NOTE TO USERS

The role of erythropoietin and its receptor in Alzheimer disease

by

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Abstract I:

Up-Regulation of Erythropoietin Receptor Expression in AD and MCI Astroglia

Background: Alzheimer Disease (AD) is a common progressive neurodegenerative disorder that results in impairment of memory, thinking and behavior (dementia). There exists ample evidence implicating oxidative stress and mitochondrial insufficiency in this condition. Erythropoietin (Epo) is a glycoprotein secreted by the kidney in response to hypoxia that stimulates erythrocyte production through interaction with Epo-receptors (EpoR). Both Epo and EpoR have been localized to brain capillaries, neurons and astroglia. Epo has been shown to confer important cytoprotective effects in various models of brain injury and disease. **Objective:** To delineate the patterns and extent of EpoR expression in the brains of patients with sporadic AD, Mild Cognitive Impairment (MCI; a frequent harbinger of AD) and normal elderly controls (NEC). **Methods:** Post-mortem tissues containing hippocampus and temporal cortex were procured from the NIH-funded Religious Orders Study. GFAP-positive astrocytes co-expressing EpoR were characterized by immunofluorescence confocal microscopy and quantified using dual label immunohistochemistry. **Results:** A) Temporal cortex: Percentages of GFAP-positive astrocytes co-expressing EpoR were (i) significantly increased in AD and MCI vs. NEC ($p<0.05$) in layers II and III, (ii) increased in MCI, but not in AD, vs. NEC in layer I, and (iii) unrelated to diagnosis ($p>0.05$) in layers IV, V and VI and the subcortical white matter. B) Hippocampus: Percentages of GFAP-positive astrocytes co-expressing EpoR were (i) significantly increased in AD and MCI vs. NEC ($p<0.05$) in the stratum oriens and pyramidal layer, (ii) increased in MCI, but not in AD, vs. NEC in the granular layer, stratum radiatum and dentate gyrus, and (iii) unrelated to diagnosis ($p>0.05$) in the

molecular layer. **Conclusions:** 1. Up-regulation of astrocyte EpoR in certain cortical and hippocampal regions is an early event in the pathogenesis of sporadic AD. 2. Based on *in vitro* and whole animal studies, glial EpoR induction may confer protection against oxidative stress in the brains of patients with MCI and AD. 3. Clinical neuroprotection trials using Epo or its derivatives in MCI/AD may be warranted.

Résumé I:

Augmentation de la synthèse des récepteurs à l'érythropoïétine dans les astrocytes d'individus atteint de la maladie d'Alzheimer et MCI

Fond: La maladie d'Alzheimer (AD) est un désordre de dégénération des neurons progressif commun qui a pour conséquence l'affaiblissement de la mémoire, de la pensée et du comportement (démence). Il existe une évidence suffisante impliquant le stress oxydatif et l'insuffisance mitochondriale de cette condition. L'érythropoïétine (Epo) est une glycoprotéine sécrétée par le rein en réponse à l'hypoxie qui stimule l'érythropoïèse par l'interaction avec les récepteurs d'érythropoïétine (EpoR). L'Epo et l'EpoR ont été localisés aux capillaires, aux neurones et aux astrocytes du cerveau. L'Epo a démontré des effets cytoprotecteur importants dans divers modèles de maladies du cerveau. **Objectif:** Tracer les modèles et l'ampleur de l'expression d'EpoR dans le cerveau de patients atteints de l'AD non héréditaire, l'affaiblissement cognitif doux (MCI ; un harbinger fréquent d'AD) et de personnes âgées en contrôle (NEC). **Méthodes:** Des tissus pris d'individus après leur mort contenants l'hippocampe et le cortex temporal ont été obtenus de l'étude d'ordres religieuse. Les astrocytes GFAP-positifs exprimant aussi l'EpoR ont été caractérisés par microscopie d'immunofluorescence confocal et mesurés en utilisant des traceurs colorants et l'immunohistochimie. **Résultats:** A) Cortex temporal: Les pourcentages des astrocytes GFAP-positifs exprimant aussi l'EpoR étaient (i) significativement accrus dans l'AD et MCI contre NEC ($p < 0.05$) dans les couches IV, V et VI et la matière blanche sous-cortical. B) Hippocampe : Les pourcentages des astrocytes GFAP-positifs exprimant aussi l'EpoR étaient (i) significativement accrus dans l'AD et MCI contre NEC ($p < 0.05$) dans la couche moléculaire. **Conclusions:** 1. L'augmentation de la synthèse des EpoR des astrocytes dans certaines régions corticales

dans certaines régions corticales et hippocampes est un événement tôt dans la pathogénie de l'AD non héréditaire. 2. Basées sur des études animales et *in vitro*, l'induction d'EpoR peut conférer une protection contre le stress oxydatif dans les cerveaux des patients atteints d'MCI et d'AD. 3. Des épreuves neuroprotectives cliniques en utilisant l'Epo ou ses dérivés dans MCI/AD peuvent être justifiées.

Abstract II:

Astroglial cytoprotection by erythropoietin pre-conditioning: Implications for ischemic and degenerative CNS disorders

Background: Erythropoietin (Epo) is a glycoprotein secreted by the kidney in response to hypoxia that stimulates erythropoiesis through interaction with cell-surface erythropoietin receptors (EpoR). Pre-treatment with Epo has been shown to protect neurons in models of ischemic injury. The mechanism responsible for this neuroprotection and the effects of Epo on astroglial and other non-neuronal cell populations remain unknown. **Objective:** To determine whether Epo pre-treatment protects neonatal rat astrocytes from apoptotic cell death resulting from treatment with nitric oxide (NO), staurosporine (STS) and arsenic (As_2O_3), and possible mechanisms mediating erythropoietin-related cytoprotection. **Methods and Results:** Epo (5-20 U/ml) significantly attenuated multiple hallmarks of apoptotic cell death in astroglia exposed to NO and STS, but not As_2O_3. Epo 20 U/ml induced mild oxidative stress as evidenced by increases in heme oxygenase-1 (HO-1) mRNA and protein expression that could be suppressed by antioxidant co-administration. Moreover, co-incubation with tin-mesoporphyrin (SnMP), a competitive inhibitor of heme oxygenase activity, abrogated the cytoprotective effects of Epo (20 U/ml) in the face of STS treatment. **Conclusion:** Induction of the *ho*-1 gene may therefore contribute to the glioprotection accruing from high-dose Epo exposure. Erythropoietin may augment astroglial resistance to certain chemical stressors by oxidative stress-dependent and –independent mechanisms.

Résumé II:

Un effet neuroprotecteur pour les astrocytes par le pré conditionnement **avec l'érythropoïétine: Implications pour des maladies neurologiques ischémiques et dégénératives du SNC**

Fond: L'érythropoïétine (Epo) est une glycoprotéine sécrétée par les reins en réponse à l'hypoxie qui stimule l'érythropoïèse par l'interaction avec les récepteurs à l'érythropoïétine (EpoR) de la surface des cellules. Le traitement antérieur avec l'Epo a montré sa capacité de protéger des neurones dans les modèles des maladies ischémiques. Le mécanisme responsable de cette protection neuronale et les effets de l'Epo sur les astrocytes et d'autres cellules non-neuronales demeurent mal connus. **Objectif:** Déterminer si le traitement antérieur avec l'Epo protège les astrocytes néonatals des rats contre la mort cellulaire (apoptose) résultant du traitement avec l'oxyde nitrique (NO), le staurosporine (STS) et l'arsenic (As_2O_3), et des mécanismes possibles résultant de la protection reliée à l'Epo. **Méthodes et résultats:** Epo (5-20 U/ml) a atténué de manière significative les cachets multiples de l'apoptose dans l'astroglia exposé au NO et au STS, mais non à l'As_2O_3. L'induction d'un stress oxydatif modéré avec 20 U/ml d'Epo a été démontré par des augmentations du mRNA de hème oxygénase -1 (HO-1) et de l'expression de protéines qui pourraient être supprimées par l'administration simultané d'antioxydant. D'ailleurs, un traitement simultanée avec l'étain-mesoporphyrin (SnMP), un inhibiteur concurrentiel d'activité de hème oxygénase, a abrogé les effets cytoprotecteur d'Epo (20 U/ml) face au traitement de STS. **Conclusion:** L'induction du gène ho-1 peut donc contribuer à la protection gliale originaire de l'exposition d'Epo à haute dose. L'Epo peut augmenter la résistance des astrocytes à certains facteurs de force chimiques en procédant par un mécanisme de stress oxydatif ou par autre procédé.

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Contributions to Original Knowledge

It is now widely accepted that Epo has a function beyond the haematopoietic system. The discovery of an Epo/EpoR system in the central nervous system (CNS) and cerebrospinal fluid has led to many studies aimed at elucidating sites of expression, the regulation and its effects on the development and maturation of the brain. Certain groups demonstrated the neurotrophic and neuroprotective functions of Epo in neuronal cells but there is a lack of knowledge on its role in astrocytes. This manuscript also explores the changes in expression of EpoR in elderly normals compared to those affected with AD. The further understanding of Epo's protective functions in the brain may lead to human recombinant Epo therapy being used in clinical practice, possibly limiting cerebral damage incurred by diseases such as cerebral ischaemia, subarachnoid haemorrhage or more chronic neurodegenerative diseases such as AD or schizophrenia.

Preface

The author was responsible for the *in vivo* staining and *in vitro* cell culture experiments. DNA fragmentation, DAPI staining, Western blotting and growth assays were performed in collaboration with Zuanel Diaz, a PhD student under the supervision of Dr. H. Schipper and Dr. W. Miller. Her continued dedication to this project allowed us to complete and submit an original article that has been accepted for publication in the *Journal of Neurochemistry*.

Alzheimer Disease

History

Throughout history, progressive mental deterioration in old age has been recognized and described. Not until the early part of the 20th century did Dr. Alois Alzheimer, a German neuropathologist and psychiatrist, first record the clinical and pathological characteristics of the disorder that now bears his name (Alzheimer 1907). In 1901, a 51-year old female was placed under the care of Dr. Alzheimer at the Frankfurt Hospital for the Mentally Ill and Epileptics. She experienced years of severe memory problems, confusion and difficulty understanding questions. During the post-mortem study of her brain, the autopsy revealed brain atrophy and a silver-staining dye uncovered structures called neurofibrillary tangles and plaques. To this day, a definitive diagnosis of Alzheimer's disease requires a neuropathological analysis demonstrating these characteristic symptoms (O'Brien 1996).

General aspects

AD is the most frequent cause of dementia and manifests with a progressive neurological decline that largely affects the basal forebrain, hippocampus, and association cortices (Selkoe 1991). The molecular hallmarks of AD are extracellular β-amyloid-containing (Aβ) senile plaques and intracellular neurofibrillary tangles that are comprised of hyper-phosphorylated tau. It is neuropathologically characterised by the accumulation of reactive astrocytes and activated microglia that up-regulate the expression of pro-inflammatory cytokines and enhance the production of reactive oxygen species (ROS) in the diseased brain (Johnstone *et al.* 1999). The loss of synapses and neurons in the AD brain ultimately results in a progressive deterioration in cognitive functioning (Cummings

et al. 1998; Vickers *et al.* 2000). The earliest symptom of this debilitating disease is memory impairment, followed by deficits in language functions, praxis, visuospatial abilities, and the capacity to independently conduct activities of daily living (Selkoe 1997; Honig and Mayeux 2001).

It is generally thought that the clinical symptoms of AD manifest themselves in response to a critical accumulation of brain lesions over one's lifetime. Appropriately, aging is considered a major risk factor for AD. Evidence exists that brain damage leading to AD may accumulate for decades before symptoms present themselves or deficits in neuropsychological measures are observed (Morris 1997). The brain's capacity to sustain and buffer such long-term injury in AD is akin to the situation in Parkinson's disease (PD) where clinical abnormalities are noted only after 80% of dopaminergic neurons in the substantia nigra pars compacta are lost (Langston *et al.* 1992). As the brain's burden of Alzheimer pathology increases, the threshold for clinical dementia is crossed and impairments become progressively more severe until patients are no longer able to speak, walk, recognize faces, or feed themselves. Death following this global cognitive deterioration is often due to infection, typically pneumonia (Honig and Mayeux 2001).

During the past two decades, the world's population has shifted from a state of high birth and death rates to one characterized by low birth and death rates. The population of Americans aged 65 years and over in 2000 was approximately 35 million and by 2050, the projected number of elderly persons is 70 million, accounting for 1 in 5 Americans (United States Administration on Aging. 2004). Sporadic AD affects approximately 5-10% of North Americans aged 65 and as many as 30-50% of those who survive to the end of their ninth decade. The term "mild cognitive impairment" is generally applied to elderly

individuals who experience gradual cognitive decline (usually memory) of at least six months duration that fails to meet the clinical criteria for AD or other dementia. A significant proportion of MCI subjects will progress to probable AD over the ensuing 3-5 years whereas other individuals exhibit a stable, non-progressive memory deficit over a long period of follow-up (Chertkow *et al.* 2001). There currently does not exist any neuroprotective therapy that unequivocally slows or arrests neuronal degeneration in established AD or prevents the conversion of MCI to AD.

Free radicals and AD

Recently, support has amassed for the idea that free radical damage may be intimately involved in the pathogenesis of AD (Markesbery 1997; Perry and Smith 1998; Nunomura *et al.* 2001). Free radicals are chemical species that contain one or more unpaired valence-shell electrons. These highly reactive agents oxidize (abstract electrons from) or reduce (donate electrons to) other molecules in order to reach a more favorable and stable energy state. Oxidative stress results from an increased formation of free radicals and/or a decreased functioning of anti-oxidant defense systems (Sies and Cadenas 1985). The brain is especially prone to oxidative damage due to its high oxygen consumption rate, its abundance of redox-active metals such as iron, its large lipid content, and its relative shortage of antioxidant defenses compared with other tissues (Coyle and Puttfarcken 1993).

The extent of oxidative damage to biological molecules in the AD brain is considerably greater than that in the normal elderly brain. Oxidative damage to nuclear DNA (Gabbita *et al.* 1998) and mitochondrial DNA (Mecocci *et al.* 1994), as measured by the presence

of oxidatively modified bases, is significantly increased in the AD brain. Such alterations result in DNA strand breakage and base substitutions (Halliwell and Gutteridge 1999). Athough AD is foremost a disease of the brain, the detection of oxidatively damaged DNA in blood lymphocytes derived from AD patients is suggestive of a peripheral component to this disorder (Mecocci *et al.* 1998). Furthermore, relative to normal elderly controls, proteins and lipids in the AD brain are subjected to increased oxidative damage, leading to amino acid abnormalities and decreased membrane fluidity, respectively (Halliwell and Gutteridge 1999). It is still unknown whether oxidative radical generation in AD is a primary (causative) event or a secondary effect of other underlying pathologies.

The strong association between oxidative stress and the cytopathological lesions in AD supports the assertion that oxidative damage is central to the disorder's pathogenesis. Heme oxygenase-1 (HO-1), a member of the stress protein superfamily, is robustly up-regulated in response to the presence of ROS and is widely accepted as a sensitive marker of oxidative stress (Vile and Tyrrell 1993). HO-1 is massively overexpressed in the neurons and astrocytes of the AD hippocampus and cerebral cortex relative to age-matched controls (Schipper *et al.* 1995). In addition, senile plaques and neurofibrillary tangles are both immunoreactive for HO-1 (Smith *et al.* 1994; Schipper *et al.* 1995). Furthermore, *in-vitro* experiments demonstrate that oxidative stress increases Aβ production (Zhang *et al.* 1997) and that Aβ itself advances free radical generation (Behl *et al.* 1992). Interestingly, Hensley *et al.* (1994) reported that the Aβ peptide exhibits a chemistry that facilitates its fragmentation into neurotoxic oligopeptide radicals. They suggest that Aβ-derived radical species may promote peptide aggregation, a process

which is considered to initiate the sequence of neuropathological events in AD. Moreover, the development of animal models of AD has allowed investigators to examine the *in-vivo* effects of Aβ overexpression. Transgenic mice harboring amyloid precursor protein mutations demonstrate abundant Aβ deposition and pervasive oxidative damage (Smith *et al.* 1998).

AD Therapeutics

In the past decade, considerable progress has been made in understanding the molecular basis of AD. These advances have facilitated research into various therapies that may act by preventing disease occurrence, deferring its onset, or slowing its progress (Cummings *et al.* 1998). Currently available treatments for AD are of two general categories: one focuses on symptom alleviation through the enhancement of cholinergic functioning, and the second centers on neuroprotection, in part, by alleviating oxidative damage.

The standard treatment for AD are cholinesterase inhibitors (Cummings and Cole 2002). In the normal brain, the major source of acetylcholine is the nucleus basalis of Meynert. Since the basal forebrain is affected early in AD, cholinesterase inhibitors are used to enhance residual cholinergic activity. Currently, four of these inhibitors are available for clinical use: tacrine, donepezil, rivastigmine, and galantamine. These drugs are the only medications approved by the US Food and Drug Administration for the treatment of AD. Tacrine, a first-generation cholinergic enhancer, is now rarely prescribed due to its significant toxicity profiles. These agents have been shown to improve cognitive functioning and prolong a patient's ability to perform activities of daily living, although efficacy varies between patients.

Antioxidant treatments have been investigated in AD to counter the harmful effects of ROS. Potent antioxidants such as alpha-tocopherol (vitamin E), and selegiline, a monoamine oxidase inhibitor, have been reported to slow the progression of AD when administered to patients with moderate disease severity (Sano *et al.* 1997). Other studies have suggested that antioxidant therapy may be a promising avenue for delaying disease onset (Zaman *et al.* 1992; Pitchumoni and Doraiswamy 1998). Such findings underscore the significance of oxidative stress in AD and suggest that an early antioxidant intervention may be particularly advantageous.

Astrocytes

The role of astrocytes

In the normal brain, astrocytes serve to maintain homeostasis. They are dynamic cells which express many receptors enabling them to respond to most neuroactive compounds namely, neurotransmitters, neuropeptides, growth factors, cytokines, small molecules and toxins. Not only are the astrocytes important in signal transduction but they also serve to protect neighboring neuronal cells. The blood brain barrier phenotype develops and is maintained by astroglia, and consists of more complex tight junctions than in other capillary endothelia, and a number of specific transport and enzyme systems which regulate molecular traffic across the endothelial cells. In addition to a role in long-term barrier induction and maintenance, astrocytes and other cells can release chemical factors that modulate endothelial permeability over a time-scale of seconds to minutes (Abbott 2002).

Astrocytes and neurodegeneration

Oxidative stress has been implicated in chronic neurodegenerative conditions as well as in acute CNS injury incurred by trauma, ischemia and exposure to various neurotoxins. This imbalance is due to surplus of ROS production and/or insufficient antioxidant capacity. Many factors can contribute to this cytotoxic ROS state; transition metals, amyloid β-peptide, inflammatory cytokines, excitotoxic amino acids, NO and mitochondrial electron transport uncoupling agents. The presence of antioxidants and antioxidant enzymes prevent the formation of ROS and can diminish the ensuing damage. The brain is at an elevated risk for oxidative damage by ROS. High O_2 and glucose consumption, peroxidizable fatty acids and increase iron content in certain brain regions contribute to the susceptibility of the brain to the formation of damaging ROS. On the other hand, the brains defence is comparatively weak, lacking sufficient antioxidant defences. The advantage of astrocytes is that they contain high antioxidant concentrations (Peuchen *et al.* 1997; Wilson 1997; Dringen *et al.* 2000) that can protect the brain environment from oxidative damage in neurons followed by apoptotic cell death.

Astrocytes secrete a number of neuroprotective substances. This is evidenced by research demonstrating that astrocyte-conditioned media supports neuronal survival *in vitro* (Liu *et al.* 1998; Wang and Cynader 1999; Eriksen and Druse 2001; Hailer *et al.* 2001; Takuma *et al.* 2004). Epo is one of the neuronal survival-promoting factors that are released from astrocytes. These secreted cytoprotective factors also promote survival and proliferation in astrocytes themselves in an autocrine manner (Takuma *et al.* 2000; Bakhiet *et al.* 2001; Albrecht *et al.* 2002; Yamamuro *et al.* 2003).

The astrocytes also serve in the clearance of synaptically released neurotransmitters, especially glutamate. The glutamate transport is an important event that protects surrounding neurons from excitotoxic damage. Astrocytic gap junction channels are associated with neuronal injury and their inhibition leads to increased damage and vulnerability to oxidative stress in the neurons.

Erythropoietin (Epo)

Epo is a naturally occurring, 30 kDa glycoprotein hormone that stimulates proliferation and differentiation of erythroid cells in response to decreased oxygen delivery to the tissues. The development of recombinant human Epo (r-HuEpo), with identical structure and biological activity to the endogenous protein, led to its clinical use for the treatment of chronic anemia in the 1980s. Epo acts by interaction with the EpoR within hematopoietic cell membranes (Lappin 2003). Epo and EpoR have recently been identified within neurons and astrocytes of the rodent and human CNS. Both Epo and EpoR mRNA and protein are expressed in the temporal cortex, hippocampus, cerebellum and amygdala and are up-regulated in these regions in experimental models of cerebral ischemia (Marti *et al.* 1996; Kalialis and Olsen 2003). Moreover, administration of Epo has been shown to have potent neuroprotective properties in *in vivo* and tissue culture models of ischemia/anoxia (Sinor and Greenberg 2000), excitotoxicity (Morishita *et al.* 1997) and subarachnoid hemorrhage (Grasso *et al.* 2002).

Epo biology

The human Epo gene, consisting of five exons and four introns, is situated on chromosome 7q11-22. The 193 amino acid, single polypeptide undergoes post-translational modification including the addition of four acidic oligosaccharides, two disulphide bonds and the cleavage of a 27 amino acid hydrophobic secretory sequence. The last amino acid (Arg-166) removed from the polypeptide is thought to occur before Epo is released into the circulation as the mature 165 amino acid protein (Jelkmann 1992). The glycosylation of Epo makes up 40% of the molecule and 22 kDa of its 30 kDa molecular mass. The disulphide bonds bridging two cysteine residues are functionally important, serving to maintain the biological activity of Epo and the ability for the molecule to bind to the EpoR. The high carbohydrate content of Epo, and particularly the sialic acid residues, allow the Epo sialoglycoprotein to remain biologically active by preventing the rapid removal of the hormone from the circulation by the liver (Ng *et al.* 2003).

Epo physiology

Epo was originally identified as the principal regulator of erythropoiesis. Initially produced in the liver, blood Epo's main site of synthesis after birth is shifted primarily to the peritubular interstitial fibroblastoid cells of the kidney, with a small amount produced in hepatocyte and interstitial fibroblasts of the liver. The kidney produces Epo in response to reduced oxygen tension of the blood. The Epo then stimulates in the bone marrow the proliferation and differentiation of red blood cell (RBC) progenitors resulting in a greater RBC mass and increased oxygen carrying capacity.

Hypoxia is the only physiologic stimulus for Epo production and subsequent erythropoiesis. Under normal conditions, there is a basal level of Epo production and plasma levels of Epo remain constant. At the onset of hypoxia, the transcription factor, hypoxia inducible factor-1 (HIF-1) is activated by a decrease in oxygen delivery to the tissues. HIF-1 activation leads to the binding of HIF-1 to the 3' flanking region of the Epo gene located on chromosome 7q11-22 (Bracken *et al.* 2003). This induces a maximal protein transcription of the Epo gene, ultimately increasing erythropoiesis in order to maintain the delivery of oxygen to the vital organs. The resultant increase in mature RBC production upon Epo stimulation takes approximately 2 weeks to begin. If the hypoxic state is severe and persistent, liver production of Epo is also up-regulated. The main function of Epo is to stabilize the levels of plasma hemoglobin (Hb) at about 14-16g/dl. An impairment in the production of Epo by the kidney can result in Hb levels of 7-8g/dl (anemia), whereas elevated levels of Epo may lead to polycythemia. The latter may increase the risk of brain damage through stroke (cerebral infarction).

Erythropoietin Receptor (EpoR) biology

The un-ligated EpoR, a 66 kDa single transmembrane receptor consisting of 507 amino acids, exists as a dimer on the surface of erythroid progenitor cells (Fig. 1). It undergoes a conformational change upon binding of Epo that triggers a signal transduction cascade resulting in the growth and differentiation of erythroid progenitors into mature hemoglobinized erythrocytes. The EpoR is a founding member of the cytokine receptor superfamily (D'Andrea *et al.* 1989; Bazan 1990; Ihle and Kerr 1995) in which all members contain an extracellular ligand binding portion, a single hydrophobic

transmembrane-spanning domain and an intracellular cytoplasmic portion lacking a kinase domain.

EpoR activation results in the phosphorylation of many kinases, adapter proteins and other molecules involved in signal transduction. These post-Epo binding phosphorylation events are dependant on the cell type acted on. The phosphoproteins may function in various pathways, delivering the Epo signal to cytoplasmic, mitochondrial and nuclear targets.

The cytoplasmic domain of the EpoR, contains a Janus kinase-2 (JAK2) domain that is activated via transphosphorylation as a result of Epo binding. JAK2 activation, in turn, initiates intracellular tyrosine phosphorylation that serves as a docking site for intracellular proteins, including STAT5. This phosphorylation cascade results in the activation of various signal transduction pathways (Mulcahy 2001). Targeted disruption of either Epo, EpoR or JAK2 results in embryonic lethality due to unsuccessful transition of the mice to definitive erythropoiesis.

Non-hematopoietic effects of Epo

For many years, Epo was considered as a hormone that has the sole function of regulating the production of RBC. However, more recently, it has become abundantly clear that Epo exerts a wide variety of actions on multiple tissues and organ systems. These include endothelial cells, the CNS (including neurons and astrocytes), the female and male reproductive systems, the heart, the gastrointestinal system, muscle cells and the kidney itself. The Epo-mediated actions are thought to be autocrine and/or paracrine, while in certain cases, the endocrine action of Epo may be involved.

Epo and neuroprotection

The earliest indication that Epo is involved in CNS functions was reported in 1992. While investigating the expression of Epo mRNA as a response to anemia and hypoxia in the kidney and liver, Tan *et al.* discovered unexpected hypoxia induction of Epo mRNA in the brain, testes and spleen (Tan *et al.* 1992).

Since the mid-90's, many research laboratories have documented the neuroprotective role of Epo. The beneficial effects of Epo administration have been observed both in *in vivo* and *in vitro* models of brain injury and disease (Olsen 2003).

Epo and EpoR are known to be expressed in neurons and astrocytes in various brain areas. Epo is produced in the brain and is up-regulated during hypoxia. When administered to healthy rats having an intact blood brain barrier, Epo concentrations in the cerebrospinal fluid (CSF) increased and peaked at 3.5 h post-injection (Cerami 2001). Epo may contribute to neuroprotection by virtue of its documented antioxidant, antiapoptotic, anti-inflammatory, neurotrophic, angiogenic and synaptogenic activity (Siren and Ehrenreich 2001). Epo has been shown to be effective in mediating neuroprotection in both *in vivo* and *in vitro* rodent stroke models. Direct intracerebroventricular (I.C.V.) infusion of Epo administered in these stroke models demonstrated neuroprotective effects. Attenuation of ischemia-induced learning disability concomitant with rescue of hippocampal CA1 neurons from ischemic damage were observed in gerbils treated with Epo (Sakanaka *et al.* 1998). The neuroprotection observed in those animals was dose-dependant and the role of Epo was confirmed using both an active form of EpoR, containing an extracellular ligand binding region, and an inactive form after an ischemic insult. Animals receiving the inactive form of the

receptor did not exhibit neuronal damage after Epo administration, whereas animals treated with the active EpoR experienced neuronal damage evidenced by a significant reduction in the neuronal density of the CA1 hippocampal region. Bernaudin *et al.* demonstrated that 24 h I.C.V. infusion of Epo resulted in a 47% reduction of cerebral infarct volume in mice following permanent left middle cerebral artery (MCA) occlusion relative to non-Epo treated controls (Bernaudin *et al.* 1999). More recently, systemic administration of Epo in a rat stroke model significantly reduced neuronal damage ranging from 24 h pre-treatment up to 6 h post-MCA occlusion. The data presented by Brines *et al.* support a therapeutic window for treatment of 6 h after ischemic injury where neuroprotection can be achieved by systemic administration of Epo (Brines *et al.* 2000). Currently, the therapeutic 'window of opportunity' for tissue plasminogen activator administration in humans after stroke is three hours.

Treatment with Epo in rodents has beneficial effects in ameliorating the extent of concussive brain injury, the immune damage as a result of experimental autoimmune encephalitis and the ensuing toxicity from kainate-induced seizures (Brines *et al.* 2000). The administration of Epo in rabbit and rat models of subarachnoid hemorrage was effective in causing a significant reduction in the cortical necrotic neuron count (Alafaci *et al.* 2000), increase in survival (Buemi *et al.* 2000) and a normalization of the autoregulation of cerebral blood flow impaired by subarachnoid hemorrage (Springborg *et al.* 2002). The neuroprotective effects mediated by Epo were demonstrated in rabbits with experimental spinal cord ischemic injury. Neurological scores from Epo treated animals were improved both acutely and within a 48 h delay (Celik *et al.* 2002).

Mechanisms of Epo-mediated neuroprotection

The mechanisms mediating Epo neuroprotection before or after metabolic or oxidative challenge are complex and numerous. Neuronal cells treated with Epo 8 h prior to glutamate exposure, an excitotoxic insult, showed significantly less attrition than non-Epo-treated control cultures. The induction of a rapid and transient Ca^{2+} influx in Epo-treated cultures is a critical initial event in enhanced resistance to glutamate toxicity (Morishita *et al.* 1997).

Neuronal apoptosis as a result of different stimuli such as hypoxia, nutrient growth factor deprivation and kainate exposure is strongly inhibited by Epo exposure (Siren *et al.* 2001). The Epo had to be administered 24 h before the apoptotic stimulus, indicating that the neuroprotective mechanism was likely dependant on gene expression. The protection from apoptosis occurred through activation of extracellular signal-regulated kinases and protein kinase Akt-1 (Siren *et al.* 2001).

Epo has anti-inflammatory properties (Brines *et al.* 2000) as well as potent antioxidant effects (Boran *et al.* 1998; Sakanaka *et al.* 1998). A unique role for Epo in the protection of the erythrocyte membrane from oxidative damage caused by exposure to copper (II)-ascorbate (Chattopadhyay *et al.* 2000), and the reduction of lipid peroxidation along with increased antioxidant activities observed in Epo-treated patients (Boran *et al.* 1998), further implicate Epo as an important antioxidant. Epo is thought to up-regulate the expression of antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase) in neurons and erythrocytes (Olsen 2003).

Future directions of Epo

The body of literature available on Epo investigation in the pre-clinical domain provides a compelling basis for exploiting the potential neuroprotective action of Epo-related cytoprotection in human CNS disorders. An important phase I/II trial is currently underway in Germany, the Göttingen Epo-stroke trial, which has shown promise for the use of Epo as a neuroprotectant in patients with acute stroke involving the MCA (Ehrenreich *et al.* 2002). In a safety study, 13 patients were administered intravenously 33000 IU of r-HuEpo once daily for three days post-ischemia and no safety issues were noted. Patients that participated in the efficacy study treated with r-HuEpo had CSF Epo concentrations 60-100 times that of untreated patients, demonstrating the capacity for r-HuEpo to cross the blood brain barrier. A reduction of infarct size was also observed in these patients using magnetic resonance imaging. At a recent meeting on Epo in Toronto (Canadian Oncology Societies Epo conference, January 2004), there were also discussions of the potential use of Epo in the management of AD, PD and schizophrenia (Ehrenreich *et al.* 2004).

Objectives

The objectives of this thesis were to test the following hypotheses: **I.** AD sub-populations of cortical and hippocampal astroglia up-regulate surface EpoR as an intrinsic neuroprotective mechanism in this neurodegenerative disorder. **II.** Erythropoietin pre-treatment activates cell survival pathways in astrocytes that attenuate apoptotic cell death accruing from subsequent exposure to chemical stressors.

Specifically, we will (1) ascertain whether EpoR protein expression is up-regulated in AD and MCI-affected brain tissues relative to normal elderly controls and (2) determine whether previous reports on the neuroprotective role of erythropoietin in neurons and retinal ganglion cells also hold true in primary rat astrocytes in the face of various apoptogens. This dissertation provides new knowledge concerning the role of erythropoietin in CNS astroglia in states of normal health and disease and contributes to the expanding literature on the role of Epo in neural tissues.

Specific Aims: 1. To examine the extent of co-localization between the EpoR and GFAP-positive astrocytes in the human brain. 2. To quantify percentages of GFAP-positive astroglial co-expression of EpoR in post-mortem temporal cortex and hippocampus derived from NEC, MCI and AD subjects. 3. To analyze potential variations in glial EpoR expression within various cortical and hippocampal sub-regions as a function of AD, MCI and normal aging. 4. To ascertain whether Epo treatment in primary rat astroglia impacts cell survival under basal growth conditions. 5. To determine whether Epo pre-treatment attenuates glial cell death (apoptosis) in the face of

NO, STS and As_2O_3 challenge. 6. To determine whether mild oxidative stress contributes to Epo pre-conditioning in apoptogen-exposed astroglia.

Materials and methods

Experiment I: EpoR up-regulation in AD brain

Tissue samples

Paraffin-embedded, post-mortem tissues containing temporal cortex and hippocampus were procured from the NIH-funded Religious Orders Study. Nine sporadic AD, 10 MCI and 9 NEC subjects were enrolled in the study. All subjects included in this study were deceased and autopsied participants in the Religious Orders Study, an ongoing longitudinal clinical-pathologic study of aging and AD. They include older catholic nuns, priests, and brothers recruited from about 40 sites across the United States. Eligibility was established at baseline and required an age of 65 years or older and the absence of a clinical diagnosis of dementia. Each subject signed an informed consent approved by the Institutional Review Board of Rush Presbyterian St. Luke's Medical Center, agreed to annual clinical evaluation, and signed an Anatomic Gift Act donating his/her brain to Rush investigators at the time of death. Since January 1994, more than 950 persons have enrolled in the Religious Orders Study and completed the baseline evaluation. Participation in the annual follow-up evaluations has exceeded 95% of survivors, and the autopsy rate exceeds 92%. Further details of the study have been previously reported (Bennett *et al.* 2002; Wilson *et al.* 2002b; Wilson *et al.* 2002a; Schneider *et al.* 2004).

Immunofluorescent labelling

Qualitative analysis was performed by confocal microscopy, including a control for the EpoR antibody (red). After application of primary polyclonal and monoclonal antibodies, FITC-conjugated secondary goat anti-mouse antibody was used for detection of GFAP-positive astrocytes and rhodamine-conjugated secondary goat anti-rabbit to visualize

EpoR. The fluorescent preparations were examined using a Bio-Rad MRC-600 laser scanning confocal imaging system. Images were scanned on two channels (red and green) and merged to produce a single profile. In this model, all regions exhibiting co-localization of red and green emitters produce yellow fluorescence.

Immunohistochemistry

Six μM tissue sections were immunostained with rabbit derived polyclonal antisera directed against human EpoR (AVARP-9008, 1:50, Aviva Antibodies, San Diego, USA) and a mouse monoclonal antibody recognizing GFAP (NCL-GFAP-GA5, 1:50, Novo Castra Laboratories, UK). Vectastain Elite ABC Kits were used to visualize the reaction product. Slides were incubated for 16 h with anti-EpoR antibody and vizualized as a black precipitate with Vector SG, followed by incubation with anti-GFAP antibody for 16 h and visualization using Vector NovaRED substrate.

Quantification of EpoR positive astrocytes

Percentages of EpoR positive astrocytes were computed in six layers of the hippocampus (stratum oriens, pyramidal layer, stratum radiatum, molecular layer, granular layer and hilus of the dentate gyrus) and in temporal cortex layers I-VI and the subcortical white matter. For each area surveyed, the numbers of GFAP-positive and EpoR-positive glial cells were counted in 400X fields with the aid of an ocular grid. The average number of cells counted per individual layer was 216.2 ± 40.0 for the hippocampus and 217.0 ± 14.8 for the temporal cortex.

Statistical analysis

Percentages of EpoR-positive astrocytes were compared using one-way ANOVA with $p<0.05$ indicating statistical significance. A Newman-Keuls test was performed to determine main effects between groups.

Experiment II: Epo protects astrocytes from apoptosis

Materials

Sodium nitroprusside (SNP), propidium iodide (PI), 4',6-diamidino-2-phenylindole (DAPI), STS and As_2O_3 were purchased from Sigma (Oakville, Canada). Diethylenetriamine nitric oxide (DETA/NO) was obtained from Alexis Biochemicals (San Diego, USA). Antibodies directed against the following proteins were procured commercially: poly ADP-ribose polymerase (PARP; dilution 1:1000; Oncogene Science, Cambridge, USA), HO-1 (dilution 1:1000; SPA-895; StressGen Biotechnologies, Victoria, Canada), Akt and phospho-Akt (Cell Signaling Technology, Beverly, USA), EpoR (R&D Systems, Minneapolis, USA). SnMP was purchased from Frontier Scientific Porphyrin Products (Logan, USA), ascorbic acid (AA) from Fisher Scientific Ltd. (Montreal, Canada), melatonin from ICN Biochemicals (Aurora, USA). Resveratrol was a gift from Pharmascience Inc. (Montreal, Canada). MDA-MB-468 cells were obtained from ATCC (Manassas, USA) and maintained in phenol red containing a-MEM (Life Technologies, Inc., Burlington, Canada) supplemented with 10% fetal bovine serum.

Astrocyte cultures and treatments

Primary neuroglial cultures were prepared by mechanoenzymatic dissociation of cerebral tissue derived from 2-day old Sprague-Dawley rats (Charles River Breeding Farms, St. Constant, Canada) as previously described (Chopra *et al.* 1995). The cells were grown in Ham's F-12 and high glucose Dubelcco's modified Eagles medium (DMEM) supplemented with 10mM HEPES, 5% heat-inactivated horse and 5% fetal calf sera, and penicillin (50 U/ml)/streptomycin (50 μg/ml). $5x10^5$ cells/ml media were plated directly onto coated 25-cm^2 plastic flasks. The cultures were incubated at 37°C in humidified 95% air-5% CO_2 for 6 h at which time the flasks were vigorously shaken by hand to remove loosely adherent oligodendrocytes and microglia followed by replacement of fresh media. The cultures were incubated under the above-mentioned conditions for an additional 6 days at which point more than 98% of the cells comprising the monolayer were astroglia as determined by immunohistochemical labeling for the astrocyte-specific marker, glial fibrillary acidic protein (Chopra *et al.* 1997).

Propidium iodide staining/flow cytometry

Quantification of cell death was performed as previously described (Hardin *et al.* 1992). Astrocytes were pre-treated with Epo for 16 h followed by exposure to STS (0.5 μM), NO (administered as 300 μM SNP or 1 mM DETA/NO) or As_2O_3 (1 μM) for 48 h. Cells were trypsinized, washed twice in buffer (PBS/ 5% FBS/ 0.01 M NaN_3) at 4° C, pelleted, and resuspended in 0.5 ml of hypotonic fluorochrome solution containing 50 μg/ml PI, 0.1% sodium citrate, and 0.1% Triton X-100. Fluorescence was measured on a Becton-Dickinson flow cytometer. Dead cell fractions (sub-G_0 compartments) were quantified using CellQUEST software.

DAPI staining

Astrocytes were seeded onto sterile coverslips, pre-treated with Epo for 16 h followed by exposure to STS, NO or As_2O_3 for 48 h. After two PBS washes, the cells were fixed with 4% paraformaldehyde for 45 min on ice. Two additional washes in cold PBS were performed and then the cells were permeabilized with 0.2% Triton X-100/PBS for 5 min followed by DAPI (2 μg/ml) staining for 5 min at 25°C. The cells were fixed onto glass slides, sealed with nail polish and allowed to dry overnight. Apoptotic nuclei were identified on morphological grounds by DAPI staining (Kulkarni and McCulloch 1994; Kawakami *et al.* 2001). Apoptotic nuclear morphology was surveyed in a minimum of 100 cells per treatment group using an Olympus BX51 fluorescent microscope at 100x magnification.

DNA fragmentation analysis

DNA fragmentation assay, a qualitative index of apoptotic cell death, was performed using agarose gel electrophoresis (Gong *et al.* 1994a). $2x10^6$ cells were fixed with 70% ethanol, stored at -20°C for 24 h and collected by centrifugation. Degraded oligonucleosomal DNA was extracted with 40 μl of phosphate-citric acid buffer at room temperature for 1 h and vacuum-dried for 15 min. The powder was resuspended in 3 μl of 0.25% Nonidet P-40 and 3 μl of 1 mg/ml RNase, and was then incubated at 37°C for 30 min. Three microliters of 1 mg/ml proteinase K was added to the solution and incubated at 37°C for another 30 min. The mixture, together with 12 μl of loading buffer, was loaded on 0.8% agarose gel containing 0.5 mg/ml ethidium bromide, and electrophoresed at 2 V/cm overnight. The DNA laddering was recorded with a ChemiImager 4000 image analyzer (Alpha Innotech Corporation, San Leandro, USA).

Northern Blotting

Northern blotting was performed to measure mRNA levels of HO-1, a sensitive marker of oxidative stress and potential determinant of astroglial survival (Schipper 2004). Astrocytes were treated with various concentrations of Epo for 16 h. RNA was isolated by acid-guanidinium thiocyanate-phenol-chloroform extraction (Mehindate *et al.* 2001). Five micrograms of RNA were size separated by gel electrophoresis, transferred on Hybond-N filter paper and covalently cross-linked to the membrane using UV light. Hybridization probe (full length rat HO-1 cDNA [EcoRI-HindIII], 1 kb, in pBluescript SKII+, a gift from Dr. S. Shibahara, Tohoku University School of Medicine, Miyagi, Japan) was prepared with the random Primer DNA labelling system (Amersham Biosciences, Baie d'Urfe, Canada). Hybridization was performed using ^{32}P-labelled denatured DNA probe. Equal loading was confirmed by hybridization with cDNA for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RNA hybridization was visualized using autoradiography.

Western Blotting

Cell extracts were washed with cold PBS and resuspended in 0.4 ml lysis buffer (5 mM NaH_2PO_4, 1mM DTT, 10% glycerol, 1mM PMSF, 10 μg/ml each of aprotinin and leupeptin, pH 7.4) at 4°C. Extracts were centrifuged at 14,000 rpm at 4°C, and supernatants transferred to new tubes. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, USA). To detect the protein of interest (PARP, HO-1, phospho-Akt, Akt, and EpoR), 50 μg of protein were added to an equal volume of 2x sample buffer and run on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Bio-Rad), stained with Ponceau S in 5%

acetic acid to ensure equal protein loading, and blocked with 5% milk in PBS containing 0.5% Triton X-100 for 1 h at room temperature. The membranes were hybridized overnight at 4°C with specific polyclonal antibodies and then washed three times with PBS and 0.5% Triton X-100, blots were incubated with the appropriate secondary antibody (1:10,000) for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence (Amersham Biosciences). Immunostaining for β-actin was used to confirm equal protein loading.

Growth Assays

Astrocytes were seeded at $1x10^5$ cells/ml in 24-well plates. Cells were pre-treated with Epo for 16 h and then treated with STS for three days. SnMP (1 μM), a competitive inhibitor of heme oxygenase activity, was administered with light shielding to obviate metalloporphyrin photoactivation as previously described (Schipper *et al.* 1999). Viable cells were counted by trypan blue exclusion on day 3 post-treatment.

Statistical Analysis

Differences between groups were determined using a one-way analysis of variance (ANOVA) with $p<0.05$ indicating significance. A Newman-Keuls post-hoc test was applied to delineate significant main effects between groups. The data were analyzed using Prism 3.0 statistical analysis software.

Results

Changes in EpoR expression in AD and MCI subjects

To delineate the pattern and extent of EpoR expression in AD, MCI and NEC patients in the temporal cortex and hippocampus, paraffin-embedded, post-mortem tissue samples were procured from the NIH-funded Religious Orders Study (Table 1). The quantification of EpoR positive astrocytes was performed and the results demonstrated a significant increase in certain cortical and hippocampal regions early in the progression of the disease (Fig. 2-4). The up-regulation of EpoR positive astrocytes was significant in MCI, but not AD in layer I of the temporal cortex and the stratum radiatum, granular layer and dentate gyrus of the hippocampus. Other regions, viz, layers II-III of the temporal cortex and the stratum oriens and pyramidal layer of the hippocampus, demonstrate significant increases in astroglial EpoR expression in both MCI and AD subjects relative to NEC values. Nonetheless, there are regions that do not show a significant change in the levels of EpoR positive astrocytes as a function of clinical diagnosis (Fig. 5).

Given our observations of augmented astroglial EpoR expression in certain cortical and hippocampal layers in AD and MCI subjects, we then proceeded to ascertain whether up-regulation of Epo confers cytoprotection to this cell compartment *in vitro*.

Epo pre-conditioning protects cultured rat astroglia from apoptosis induced by NO and STS, but not As_2O_3

We examined the effects of different apoptosis inducers, independently and in combination with Epo, on rates of cell death in cultured rat astroglia. Apoptosis was evaluated by PI, DAPI staining, PARP cleavage and DNA fragmentation. As shown in

Fig. 6A, treatment with STS markedly attenuated the density of the astroglial monolayers. Similar cytotoxicity was obtained with NO exposure but not with As_2O_3 (data not shown). Pre-treatment with Epo 20 U/ml for 16 h ameliorated the rarefaction of the glial monolayers induced by STS an NO. As depicted in Fig. 6B and C, flow cytometric analysis of PI stained cells revealed that STS (0.5 μM) induced apoptosis in 66% ± 1.42 of the cells vs. 6.7% ± 0.34 in the untreated control cultures. Pre-treatment of astrocytes with either 5 or 20 U/ml Epo for 16 h significantly reduced STS-induced glial cell death (Fig. 6C i). Epo protection against STS-mediated apoptosis was dose-dependent. Lower concentrations of Epo (0.5 and 2 U/ml) conferred no glioprotection, whereas Epo (5 and 20 U/ml) conferred protection with pre-incubation periods as short as three hours (data not shown). NO (administered either as SNP or DETA/NO) increased by more than 30% the number of cells undergoing apoptosis. As in the case of STS, cell death induced by both NO donors was significantly attenuated by Epo 5 U/ml pre-treatment but there was no further increase in Epo-mediated cytoprotection when the cells where pre-treated with 20 U/ml Epo (Fig. 6C ii). As_2O_3 also induced apoptosis in rat astrocytes. However, in contradistinction to STS and NO, Epo pre-treatment had no effect on glial apoptosis induced by As_2O_3 in our model (Fig. 6C iii). Epo alone did not significantly increase apoptosis at any time point or concentration used. To further demonstrate the cytoprotective effect of Epo, nuclear morphology of cultured rat astroglia was analyzed by DAPI staining following apoptogen challenge (Fig. 7A). Fig. 7B shows that STS and NO increased apoptosis by 38% ± 1.72 and 29% ± 2.35, respectively. Pre-treatment with Epo significantly decreased numbers of apoptotic cells in the face of STS and NO challenge. Consistent with the flow cytometric data, Epo pre-treatment had no effect on glial apoptosis induced by As_2O_3. In addition, we evaluated PARP cleavage and DNA

fragmentation, qualitative hallmarks of apoptosis (Fig. 8A,B). STS, and to a lesser extent NO and As_2O_3, elicited proteolysis of the nuclear enzyme PARP, as evidenced by a decline in total protein levels relative to control and Epo-treated cells. Pre-treatment of STS- or NO-, but not As_2O_3-challenged cells with Epo substantially decreased PARP cleavage. Furthermore, there was significant DNA fragmentation in STS-, NO- and As_2O_3-treated cultures (Fig. 8B). Pre-treatment with Epo reduced the amount of DNA fragmentation observed after STS and NO, but not As_2O_3 treatment. Finally, all doses of Epo utilized in this study promoted up-regulation of the astroglial EpoR as determined by Western blotting (data not shown). Collectively, our data indicate that Epo pre-conditioning induces the EpoR and significantly attenuates multiple indices of apoptotic cell death in astroglia exposed to NO and STS, but not As_2O_3.

Role of HO-1 in Epo pre-conditioning of cultured astroglia

Epo has been shown to enhance superoxide formation in leukocytes and promote oxidative stress in erythrocytes (Zachee *et al.* 1993; Chen *et al.* 1997). Therefore, we investigated the role of oxidative stress in Epo pre-conditioning of cultured astroglia. HO-1 expression is widely accepted as a sensitive and reliable marker of cellular oxidative stress (Schipper 2004). Moreover, the products of the heme oxygenase reaction, free iron, CO and biliverdin/bilirubin, are biologically active molecules that can directly impact cellular redox homeostasis (Dennery 2000; Ryter and Tyrrell 2000). As depicted in Fig. 9A and B, low concentrations of Epo (0.5-5 U/ml) had no apparent effect on HO-1 mRNA and protein levels in the cultured astroglia. In contrast, 20 U/ml Epo, a clinically achievable concentration of the hormone (Assandri *et al.* 1999; Ashley *et al.* 2002), induced the expression of HO-1 mRNA and protein in these cells. These data

indicate that exposure to high dose Epo induces oxidative stress in these glial cultures implicating a possible pre-conditioning effect of HO-1. Considering that the protection against STS-mediated apoptosis were increased when higher Epo concentrations were used (50.5% STS + Epo 20 U/ml vs 37.9% STS + Epo 5 U/ml) and that Epo 20 U/ml induces HO-1 mRNA and protein levels, we queried whether inhibition of heme oxygenase activity would abrogate the added protection conferred by Epo 20 U/ml. At 1-5 μM concentrations, the metalloporphyrin, SnMP competitively blocks heme oxygenase activity without interfering with the action of nitric oxide synthase (Schipper 1999). As shown in Fig. 10A, co-incubation with 1 μM SnMP (light-shielded to prevent potential metalloporphyrin photoactivation), abrogated the cytoprotective effects of high dose Epo pre-conditioning in the face of STS treatment for 72 h. Further experiments were conducted to determine whether ROS generation and oxidative stress contribute to Epo-related pre-conditioning in our glial cultures. Cells were pre-treated with Epo 20 U/ml and AA (200 μM) or melatonin (100 μM) for 16 h followed by STS challenge for an additional 72 h. Fig. 10B and C show that AA or melatonin administered alone did not significantly impact cellular viability as determined by PI staining and flow cytometry. As expected, STS increased glial cell death by more than 60% relative to unchallenged cultures, and pre-treatment with Epo significantly protected the cells from STS-mediated toxicity. Co-administration of AA, melatonin (Fig. 10B,C) or the potent antioxidant, resveratrol (data not shown) during Epo pre-treatment significantly attenuated the cytoprotective effects of Epo in these cells. Furthermore, AA or melatonin administration suppressed the elaboration of HO-1 protein accruing from exposure to Epo 20 U/ml (Fig. 10D). Taken together, our findings indicate that mild oxidative stress induced by Epo

pre-conditioning is responsible, at least in part, for the observed Epo-mediated cytoprotection.

Effects of Epo on the Akt signaling pathway

We examined the effects of Epo on the Akt signaling pathway because Akt activation has been implicated in Epo-related cytoprotection in several cell types (Ihle and Kerr 1995; Ratajczak *et al.* 2001; Weishaupt *et al.* 2004). Astrocytes were treated with various concentrations of Epo. Western blotting was performed using antibodies for Akt and PhosphoAkt. As shown in Fig. 11, treatment with Epo in doses ranging from 0.5 to 20U/ml and exposure times of 1-16 h had no appreciable effect on total Akt levels or Akt phosphorylation (activation). Whole cell extracts from MDA-MB-468 breast cancer cells that express constitutively active Akt (Clark *et al.* 2002) were used as positive controls. Similar results were obtained following Epo incubation for 24 to 72 h (data not shown).

Discussion

EpoR up-regulation in AD brain

Cognitive abilities in the population aged 65 and over range from normal, to mild impairment, to obvious dementia. The term MCI is generally applied to elderly individuals who experience gradual cognitive decline (usually memory) that fail to meet the clinical criteria for AD or other dementia. MCI is increasingly recognized as an important public health problem because it is highly prevalent and confers a significantly augmented risk of progression to incipient AD relative to age-matched, cognitively-intact individuals.

There have been relatively few large longitudinal studies that have directly compared change in different cognitive abilities and other key clinical milestones in persons with MCI to those without cognitive impairment (Rubin *et al.* 1989; Chertkow *et al.* 2001). The Religious Orders Study is an ongoing longitudinal clinical–pathologic study of aging and AD in older Catholic clergy, to examine the natural history of MCI (Bennett *et al.* 2002). Subjects enrolled in this study underwent a uniform structured clinical evaluation, including a medical history, neurologic examination, cognitive performance testing, and review of a brain scan when available. Twenty-one cognitive performance tests were administered that assessed a broad range of cognitive abilities commonly affected by aging and AD, as previously described (Bennett *et al.* 2002; Wilson *et al.* 2002b). Cognitive test results were reviewed by a board-certified neuropsychologist, and participants were evaluated in person by a physician with expertise in the assessment of older persons with and without dementia. On the basis of this evaluation, participants were classified with respect to AD and other common conditions with the potential to affect cognitive function. Details of the clinical evaluation have been previously

published (Schneider *et al.* 2003). An annual follow-up evaluation was performed by examiners that were blinded to data collected previously. At the time of death, all available clinical data were reviewed, and a summary opinion was rendered regarding the most likely clinical diagnosis at the time of death. The reviewers were also blinded to all postmortem data (Bennett *et al.* 2004).

There are currently many published pre-clinical studies outlining the potential neuroprotective properties of Epo in neonatal and adult models of various human central and peripheral nervous system disorders. Erythropoietin has been demonstrated to be cytoprotective in retinal ganglion cells, neurons and astrocytes following conditions of metabolic as well as oxidative stress. The up-regulation of the EpoR observed in this study may mediate to some degree the cytoprotection observed in the various *in vitro* models. Further work will be required to determine whether EpoR expression in other cellular compartments (e.g. neurons, microglia) is also affected in Alzheimer-diseased CNS tissues. Since 1986, epoetin alfa has proven safe and effective for management of anemia in adult and pediatric patients with chronic kidney disease (Winearls *et al.* 1986; Henry *et al.* 2004). In light of the findings presented herein, and given that Epo is produced endogenously, has a positive safety profile, and is generally well tolerated, the advent of clinical trials using Epo as a potential neuroprotective agent in AD patients may be warranted.

These observations are the first to demonstrate altered levels of EpoR expression in the brain of MCI and AD subjects relative to the NEC. These findings may lead to significant developments in the understanding of the role of erythropoietin in the brain and implications for treatment of chronic neurodegenerative diseases with r-HuEpo.

Whether the up-regulation of EpoR in the astrocytes is specific to Alzheimer pathology or is a non-specific indication of reactive gliosis remains to be determined.

Epo protects astrocytes from apoptosis

Previous reports have documented Epo-related cytoprotection of CNS neurons, retinal ganglion cells, dorsal root ganglion cells, and microglia *in vivo* and *in vitro* (Sadamoto *et al.* 1998; Bernaudin *et al.* 1999; Brines *et al.* 2000; Lewczuk *et al.* 2000; Sinor and Greenberg 2000; Kawakami *et al.* 2001; Celik *et al.* 2002; Junk *et al.* 2002; Vairano *et al.* 2002; Weber *et al.* 2002; Campana and Myers 2003). To our knowledge, the current study is the first to demonstrate astroglial cytoprotection by Epo pre-conditioning. In this study, astroglial apoptosis was induced by three unrelated stressors: NO, STS and As_2O_3. Epo pre-conditioning at doses ranging from 5 to 20 U/ml produced significant glioprotection following exposure to NO and STS, but had no effect on As_2O_3-induced cell death.

The differential effects of Epo glioprotection likely reflect disparate signalling pathways mediating glial apoptosis in response to the various apoptotic stimuli utilized in this study. NO, STS and As_2O_3 reduce cell viability and induce apoptosis, as evidenced by nuclear fragmentation and condensation, DNA degradation to oligonucleosomes, decrease in mitochondrial membrane potential, cytochrome c release and caspase activation. NO has been shown to promote apoptosis via mitochondria-dependent and -independent apoptotic signaling pathways (Beltran *et al.* 2000). STS prevents the binding of ATP to a broad spectrum of kinases that play essential roles in cell survival (Meggio *et al.* 1995). STS arrests progression of normal non-transformed cells in the G_1 phase of the cell cycle suggesting that cell cycle associated-kinases essential for cell transit through G_1 are

sensitive to STS (Gong *et al.* 1994b). Epo may confer cytoprotection to astrocytes in the face of STS and NO challenge by interfering with one or more of these signaling pathways. Arsenic selectively activates p38 and JNK3 (a neural-specific isoform), but not JNK1 or JNK2 in cerebellar neurons. Blockade of p38 or JNK signaling pathways using specific inhibitors protected cerebellar neurons against As_2O_3-induced apoptosis suggesting that activation of p38 and JNK3 MAP kinases plays an important role in arsenite neurotoxicity (Namgung and Xia 2001). In contrast to STS and NO, As_2O_3-mediated gliotoxicity in the current study was not ameliorated by Epo pre-treatment suggesting that Epo exerts little or no effect on the p38/JNK3 signalling in rat astroglia. These disparate effects do not appear to be dependant on the dose or toxicity of the apoptotic inducer utilized. Despite the fact that both NO and As_2O_3 were equally toxic to the glial cultures (approximately 30% cell death), Epo protected these cells against NO- but not As_2O_3-mediated apoptosis. Furthermore, Epo pre-conditioning was effective in mediating cytoprotection in the face of the relatively more toxic STS challenge that, when administered alone, resulted in 66% cell death.

The mechanism by which Epo protects neuronal cells from apoptosis has not been completely elucidated; however, it has been shown that Epo activation of EpoR prevents apoptosis of CNS neurons by triggering cross-talk between the Epo-induced JAK2 activation and several downstream signaling pathways, including Ras-mitogen-activated protein kinase (MAPK) and PI-3-kinase/Akt (Ihle 1995). Phosphorylation of the latter, in turn, interrupts several cell death pathways, including those involving glycogen synthase kinase 3β, caspases-9, BAD (BCL-2 antagonist of cell death), and forkhead transcription factor. Chong *et al.* (2003) demonstrated that in neurons obtained from the hippocampi

of E-19 Sprague-Dawley rat pups, Epo (10 ng/ml) substantially increased levels of phospho-Akt, an effect that could be prevented by exposure to wortmannin. Digicaylioglu and Lipton (2001) observed PI-3-kinase activation after 30 min of Epo (10 U/ml) exposure in cortical neurons. In addition, Weishaupt *et al.* (2004) demonstrated that Epo-induced Akt phosphorylation and survival-promoting Epo effects in immunopurified retinal ganglion cells are completely abolished by inhibition of PI-3-kinase. However, we did not observe any change in total Akt at any time or dose used. Nor was the protein activated as indicated by failure to detect increased levels of phosphorylated Akt. Taken together, these results indicate that the mechanism of Epo protection at these doses is not related to Akt activation. Although it is known that Epo activates phosphatidylinositol 3-kinase in Ba/F3 cells (Damen *et al.* 1993) and inhibits apoptosis through an Akt-dependent pathway in endothelial cells (Chong *et al.* 2002), we demonstrated that this mechanism is not activated in rat astrocytes. Astrocytes express EpoR (Nagai *et al.* 2001) and, in the present study, the latter was up-regulated by Epo at all doses tested. In neurons, EpoR-mediated activation of JAK2 leads to phosphorylation of the inhibitor of NF-kB, subsequent translocation of the transcription factor to the nucleus and NF-kB-dependent transcription of neuroprotective genes (Digicaylioglu and Lipton 2001). Whether EpoR-mediated activation of NF-kB signaling contributes to cell survival in astrocytes as well remains to be determined. Augmentation of cellular glutathione peroxidase activity and other antioxidant defenses (Genc *et al.* 2002) may also have contributed to the hormone's glioprotective action in our model.

Finally, very short-term Epo exposure (minutes) has been shown to protect cardiomyocytes by raising the threshold for mitochondrial permeability transition (pore

opening) and attendant ROS generation in these cells (Juhaszova *et al.* 2004). Whether similar exposure to Epo impacts permeability transition in astrocytes and other cell types remains unknown.

Epo-mediated cytoprotection may not be entirely due to the hormone's anti-apoptotic effects. For example, Epo has been implicated in the proliferation and differentiation of erythrocytes (Krantz 1991; Sawyer 1994) and enhances BrdU-incorporation (indication of cells in S phase) in rat neonatal astrocytes (Sugawa *et al.* 2002). Thus, both anti-proliferative and anti-apoptotic actions of Epo may mediate astroglial cytoprotection *in vitro.*

In both neuronal and non-neuronal brain cells, oxidative stress and other noxious stimuli induce the rapid up-regulation of HO-1 at the transcriptional level (Dwyer *et al.* 1995; Manganaro *et al.* 1995). We observed that 20 U/ml Epo, but not lower doses of the hormone, up-regulated HO-1 mRNA and protein levels, indicating that elevated but clinically relevant concentrations of Epo may subject astrocytes to oxidative stress. Attenuation of both glial HO-1 protein synthesis and the protective effect of high-dose Epo by antioxidant co-administration during the pre-conditioning phase further supports this contention. The oxidative stress accruing from exposure to Epo (20 U/ml) must be relatively mild in so far as astroglial viability was not compromised when Epo (20 U/ml) was administered alone. In astrocytes exposed to NO, HO-1 up-regulation had no appreciable effect on cell survival. On the other hand, HO-1 induction resulting from high-dose Epo pre-treatment contributed significantly to protection against STS toxicity. Enhanced HO-1 biosynthesis may have conferred cytoprotection by catalyzing the degradation of pro-oxidant heme to the radical scavengers, biliverdin and bilirubin

(Stocker *et al.* 1987; Nakagami *et al.* 1993; Llesuy and Tomaro 1994; Dore *et al.* 1999; Baranano and Snyder 2001). Previous studies have shown that Epo increases HO-1 mRNA levels in blood monocytes and augments plasma antioxidant capacity in hemodialysis patients (Calo *et al.* 2003). Thus, mild antecedent oxidative stress may be a common mechanism mediating the cytoprotective effects of Epo pre-conditioning in disparate cell types. As such, Epo pre-conditioning may be analogous to induction of a cellular heat shock response that confers a degree of tolerance to subsequent stressors (Verbeke *et al.* 2001).

Epo-related glioprotection may also play an important role in human neurodegenerative and ischemic CNS disorders. It was observed that the proportions of GFAP-positive astrocytes co-expressing immunoreactive EpoR are significantly increased in the temporal cortex and hippocampus of subjects with sporadic AD and MCI, a frequent harbinger of incipient AD (Assaraf *et al.* 2004). These findings suggest that induction of the astroglial EpoR gene is a very early event in the pathogenesis of this common dementing disorder. Of note, glial HO-1 overexpression and other markers of oxidative stress are also augmented in the brains of patients with MCI and early AD (Schipper *et al.* 1995; Nunomura *et al.* 2001; Pratico *et al.* 2002). Expression of both Epo and its receptor is also augmented in the brains of patients with ischemic stroke (infarction) and cerebral hypoxia complicating cardiorespiratory arrest relative to neurohistologically normal subjects (Siren *et al.* 2001). Finally, enrichment for glial EpoR has recently been reported in the brains of schizophrenic patients (Ehrenreich *et al.* 2004). These human neuropathological studies implicate astroglia as major substrates of Epo and EpoR up-regulation under a host of adverse conditions. In light of the *in vitro* data presented

herein, it is conceivable that astroglial Epo secretion confers cytoprotection to this cellular compartment by autocrine or paracrine regulation of key apoptotic cell death pathways. In degenerative and ischemic brain disorders, protection of the astroglial compartment may have a profound impact on the survival of indigent neuronal populations. For example, glutamate uptake by astrocytes normally prevents the accumulation of this neurotransmitter to excitotoxic levels in brain extracellular space, a critical determinant of neuronal survival in the ischemic penumbra (Swanson *et al.* 2004). Astrocytes also influence neuronal redox homeostasis through release of AA and uptake of its oxidized form, dehydroascorbate, and by indirectly supporting neuronal glutathione metabolism (Swanson *et al.* 2004).

In a completed phase I/II trial for acute ischemic stroke, intravenous high-dose r-HuEpo was reportedly well tolerated and associated with improvement in clinical outcome at one month (Ehrenreich *et al.* 2002). A caveat to the use of Epo as a neurotherapeutic modality is the potentially deleterious expansion of the red cell mass (hematocrit) and increased platelet aggregability. AsialoEpo, a de-glycosylated congener of Epo, crosses the blood brain barrier, provides neuroprotection but does not significantly affect the hematocrit (Erbayraktar *et al.* 2003; Wang *et al.* 2004). Further experiments will be required to determine whether asialoEpo (or other Epo derivatives) pre-conditioning recapitulates the glioprotective effects of Epo observed in the current study.

Conclusions

1. EpoR co-localizes to a proportion of GFAP-positive astrocytes in the adult human temporal cortex and hippocampus, corroborating earlier reports (Sugawa *et al.* 2002).

2. In AD and MCI, there are significant increases in the proportion of astroglia expressing EpoR in layers II-III in the temporal cortex as well as the stratum oriens and pyramidal layer of the hippocampus relative to NEC values. In certain regions, viz, layer I of the temporal cortex and the stratum radiatum, granular layer and dentate gyrus of the hippocampus, glial EpoR expression is augmented in MCI relative to NEC, whereas AD values do not differ significantly from the normal controls. No differences in glial EpoR expression among the three groups were discerned in the molecular layer.

3. Our findings indicate that astroglial EpoR is induced in certain cortical and hippocampal regions in patients with sporadic AD. In some regions (Layer I of the temporal cortex and stratum radiatum, granular layer and dentate gyrus of the hippocampus), the EpoR response may be transient with return to baseline (NEC) levels with advancing disease.

4. Epo pre-conditioning protects cultured rat astroglia from apoptosis induced by NO and STS, akin to the cytoprotection previously reported in neurons (Chong *et al.* 2003). In contrast, Epo has no effect on glial apoptosis induced by As_2O_3 in our model. Furthermore, the protection against STS-mediated apoptosis conferred by Epo (5-20 U/ml) pre-conditioning was dose dependent, whereas protection against

NO-mediated apoptosis by Epo plateaued at 2 U/ml. The differential effects of Epo glioprotection likely reflect disparate signalling pathways mediating apoptosis in response to various apoptogens utilized in this study. For example, STS prevents the binding of ATP to a broad spectrum of kinases that play essential roles in cell survival (Meggio *et al.* 1995). NO induces apoptosis through genomic DNA fragmentation and inhibition of mitochondrial respiration (Beltran *et al.* 2000), and As_2O_3 induces apoptosis by JNK activation and generation of ROS (Miller *et al.* 2002).

5. HO-1 is a sensitive marker of cellular oxidative stress and, under certain circumstances, contributes to cell survival (Schipper 1999). Epo 2 and 5 U/ml had no effect on HO-1 mRNA and protein levels in rat astroglia suggesting that, contrary to hypothesis, (i) pre-conditioning via mild oxidative stress is not responsible for Epo glioprotection and (ii) HO-1 plays little or no role in Epo-related astroglial survival at low Epo concentrations.

6. Epo 20 U/ml, a high but clinically achievable concentration (Assandri *et al.* 1999; Ashley *et al.* 2002), up-regulates HO-1 mRNA and protein expression in cultured astroglia. Our findings suggest that, in contrast to the effects of lower Epo doses, (i) Epo 20 U/ml induces mild oxidative stress in cultured astroglia and (ii) the up-regulation of HO-1 in these cells may contribute to the cytoprotection observed in the face of STS, but not NO, challenge.

7. The up-regulation of EpoR in AD-affected cortical and hippocampal astroglia together with the cytoprotective role of Epo pre-treatment on rat astrocytes may

contribute to the beneficial effects of Epo/EpoR interaction observed in multiple models of CNS injury and disease (Bernaudin *et al.* 1999; Digicaylioglu and Lipton 2001; Agnello *et al.* 2002; Cerami *et al.* 2002). If confirmed, clinical studies using Epo as a potential neuroprotectant in early AD may be warranted.

Future Directions

Future studies should (i) establish the relation between Epo/EpoR protein expression in brain regions affected by MCI and AD, (ii) further delineate the role of HO-1 in Epo-mediated glioprotection and (iii) determine whether systemically- or centrally-administered Epo impacts astroglial survival *in vivo*.

References

Abbott N. J. (2002) Astrocyte-endothelial interactions and blood-brain barrier permeability. *J Anat* **200,** 629-638.

Agnello D., Bigini P., Villa P., Mennini T., Cerami A., Brines M. L. and Ghezzi P. (2002) Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. *Brain Res* **952,** 128-134.

Alafaci C., Salpietro F., Grasso G., Sfacteria A., Passalacqua M., Morabito A., Tripodo E., Calapai G., Buemi M. and Tomasello F. (2000) Effect of recombinant human erythropoietin on cerebral ischemia following experimental subarachnoid hemorrhage. *Eur J Pharmacol* **406,** 219-225.

Albrecht P. J., Dahl J. P., Stoltzfus O. K., Levenson R. and Levison S. W. (2002) Ciliary neurotrophic factor activates spinal cord astrocytes, stimulating their production and release of fibroblast growth factor-2, to increase motor neuron survival. *Exp Neurol* **173,** 46-62.

Ashley R. A., Dubuque S. H., Dvorak B., Woodward S. S., Williams S. K. and Kling P. J. (2002) Erythropoietin stimulates vasculogenesis in neonatal rat mesenteric microvascular endothelial cells. *Pediatr Res* **51,** 472-478.

Assandri R., Egger M., Gassmann M., Niggli E., Bauer C., Forster I. and Gorlach A. (1999) Erythropoietin modulates intracellular calcium in a human neuroblastoma cell line. *J Physiol* **516 (Pt 2),** 343-352.

Assaraf M. I., Liberman A., Bennett D., Miller W. H. and Schipper H. M. (2004) Up-Regulation of Erythropoietin Receptor Expression in AD and MCI Astroglia. *Neurobiology of Aging* **25,** S546.

Bakhiet M., Tjernlund A., Mousa A., Gad A., Stromblad S., Kuziel W. A., Seiger A. and Andersson J. (2001) RANTES promotes growth and survival of human first-trimester forebrain astrocytes. *Nat Cell Biol* **3,** 150-157.

Baranano D. E. and Snyder S. H. (2001) Neural roles for heme oxygenase: contrasts to nitric oxide synthase. *Proc Natl Acad Sci U S A* **98,** 10996-11002.

Bazan J. F. (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci U S A* **87,** 6934-6938.

Behl C., Davis J., Cole G. M. and Schubert D. (1992) Vitamin E protects nerve cells from amyloid beta protein toxicity. *Biochem Biophys Res Commun* **186,** 944-950.

Beltran B., Mathur A., Duchen M. R., Erusalimsky J. D. and Moncada S. (2000) The effect of nitric oxide on cell respiration: A key to understanding its role in cell survival or death. *Proc Natl Acad Sci U S A* **97,** 14602-14607.

Bennett D. A., Schneider J. A., Wilson R. S., Bienias J. L. and Arnold S. E. (2004) Neurofibrillary tangles mediate the association of amyloid load with clinical Alzheimer disease and level of cognitive function. *Arch Neurol* **61,** 378-384.

Bennett D. A., Wilson R. S., Schneider J. A., Evans D. A., Beckett L. A., Aggarwal N. T., Barnes L. L., Fox J. H. and Bach J. (2002) Natural history of mild cognitive impairment in older persons. *Neurology* **59,** 198-205.

Bernaudin M., Marti H. H., Roussel S., Divoux D., Nouvelot A., MacKenzie E. T. and Petit E. (1999) A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *J Cereb Blood Flow Metab* **19,** 643-651.

Boran M., Kucukaksu C., Balk M. and Cetin S. (1998) Red cell lipid peroxidation and antioxidant system in haemodialysed patients: influence of recombinant human erythropoietin (r-HuEPO) treatment. *Int Urol Nephrol* **30,** 507-512.

Bracken C. P., Whitelaw M. L. and Peet D. J. (2003) The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. *Cell Mol Life Sci* **60,** 1376-1393.

Brines M. L., Ghezzi P., Keenan S., Agnello D., de Lanerolle N. C., Cerami C., Itri L. M. and Cerami A. (2000) Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci U S A* **97,** 10526-10531.

Buemi M., Grasso G., Corica F., Calapai G., Salpietro F. M., Casuscelli T., Sfacteria A., Aloisi C., Alafaci C., Sturiale A., Frisina N. and Tomasello F. (2000) In vivo evidence that erythropoietin has a neuroprotective effect during subarachnoid hemorrhage. *Eur J Pharmacol* **392,** 31-34.

Calo L. A., Stanic L., Davis P. A., Pagnin E., Munaretto G., Fusaro M., Landini S., Semplicini A. and Piccoli A. (2003) Effect of epoetin on HO-1 mRNA level and plasma antioxidants in hemodialysis patients. *Int J Clin Pharmacol Ther* **41,** 187-192.

Campana W. M. and Myers R. R. (2003) Exogenous erythropoietin protects against dorsal root ganglion apoptosis and pain following peripheral nerve injury. *Eur J Neurosci* **18,** 1497-1506.

Celik M., Gokmen N., Erbayraktar S., Akhisaroglu M., Konakc S., Ulukus C., Genc S., Genc K., Sagiroglu E., Cerami A. and Brines M. (2002) Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. *Proc Natl Acad Sci U S A* **99,** 2258-2263.

Cerami A. (2001) Beyond erythropoiesis: novel applications for recombinant human erythropoietin. *Semin Hematol* **38,** 33-39.

Cerami A., Brines M., Ghezzi P., Cerami C. and Itri L. M. (2002) Neuroprotective properties of epoetin alfa. *Nephrol Dial Transplant* **17 Suppl 1,** 8-12.

Chattopadhyay A., Choudhury T. D., Bandyopadhyay D. and Datta A. G. (2000) Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical. *Biochem Pharmacol* **59,** 419-425.

Chen H. C., Tsai J. C., Tsai J. H. and Lai Y. H. (1997) Recombinant human erythropoietin enhances superoxide production by FMLP-stimulated polymorphonuclear leukocytes in hemodialysis patients. *Kidney Int* **52,** 1390-1394.

Chertkow H., Bergman H., Schipper H. M., Gauthier S., Bouchard R., Fontaine S. and Clarfield A. M. (2001) Assessment of suspected dementia. *Can J Neurol Sci* **28 Suppl 1,** S28-41.

Chong Z. Z., Kang J. Q. and Maiese K. (2002) Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. *Circulation* **106,** 2973-2979.

Chong Z. Z., Kang J. Q. and Maiese K. (2003) Erythropoietin: cytoprotection in vascular and neuronal cells. *Curr Drug Targets Cardiovasc Haematol Disord* **3,** 141-154.

Chopra V. S., Chalifour L. E. and Schipper H. M. (1995) Differential effects of cysteamine on heat shock protein induction and cytoplasmic granulation in astrocytes and glioma cells. *Brain Res Mol Brain Res* **31,** 173-184.

Chopra V. S., Moozar K. L., Mehindate K. and Schipper H. M. (1997) A cellular stress model for the differential expression of glial lysosomal cathepsins in the aging nervous system. *Exp Neurol* **147,** 221-228.

Clark A. S., West K., Streicher S. and Dennis P. A. (2002) Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. *Mol Cancer Ther* **1,** 707-717.

Coyle J. T. and Puttfarcken P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262,** 689-695.

Cummings J. L. and Cole G. (2002) Alzheimer disease. *Jama* **287,** 2335-2338.

Cummings J. L., Vinters H. V., Cole G. M. and Khachaturian Z. S. (1998) Alzheimer's disease: etiologies, pathophysiology, cognitive reserve, and treatment opportunities. *Neurology* **51,** S2-17; discussion S65-17.

Damen J. E., Mui A. L., Puil L., Pawson T. and Krystal G. (1993) Phosphatidylinositol 3-kinase associates, via its Src homology 2 domains, with the activated erythropoietin receptor. *Blood* **81,** 3204-3210.

D'Andrea A. D., Fasman G. D. and Lodish H. F. (1989) Erythropoietin receptor and interleukin-2 receptor beta chain: a new receptor family. *Cell* **58,** 1023-1024.

Dennery P. A. (2000) Regulation and role of heme oxygenase in oxidative injury. *Curr Top Cell Regul* **36,** 181-199.

Digicaylioglu M. and Lipton S. A. (2001) Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. *Nature* **412,** 641-647.

Dore S., Takahashi M., Ferris C. D., Zakhary R., Hester L. D., Guastella D. and Snyder S. H. (1999) Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. *Proc Natl Acad Sci U S A* **96,** 2445-2450.

Dringen R., Gutterer J. M. and Hirrlinger J. (2000) Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. *Eur J Biochem* **267,** 4912-4916.

Dwyer B. E., Nishimura R. N. and Lu S. Y. (1995) Differential expression of heme oxygenase-1 in cultured cortical neurons and astrocytes determined by the aid of a new heme oxygenase antibody. Response to oxidative stress. *Brain Res Mol Brain Res* **30,** 37-47.

Ehrenreich H., Degner D., Meller J., Brines M., Behe M., Hasselblatt M., Woldt H., Falkai P., Knerlich F., Jacob S., von Ahsen N., Maier W., Bruck W., Ruther E.,

Cerami A., Becker W. and Siren A. L. (2004) Erythropoietin: a candidate compound for neuroprotection in schizophrenia. *Mol Psychiatry* **9,** 42-54.

Ehrenreich H., Hasselblatt M., Dembowski C., Cepek L., Lewczuk P., Stiefel M., Rustenbeck H. H., Breiter N., Jacob S., Knerlich F., Bohn M., Poser W., Ruther E., Kochen M., Gefeller O., Gleiter C., Wessel T. C., De Ryck M., Itri L., Prange H., Cerami A., Brines M. and Siren A. L. (2002) Erythropoietin therapy for acute stroke is both safe and beneficial. *Mol Med* **8,** 495-505.

Erbayraktar S., Grasso G., Sfacteria A., Xie Q. W., Coleman T., Kreilgaard M., Torup L., Sager T., Erbayraktar Z., Gokmen N., Yilmaz O., Ghezzi P., Villa P., Fratelli M., Casagrande S., Leist M., Helboe L., Gerwein J., Christensen S., Geist M. A., Pedersen L. O., Cerami-Hand C., Wuerth J. P., Cerami A. and Brines M. (2003) Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. *Proc Natl Acad Sci U S A* **100,** 6741-6746.

Eriksen J. L. and Druse M. J. (2001) Astrocyte-mediated trophic support of developing serotonin neurons: effects of ethanol, buspirone, and S100B. *Brain Res Dev Brain Res* **131,** 9-15.

Gabbita S. P., Lovell M. A. and Markesbery W. R. (1998) Increased nuclear DNA oxidation in the brain in Alzheimer's disease. *J Neurochem* **71,** 2034-2040.

Genc S., Akhisaroglu M., Kuralay F. and Genc K. (2002) Erythropoietin restores glutathione peroxidase activity in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine-induced neurotoxicity in C57BL mice and stimulates murine astroglial glutathione peroxidase production in vitro. *Neurosci Lett* **321,** 73-76.

Gong J., Traganos F. and Darzynkiewicz Z. (1994a) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal Biochem* **218,** 314-319.

Gong J., Traganos F. and Darzynkiewicz Z. (1994b) Staurosporine blocks cell progression through G1 between the cyclin D and cyclin E restriction points. *Cancer Res* **54,** 3136-3139.

Grasso G., Buemi M., Alafaci C., Sfacteria A., Passalacqua M., Sturiale A., Calapai G., De Vico G., Piedimonte G., Salpietro F. M. and Tomasello F. (2002) Beneficial

effects of systemic administration of recombinant human erythropoietin in rabbits subjected to subarachnoid hemorrhage. *Proc Natl Acad Sci U S A* **99,** 5627-5631.

Hailer N. P., Wirjatijasa F., Roser N., Hischebeth G. T., Korf H. W. and Dehghani F. (2001) Astrocytic factors protect neuronal integrity and reduce microglial activation in an in vitro model of N-methyl-D-aspartate-induced excitotoxic injury in organotypic hippocampal slice cultures. *Eur J Neurosci* **14,** 315-326.

Halliwell B. and Gutteridge J. M. C. (1999) *Free radicals in biology and medicine,* 3rd Edition, pp xxxi, 936. Clarendon Press ; Oxford University Press, Oxford New York.

Hardin J. A., Sherr D. H., DeMaria M. and Lopez P. A. (1992) A simple fluorescence method for surface antigen phenotyping of lymphocytes undergoing DNA fragmentation. *J Immunol Methods* **154,** 99-107.

Henry D. H., Bowers P., Romano M. T. and Provenzano R. (2004) Epoetin alfa. Clinical evolution of a pleiotropic cytokine. *Arch Intern Med* **164,** 262-276.

Hensley K., Carney J. M., Mattson M. P., Aksenova M., Harris M., Wu J. F., Floyd R. A. and Butterfield D. A. (1994) A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. *Proc Natl Acad Sci U S A* **91,** 3270-3274.

Honig L. S. and Mayeux R. (2001) Natural history of Alzheimer's disease. *Aging (Milano)* **13,** 171-182.

Ihle J. N. (1995) Cytokine receptor signalling. *Nature* **377,** 591-594.

Ihle J. N. and Kerr I. M. (1995) Jaks and Stats in signaling by the cytokine receptor superfamily. *Trends Genet* **11,** 69-74.

Jelkmann W. (1992) Erythropoietin: structure, control of production, and function. *Physiol Rev* **72,** 449-489.

Johnstone M., Gearing A. J. and Miller K. M. (1999) A central role for astrocytes in the inflammatory response to beta-amyloid; chemokines, cytokines and reactive oxygen species are produced. *J Neuroimmunol* **93,** 182-193.

Juhaszova M., Zorov D. B., Kim S. H., Pepe S., Fu Q., Fishbein K. W., Ziman B. D., Wang S., Ytrehus K., Antos C. L., Olson E. N. and Sollott S. J. (2004) Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* **113,** 1535-1549.

Junk A. K., Mammis A., Savitz S. I., Singh M., Roth S., Malhotra S., Rosenbaum P. S., Cerami A., Brines M. and Rosenbaum D. M. (2002) Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* **99,** 10659-10664.

Kalialis L. V. and Olsen N. V. (2003) [Erythropoietin--a new therapy in cerebral ischemia?]. *Ugeskr Laeger* **165,** 2477-2481.

Kawakami M., Sekiguchi M., Sato K., Kozaki S. and Takahashi M. (2001) Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia. *J Biol Chem* **276,** 39469-39475.

Krantz S. B. (1991) Erythropoietin. *Blood* **77,** 419-434.

Kulkarni G. V. and McCulloch C. A. (1994) Serum deprivation induces apoptotic cell death in a subset of Balb/c 3T3 fibroblasts. *J Cell Sci* **107 (Pt 5),** 1169-1179.

Langston J. W., Widner H., Goetz C. G., Brooks D., Fahn S., Freeman T. and Watts R. (1992) Core assessment program for intracerebral transplantations (CAPIT). *Mov Disord* **7,** 2-13.

Lappin T. (2003) The cellular biology of erythropoietin receptors. *Oncologist* **8 Suppl 1,** 15-18.

Lewczuk P., Hasselblatt M., Kamrowski-Kruck H., Heyer A., Unzicker C., Siren A. L. and Ehrenreich H. (2000) Survival of hippocampal neurons in culture upon hypoxia: effect of erythropoietin. *Neuroreport* **11,** 3485-3488.

Liu Q. Y., Schaffner A. E., Chang Y. H. and Barker J. L. (1998) Astrocyte-conditioned saline supports embryonic rat hippocampal neuron differentiation in short-term cultures. *J Neurosci Methods* **86,** 71-77.

Llesuy S. F. and Tomaro M. L. (1994) Heme oxygenase and oxidative stress. Evidence of involvement of bilirubin as physiological protector against oxidative damage. *Biochim Biophys Acta* **1223,** 9-14.

Manganaro F., Chopra V. S., Mydlarski M. B., Bernatchez G. and Schipper H. M. (1995) Redox perturbations in cysteamine-stressed astroglia: implications for inclusion formation and gliosis in the aging brain. *Free Radic Biol Med* **19,** 823-835.

Markesbery W. R. (1997) Oxidative stress hypothesis in Alzheimer's disease. *Free Radic Biol Med* **23,** 134-147.

Marti H. H., Wenger R. H., Rivas L. A., Straumann U., Digicaylioglu M., Henn V., Yonekawa Y., Bauer C. and Gassmann M. (1996) Erythropoietin gene expression in human, monkey and murine brain. *Eur J Neurosci* **8,** 666-676.

Mecocci P., MacGarvey U. and Beal M. F. (1994) Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann Neurol* **36,** 747-751.

Mecocci P., Polidori M. C., Ingegni T., Cherubini A., Chionne F., Cecchetti R. and Senin U. (1998) Oxidative damage to DNA in lymphocytes from AD patients. *Neurology* **51,** 1014-1017.

Meggio F., Donella Deana A., Ruzzene M., Brunati A. M., Cesaro L., Guerra B., Meyer T., Mett H., Fabbro D., Furet P. and et al. (1995) Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2. *Eur J Biochem* **234,** 317-322.

Mehindate K., Sahlas D. J., Frankel D., Mawal Y., Liberman A., Corcos J., Dion S. and Schipper H. M. (2001) Proinflammatory cytokines promote glial heme oxygenase-1 expression and mitochondrial iron deposition: implications for multiple sclerosis. *J Neurochem* **77,** 1386-1395.

Miller W. H., Jr., Schipper H. M., Lee J. S., Singer J. and Waxman S. (2002) Mechanisms of action of arsenic trioxide. *Cancer Res* **62,** 3893-3903.

Morishita E., Masuda S., Nagao M., Yasuda Y. and Sasaki R. (1997) Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. *Neuroscience* **76,** 105-116.

Morris J. C. (1997) Clinical dementia rating: a reliable and valid diagnostic and staging measure for dementia of the Alzheimer type. *Int Psychogeriatr* **9 Suppl 1,** 173-176; discussion 177-178.

Mulcahy L. (2001) The erythropoietin receptor. *Semin Oncol* **28,** 19-23.

Nagai A., Nakagawa E., Choi H. B., Hatori K., Kobayashi S. and Kim S. U. (2001) Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia, and oligodendrocytes grown in culture. *J Neuropathol Exp Neurol* **60,** 386-392.

Nakagami T., Toyomura K., Kinoshita T. and Morisawa S. (1993) A beneficial role of bile pigments as an endogenous tissue protector: anti-complement effects of biliverdin and conjugated bilirubin. *Biochim Biophys Acta* **1158,** 189-193.

Namgung U. and Xia Z. (2001) Arsenic induces apoptosis in rat cerebellar neurons via activation of JNK3 and p38 MAP kinases. *Toxicol Appl Pharmacol* **174,** 130-138.

Ng T., Marx G., Littlewood T. and Macdougall I. (2003) Recombinant erythropoietin in clinical practice. *Postgrad Med J* **79,** 367-376.

Nunomura A., Perry G., Aliev G., Hirai K., Takeda A., Balraj E. K., Jones P. K., Ghanbari H., Wataya T., Shimohama S., Chiba S., Atwood C. S., Petersen R. B. and Smith M. A. (2001) Oxidative damage is the earliest event in Alzheimer disease. *J Neuropathol Exp Neurol* **60,** 759-767.

O'Brien C. (1996) Auguste D. and Alzheimer's disease. *Science* **273,** 28.

Olsen N. V. (2003) Central nervous system frontiers for the use of erythropoietin. *Clin Infect Dis* **37 Suppl 4,** S323-330.

Perry G. and Smith M. A. (1998) Is oxidative damage central to the pathogenesis of Alzheimer disease? *Acta Neurol Belg* **98,** 175-179.

Peuchen S., Bolanos J. P., Heales S. J., Almeida A., Duchen M. R. and Clark J. B. (1997) Interrelationships between astrocyte function, oxidative stress and antioxidant status within the central nervous system. *Prog Neurobiol* **52,** 261-281.

Pitchumoni S. S. and Doraiswamy P. M. (1998) Current status of antioxidant therapy for Alzheimer's Disease. *J Am Geriatr Soc* **46,** 1566-1572.

Pratico D., Clark C. M., Liun F., Rokach J., Lee V. Y. and Trojanowski J. Q. (2002) Increase of brain oxidative stress in mild cognitive impairment: a possible predictor of Alzheimer disease. *Arch Neurol* **59,** 972-976.

Ratajczak J., Majka M., Kijowski J., Baj M., Pan Z. K., Marquez L. A., Janowska-Wieczorek A. and Ratajczak M. Z. (2001) Biological significance of MAPK, AKT and JAK-STAT protein activation by various erythropoietic factors in normal human early erythroid cells. *Br J Haematol* **115,** 195-204.

Rubin E. H., Morris J. C., Grant E. A. and Vendegna T. (1989) Very mild senile dementia of the Alzheimer type. I. Clinical assessment. *Arch Neurol* **46,** 379-382.

Ryter S. W. and Tyrrell R. M. (2000) The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free Radic Biol Med* **28,** 289-309.

Sadamoto Y., Igase K., Sakanaka M., Sato K., Otsuka H., Sakaki S., Masuda S. and Sasaki R. (1998) Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery. *Biochem Biophys Res Commun* **253,** 26-32.

Sakanaka M., Wen T. C., Matsuda S., Masuda S., Morishita E., Nagao M. and Sasaki R. (1998) In vivo evidence that erythropoietin protects neurons from ischemic damage. *Proc Natl Acad Sci U S A* **95,** 4635-4640.

Sano M., Ernesto C., Thomas R. G., Klauber M. R., Schafer K., Grundman M., Woodbury P., Growdon J., Cotman C. W., Pfeiffer E., Schneider L. S. and Thal L. J. (1997) A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N Engl J Med* **336,** 1216-1222.

Sawyer S. T. (1994) Introduction: the erythropoietin receptor and signal transduction. *Ann N Y Acad Sci* **718,** 185-190.

Schipper H. M. (1999) Glial HO-1 expression, iron deposition and oxidative stress in neurodegenerative diseases. *Neurotox Res* **1,** 57-70.

Schipper H. M. (2004) Heme oxygenase-1: transducer of pathological brain iron sequestration under oxidative stress. *Ann N Y Acad Sci* **1012,** 84-93.

Schipper H. M., Cisse S. and Stopa E. G. (1995) Expression of heme oxygenase-1 in the senescent and Alzheimer-diseased brain. *Ann Neurol* **37,** 758-768.

Schipper H. M., Bernier L., Mehindate K. and Frankel D. (1999) Mitochondrial iron sequestration in dopamine-challenged astroglia: role of heme oxygenase-1 and the permeability transition pore. *J Neurochem* **72,** 1802-1811.

Schneider J. A., Wilson R. S., Bienias J. L., Evans D. A. and Bennett D. A. (2004) Cerebral infarctions and the likelihood of dementia from Alzheimer disease pathology. *Neurology* **62,** 1148-1155.

Schneider J. A., Wilson R. S., Cochran E. J., Bienias J. L., Arnold S. E., Evans D. A. and Bennett D. A. (2003) Relation of cerebral infarctions to dementia and cognitive function in older persons. *Neurology* **60,** 1082-1088.

Selkoe D. J. (1991) The molecular pathology of Alzheimer's disease. *Neuron* **6,** 487-498.

Selkoe D. J. (1997) Alzheimer's disease: genotypes, phenotypes, and treatments. *Science* **275,** 630-631.

Sies H. and Cadenas E. (1985) Oxidative stress: damage to intact cells and organs. *Philos Trans R Soc Lond B Biol Sci* **311,** 617-631.

Sinor A. D. and Greenberg D. A. (2000) Erythropoietin protects cultured cortical neurons, but not astroglia, from hypoxia and AMPA toxicity. *Neurosci Lett* **290,** 213-215.

Siren A. L. and Ehrenreich H. (2001) Erythropoietin--a novel concept for neuroprotection. *Eur Arch Psychiatry Clin Neurosci* **251,** 179-184.

Siren A. L., Fratelli M., Brines M., Goemans C., Casagrande S., Lewczuk P., Keenan S., Gleiter C., Pasquali C., Capobianco A., Mennini T., Heumann R., Cerami A., Ehrenreich H. and Ghezzi P. (2001) Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc Natl Acad Sci U S A* **98,** 4044-4049.

Smith M. A., Hirai K., Hsiao K., Pappolla M. A., Harris P. L., Siedlak S. L., Tabaton M. and Perry G. (1998) Amyloid-beta deposition in Alzheimer transgenic mice is associated with oxidative stress. *J Neurochem* **70,** 2212-2215.

Smith M. A., Kutty R. K., Richey P. L., Yan S. D., Stern D., Chader G. J., Wiggert B., Petersen R. B. and Perry G. (1994) Heme oxygenase-1 is associated with the neurofibrillary pathology of Alzheimer's disease. *Am J Pathol* **145,** 42-47.

Springborg J. B., Ma X., Rochat P., Knudsen G. M., Amtorp O., Paulson O. B., Juhler M. and Olsen N. V. (2002) A single subcutaneous bolus of erythropoietin normalizes cerebral blood flow autoregulation after subarachnoid haemorrhage in rats. *Br J Pharmacol* **135,** 823-829.

Stocker R., Yamamoto Y., McDonagh A. F., Glazer A. N. and Ames B. N. (1987) Bilirubin is an antioxidant of possible physiological importance. *Science* **235,** 1043-1046.

Sugawa M., Sakurai Y., Ishikawa-Ieda Y., Suzuki H. and Asou H. (2002) Effects of erythropoietin on glial cell development; oligodendrocyte maturation and astrocyte proliferation. *Neurosci Res* **44,** 391-403.

Swanson R. A., Ying W. and Kauppinen T. M. (2004) Astrocyte influences on ischemic neuronal death. *Curr Mol Med* **4,** 193-205.

Takuma K., Baba A. and Matsuda T. (2004) Astrocyte apoptosis: implications for neuroprotection. *Prog Neurobiol* **72,** 111-127.

Takuma K., Yoshida T., Lee E., Mori K., Kishi T., Baba A. and Matsuda T. (2000) CV-2619 protects cultured astrocytes against reperfusion injury via nerve growth factor production. *Eur J Pharmacol* **406,** 333-339.

Tan C. C., Eckardt K. U., Firth J. D. and Ratcliffe P. J. (1992) Feedback modulation of renal and hepatic erythropoietin mRNA in response to graded anemia and hypoxia. *Am J Physiol* **263,** F474-481.

United States Administration on Aging. (2004) The Growth of America's Older Population. Retrieved Jan 21, 2004 from the World Wide Web: http://www.aoa.gov/naic/may2000/factsheets/growth.html.

Vairano M., Dello Russo C., Pozzoli G., Battaglia A., Scambia G., Tringali G., Aloe-Spiriti M. A., Preziosi P. and Navarra P. (2002) Erythropoietin exerts anti-apoptotic effects on rat microglial cells in vitro. *Eur J Neurosci* **16,** 584-592.

Verbeke P., Fonager J., Clark B. F. and Rattan S. I. (2001) Heat shock response and ageing: mechanisms and applications. *Cell Biol Int* **25,** 845-857.

Vickers J. C., Dickson T. C., Adlard P. A., Saunders H. L., King C. E. and McCormack G. (2000) The cause of neuronal degeneration in Alzheimer's disease. *Prog Neurobiol* **60,** 139-165.

Vile G. F. and Tyrrell R. M. (1993) Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. *J Biol Chem* **268,** 14678-14681.

Wang X., Zhu C., Gerwien J. G., Schrattenholz A., Sandberg M., Leist M. and Blomgren K. (2004) The nonerythropoietic asialoerythropoietin protects against neonatal hypoxia-ischemia as potently as erythropoietin. *J Neurochem* **91,** 900-910.

Wang X. F. and Cynader M. S. (1999) Effects of astrocytes on neuronal attachment and survival shown in a serum-free co-culture system. *Brain Res Brain Res Protoc* **4,** 209-216.

Weber A., Maier R. F., Hoffmann U., Grips M., Hoppenz M., Aktas A. G., Heinemann U., Obladen M. and Schuchmann S. (2002) Erythropoietin improves synaptic transmission during and following ischemia in rat hippocampal slice cultures. *Brain Res* **958,** 305-311.

Weishaupt J. H., Rohde G., Polking E., Siren A. L., Ehrenreich H. and Bahr M. (2004) Effect of erythropoietin axotomy-induced apoptosis in rat retinal ganglion cells. *Invest Ophthalmol Vis Sci* **45,** 1514-1522.

Wilson J. X. (1997) Antioxidant defense of the brain: a role for astrocytes. *Can J Physiol Pharmacol* **75,** 1149-1163.

Wilson R. S., Mendes De Leon C. F., Barnes L. L., Schneider J. A., Bienias J. L., Evans D. A. and Bennett D. A. (2002a) Participation in cognitively stimulating activities and risk of incident Alzheimer disease. *Jama* **287,** 742-748.

Wilson R. S., Beckett L. A., Barnes L. L., Schneider J. A., Bach J., Evans D. A. and Bennett D. A. (2002b) Individual differences in rates of change in cognitive abilities of older persons. *Psychol Aging* **17,** 179-193.

Winearls C. G., Oliver D. O., Pippard M. J., Reid C., Downing M. R. and Cotes P. M. (1986) Effect of human erythropoietin derived from recombinant DNA on the anaemia of patients maintained by chronic haemodialysis. *Lancet* **2,** 1175-1178.

Yamamuro A., Ago Y., Takuma K., Maeda S., Sakai Y., Baba A. and Matsuda T. (2003) Possible involvement of astrocytes in neuroprotection by the cognitive enhancer T-588. *Neurochem Res* **28,** 1779-1783.

Zachee P., Ferrant A., Daelemans R., Coolen L., Goossens W., Lins R. L., Couttenye M., De Broe M. E. and Boogaerts M. A. (1993) Oxidative injury to erythrocytes, cell rigidity and splenic hemolysis in hemodialyzed patients before and during erythropoietin treatment. *Nephron* **65,** 288-293.

Zaman Z., Roche S., Fielden P., Frost P. G., Niriella D. C. and Cayley A. C. (1992) Plasma concentrations of vitamins A and E and carotenoids in Alzheimer's disease. *Age Ageing* **21,** 91-94.

Zhang X., Rosenstein B. S., Wang Y., Lebwohl M. and Wei H. (1997) Identification of possible reactive oxygen species involved in ultraviolet radiation-induced oxidative DNA damage. *Free Radic Biol Med* **23,** 980-985.

Figure Legends:

Figure 1: Hypothetical structure of the EpoR dimer and its interaction with Epo.

Figure 1.

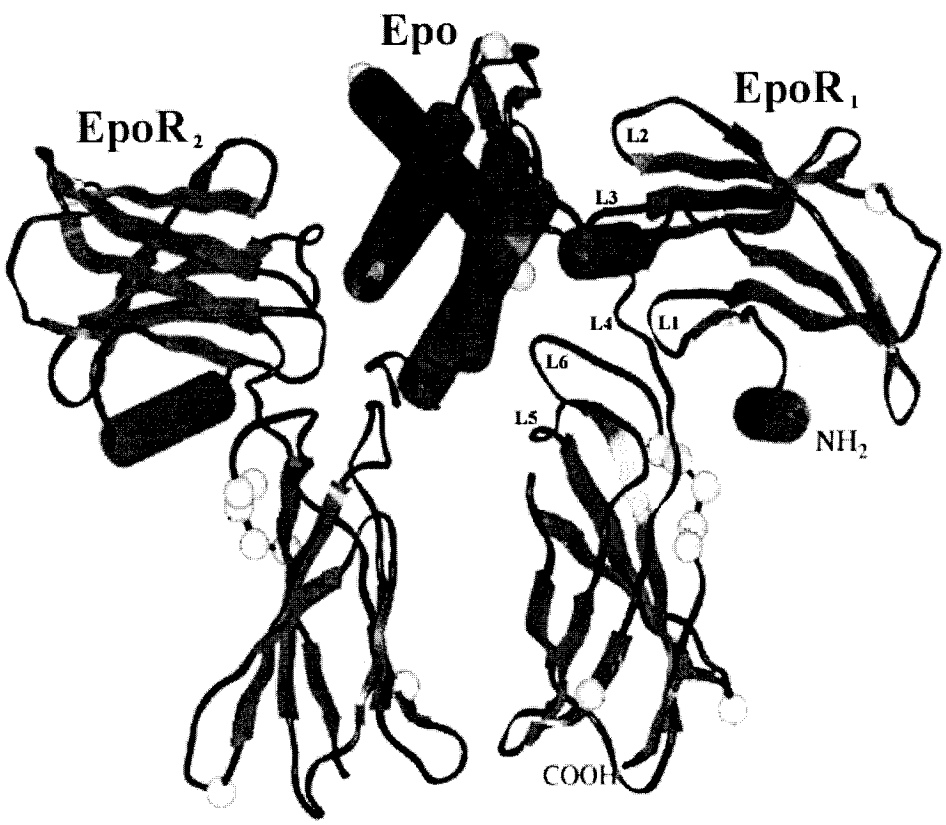


Table 1. Demographic information.

	All (n=28)	**NEC** (n=9)	**MCI** (n=10)	**AD** (n=9)
Age, mean years (SD, range)	83.5 (5.8, 72-97)	80.1 (5.9, 72-90)	84.5 (6.1, 76-97)	85.7 (3.9, 80-91)
Sex (Male/Female)	50% / 50%	77.8% / 22.2%	40% / 60%	33.3% / 66.7%
Education, mean years (SD, range)	18.5 (3.0, 14-25)	19.9 (2.8, 16-25)	19.0 (3.0, 15-24)	16.7 (2.1, 14-21)
MMSE, mean (SD, range)	22.7 (8.2, 0-30)	28.4 (1.3, 26-30)	26.9 (1.8, 25-30)	12.2 (6.3, 0-18)

Figure 2: Representation of layers analyzed in the adult human hippocampus.

Figure 2.

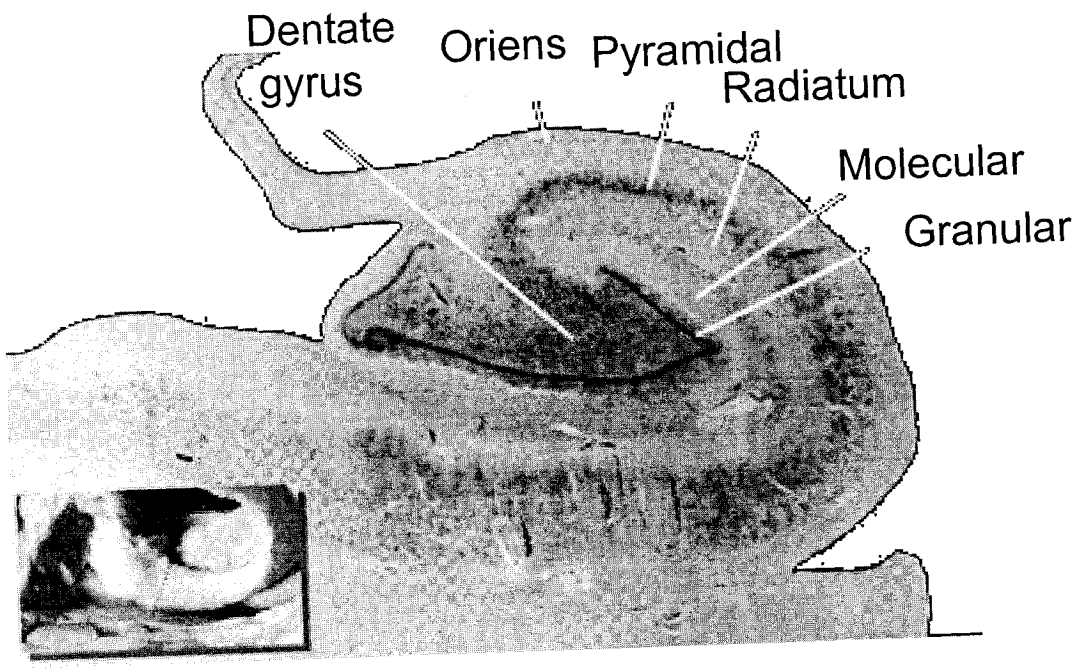

Dentate gyrus
Oriens
Pyramidal
Radiatum
Molecular
Granular

Figure 3: EpoR/GFAP co-localization in human hippocampus.

A. Control for anti-EpoR (red) antibody. The astrocytes fluoresce green following staining with anti-GFAP antibody and FITC. In Fig. 1, A-D, bars = 25 μm.

B. NEC subject. GFAP-positive astrocytes are depicted (green). EpoR staining (red) does not co-localize with the astroglia.

C. MCI patient. Yellow fluorescence (arrow) indicates extensive co-localization of EpoR (red) to astrocytes (green). Arrowhead denotes EpoR-positive blood vessel.

D. AD patient. There is co-localization of EpoR (red) to astroglia (green) yielding yellow fluorescence (arrow).

Figure 3.

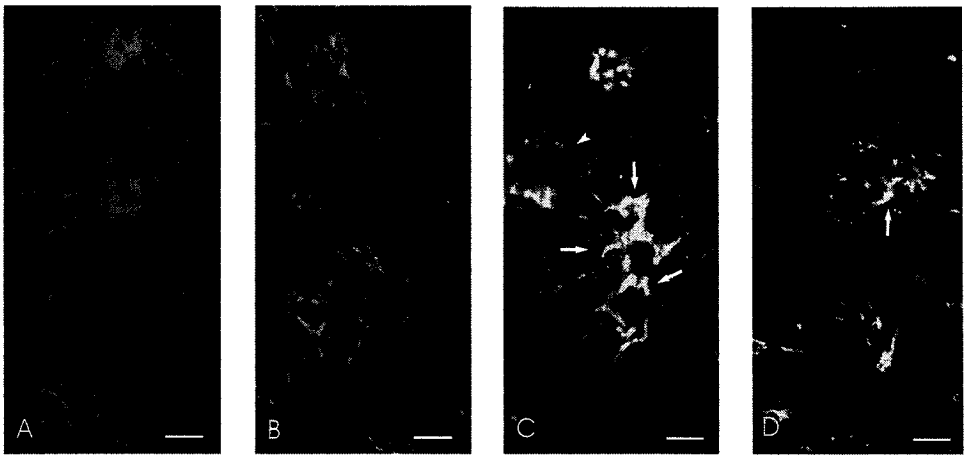
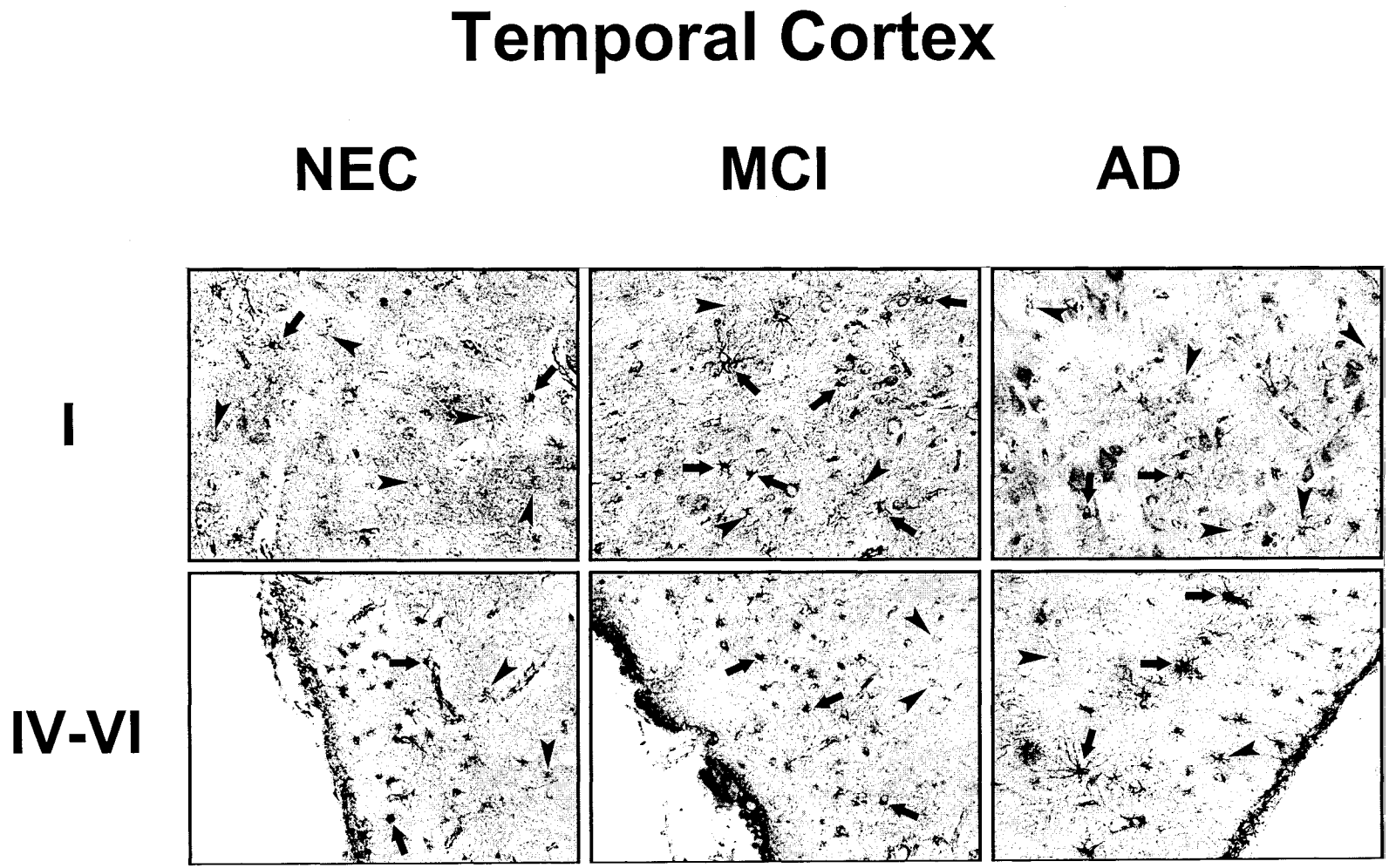


Figure 4: Dual-label EpoR/GFAP immunohistochemical localization in human temporal cortex and hippocampus.

Arrows denote EpoR positive astrocytes, whereas arrowheads indicate EpoR negative astrocytes. Full figures are shown at 400x magnification. Insets represent 1000x magnification.

Figure 4.



Hippocampus

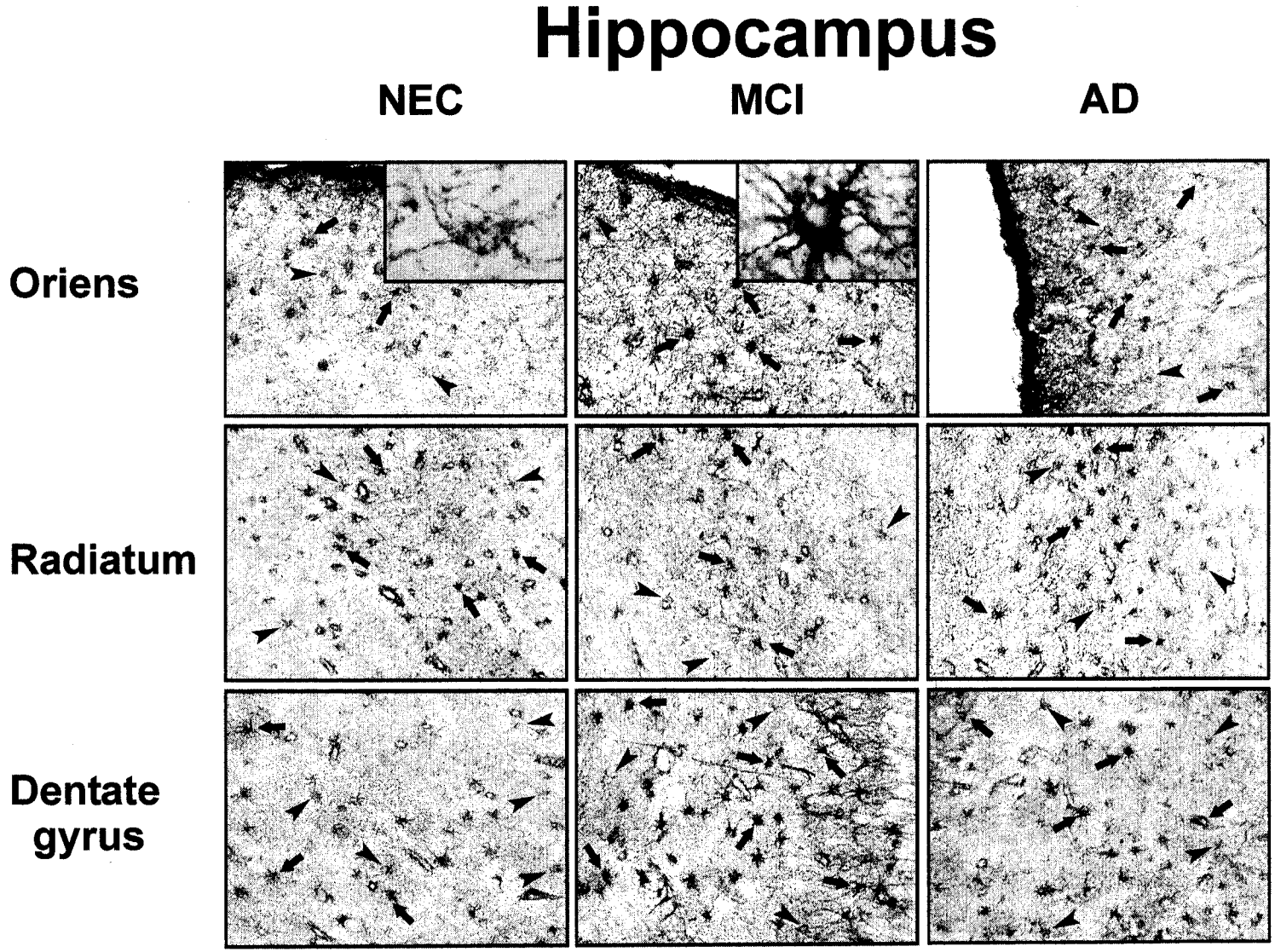


Figure 5: EpoR expression in human cortical (A) and hippocampal (B) astrocytes.

Differential expression of EpoR within the layers of the human temporal cortex and hippocampus in NEC, MCI and AD subjects. In all figures, * denotes a significance of $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, relative to the NEC cases. ( ) = # of cases per group.

Figure 5.

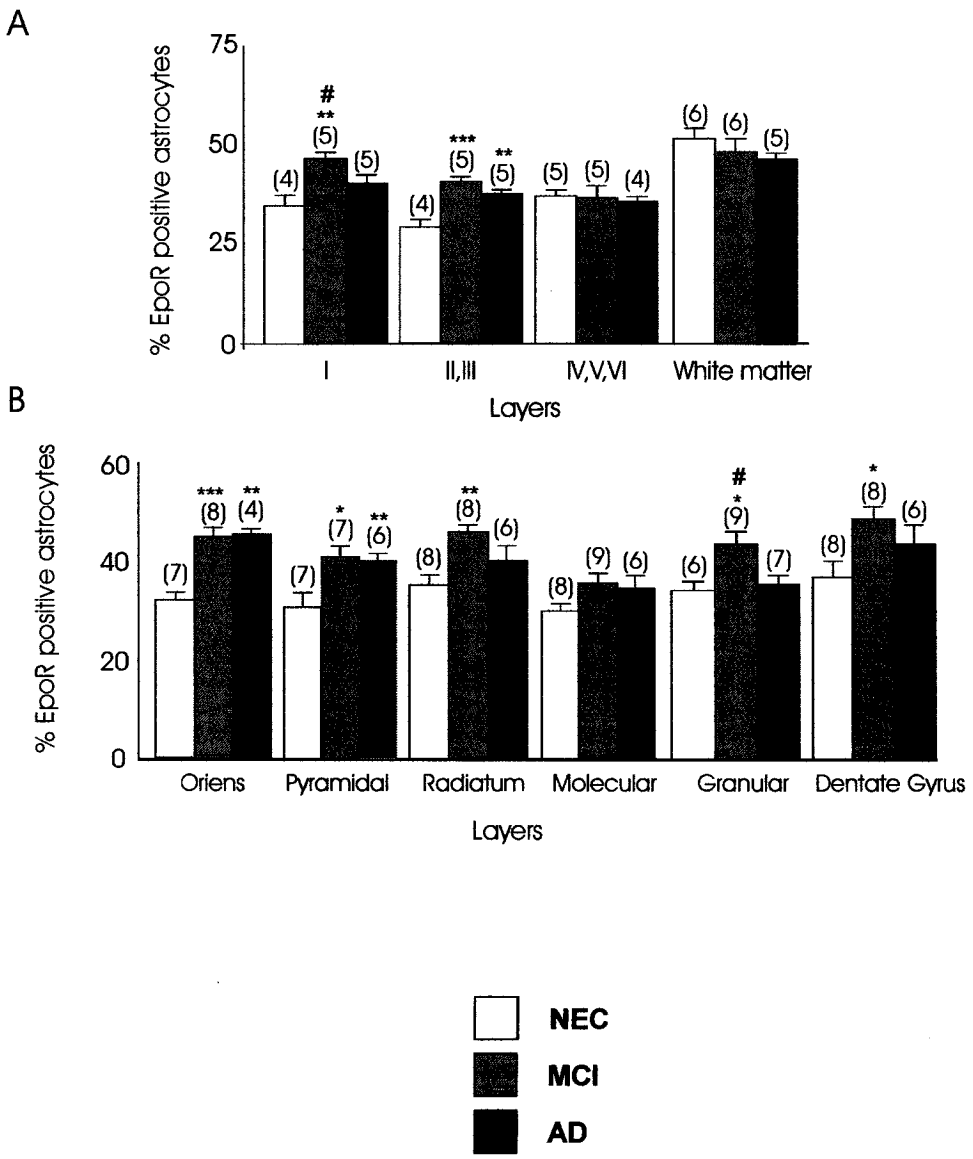

A
% EpoR positive astrocytes
75
50
25
0
I
II,III
IV,V,VI
White matter
Layers
B
% EpoR positive astrocytes
60
40
20
0
Oriens
Pyramidal
Radiatum
Molecular
Granular
Dentate Gyrus
Layers
NEC
MCI
AD

Figure 6: Effects of Epo pre-conditioning on astroglial cell death induced by NO, STS and As2O3.

A. Light micrographs of astroglial monolayers under various treatment conditions (40x magnification).

B. Flow cytometric analysis of PI-positive cells. Astrocytes were pre-treated with Epo for 16 h, followed by exposure to STS (0.5 μM), NO (administered as 300 μM SNP or DETA/NO 1 mM) or As_2O_3 (1 μM) for 48 h.

C. Percentages of sub-G_0 (dead) cells treated as described in B. Graphs depict means of three independent samples. Vertical lines denote standard deviations. Asterisks indicate significant differences ($p<0.001$) from NO or STS-treated cells. Pound signs indicate significant differences ($p<0.01$) from STS + Epo 5 U/ml-treated cells.

Figure 6 A.

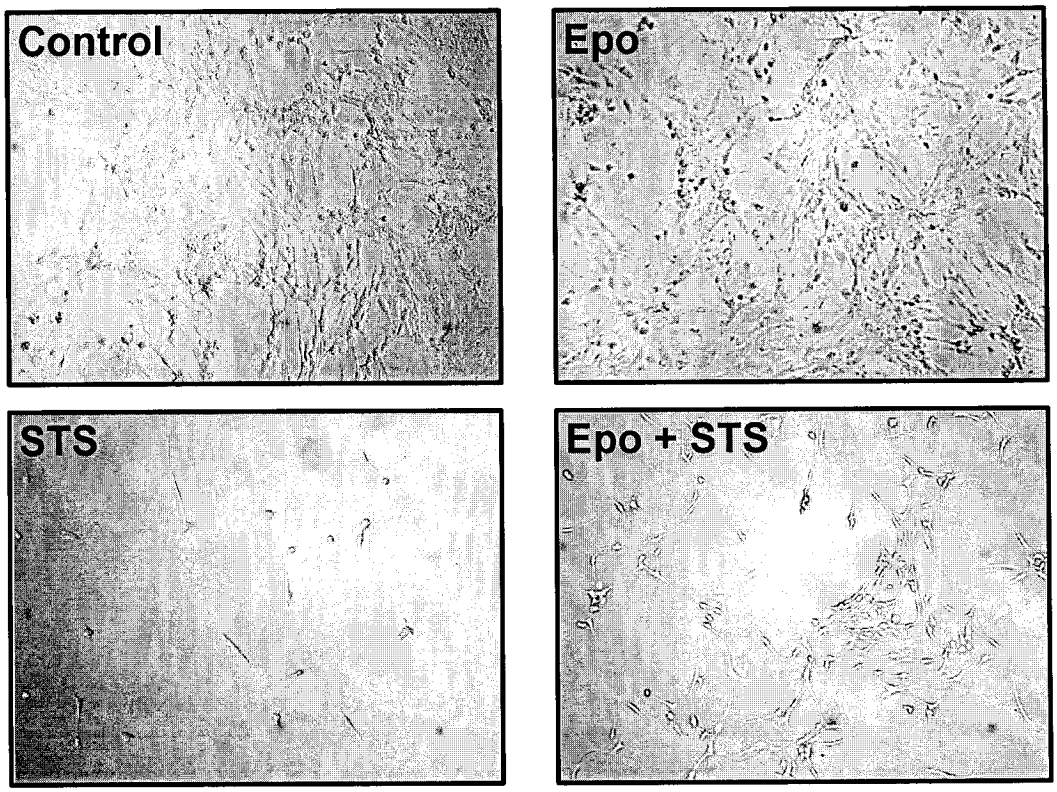


Figure 6 B.

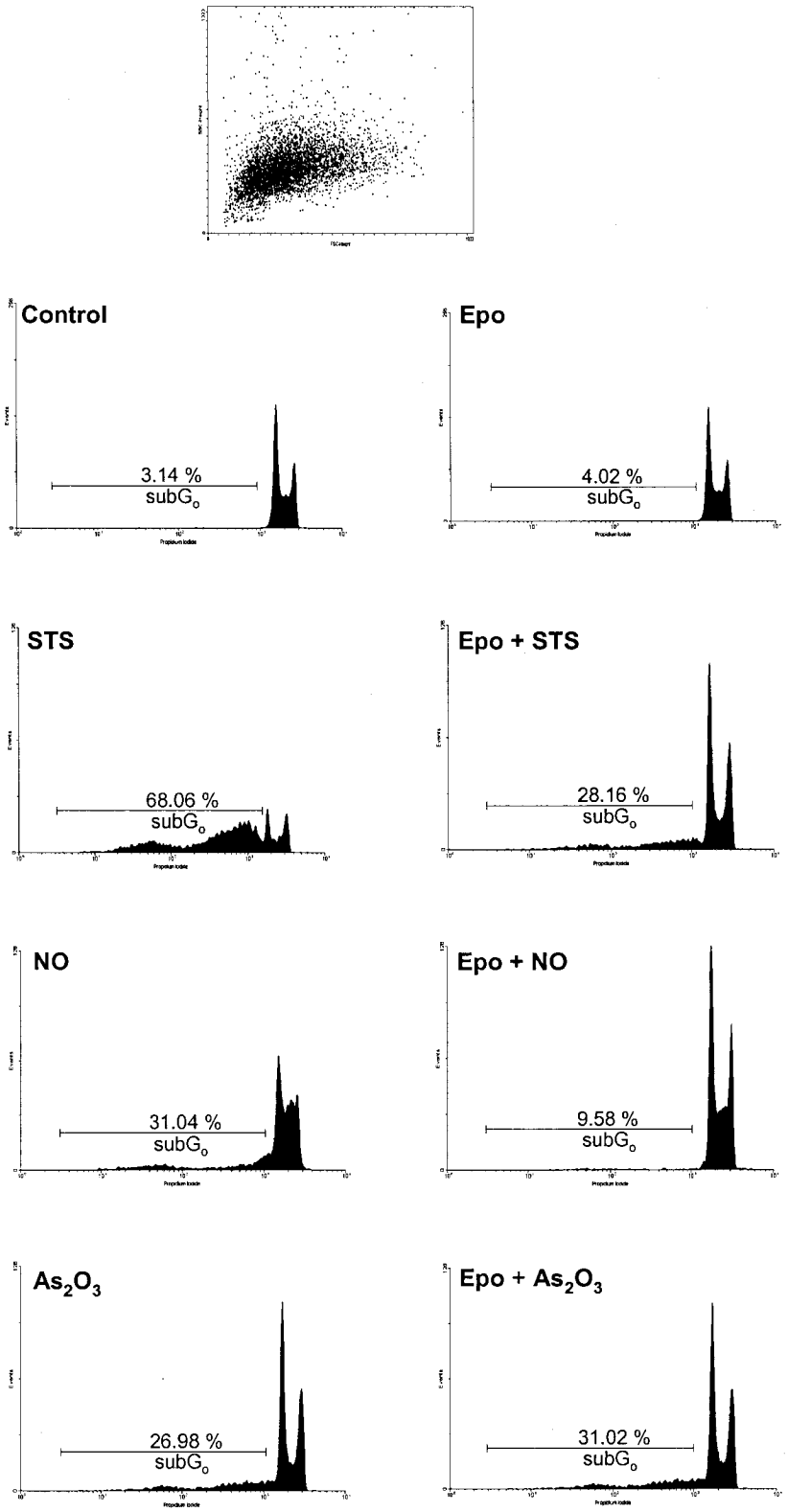
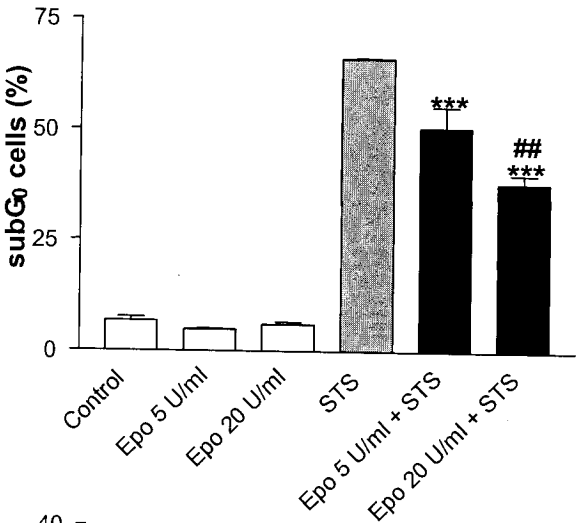
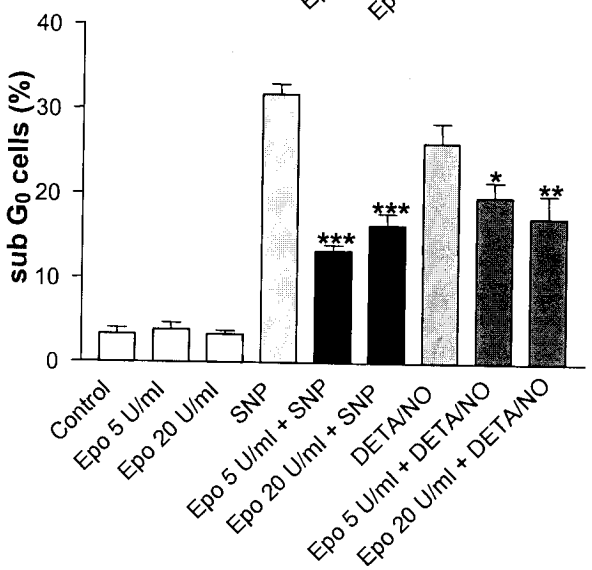

Control
3.14 %
subG0
Epo
4.02 %
subG0
STS
68.06 %
subG0
Epo + STS
28.16 %
subG0
NO
31.04 %
subG0
Epo + NO
9.58 %
subG0
As2O3
26.98 %
subG0
Epo + As2O3
31.02 %
subG0

Figure 6 C.

i.



ii.



iii.

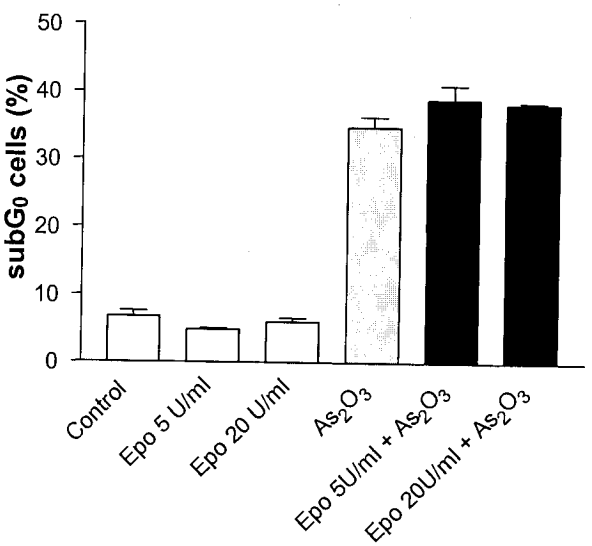


Figure 7: Effects of Epo pre-conditioning on NO and STS-mediated astroglial apoptosis.

A. Astrocytes were seeded onto sterile coverslips, pre-treated with Epo 20 U/ml for 16 h followed by exposure to STS (0.5 μM), NO (administered as 300 μM SNP) or As_2O_3 (1 μM) for 48 h. Micrographs (60x magnification) are representative of two independent experiments, each performed in triplicate.

B. Quantification of DAPI-positive apoptotic cells in a minimum of 100 cells per treatment. Each bar represents an average of two independent samples. Vertical lines denote standard deviations. Asterisks indicate significant differences from NO ($p<0.001$) or STS-treated cells ($p<0.01$).

Figure 7

A.

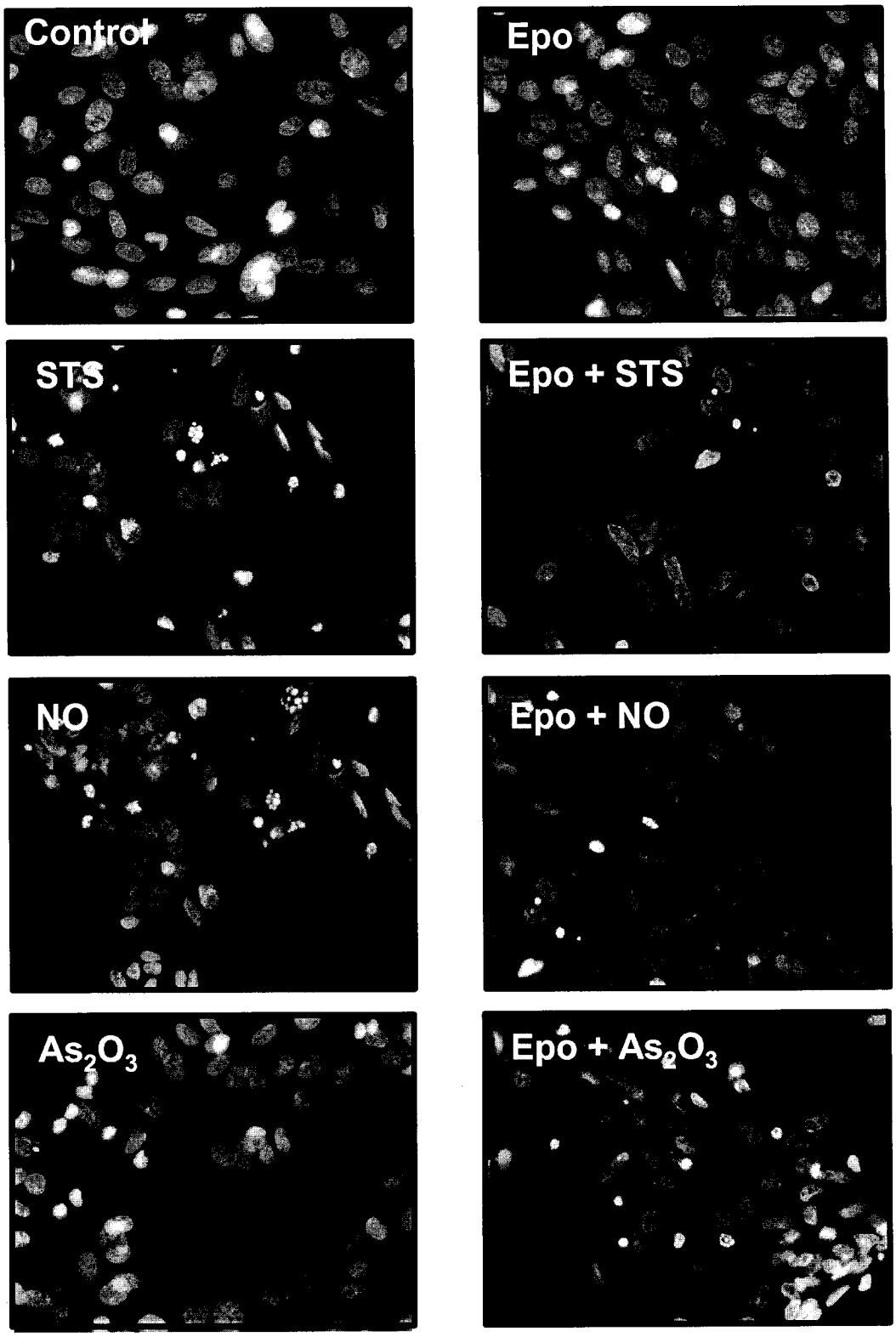


Figure 7

B.

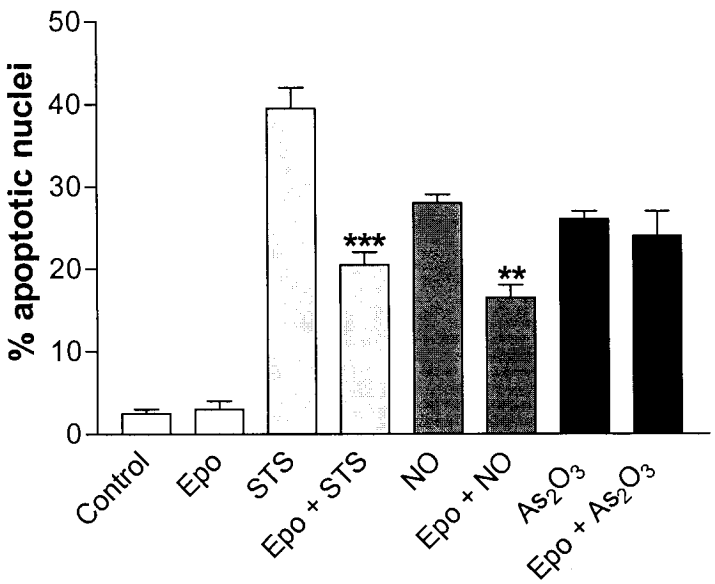

% apoptotic nuclei
50
40
30
20
10
0
***
**
Control
Epo
STS
Epo + STS
NO
Epo + NO
As2O3
Epo + As2O3

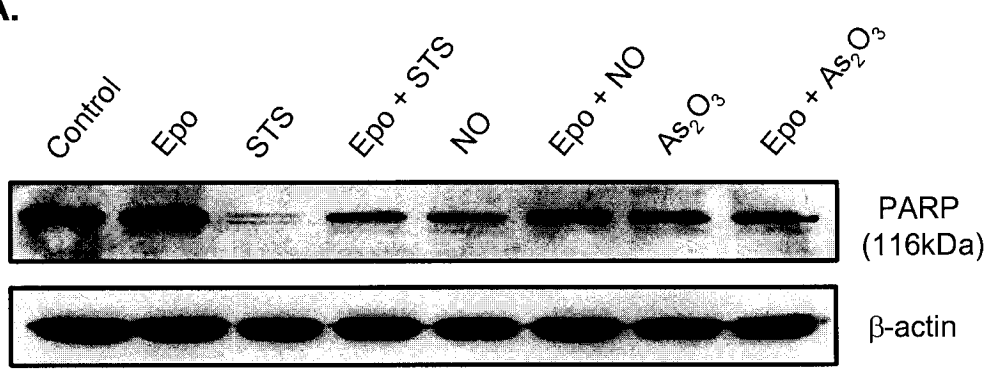
Figure 8: Effects of Epo pre-conditioning on PARP cleavage and DNA fragmentation in cultured astroglia.

A. Western blotting for PARP cleavage. β-actin staining was used as a loading control. Results are representative of three independent experiments, each performed in duplicate.

B. DNA fragmentation assay. Oligonucleosomes were isolated as described in Materials and Methods. The figure is representative of results obtained in three independent experiments.

Figure 8.

A.


Control
Epo
STS
Epo + STS
NO
Epo + NO
As_2O_3
Epo + As_2O_3
PARP
(116kDa)
β-actin

B.

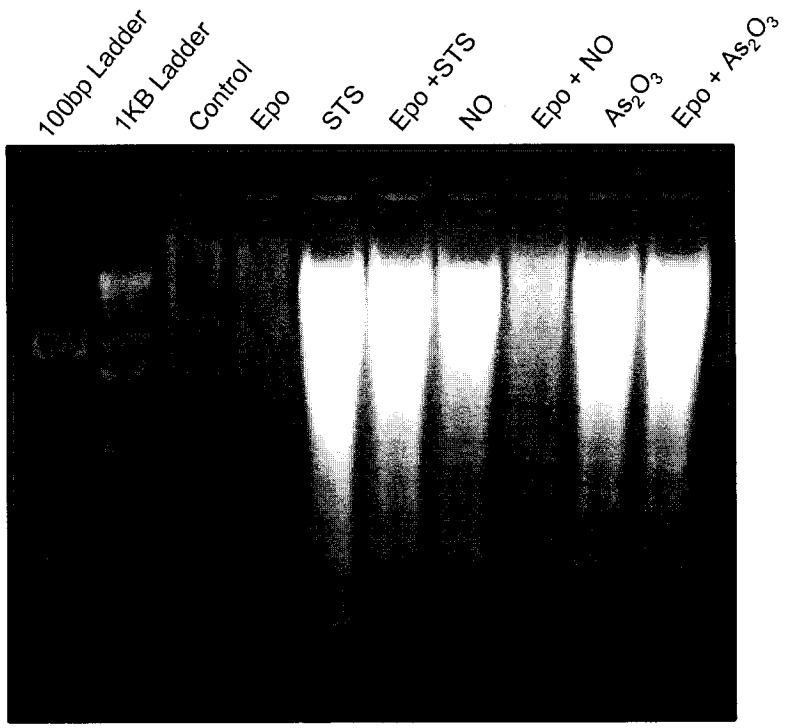

100bp Ladder
1KB Ladder
Control
Epo
STS
Epo +STS
NO
Epo + NO
As_2O_3
Epo + As_2O_3

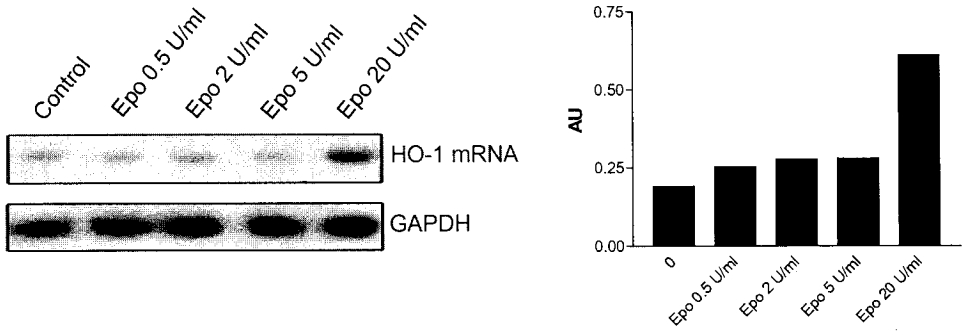
Figure 9: Effects of Epo on HO-1 mRNA and protein levels in cultured astroglia.

A. Northern blot for HO-1 mRNA levels. GAPDH mRNA served as loading control. Data is representative of three independent experiments. Graph depict analyse of HO-1 / β-actin band ratio in arbitrary units (AU).

B. Western blot for HO-1 protein levels. β-actin staining was used as loading control. Results are representative of three independent experiments. Graph depict analyse of HO-1 / β-actin band ratio in arbitrary units (AU).

Figure 9.

A.


Control
Epo 0.5 U/ml
Epo 2 U/ml
Epo 5 U/ml
Epo 20 U/ml
HO-1 mRNA
GAPDH
AU
0.75
0.50
0.25
0.00
0
Epo 0.5 U/ml
Epo 2 U/ml
Epo 5 U/ml
Epo 20 U/ml

B.

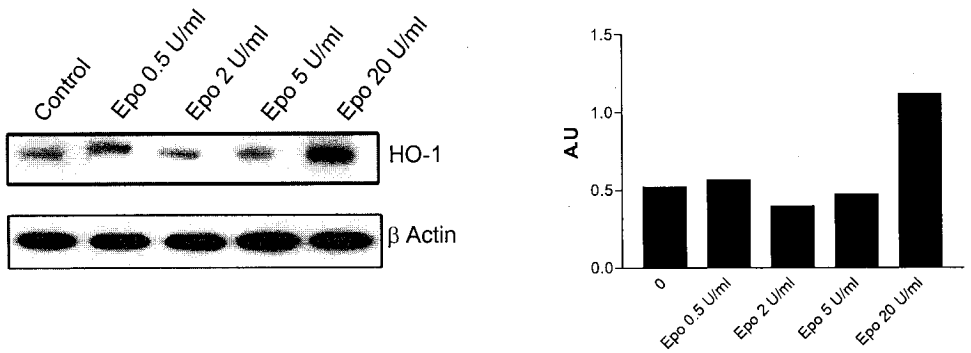

Control
Epo 0.5 U/ml
Epo 2 U/ml
Epo 5 U/ml
Epo 20 U/ml
HO-1
β Actin
A.U
1.5
1.0
0.5
0.0
0
Epo 0.5 U/ml
Epo 2 U/ml
Epo 5 U/ml
Epo 20 U/ml

Figure 10: Role of HO-1 and reactive oxygen species in Epo-mediated glioprotection against STS toxicity.

A. Rat astrocytes were left untreated (Control) or were pre-treated with Epo 20 U/ml, or SnMP 1 mM, and then treated with STS (0.5 μM) for 72 h as indicated. Cell viability was evaluated on day 3 by trypan blue exclusion. Values represent the means ± SD of three independent experiments, each performed in triplicate. Asterisks denote significant differences ($p<0.001$) from Epo + STS-treated cells.

B,C. Effects of antioxidants on Epo-mediated glioprotection. Rat astrocytes were pre-treated with Epo 20 U/ml and AA (200 μM) or melatonin (100 μM), followed by exposure to STS for 48 h. PI staining and flow cytometric analysis were performed as described in Materials and Methods. Values represent the means ± SD of two independent experiments, each performed in triplicate. Asterisks indicate significant differences from STS-treated cells (**$p<0.01$, ***$p<0.001$). Pound signs denote significant differences ($p<0.01$) from Epo + STS -treated cells.

D. Western blot for HO-1 protein levels. β-actin staining was used as a loading control. Results are representative of three independent experiments.

Figure 10.

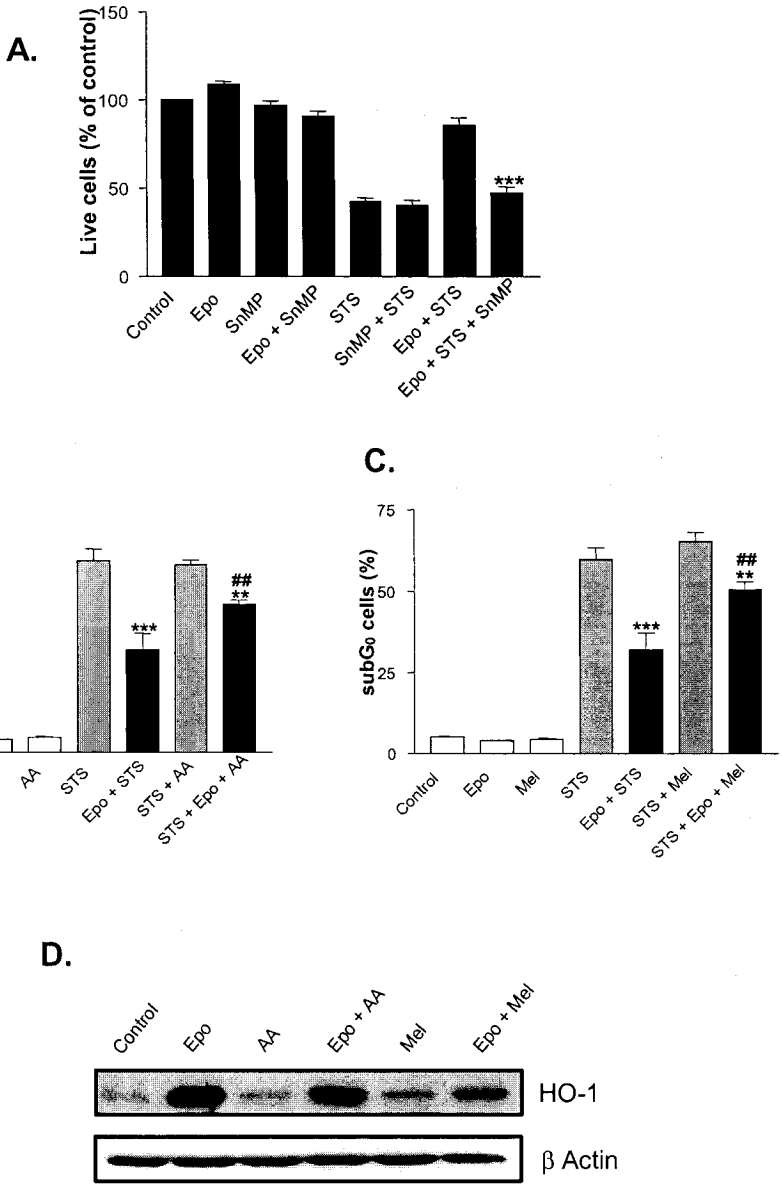
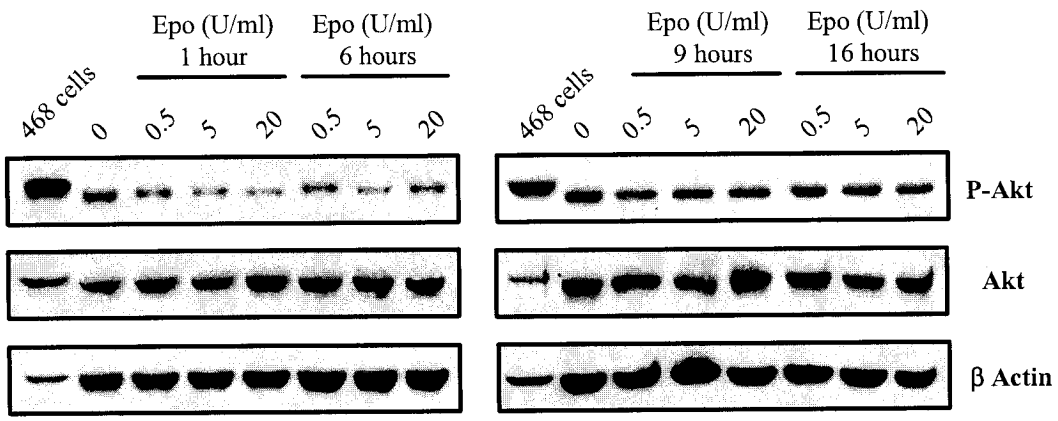

A.
Live cells (% of control)
150
100
50
0
***
Control
Epo
SnMP
Epo + SnMP
STS
SnMP + STS
Epo + STS
Epo + STS + SnMP
B.
subG0 cells (%)
75
50
25
0
***
##
**
Control
Epo
AA
STS
Epo + STS
STS + AA
STS + Epo + AA
C.
subG0 cells (%)
75
50
25
0
***
##
**
Control
Epo
Mel
STS
Epo + STS
STS + Mel
STS + Epo + Mel
D.
Control
Epo
AA
Epo + AA
Mel
Epo + Mel
HO-1
β Actin

Figure 11: Effects of Epo on Akt activation.

Rat astrocytes were left untreated (Control) or were treated with Epo as indicated. Western Blotting was performed for total Akt or activated Akt (phospho-Akt). Whole cell extracts from MDA-MB-468 breast cancer cells were used as positive controls. β-actin staining was used as a loading control. Results are representative of three independent experiments.

gure 11.


Epo (U/ml)
1 hour
Epo (U/ml)
6 hours
Epo (U/ml)
9 hours
Epo (U/ml)
16 hours
468 cells
0
0.5
5
20
0.5
5
20
468 cells
0
0.5
5
20
0.5
5
20
P-Akt
Akt
β Actin