cultured astrocytes.

Panel A) Effect of CSH-treatment on MnSOD mRNA levels. Lanes a-f were control astrocytes and lanes g-l were CSH-treated astrocytes. Lanes a and g: 30 min, lanes b and h: 1 hr, lanes c and i: 3 hr, lanes d and j: 6 hr, lanes e and k: 9 hr, lanes f and l: 12 hr. Total cellular RNA (15 µg) was loaded per lane.

Panel B) Effect of CSH-treatment on HO-1 mRNA levels.

Panel C) The blot was also probed with ^{32}P labeled 18s ribosomal RNA.

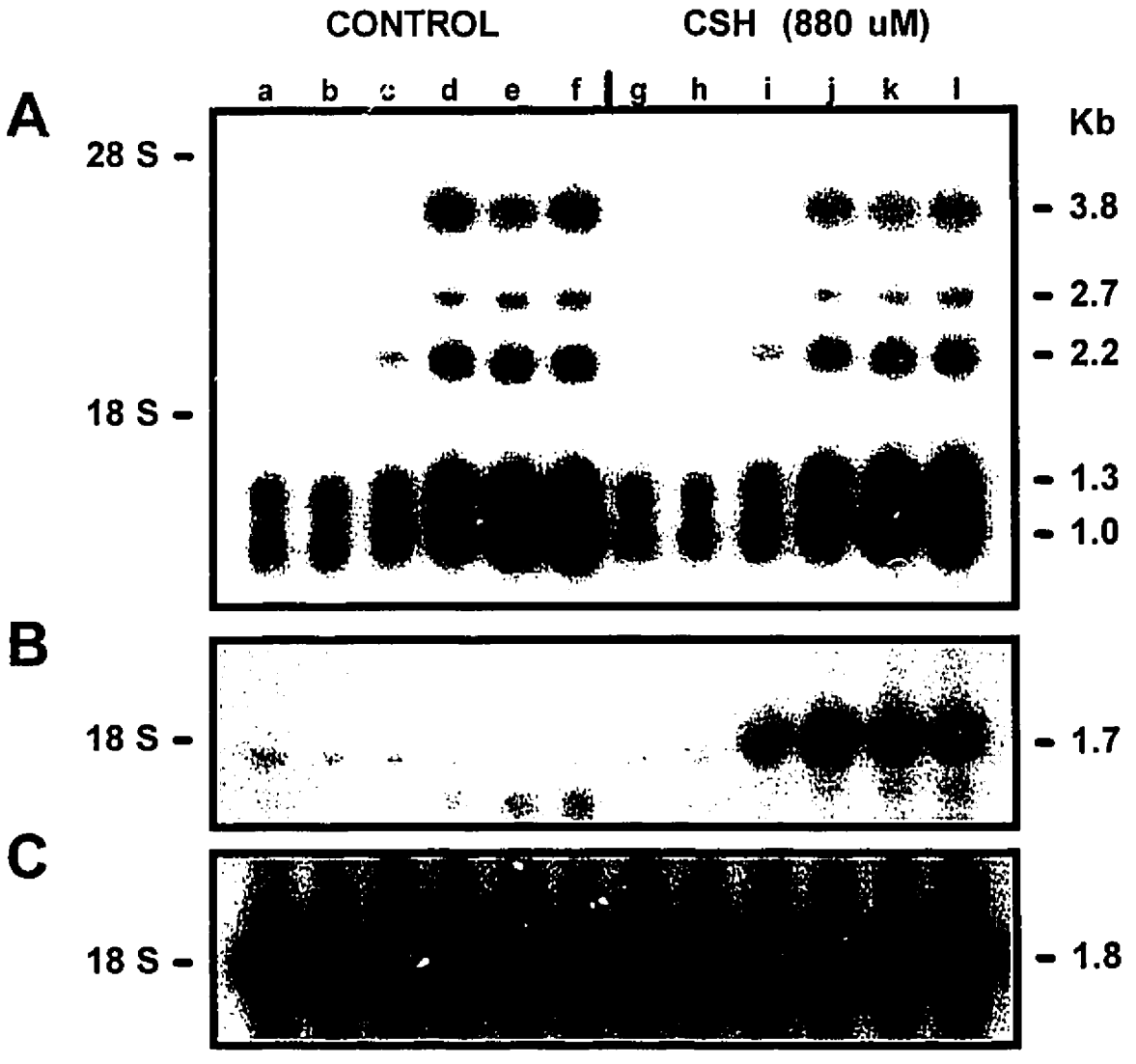

CONTROL
CSH (880 uM)
a b c d e f g h i j k l
A
28 S
18 S
Kb
3.8
2.7
2.2
1.3
1.0
B
18 S
1.7
C
18 S
1.8

Fig. 13: Northern analysis of the long term effects of CSH on MnSOD and HO-1 mRNA levels in cultured astrocytes.

Panel A) Effect of CSH-treatment on MnSOD mRNA levels. Lanes a-e denote control astrocytes and lanes f-j denote CSH-treated astrocytes. Lanes a and f: 1 day, lanes b and g: 3 days, lanes c and h: 6 days, lanes d and i: 9 days, lanes e and j: 12 days. 15 µg of cellular RNA were loaded per lane.

Panel B) Effect of CSH-treatment on HO-1 mRNA levels.

Panel C) Blot depicted in (A) and (B) after re-probing with ^{32}P labeled 18s ribosomal RNA.

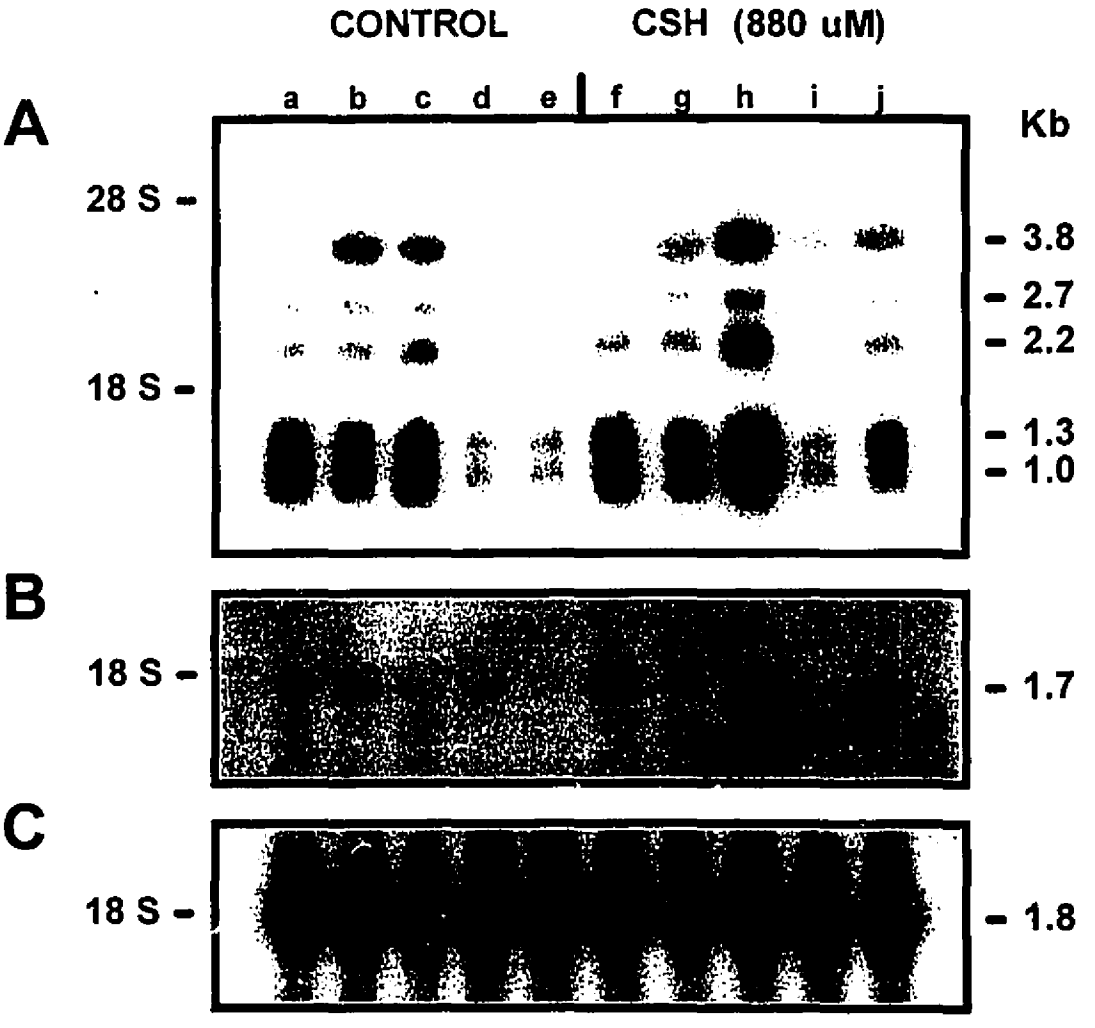
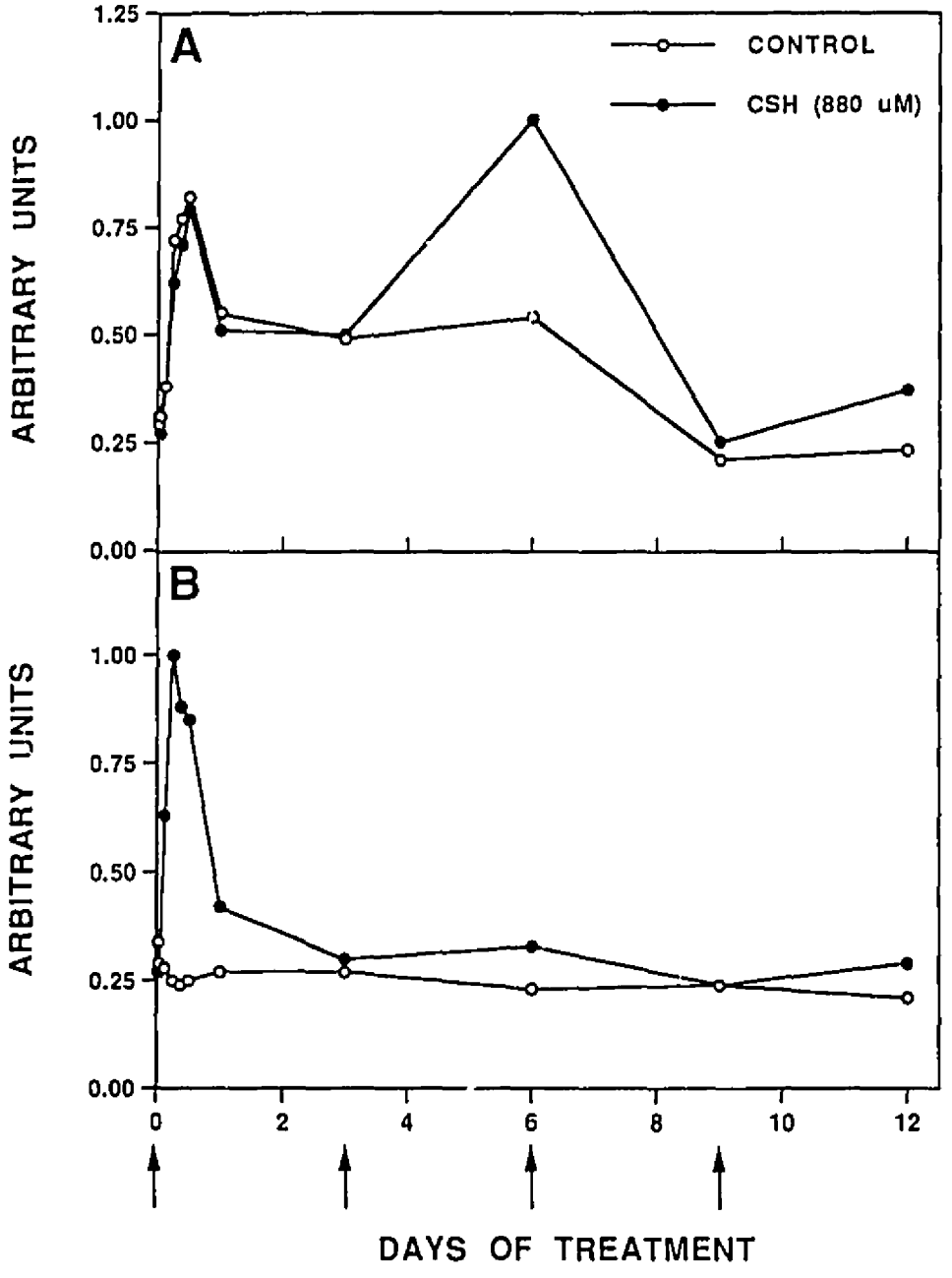

CONTROL
CSH (880 uM)
a b c d e f g h i j
A
Kb
28 S
18 S
3.8
2.7
2.2
1.3
1.0
B
18 S
1.7
C
18 S
1.8

Fig. 14: Quantitation of MnSOD (A) and HO-1 (B) mRNA. mRNA levels were evaluated by densitometric scanning of Northern blots depicted in fig. 12 and 13 and were expressed in arbitrary units relative to 18s ribosomal RNA. Arrows indicate time of feeding of cells in culture.


A
B
CONTROL
CSH (880 uM)
ARBITRARY UNITS
ARBITRARY UNITS
1.25
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
10
12
DAYS OF TREATMENT

Discussion

CSH, X-irradiation, and chronic estrogenization have been shown to induce a massive accumulation of Gomori-granules in cultured astrocytes and/or in the intact rat brain, that are morphologically and histochemically identical to those which spontaneously arise in limbic and periventricular astrocytes as a function of advancing age (Schipper, 1991). The current project was undertaken based on the premise that careful delineation of the mechanisms responsible for inclusion biogenesis in a cell culture model of Gomori astroglia would shed light on the cellular and molecular events promoting their formation in the normal aging brain and under pathological conditions. The results of this study demonstrate that the aminothiol compound, cysteamine (CSH), rapidly augments stress protein mRNA levels followed by a relatively late increase in MnSOD mRNA in cultured astrocytes. As previously observed, CSH induces the formation of autofluorescent, peroxidase-positive cytoplasmic inclusions in these cells. CSH was not capable of inducing either cytoplasmic granulation or heat shock protein expression in the astrocyte-derived C6 glioma cell line.

Inclusion Biogenesis in Astrocytes and C6 Glioma Cells

It has recently been shown that the CSH-induced inclusions arise from swollen, iron-laden mitochondria engaged in a macroautophagic process (Brawer et al., 1994a). Transmission electron microscopy, in conjunction with DAB cytochemistry and microprobe analysis, revealed that within 3 days of CSH exposure, many astrocyte mitochondria become swollen, devoid of cristae, replete with redox-active iron (subcompartmentalized), and occasionally undergo fusion with acid phosphatase-positive lysosomes (IBID). Furthermore, using confocal microscopy and organelle-specific antibodies, Schipper et al. (1993a) have shown that the Gomori-granules partially colocalize to lysosomes and, to a lesser extent rough endoplasmic reticulum (rER) and

early endosomes. Thus, CSH appears to accelerate a senescence-related mitochondrial dystrophy in astroglia reminiscent of changes previously described in liver and other non-neural tissues under various pathological and experimental conditions (Hoppel and Tandler, 1993).

It was initially believed that the autofluorescence and peroxidase activity in these astroglia were due to porphyrins and heme ferrous iron, respectively (Goldgefter et al., 1980; Schipper, 1991). However, Dr. Schipper's laboratory recently demonstrated that CSH suppresses the incorporation of heme precursors into porphyrin and heme in primary astrocyte cultures prior to and during the time when increased iron content is detectable in swollen astrocyte mitochondria by microprobe analysis (Brawer et al., 1994a; Wang et al., 1995). These findings suggest that the autofluorescence, pseudoperoxidase activity, and increased mitochondrial iron content in the CSH-induced and naturally occurring Gomori-positive granules are not due to increased biosynthesis of porphyrins and heme. It is possible that orange-red autofluorescence is due to oxidized mitochondrial flavoproteins which are known to emit fluorescence spectra that are similar to porphyrins (Kohler and Fromter, 1985; Duchen and Biscoe,1992). Since CSH is known to generate pro-oxidant species, it is possible that this compound is responsible for the oxidation of the flavoproteins. Wang et al. (1995) have also demonstrated that CSH promoted the accumulation of iron within astroglial mitochondria but did not affect the total cellular uptake of iron nor the incorporation of iron into the lysosomal fraction. This CSH effect was observed using inorganic $FeCl_3$ as the metal source but not with high molecular weight diferric transferrin. These results are consistent with that of Adams et al. (1989) who demonstrated that inhibition of heme biosynthesis results in the transport of low-molecular weight iron from the cytoplasm to the mitochondria of rat reticulocytes. The accumulation of ferrous iron within astroglial mitochondria is felt to be responsible for the pseudoperoxidase activity in these cells. Indeed, McLaren et al. (1992) have demonstrated that the presence and concentration of elemental iron in CSH-

treated astroglia closely correlated with the presence and intensity of DAB staining, indicating that redox-active iron is the likely source of the pseudoperoxidase activity manifest in these cells. In addition to iron, the Gomori-positive astrocytes have also been shown to accumulate other transition metals including copper and chromium, and express the metal-binding protein metallothionein (Brawer et al., 1994, 1995; Young et al., 1991).

In marked contrast to the primary astrocytes, CSH did not elicit cytoplasmic granulation (mitochondrial damage) in the C6 glioma cell line. The C6 glioma exhibited yellow autofluorescent bodies characteristic of lipofuscin (Collins and Brunk, 1979) which were not appreciably altered by CSH exposure. Furthermore, the glioma cells occasionally exhibited faint, diffuse DAB staining of their cytoplasm (but no discrete DAB-positive inclusions) which was unaffected by CSH exposure. Lipofuscin is known to share several histochemical and morphological properties with Gomori-positive granules. For example, both types of inclusions increase with aging, stain positively for Gomori stains, and may exhibit non-enzymatic peroxidase activity (Schipper et al., 1990b). In addition, lipofuscin, like Gomori-positive granules, may also contain heavy metals (Collins and Brunk, 1979). Some investigators have postulated that the formation of lipofuscin is a result of autophagocytosis of cellular structures such as mitochondria and the endoplasmic reticulum. It is thought that during the breakdown of these cellular constituents, some material may escape lysosomal degradation by polymerization and covalent binding to proteins, with resultant formation of chemically heterogeneous, non-degradable lipofuscin (Collins and Brunk, 1979). Gomori-positive glial inclusions were once regarded as a form of lipofuscin (Lofgreen et al, 1961). However, Gomori-positive granules, unlike lipofuscin, exhibit unique red autofluorescence, no visible pigment under light microscopy, and the absence of stainable lipid. Under transmission electron microscopy, lipofuscin is highly variegated in texture containing both electron-dense and lucent (lipid) components whereas Gomori granules are intensely and homogeneously

osmiophilic (Brawer et al., 1975). In the present study, CSH was incapable of inducing classical lipofuscin in primary astrocytes or any further accumulation of lipofuscin within the C6 glioma cells. Furthermore, in contradistinction to primary astroglia, CSH did not stimulate the transformation of normal mitochondria to red autofluorescent granules in the glioma cells. Thus, the mechanism(s) which lead to the formation of Gomori-granules may differ fundamentally from those responsible for lipofuscinogenesis. As discussed below, glioma cells appear to have specific biochemical defense mechanisms which render their mitochondria resistant to the toxic effects of CSH.

The Cellular Stress Response in Astrocytes and C6 Glioma Cells

Recent evidence from Dr. Schipper's laboratory indicates that activation of the cellular heat shock (stress) response may play an important role in the biogenesis of the mitochondria-derived, Gomori-positive granules. On DIV 6, untreated astroglial cultures exhibit faint or no immunoreactive HSP 27, 72, 90 or HO-1. Within 6 hours of CSH exposure, there is robust expression of these stress proteins as determined by Western blotting and immunofluorescence microscopy (Mydlarski et al., 1993, Mydlarski and Schipper, 1993). In the present study, we determined that CSH treatment resulted in an increase in HSP 27, HSP 90 and HO-1 at both the protein and mRNA level using Western blotting, slot-blot and Northern hybridization assays. The genes coding for these proteins contain heat shock elements in their promoter regions and are typically up-regulated by hyperthermia, oxidative stress, and heavy metal exposure (Applegate et al., 1991; Donati et al., 1990; Dwyer et al., 1992; Schlesinger et al., 1982). Under acute stress, HSPs may protect cells by preventing damage to translational machinery (Liu et al., 1992), accelerating degradation of denatured or abnormal proteins (Ananthan et al., 1986) and maintaining the integrity of lipid membranes (Burdon et al., 1987). In addition, HO-1 may confer cytoprotection by degrading pro-oxidant metalloporphyrins (heme) to biliverdin and bilirubin, catabolites with antioxidant properties (Applegate et

al., 1991). Moreover, immunofluorescence confocal microscopy has demonstrated partial or complete co-localization of HSP 27, HSP 72, HO-1, ubiquitin, and GRP 94 (but not HSP 90 or α-B-crystallin) to the red autofluorescent astrocyte granules both in CSH-treated cultures and in the aging periventricular brain (Mydlarski and Schipper, 1993). These findings further demonstrate the importance of the cellular stress response to the biogenesis of the astrocytic inclusions and suggest that the latter are "heat shock" granules similar to those reported in other tissues subjected to sustained stress. As has been hypothesized for the latter, astroglial granules may protect various mRNAs and critical proteins from stress-related degradation (Nover et al., 1989; Pelham, 1986). Thus, the HSP expression and granulation may be a cytoprotective mechanism which facilitates astrocyte survival in the face of subsequent stressors. Indeed, CSH-pretreated astrocytes exhibit enhanced tolerance to H_2O_2 toxicity and mechanoenzymatic trauma (trypsinization) providing physiological evidence of an antecedent stress response in these cells (Mydlarski et al., 1993a).

The present results demonstrate that CSH is incapable of inducing a generalized cellular stress response in C6 glioma cells. Unlike primary astrocytes, the control (untreated) C6 glioma cells demonstrated low-level, constitutive expression of immunoreactive HSP 27, HSP 90 and HO-1. Furthermore, in contradistinction to the astrocytes, HSP 27 and HSP 90 mRNA and protein could not be up-regulated in the glioma cells by CSH treatment. In the case of HO-1, the mRNA levels in the glioma cells did not increase upon CSH exposure using slot blot hybridization analysis but did appear to be augmented using Northern analysis. In support of the data obtained by Northern analysis, the Western blots demonstrated that HO-1 protein is induced in C6 glioma by CSH exposure. One explanation for these disparate results may be that C6 glioma cell line expresses a yet unidentified mRNA species which can cross hybridize with HO-1. The expression pattern of this unknown gene(s) in glioma cells may obscure any concomitant changes in HO-1 gene expression in response to CSH. Thus, while slot blot

hybridization is useful in rapidly examining the expression of multiple genes simultaneously (Chalifour et al., 1994), corroborative Northern analyses may be necessary to rule out the possibility that unidentified gene products may cross-hybridize with the cDNA of interest.

Taken together with the results of earlier studies (Mydlarski et al., 1993, Mydlarski and Schipper et al., 1993), the present findings further support the notion that activation of the cellular stress response is a prerequisite for the formation of peroxidase-positive cytoplasmic granules in CSH-treated astroglial cultures and in astrocytes of the aging periventricular brain. Also consistent with this conclusion is the fact that following systemic CSH administration, HSP expression in GFAP-positive astroglia precedes accelerated astrocyte granulation in limbic and periventricular regions of the intact rat brain (Schipper et al., 1993a). Since CSH upregulated HO-1 in the glioma cells without inducing granulation, it would seem unlikely that HO-1 induction in CSH-exposed astrocytes (with attendant dysregulation of porphyrin-heme metabolism) is sufficient for subsequent granulation to occur as had been previously conjectured (Schipper et al., 1990).

CSH-mediated Oxidative Stress

The exact mechanism by which CSH induces HSP expression and Gomori-granules formation within primary astrocyte cultures and in vivo is unknown. Several lines of evidence suggest that oxidative stress may be the "final common pathway" responsible for the up-regulation of the stress proteins which in turn, participate in the biogenesis of mitochondria-derived astrocytic inclusions *in vitro* and in the aging brain. For example, it has been shown that H_2O_2 induces HSP and HO-1 expression in rat astrocytes and also stimulates the accumulation of peroxidase-positive granules in primary astrocyte cultures (Mydlarski et al., 1993). Also, Srebro (1971) demonstrated that ionizing radiation, a known generator of intracellular pro-oxidant intermediates,

increases the numbers of peroxidase-positive glial granules in the rat hypothalamus. It has been established that oxidative stress can induce the expression of HSPs (Kochevar et al., 1991; Pelham, 1986) with the exception of GRP 94, which is inducible by stimuli such as glucose deprivation and calcium ionophores (Craig and Lindquist, 1988). Similarly, CSH can induce the accumulation of HSPs which normally respond to oxidative stress (HSP 27, HSP 72, HSP 90) but has virtually no direct effect on GRP 94 *in vitro* (Mydlarski et al., 1993). CSH, in the presence of transition metals, undergoes redox cycling with the generation of pro-oxidant species including thiyl radicals, $O_2\cdot$, H_2O_2 and HO· (Munday, 1989). Recently, Manganaro et al. (1995) have demonstrated that CSH causes lipid peroxidation in isolated mitochondria which can be completely inhibited by the addition of catalase. This suggests that the autoxidation of CSH results in the formation of H_2O_2 which in turn causes lipid peroxidation of the mitochondria. Also, it may be possible that the iron normally present in the mitochondria may be catalyzing the conversion of CSH into the pro-oxidant species. Indeed, Svensson et al. (1993) have demonstrated that iron-mediated peroxidase activity of abundant mitochondrial hemoproteins is capable of oxidizing CSH to H_2O_2 and other free radical intermediates. Paradoxically, CSH-treated astrocytes show lower indices of lipid peroxidation as a function of time relative to control cultures (Manganaro et al., 1995). These seemingly contradictory effects of CSH on lipid peroxidation may be due to the fact that CSH and other aminothiols may behave as either pro- or anti-oxidants depending upon their redox microenviroment (Munday, 1989; Weiss and Kumar , 1994). Hwang et al. (1992) have shown that redox potentials within the a cell can vary from one subcompartment to another. Another explanation is that CSH is capable of increasing the concentrations of reduced GSH (see below) in astrocytes, and thereby mitigate against subsequent oxidative damage to lipids and other cellular substrates.

Several lines of evidence suggests that CSH may influence the activity and expression of various antioxidant defense mechanisms (see Dahn et al., 1983; Krizala et

al., 1982; Kovarova and Pulpanova, 1979). Manganaro et al (1995) have demonstrated that after 48 hours of CSH exposure there is a suppression of catalase and glutathione reductase activities in primary rat astrocyte cultures and in the intact rat diencephalon. In primary astroglial cultures, CSH induces a transient increase in glutathione peroxidase activity during the first 48 hours of treatment followed by a subsequent decline in this activity to below control levels. In addition, they demonstrated that MnSOD activity increased in cultured astrocytes, after 7 days CSH treatment, relative to control cultures. Similarly, systemic CSH administration increased MnSOD activity within the intact rat diencephalon. However, CSH exposure did not seem to have any effect on CuZnSOD activity. Finally, CSH induced a transient, 6-fold increase in GSH concentrations in cultured astroglia. This observation is in accord with previous studies demonstrating GSH accumulation in Chinese Hamster Ovary cells (Issels et al., 1988), OvCa cells (Issels and Nagele, 1990) and mouse fibroblasts (Djurhuus et al., 1990) following CSH exposure. CSH induces the synthesis of GSH by 1) forming mixed disulfides with extracellular cystine thereby generating the GSH precursor, cysteine and 2) promoting the transport of cysteine into cells where it can be utilized for *de novo* GSH biosynthesis (Issels et al., 1988). The rise in GSH may initially protect astrocytes from CSH-induced mitochondrial damage and lipid peroxidation. However, the GSH levels return to baseline within several days and subsequent GSH responses to CSH are greatly reduced. Thus, it is unlikely that GSH is entirely responsible for the enhanced astrocyte cytoprotection against H_2O_2 and trypsinization stress observed after 12 days of CSH exposure (Mydlarski et al., 1993). A puzzling finding by Manganaro et al. (1995) is that systemic CSH administration appeared to influence anti-oxidant enzyme activities in the intact diencephalon while other regions remained unaffected. The susceptibility of this brain region to the action of CSH may be related to the fact that during normal aging, astroglia in periventricular diencephalic nuclei (such as the hypothalamic arcuate nucleus) exhibit a greater propensity to granulate than astrocytes elsewhere in the CNS

(Schipper, 1991). It may be possible that the attenuated blood-brain barrier in the basal hypothalamus-median eminence region permits unidentified blood-borne stressors (simulated by systemic CSH administration) to perturb antioxidant defenses and induce mitochondrial damage (granulation) in the indigent glia.

In the present study, Western blot analysis demonstrated that CSH-treated (3 and 5 days) astrocyte cultures contained higher levels of MnSOD protein than time-matched controls. In addition, Northern blot analysis revealed that the MnSOD mRNA levels of both control and CSH treated increased during the first 12 hours of treatment. Conceivably, cytokines and/or other unidentified factors present in the media may have been responsible for the initial transient induction of MnSOD observed in the control cells. However, after 6 days CSH exposure, the MnSOD mRNA levels in treated primary cultures were approximately double that of controls. Thereafter, the MnSOD mRNA levels in treated astrocyte cultures decreased progressively to control levels. Northern analysis revealed 5 discrete MnSOD mRNA transcripts, each of which can give rise to the mature, functional protein (Hurt et al., 1992). The results of the present study suggest that late augmentation of MnSOD by CSH occurs at the level of *de novo* mRNA and protein synthesis. Interestingly, however, Western blots demonstrated that 3 day CSH treatment results in increased levels of MnSOD protein, prior to any detectable increase in MnSOD mRNA levels. Moreover, CSH elevates MnSOD enzyme activity within 1 day of CSH administration.. Taken together these results suggest that prior to induction of the MnSOD gene, initial stimulation of MnSOD activity by CSH is mediated by translational or post-translational control.

Transcriptional control of MnSOD by intracellular oxidants has been documented in bacteria and, to a lesser extent, in mammalian cells (Wong et al., 1992; Liochev et al., 1992). In the present studies Northern blots of HO-1 were performed in conjunction with those for MnSOD. It was found that HO-1 mRNA levels increased rapidly in response to CSH but decreased to control levels after 1 day of treatment. This finding is consistent

with the hypothesis that CSH rapidly induces a HSP response in astrocyte cultures (Mydlarski et al., 1993, Mydlarski and Schipper et al., 1993; present study). However, the relatively late induction of MnSOD mRNA and protein by CSH indicates that MnSOD is not regulated as part of a generalized cellular stress response. Taken together, the present findings suggest that CSH-induced oxidative stress may be a signal promoting both an early stress response and delayed MnSOD gene expression. The early HSP response may play a protective role by mechanisms mentioned previously. In the case of MnSOD, it is possible that mitochondrial damage incurred by CSH (en route to the formation of Gomori-positive granules) may stimulate a compensatory increase in this mitochondrial enzyme. MnSOD up-regulation may serve as an attempt to protect astroglial mitochondria from CSH-derived free radicals intermediates. Cytoprotective properties of MnSOD have been demonstrated by Wong et al. (1992). They found that MnSOD can be induced by various cytotoxins and immunoregulatory proteins in mammalian cells, and then showed that the overexpression of MnSOD or its anti-sense mRNA renders mammalian cells excessively resistant or sensitive, respectively, to various cytotoxic insults.

Preliminary results from Dr. Schipper's laboratory have indicated that untreated C6 glioma have higher baseline levels of GSH, glutathione reductase, and catalase than primary astrocytes cultured for identical lengths of time. In fact, GSH is present in approximately 3 fold greater concentrations in untreated C6 glioma cells relative primary astrocyte cultures. The high GSH levels and perhaps other differences in baseline antioxidant enzyme activities may effectively scavenge CSH-derived free radicals in the glioma cells and thereby preclude both mitochondrial oxidative injury (and subsequent granulation) and the initiation of an acute cellular stress response (present study). Furthermore, low-level constitutive expression of HSP 27 and HSP 90 in untreated glioma cells observed in the present study indicates that the latter are experiencing mild, chronic stress of uncertain etiology, or overexpress HSPs as paraproteins. In either case,

the elevated stress protein levels in glioma cells may confer cytoprotection against a host of subsequent stressors including CSH. Dr. Schipper's laboratory has also shown that CSH treatment of C6 glioma cells results in a minimal elevation in glutathione levels, inhibition of catalase, glutathione reductase, and glutathione peroxidase activities, and no change in MnSOD or CuZnSOD activities (unpublished findings). The absence of a MnSOD response to CSH in the glioma cells contrasts sharply with the behaviour of primary astroglia (Manganaro et al., 1995; present study) and further argues that CSH does not present a significant oxidative challenge to the former population. Since the activity of MnSOD in C6 glioma cells was unaltered by CSH, Western and Northern blots of MnSOD protein and mRNA were not performed in the present study.

The Role of Gomori-Positive Astrocytes in Brain Aging and Neurodegeneration

It has been suggested that Gomori-positive astrocytes may play a role in the development of reproductive senescence (persistent estrus) in female rats. The non-enzymatic peroxidase activity of the Gomori-granules has been shown to oxidize catecholestrogens to ortho-semiquinones and other reactive oxygen species which, in turn, could promote axo-dendritic damage within the neuroendocrine hypothalamus and ensuing reproductive failure (Schipper et al., 1990a; Schipper et al.,1991; Schipper, 1993). Similarly, the pseudoperoxidase activity of these granules is capable of converting dopamine into neurotoxic dopamine-o-semiquinone derivatives (Schipper et al., 1991). It is therefore conceivable that the oxidation of dopamine and other catechols to semiquinones and oxyradicals by peroxidase-positive astrocytes may play an important role in the pathogenesis of Parkinson's disease (PD) in accord with the prevailing free radical hypothesis of this disorder. For example, Dexter et al. (1988) have demonstrated that there is greater lipid peroxidation in the substantia nigra of post-mortem human PD brain than in non-parkinsonian controls matched for age and post-mortem interval. In addition, subjects with PD have elevated levels of tissue iron within the substantia nigra

and basal ganglia which have been heavily implicated in the generation of local oxidative stress (Youdim et al., 1993). As described above, during the formation of Gomori-positive granules, astrocyte mitochondria (granule precursors) accumulate redox-active iron; a similar mechanism of iron sequestration may be occurring in astrocytes of the substantia nigra and basal ganglia in subjects with PD (Olanow et al., 1992; Connor et al., 1990; Jellinger et al., 1990, Wang et al., 1995). In addition, the redox-active iron in Gomori astrocyte granules may oxidize the pro-toxin, MPTP, to the dopaminergic toxin, MPP+ in the presence of monoamine oxidase inhibitors (DiMonte et al., 1995), suggesting that astroglial iron may play a role in the development of MPTP-induced parkinsonism. Finally, Gomori-positive astrocytes may contribute to the pathogenesis of Alzheimer's disease in light of the fact that glial iron concentrations and lipid peroxidation appear to be augmented in the brains of Alzheimer subjects (Subbarao et al., 1990; Connor et al., 1990).

The results of the present study appear to recapitulate certain biochemical changes which have been documented in the normal senescent brain and in aging-related neurodegenerative disorders. The results of several studies indicate that glutathione reductase and catalase activities progressively decrease in rat brain as a function of advancing age (Semsei et al., 1991; Del Maestro and McDonald, 1987; Mizuno and Ohta, 1986). Conversely, MnSOD activity is reportedly augmented in senescent rodent brain relative to younger controls(Semsei et al., 1991). Similarly, it has been reported that antioxidant defenses, such as catalase and reduced GSH, are compromised in patients with PD. In contrast, MnSOD (but not CuZnSOD) levels are elevated in the basal ganglia of PD patients (Saggu et al., 1989; Kalra et al., 1992). Immunoreactive MnSOD is selectively increased in granular structures (damaged mitochondria?) of reactive astrocytes surrounding senile plaques in Alzheimer-diseased hippocampus (Shibata et al., 1993). Many of these aging and disease-related changes in antioxidant enzyme activities are recapitulated in primary astrocyte cultures and in the intact diencephalon as a result

of CSH exposure (Manganaro et al., 1995; present study). Thus, CSH-treated astrocytes may serve as a useful model for investigating the (dys)regulation of MnSOD and other antioxidant enzymes in the aging and degenerating nervous system. Furthermore, systemic CSH administration elicits robust astrocyte hypertrophy and GFAP synthesis in hippocampus, striatum, and other brain regions *in situ* (reactive gliosis). Astrogliosis is a characteristic feature of many neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Beach et al., 1989; Duffy et al., 1980; Kushner et al., 1991; Schipper et al., 1993) and occurs, to a lesser extent, in the course of normal aging (Linneman et al., 1994; Peters and Vaughn, 1981; Schipper and Wang, 1990). Conceivably, stress-related up-regulation of HSPs and antioxidant enzymes in these cells (simulated by CSH exposure) promotes astroglial survival and the establishment of gliosis in the face of concomitant neuronal degeneration.

In summary, CSH exposure appears to accelerate a spectrum of pathophysiological processes in astroglia germane to normal brain aging and various senescence-related neurodegenerative disorders including: 1) the transformation of astroglial mitochondria to Gomori-positive inclusions, 2) stress-related sequestration of redox-active glial iron, 3) dysregulation of MnSOD and other antioxidant enzymes, and 4) the establishment of reactive gliosis.

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