Glutathione Dependent and Thioredoxin Dependent Peroxidase Systems in Neural Cells

A Thesis Submitted to the College of Graduate Studies and Research in
Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the
Department of Anatomy and Cell Biology
University of Saskatchewan
Saskatoon

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Abstract:

Amongst the different reactive oxygen species (ROS) that are produced during normal physiologic and pathologic conditions in the brain, peroxides are of special interest. Interaction of transition metal ions such as Fe\(^{2+}\) and Cu\(^{+}\) with peroxides produces free radicals such as OH\(^cdot\), which is highly reactive. Although ROS play important roles in gene regulation and signaling systems, their over-production or failure to be properly scavenged leads to oxidation of vital cellular systems and can cause cell injury. Two important thiol systems are responsible for peroxide scavenging the glutathione (GSH) and thioredoxin (Trx) dependent systems. Sulhydryl groups of GSH and Trx can buffer the ROS-mediated oxidative disturbances by direct interaction with oxidized proteins and also by scavenging peroxides through related enzymatic systems. Neurons are generally known to have lower resistance against oxidative stress than astrocytes. In these studies I hypothesized that neuronal susceptibility is mainly due to lower GSH as well as Trx dependent peroxidase systems. It seems possible that upregulation of these endogenous antioxidants is a potential way to inhibit/decrease ROS-mediated effects during oxidative stress.

I found that the ability of astrocytes to withstand oxidative stress better than neurons is related to: 1) A higher GSH content as well as higher glutathione reductase activity and glutathione peroxidase (GSH-Px) activity in astrocytes. 2) Higher Trx, Trx reductase (Trx-R) and Trx-dependent peroxidase (peroxiredoxin [Prx]) activities in astrocytes. Examination of the distribution of three isoforms of Prx showed that astrocytes have a higher content of Prx1 and Prx3 while neurons have a higher content of Prx2. GSH-Px but not Prx activity can be increased in astrocytes by nerve growth factor and epidermal growth factor. The phase 2 enzyme inducer tertiary-butylhydroquinone (tBHQ) increased both the GSH- and Trx-dependent peroxide scavenging systems in astrocytes, but not in neurons. Glutathione reductase activity and GSH content of astrocytes, but not neurons, can be increased upon exposure to the phase 2 enzyme inducer tertiary-butylhydroquinone (tBHQ). I also demonstrated that protein content and activity was increased for both the cytosolic and mitochondrial isoforms of Trx-R. Induction of phase 2 enzymes was associated with a higher
cytosolic cysteine:cystine ratio, indicating that tBHQ increased the reducing capacity of astrocytes. Induction of phase 2 enzymes in astrocytes increased the ability to withstand tertiary-butylhydroperoxide-induced oxidative stress as determined by lower release of the cytosolic marker lactate dehydrogenase and better retention of mitochondrial membrane potentials. To determine whether the GSH- or the Trx-dependent played a larger role in this enhanced ability to cope with oxidative stress, I selectively inactivated critical components of each system and then examined the ability of astrocytes to cope with peroxide-induced stress. Depleting GSH with diethylmaleate markedly decreased the ability of astrocytes to withstand oxidative stress, whereas inactivating Trx-R with cis-diaminedichloro-platinum (CDDP) had no effect on the ability of astrocytes to cope with peroxides, indicating the importance of the GSH-dependent peroxide scavenging system in astrocytes. To delineate the role of Trx system to cope with thiol oxidation in astrocytes. Trx-R activity was inhibited with CDDP and then exposed to thimerosal, a thiol oxidant. Inhibition of Trx-R resulted in a more rapid drop of mitochondrial membrane potential than in vehicle-treated control astrocytes. The conclusion is that upregulation of phase 2 enzymes better enabled astrocytes to cope with a variety of oxidative insults.

The final question addressed in this thesis was whether astrocytes release factors that enable neurons to better cope with oxidative stress. Transient co-cultures of astrocytes with neurons were established and neuronal capacity to scavenge hydrogen peroxide was examined. A 24 hr period of co-culture of neurons with astrocytes greatly increased the hydrogen peroxide scavenging ability of astrocytes. A longer period of co-culture with astrocytes or a 24 hr co-culture of neurons with astrocytes whose phase 2 enzymes were induced enhanced further the ability of neurons to scavenge hydrogen peroxide. Co-culture of neurons with astrocytes increased neuronal GH content, partially accounting for the enhanced ability of neurons to scavenge hydrogen peroxide. This increase in neuronal GSH was correlated with release by astrocyte of the compounds cysteine and glutamyl-cysteine that promote neuronal GSH synthesis.
My results have shown that upregulation of the GSH and Trx-dependent systems in astrocytes not only better enables astrocytes to cope with oxidants but also better enables neurons to cope with oxidants.
ACKNOWLEDGEMENTS

This thesis is dedicated to the memory of my loving father.

And to my dearest: my mother, my wife Soheila, and to Amir, our beloved son.

I would like to appreciate deeply my supervisor Dr. Bernhard H. J. Juurlink for his patience, his kindness, and the vast ocean of knowledge, which was always available to me. I appreciate all of your efforts and time that you dedicated to my project. I will miss working with you very much.

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<tbody>
<tr>
<td>ADF</td>
<td>adult T-cell derived factor</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine dinucleotide phosphate</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>ARE/EpRE</td>
<td>antioxidant response element/electrophile responsive element</td>
</tr>
<tr>
<td>ASK-1</td>
<td>apoptosis signal kinase-1</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BSO</td>
<td>butathione sulfoxide</td>
</tr>
<tr>
<td>CDDP</td>
<td>cis-diaminedichloroplatinum (II)</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphat</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding</td>
</tr>
<tr>
<td>Cu/ZnSOD</td>
<td>copper-zinc superoxide dismutase</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>CysGly</td>
<td>cysteinyl glycine</td>
</tr>
<tr>
<td>CysS-O2H</td>
<td>sulfinic acid</td>
</tr>
<tr>
<td>CysS-OH</td>
<td>cysteine sulfenic acid</td>
</tr>
<tr>
<td>CysSO3H</td>
<td>sulfonic acid</td>
</tr>
<tr>
<td>DCFH</td>
<td>5−- (and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein</td>
</tr>
<tr>
<td>DCNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>DEM</td>
<td>diethylmaleate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleotide acid</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5′-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>EGF</td>
<td>epithelial growth factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FADH2</td>
<td>flavin adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GCS</td>
<td>γ-glutamylcysteine synthase</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>glutathione Peroxidase</td>
</tr>
<tr>
<td>GSSG</td>
<td>oxidized glutathione</td>
</tr>
<tr>
<td>GSSG-R</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HEPES</td>
<td>(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acis]</td>
</tr>
<tr>
<td>HNE</td>
<td>hydroxynonenal</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate Dehydrogenase</td>
</tr>
<tr>
<td>LOOH</td>
<td>lipid peroxide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotine amide dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NFkB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NKEF</td>
<td>natural killer cell-enhancing factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO°</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Nrt2</td>
<td>nuclear factor erythroid 2-related protein</td>
</tr>
<tr>
<td>OCI-</td>
<td>hypochlorite</td>
</tr>
<tr>
<td>OH°</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>ONOO°</td>
<td>peroxynitrite</td>
</tr>
</tbody>
</table>
O²⁻  superoxide anion
PC12 Cells  pheochromocytoma cells
PDGF  plattlet derived growth factor
PepT2  peptide transporter 2
PHGPx  phospholipid hydroperoxide glutathione peroxidase
Prx  peroxiredoxin
PTK  protein tyrosine kinase
QR  quinone reductase
RNS  reactive nitrogen species
ROOH  organic peroxide
ROS  reactive oxygen species
SAPK  stress activated protein kinase
SECIS  selenocysteine insertion sequence
SELB  selenocysteinyl-tRNA specific elongation binding protein
SOD  superoxide dismutase
tBHQ  tertiary butylhydroquinone
tBOOH  tertiary butylhydroperoxidase
TNF  tumor necrosis factor
TPA  tetradenoic phorbol acetate
TPx  thioredoxin peroxidase
tRNAsec  selenocysteine specific tRNA
Trx(S)2  oxidized thioredoxin
Trx(SH)2  reduced thioredoxin
Trx-R  thioredoxin reductase
TSA  thiol specific antioxidant
Introduction:

Since the introduction of oxygen in the atmosphere living organisms have been exposed to toxic effects of oxygen. Gerschman and her colleagues (Gerschman et al., 1954) were the first to suggest the theory of free radical production by oxygen. Free radicals can be any molecular species with capability of independent existence that have one or more unpaired electrons in their outermost orbitals (McCord, 2000). Oxygen and nitrogen free radicals are of great importance in biological systems. Oxygen free radicals and its non-radical derivatives are referred to as reactive oxygen species (ROS), while nitrogen-based radicals are referred to as reactive nitrogen species (RNS). All these reactive chemical species are known to play central roles in a wide variety of diseases, including neurodegenerative diseases, ischemia, stroke, cancer (Sen, 1998). Oxygen free radicals are generated during normal physiologic pathways and play important roles in normal cell activities such as cell division, gene regulation and cellular defense against bacteria or other antigens (Maher and Schubert, 2000). Examples of free radicals are superoxide anion ($O_2^{-}$), hydroxyl radical ($OH^\cdot$) and nitric oxide ($NO^\cdot$). Hydrogen peroxide, which is a natural byproduct of superoxide anion dismutation by superoxide dismutase (SOD), can also lead to free radical generation upon interaction with transitional metal ions (reviewed in Juurlink, 1999). Once free radicals are formed, they can initiate chain reactions that lead to production of more free radicals (Hawkins and Davies, 2001). These can reduce (donate electrons) or oxidize (extract electrons) all neighboring molecules including protein phosphatases, protein kinases and transcription factors, therefore affect all
aspects of cellular machinery including intracellular or intercellular signaling pathways which may be protective or destructive for the cells (Castro and Freeman, 2001). To protect themselves against free radicals, or to control their physiologic functions, cells have developed a variety of antioxidant molecules to control free radical reactions. The antioxidant systems consist of several nonenzymatic and enzymatic systems, such as vitamins E and C, glutathione (GSH), superoxide dismutase (SOD), catalase (Young and Woodside, 2001). These molecules must control free radical concentration so that the reduction/oxidation (redox) balance under normal conditions remains constant. When this balanced is disrupted, oxidative stress occurs (Juurink and Paterson, 1998). Amongst different ROS, superoxide and hydroxyl radical play central roles in generation of reactive molecules and are known to be involved in many diseases.

1.1. Mechanisms producing reactive chemical species:

Free radicals are unstable reactive compounds that undergo a variety of reactions including hydrogen abstraction, electron transfer, addition, rearrangement with their substrates. They are formed through a series of self-propagating reactions. Figure 1.1 illustrates the potential role of $\text{O}_2^\cdot$ and $\text{OH}^\cdot$ in free radical production and their potential interactions to generate more reactive compounds. Here the basic mechanisms of free radical production in living organisms are reviewed.

1.1.1. Superoxide anion: Oxidative phosphorylation in mitochondria is the main source of $\text{O}_2^\cdot$ production, during which electrons flow from NADH or FADH2
Figure 1.1. Interaction of free radicals, potential biological and pathological effects, and role of antioxidants.
to oxygen is a step-by-step fashion through protein complexes located in the inner membrane of the mitochondria. Oxygen is reduced during the electron transport chain and the released free energy is used to generate ATP for cellular metabolic activities. The electron carriers in the respiratory assembly are organized in four complexes: NADH-Q reductase, succinate-Q reductase, cytochrome reductase, and cytochrome oxidase (Stryer. 1995). The organization of these complexes minimizes the release of partially reduced intermediates of oxygen, but ~3% of consumed oxygen is converted to superoxide anion O$_2^-$ (Fridovich. 1986). Although it is still controversial, complex 1 is believed to be the main site of superoxide generation (Turrens and Boveris. 1980). Complex 2, and also complex 3 with a less substantial contribution than complex 2, are other superoxide formation systems in the mitochondrial inner membrane (reviewed in Maher and Schubert. 2000). O$_2^-$ is not a powerful oxidant in aqueous solutions (Halliwell. 1999), but it can convert to more damaging species such as hydroxyl radical, peroxynitrite and H$_2$O$_2$ (Fig. 1.1). O$_2^-$ is also generated during arachidonic acid metabolism by lipoxygenase and cyclooxygenase in the presence of NADH or NADPH, which results in production of leukotrienes and prostaglandins (Deby and Goutier. 1990; Kukreja et al.. 1986). Also auto-oxidation of cysteine (Saez et al.. 1991), oxidation of catecholamines (Kalyanaraman. 1989), and xanthine oxidase activity (Sussman and Bulkley. 1990) are other sources of O$_2^-$ production. The rate of superoxide production is increased with aging (Sawada and Carlson. 1987).
Superoxide radical is scavenged by superoxide dismutase (SOD). During superoxide dismutase activity hydrogen peroxide and molecular oxygen are produced:

\[ 2 \text{O}_2^\cdot + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \frac{1}{2}\text{O}_2 \]

1.1.2. **Hydrogen Peroxide:** The inner mitochondrial membrane is the main site of superoxide production; superoxide is converted to \(\text{H}_2\text{O}_2\). Monoamine oxidase located in the outer mitochondrial membrane is another source of \(\text{H}_2\text{O}_2\) through oxidative deamination of biogenic amines via a direct two-electron transfer to molecular oxygen (Hauptmann et al., 1996). \(\text{H}_2\text{O}_2\) is also a natural byproduct of peroxisomal oxidoreductase enzymes such as D-amino acid oxidase and acetylCoA oxidase (van den Bosch et al., 1992). Although \(\text{H}_2\text{O}_2\) is a weak oxidant, in biological systems it is very damaging, because it can be converted to the hydroxyl radical. The hydroxyl radical can extract two electrons from all biomolecules, making it a cytotoxic oxidant. In the presence of iron or copper, \(\text{H}_2\text{O}_2\) generates the potent hydroxyl radical through the Fenton reaction (reviewed by Hawkins and Davies, 2001; Juurlink, 1999):

\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^\cdot + \text{Fe}^{3+} + \text{OH}^- \]

\(\text{Fe}^{3+}\) can be reduced by \(\text{O}_2^\cdot\), and the resulted \(\text{Fe}^{2+}\) continues hydroxyl radical formation from \(\text{H}_2\text{O}_2\):

\[ \text{O}_2^\cdot + \text{Fe}^{3+} \rightarrow \text{O}_2 + \text{Fe}^{2+} \]

\[ \text{Fe}^{2+}\text{H}_2\text{O}_2 \rightarrow \text{OH}^\cdot + \text{OH}^- + \text{Fe}^{3+} \]

The last two reactions are known as Harber-Weiss reaction.
1. 1. 3. Hydroxyl radical: OH\(^{\circ}\) is highly electrophilic and can extract electrons from proteins (reviewed by Hawkins and Davies. 2001) as well as polyunsaturated fatty acids (Juurlink and Sweeney. 1997) in membranous systems of the cell. that are easily accessible to this free radical. Hydrogen extraction results in production of carbon centered free radicals (R\(^{\circ}\)).

\[ RH + OH^{\circ} \rightarrow R^{\circ} + HOH \]

The carbon centered radical can start a set of chain reactions that result in production of peroxides (ROOH) as well as peroxyl (ROO\(^{\circ}\)) and alkoxy (RO\(^{\circ}\)) radicals.

\[ R^{\circ} + O_2 \rightarrow ROO^{\circ} \]
\[ ROO^{\circ} + RH \rightarrow ROOH + R^{\circ} \]
\[ ROOH + Fe^{3+} \rightarrow Fe^{2+} + ROO^{\circ} \]
\[ ROOH + Fe^{2+} \rightarrow Fe^{3+} + RO^{\circ} + OH^{-} \]

Although many amino acid residues are susceptible to oxidative modification, the sulphydryl groups of cysteine are especially accessible to hydroxyl radical attacks (Hawkins and Davies. 2001). Oxidation of proteins can lead to their inactivation. Lipid oxidation results in loss of membrane functional integrity and edema after brain injury (Hall, 1993). Nucleic acids are also attacked by ROS, which can cause DNA mutation, single-strand breaks and chromosomal shortening (Oexle and Zwisler, 1997). OH\(^{\circ}\) is also produced by ionizing radiation of water which generates several reactive species such as H\(^{\circ}\), H\(_3\)O\(^{+}\), H\(_2\), H\(_2\)O\(_2\) (Maher and Schubert. 2000).
1.1.4. **Singlet Oxygen**: \( ^1\text{O}_2 \), a powerful oxidant, can be released by hydrogen peroxide upon interaction with hypochlorite in stimulated neutrophiles (\( \text{OCl}^- \)) (Steinbeck et al., 1992). nitric oxide (\( \text{NO}^\circ \)) (Noronha-Dutra et al., 1993), and peroxynitrite (\( \text{ONOO}^- \))(Di Mascio et al., 1994).

\[
\text{HOOH} + \text{OCl}^- \rightarrow \text{H}_2\text{O} + \text{Cl}^- + ^1\text{O}_2 \\
\text{HOOH} + \text{ONOO}^- \rightarrow \text{NO}_2 + \text{HOH} + ^1\text{O}_2 \\
\text{HOOH} + \text{O}_2^\circ \rightarrow \text{OH}^\circ + \text{OH}^- + ^1\text{O}_2 
\]

1.1.5. **Hypochlorite**: Hydrogen peroxide is produced during the respiratory burst in neutrophils and macrophages and is a cytotoxic agent against bacteria or other organisms. In these leukocytes, the \( \text{H}_2\text{O}_2 \) is scavenged by chloride ion and hypochlorite is produced. This reaction is catalyzed by myeloperoxidase (Weiss, 1989).

\[
\text{Cl}^- + \text{H}_2\text{O}_2 \rightarrow \text{OCl}^- + \text{H}_2\text{O}
\]

* Hypochlorous acid is a very powerful oxidant.

1.1.6. **Reactive Nitrogen Species**: RNS also play important roles in cell physiology and pathology. \( \text{NO}^\circ \) is produced by nitric oxide synthase (NOS) from arginine and citrulline is formed (Dawson and Snyder, 1994). Three isoforms of NOS have been identified: 1) inducible NOS (iNOS). 2) endothelial NOS (eNOS) and 3) neuronal NOS (nNOS). While iNOS activity is increased through cytokine induction of second messengers (Bolanos et al., 1997b). eNOS and nNOS activity is \( \text{Ca}^{2+} \)/calmodulin dependent (Forstermann et al., 1994). \( \text{NO}^\circ \) is an uncharged
molecule that can diffuse freely across membranes and may work as a neurotransmitter. \( \text{NO}^\circ \) is different from the other conventional neurotransmitters: it has no storage mechanism, lacks excitocytic release process and also is inactivated spontaneously to nitrite with a short half life (~30 s) (Zigmond, 1999). In some hippocampal neurons, nitric oxide release has been linked to NMDA activation and production of long-term potentiation (Schuman and Madison, 1994). \( \text{NO}^\circ \) targets cGMP synthase in neurons as well as in glial cells (Bredt and Snyder, 1989) which affects neurotransmitter release from presynaptic neurons. It has been reported that \( \text{NO}^\circ \) from endothelial cells increases cGMP activation in vascular smooth muscles which results in vasodilation (Furchgott and Zawadzki, 1980). \( \text{NO}^\circ \) has been reported to be involved in neurite outgrowth induction in hippocampal neurons as well as cells in a cGMP-dependent manner (Hindley et al., 1997). Cytotoxic effects of \( \text{NO}^\circ \) might also be due to its potential to interact with superoxide and production of peroxynitrite (\( \text{ONOO}^- \)) radical:

\[
\text{NO}^\circ + \text{O}_2^\circ^- \rightarrow \text{ONOO}^-
\]

It has been shown that the above reaction can occur in mitochondria (Packer et al., 1996), which are the major source of \( \text{O}_2^\circ^- \). Peroxynitrus acid, the protonated form of peroxynitrite can produce hydroxyl radical and nitrogen dioxide radical, which are highly reactive (Beckman et al., 1990; Crow et al., 1994; Denicola et al., 1995):

\[
\text{ONOO}^- + \text{H}^+ \rightarrow \text{ONOOH}
\]
\[
\text{ONOOH} \rightarrow \text{OH}^\circ^- + \text{NO}_2^\circ
\]
1. 1. 7. **Other sources of free radical formation:** Although free radicals are generated during the above-mentioned endogenous mechanisms, exogenous environmental factors can also cause free radical formation. Ultra violet radiation may lead to production of singlet oxygen and other ROS in the skin (reviewed by McCaughan, 1999). Also ozone and NO\textsubscript{2\circ} may cause ROS formation and antioxidant depletion in the lung lining (Kelly et al., 1995; Kelly and Tetley, 1997). Many antineoplastic drugs eliminate tumor cells by inducing ROS formation and cell death (Thompson, 1995).

1. 2. **Effects of Reactive Oxygen and Nitrogen Species on Biological Systems:** Production of ROS/RNS is an inevitable process in biological systems. Although the harmful effects of free radicals was first suggested by Gerschman et al. (1954), and then led to the Denham Harman’s free radical theory of aging (Harman, 1956), during the last two decades our knowledge of vital roles of oxygen/nitrogen free radicals in maintaining tissue homeostasis has expanded rapidly. It has been shown that physiologically low levels of ROS regulate many critical molecular mechanisms, e.g., cell signaling, gene regulation, neurotransmitter actions, cell proliferation and death (reviewed by Castro and Freeman, 2001; Davis et al., 2001; Sen et al., 2001; Maher and Schubert, 2000; Ullrich and Bachschmid, 2000; Sen, 1998; Sen, 2001; Sen and Parker, 1996). It is difficult to differentiate the degree of the overlap between these physiological pathways and those involved in damaging mechanisms. However the basic mechanism of activation of any signaling pathways by ROS involves the modification of a protein or other key elements by ROS.
Reversible oxidation/reduction of these key proteins by direct effects of ROS or through glutathione and thioredoxin leads to alteration of the signaling pathways.

The list of redox-sensitive signal transduction pathways is steadily growing. Sulfhydryl groups in cysteinyl residues of these molecules is the principal oxidation site by free radicals (Hawkins and Davies. 2001). For example H₂O₂, which has been implicated in oxidative stress as well as cell signaling, oxidizes sulfhydryl groups to produce cysteine sulfenic acid (CysS-OH) or disulfide bonds, which can be reduced by cellular reductants. However, following prolonged oxidation or more severe oxidative conditions, the sulfenic acid can undergo further oxidation to either sulfenic acid (Cys-S-O₂H) or sulfonic acid (CysSO₃H), which are irreversible (Rhee. 1999). Although many proteins contain cysteine residues, since H₂O₂ is a mild oxidant, only proteins with the pKₐ value below 7.0 might be affected by H₂O₂ oxidation. For example, thioredoxin, peroxiredoxins, protein disulfide isomerase, cysteine proteases and tyrosine phosphatases that contain Cys-SH groups at their active sites and have low pKa are susceptible to oxidation by H₂O₂ and regulated by cellular redox status (Rhee. 1999). The pKₐ value is an indicator of how readily the protein can release electrons (Stryer. 1995), and most of the cysteine groups of proteins are higher than 8.0 (Rhee. 1999).

Production of free radicals occurs continuously in all cells. However excess free radical production originating from exogenous or endogenous sources might play some roles in many diseases. A short summary of our recent knowledge of their roles in health as well as diseases is reviewed in this section.
1.2.1. ROS are part of the cellular defensive system: Superoxide as well as H$_2$O$_2$ are produced during the respiratory oxidase burst process in macrophages and neutrophils (Chanock et al., 1994) as a defense mechanism against invading bacteria. NADPH oxidase is responsible for O$_2^{\cdot-}$ production in these cells (Babior et al., 1973). NADPH oxidase activity in microglia is a main source of ROS formation in the CNS (Maher and Schubert, 2000).

1.2.2. ROS can regulate cell division: O$_2^{\cdot-}$ can also regulate cell division, as it has been shown that during the wound healing process this radical stimulates cell division in fibroblasts (Maher and Schubert, 2000). Abnormal cell proliferation in some forms of cancer has been linked to increased ROS activity as low levels of manganese superoxide dismutase (MnSOD) activity in many types of human cancer cells have been reported (Oberley and Buettner, 1979). Transfection of these cell lines with MnSOD gene can reverse the malignant phenotype of the tumor cell by lowering superoxide levels in these cells, supporting a role for this free radical as a mitogenic factor (Li et al., 1995; St Clair et al., 1994) and an evidence for redox regulation of cell proliferation. Mutations in the proximal region of the GC-rich region of the MnSOD promoter in five human tumor cell lines change the binding pattern of AP-2 and lead to a reduction in MnSOD transcription (Xu et al., 1999).

1.2.3. ROS are important regulators of cell signaling and gene expression: ROS-mediated intracellular signaling through oxidation of protein tyrosine kinase (PTK) as well as serine/threonine kinases (S/TK) have been implicated in many systems. Both receptor and nonreceptor PTKs can be modulated by ROS.
(Adler et al., 1999b; Kamata and Hirata, 1999). For example, epidermal growth factor (EGF) and platelet derived growth factor (PDGF) receptors are activated by tyrosine phosphorylation that can be mediated by an indirect mechanism that inhibits their dephosphorylation rather than direct ROS-mediated activation of kinases (Knebel et al., 1996). PDGF receptors are also shown to be sensitive to redox status of the cell and glutathione levels are especially important for PDGF receptor regulation in fibroblasts (Rigacci et al., 1997).

H₂O₂ as well as other oxidants can activate nonreceptor PTKs, such as app60src (Devary et al., 1992) or p56lck (Hardwick and Sefton, 1995; Nakamura et al., 1993). These effects can also be direct or through the inhibition of phosphatases (Hardwick and Sefton, 1995) or by direct activation of kinases (Maher and Schubert, 2000). The consequences of the ROS-mediated PTK activation are not quite clear, sometimes it might be a protective response. For example UV irradiation of HeLa cells increases ROS formation which leads to PTKpp60src activation, whereas inhibition of pp60src activity potentiates cell killing by UV (Devary et al., 1992).

Serine/Threonine kinases are also targeted by ROS (Maher and Schubert, 2000): mitogen activated protein kinases (MAPK) are the best examples for this group of proteins. These proteins are part of major intracellular signaling pathways. In mammalian cells there are three types of protein kinases: ERKs, stress activated protein kinase (JNK), and p38 MAPK (reviewed in Robinson and Cobb, 1997). ERKs are implicated in growth factor pathways in cell proliferation and differentiation, while the two other systems are involved with cellular responses to inflammatory cytokines and cell cycle regulation. To activate the above mentioned MAPKs, upstream kinases
known as MAPKKKs. and MAKKs must be phosphorylated, which then result in MAPKs phosphorylation (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997). \(H_2O_2\) and other oxidants can activate all three members of the MAPK family, i.e., ERK, JNK, p38 (Bass et al., 1983; Guyton et al., 1996). The effective ROS that induce MAPK activity might be cell type dependent, i.e., \(H_2O_2\) in some cells (Guyton et al., 1996) and \(O_2^-\)-in others (Baas and Berk, 1995). Activation of ERK by \(H_2O_2\) seems to be indirect as it has been shown to be controlled by an upstream kinase known as Ras which is responsive to the redox status of the cell (Lander et al., 1996; Lander et al., 1995). A role for ERK activation in cell death inhibiting has been shown for neural cells (Guyton et al., 1996). These authors showed that in PC12 cells, activation of Ras is necessary for ERK activation; while inducible or constitutive expression of the dominant negative Ras abolished the ERK activation by \(H_2O_2\). Transfected cells with dominant negative Ras were more sensitive to ROS than controls. Activation of ERK by transfection of NIH3T3 cells with MEK, the MAPK kinase responsible for ERK activation, increased resistance to \(H_2O_2\). While the dominant negative mutant of MEK induced the \(H_2O_2\) susceptibility (Guyton et al., 1996). JNK and p38 MAPK are also regulated by ROS inactivation of phosphatases (Meriin et al., 1999) which results in inhibition of dephosphorylation of JNK and p38 MAPK and thus, inactivation. Activation of JNK and p38 MAPK is also a downstream event of ASK1 (apoptosis signal kinase-1) activation, which is a MAPKKK (Saitoh et al., 1998). ASK1 can be activated by \(H_2O_2\)-induced dimerization (Gotoh and Cooper, 1998) or by a thioredoxin-mediated mechanism to promote programmed cell death (Nakamura et al., 1997). The role of thioredoxin in
redox regulation of molecules will be discussed later in this chapter. Glutathione transferase Pi (GSTp) was also shown to be involved in JNK regulation (Adler et al., 1999a), since increased ROS causes GSTp disassociation from the GSTp-JNK complex, leading to JNK activation. Adler et al., (1999) reported that GSTp null mice have higher JNK basal activity than their normal counterparts, and GSTp transfection decreases JNK activity. The role of JNK in promoting cell death is still controversial. but p38 MAPK activation has been shown to cause cell death (for review see Maher and Schubert, 2000). Recently it was shown that phosphorylation of p38 MAPK is required for cell death induction in neuronal cultures exposed to 2,2'-dithiodipyridine (DTDP), a sulfhydryl oxidizing agent (McLaughlin et al., 2001). These authors have shown that p38 MAPK phosphorylation is upstream from voltage-gated potassium currents, which are necessary for caspase activation. They postulated the following model for p38-induced cell death in neuronal cultures: Within 10 min after DTDP-induced oxidative stress, zinc homeostasis in disturbed due to zinc release from metalloproteins (Aizenman et al., 2000). This osmotic stress causes p38 activation as soon as 30 min following oxidative stress. K+ release from voltage gated channels occurs later, but is not lethal. Energy failure due to activation of PARS (Poly(adenosine 5'-diphosphoribose) synthetase) as well as inhibition of mitochondrial electron transport chain are late events in this cascade that lead to cytochrome C release and caspase activation (McLaughlin et al., 2001).

Protein kinase C (PKC) are also responsive to ROS as they all are phosphorylated on tyrosine residues and activated under oxidative conditions (Konishi et al., 1997). This is usually an indirect effect of ROS, as ROS inhibit the phosphatases that dephosphorylate the tyrosine kinases (Maher and Schubert, 2000).
The active site of these protein phosphatases contains a cysteine residue which is sensitive to the redox status of the cell and can be inactivated by low concentrations of ROS (Denu et al., 1996).

ROS are also involved in regulation of transcription factors and therefore gene regulation. The redox sensitivity of these transcription factors determines their DNA binding properties and therefore the rate of gene regulation. Nuclear factor kappa B (NF-kB) and activator protein-1 (AP-1) are examples of such transcription factors that regulate transcription of a large variety of genes that are directly involved in the pathogenesis of diseases, e.g., AIDS, cancer, atherosclerosis and diabetic complications (Sen and Packer, 1996). Many reports of AP-1 activation during a wide variety of oxidative conditions have been reported, such as UV irradiation, X-rays, cytokines and chemotherapeutic drugs (reviewed in Fanger et al., 1997) as well as treatment with H$_2$O$_2$ (Ishikawa et al., 1997), hypoxia (Rupec and Baueuerle, 1995), NO$^\circ$ (Janssen et al., 1997) and ONOO$^-$ (Muller et al., 1997). The AP-1 pathway is quite complicated and the ROS-mediated effects on this transcription factor depends upon the context. This is partly due to the fact that AP-1 is not a single transcription factor, but rather a group of related dimeric complexes composed mainly of Jun homodimers and Jun-Fos heterodimers. Jun and Fos families consist of several different members with distinct transcriptional properties (reviewed in Herdegen et al., 1997; Leppa et al., 1997). DNA binding is activated by dimerization of these proteins, followed by interaction with the tetradecanoic phorbol acetate (TPA)-response elements in the promoter regions of a variety of genes. Several different signaling pathways can regulate different aspects of the activity of these transcription factors through activation domains. (Klotz et al., 2000). Therefore the effects of ROS on AP-
Activation can be mediated through interaction with different signaling pathways. For example, activation of AP-1 by "c-fos" might occur through activation of (ERK/p38→Elk-1→c-fos: or ERK/p38 →MAPKAP-K1/2 → CREB → c-fos) pathways or by "c-jun" through (JNK → c-Jun → c-jun: or JNK/p38 → ATF2 → c-jun) pathways, both ending in AP-1 activation (Klotz et al., 2000), but in different contexts.

The above examples show the complexity of ROS signaling through manipulation of proteins. Therefore more than one pattern of activation of upstream transcription factors or kinases can lead to the same effect. Since any reactive species may lead to activation of different MAP kinases, the overall effects on gene regulation are not necessarily distinguishable for different reactive species. For example, in NIH 3T3 cells, H₂O₂ has been shown to activate ERK but not JNK (Kyriakis et al., 1994), while Baas and Berk (1995) did not observe ERK activation in the rat aortic smooth muscles. In another study by Guyton et al. (1996), H₂O₂ activated ERK, JNK as well as p38. Even the response of the cell to activation of the same kinases can be quite different. Activation of JNK/SAPK (c-Jun N-terminal kinase/stress activated protein kinase) has been shown to induce programmed cell death or cell proliferation, depending on the duration of its activation (reviewed by Davis et al., 2001). ROS and/or RNS are known to modulate redox regulation in relation to glucose transport and regulation of carbohydrate metabolism in skeletal muscle (Balon et al., 2001). The latest manuscript shows that the proto-oncogene p21ras, a G protein which mediates glucose transport in muscle cells, is activated by ROS and RNS and causes NF-kB activation. Activation of MAP kinases (ERK, JNK, p38) is a downstream
signaling event in the p21\textsuperscript{ras} pathway that may lead to regulation of glucose transport (Lander et al., 1996).

NRF2 (nuclear factor erythroid 2-related protein) is another example of a transcription factor that might be activated by ROS and play an important role in the upregulation of defensive enzymatic systems (Itoh et al., 1999a). This system will be discussed later in this chapter.

\[ \text{Ca}^{2+} \text{ signaling is another important signaling mechanism and is also sensitive to reactive oxygen species. ROS exposure can change Ca}^{2+} \text{ signaling not always as a threat to cells but to modulate physiological signaling pathways. The effects of reactive oxygen species may be caused by modification of Ca}^{2+} \text{ channels, exchangers, pumps or Ca}^{2+} \text{ binding proteins (Suzuki et al., 1997). Many of the above-mentioned effects of oxidants on signaling systems can be mediated by Ca}^{2+} \text{ homeostasis. Sarcoplasmic reticulum preparations treated with } \text{H}_2\text{O}_2 \text{ (Boraso and Williams, 1994) and singlet oxygen plus a superoxide anion generating system (Holmberg et al., 1991; Xiong et al., 1992) showed an increase in the probability of the opening of Ca}^{2+} \text{ channels. When channel proteins were reconstituted in artificial membranes and treated with hypoxanthine/ xanthine oxidase, an IP3 increase as well as Ca}^{2+} \text{ ATPase inhibition were observed. Superoxide and not hydrogen peroxide was responsible for this effect (Suzuki and Ford, 1992). This demonstrates the differential sensitivity of Ca}^{2+} \text{ channels/pumps to different reactive species.} \]

1.2.4. ROS and RNS act as second messengers: NO\textsuperscript{o} has been proposed as a second messenger involved in long term potentiation in hippocampal neurons
(Schuman and Madison, 1994). The NO role was implicated in relaxation signaling mechanism from endothelial cells to smooth muscle cells in 1979 (Gruetter et al., 1979). Intact endothelium was reported to be essential for this effect (Furchgott and Zawadzki, 1980). In 1987 experimental evidence was reported by Ignarro et al., (1987). NO in NOS positive regions of the brain alters gene expression in both cGMP-dependent and independent manners. For example Sin-1 molsidomine (NO donor). 8-bromo-cyclic GMP (a cGMP analog) or NMDA can trigger a switch in opioid gene expression by downregulating prodynorphin mRNA and upregulating proenkephalin mRNA, thus aiding in the long-term regulation of hippocampal excitability (Johnston and Morris, 1994). In cells RNS donors have been found to stimulate immediate early genes such as c-fos and jun-B by NF-κB activation in a cGMP independent manner (Morris et al., 1995).

It has been shown that ROS are second messengers involved in apoptosis induction in response to tumor necrosis factor (TNF). H₂O₂ as well as UV radiation; this involves sphingomyelinase enzyme activation to increase intracellular ceramide (Mansat-de Mas et al., 1999). Ceramide activates the JNK/SAPK pathway leading to apoptosis in human leukemia cells under environmental stress and also inactivates protein kinase C δ and ε by translocating the protein kinase C from membrane to the cytoplasm (Sawai et al., 1997). Antioxidant therapy with N-acetylcysteine inhibited activation of sphingomyelinase and ceramide release, and therefore apoptosis (Singh et al., 1998).
1.2.5. Damaging effects of ROS in CNS: $O_2^{\ast}$ increases in stroke or traumatic CNS injury (reviewed in Juurlink and Paterson, 1998; Juurlink and Sweeney, 1997; Sweeney et al., 1995), which is largely due to $Ca^{2+}$-mediated mechanisms. The initial decrease in blood flow causes ATP depletion due to mitochondrial failure and leads to membrane depolarization (Folbergrova et al., 1997). This may open voltage-dependent $Ca^{2+}$ channels and increase $[Ca^{2+}]_i$ (Barone et al., 1994). High levels of $Ca^{2+}$ can also be due to physical disruptions of the plasmalemma and may cause glutamate release (Katayama et al., 1995). Glutamate accumulation in the extracellular space is also increased, due to inactivation of astrocyte glutamate transporters by $H_2O_2$ (Trotti et al., 1998). This causes activation of N-methyl-D-aspartate (NMDA) receptors (Mody and MacDonald, 1995) as well as D.L-amino-3-hydroxy-5-methyl-4-isozazolepropionate (AMPA)/kinate receptors (Carriedo et al., 1998) and a further rise in intracellular $Ca^{2+}$. Increased cytosolic $Ca^{2+}$ can induce calpain activation (Kampfl et al., 1997) that results in conversion of xanthine dehydrogenase to xanthine oxidase, the enzymes involved in uric acid metabolism, with subsequent production of $O_2^{\ast}$ (Sussman and Bulkley, 1990). Mitochondrial $Ca^{2+}$ cycling is also disrupted after cytosolic $Ca^{2+}$ elevation, which increases superoxide generation by mitochondrial electron transport (Richter and Kass, 1991) due to structural alteration in mitochondrial membrane and electron transport chain organization (Kowaltowski et al., 1995). Mitochondrial $Ca^{2+}$ disturbance also triggers the induction of permeability transition pores in the inner mitochondrial membrane (Zoratti and Szabo, 1995); the induction of permeability transition pores is
inhibited by cyclosporine A (Schinder et al., 1996). Ca\(^{2+}\) also activates phospholipases, e.g., phospholipase C (Wei et al., 1982) leading to arachidonic acid release. Arachidonic acid then can be metabolized to inflammatory eicosanoids (Demediuk et al., 1985). These inflammatory intermediates cause neutrophil invasion and production of strong oxidants (Bazan et al., 1995). O\(_2\)\(^{-}\) can deplete NAD(P)H through interaction with NADH bound to lactate dehydrogenase. NAD radical is formed here and can react with molecular oxygen causing more superoxide production thereby a chain reaction starts that depletes NAD(P)H stores (Fridovich, 1986).

Proteins are also vulnerable to superoxide which can interfere with their biological functions. More than a dozen important mammalian enzymes are inactivated by superoxide radicals, including aconitase (Halliwell, 1999), catalase, glutathione peroxidase, glutamine synthase, creatine phosphokinase, and myofibrilar ATPase (reviewed by McCord, 2000). Oxidation of cysteine groups in glyceraldehyde-3 phosphate dehydrogenase by OH\(^o\) or ONOO\(^-\) causes its inactivation, therefore impairing the glycolytic process (Souza and Radi, 1998). Singlet oxygen can oxidize any accessible tyrosine, cysteine, tryptophan, methionine and histidine residues in proteins (Michaeli and Feitelson, 1994; Michaeli and Feitelson, 1995). Examples of proteins inactivated by singlet oxygen are superoxide dismutase and catalase, two important free radical scavengers (Escobar et al., 1996).

Transition metals play an important role in ROS mediate cell injury. Their interaction with H\(_2\)O\(_2\) and other peroxides leads to OH\(^o\) generation through the Fenton reaction. OH\(^o\) interact with other macromolecules, such as lipids and proteins and nucleic acids. This not only changes their biological properties but also may
interfere with their normal physiology. (Juurlink and Paterson, 1998). Oxidation of proteins leads to carbonyl formation and this is a useful marker for assessment of ROS-mediated protein modification. Protein oxidation has been reported in aging and neurodegenerative diseases (Carney and Carney, 1994) as well as (Hall et al., 1995) in ischemia/reperfusion induced changes. The reaction between ROS and polyunsaturated fatty acids results in lipid peroxidation. This is a series of self-propagating reactions that leads to formation of lipid radicals as well as lipid peroxides (Juurlink and Paterson, 1998).

\[
\begin{align*}
L^\circ + OH^- \rightarrow L^o + HOH \\
L^o + O_2 \rightarrow LOO^o \\
LOO^o + RH \rightarrow LOOH + L^o \\
LOOH + Fe^{3+} \rightarrow Fe^{2+} + LOO^o \\
LOOH + Fe^{2+} \rightarrow Fe^{3+} + LO^o + OH^- 
\end{align*}
\]

Lipid peroxidation is responsible for loss of membrane integrity function and edema formation (Hall, 1993). Lipid peroxidation can be assayed by measuring malondialdehyde (MDA) levels by thiobarbituric acid-reacting substances assay. 4-hydroxynonenal (HNE) is also a marker as well as a cytotoxic product of lipid oxidation (Esterbauer et al., 1991).

Nucleic acids are also subject to oxidative damage. Both mitochondrial and nuclear DNA can be attacked by the OH\(^-\). 8-hydroxy-2'-deoxyguanosine is the most commonly used biomarker for DNA oxidation (Rehman et al., 1998). The aged brain mitochondrial DNA contain a 15-fold increase in oxidized DNA compared to young brain (Mecocci et al., 1994). Lack of protective histone proteins in the mitochondrial DNA and the close proximity of their DNA to oxidants, make mitochondrial DNA
more susceptible to ROS than nuclear DNA. (Yakes and Van Houten, 1997). After traumatic brain injury, the rate of mitochondrial DNA deletion is increased. The oxidized DNA may be repaired by apurinic/apyrimidinic endonuclease (Chopp et al., 1996). A decrease in apurinic/apyrimidinic endonuclease expression in ischemic regions of the brain may be responsible for DNA fragmentation in these areas (Fujimura et al., 1999; Kawase et al., 1999). Singlet oxygen has also been shown to cause DNA damage by inducing breaks that could be protected by singlet oxygen scavengers (Li and Trush, 1994). $^{1}\text{O}_2$ is responsible for DNA high mutation rates via oxidation of guanine residues mainly to 7-hydro-8-oxodeoxyguanosine moieties (Artuso et al., 1991). These mutations are repaired efficiently (Anson et al., 1998).

Oxidation of thiol containing proteins, which results in disruption of vital functions of membranous system such as ion homeostasis, ATPase activity and glucose and amino acid uptake systems (Schraufstatter et al., 1990) has been reported for low concentration (10-20 $\mu$M) of the strong oxidant, hypochlorite. At higher doses (200 $\mu$M) hypochlorite also causes lipid oxidation (Panasenko et al., 1995).

1. 2. 6. **ROS and neurodegenerative diseases:** There is a large body of literature dealing with the role of oxidative stress resulting in neuronal death in neurodegenerative diseases. These diseases are exemplified by illnesses such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), hereditary spastic paraplegia and cerebellar degeneration (Simonian and Coyle, 1996). The increased production of ROS in these diseases is considered an important mediator of neuronal death in these diseases (Beal, 1995). In PD (reviewed in Schulz et al., 2000), enzymatic oxidation of dopamine by
monoamine oxidase leads to increased $H_2O_2$ production. Auto-oxidation of dopamine results in neuromelanin formation and can generate quinone and semiquinone and other ROS. The activity of mitochondrial complex 1 is decreased so that the rate of superoxide formation is increased. In AD, decreased activity of cytochrome oxidase C (Ojaimi et al., 1999) can enhance ROS production in affected cells. The level of lipid oxidation is also increased in this disease, which further confirms the oxidative damage associated with ROS activities (Lovell et al., 1995; Marcus et al., 1998). In some cases of familial ALS a mutation in Cu/Zn superoxide dismutase enzyme has been reported (Ferrante et al., 1997). This mutation leads to an ALS phenotype in mouse (Brown, 1995). Mutations of the SOD gene may reduce its superoxide dismutase activity, thereby elevating free radical levels (Liu, 1996). In some cases, the mutant SOD may have normal activity but the protein may function as a peroxidase to oxidize cellular components. and it may also react with peroxynitrite, a product of the reaction between superoxide and nitric oxide-to ultimately form nitrate proteins (Hall et al., 1998; Liu, 1996). Levels of 3-nitrotyrosine, a marker of peroxynitrite formation is increased in cortex, spinal cord and CSF of ALS patients has been detected (Beal et al., 1997; Bruijn et al., 1997; Tohgi et al., 1999).

1.2.7. **Damage of CNS with RNS:** Although NO$^\circ$ is relatively innocuous, it can react with proteins and change normal protein function. Depending on the redox state of the cell this effect can be neuroprotective or neurodestructive. The reaction of the NO$^\circ$ group with cysteine sulfhydryls, known as S-nitrosylation, on the NMDA receptor leads to a decrease in receptor/channel activity, interfering with
excessive Ca$^{2+}$ entry, and thus is neuroprotective (Lipton et al., 1998). S-nitrosylation of poly (ADP-ribose) synthase is neurodestructive (Zhang et al., 1994). NO$^\circ$ and its derivative peroxynitrite (ONOO$^-$) inhibit mitochondrial respiration; this inhibition may contribute to both the physiological and cytotoxic actions of NO$^\circ$ (Brown and Borutaite, 1999). Oxidation of mitochondrial proteins involved in electron transport chain by (ONOO$^-$) leads to decreased ATP production and increased O$_2$$^{\circ-}$ formation (Radi et al., 1994). NO$^\circ$ can nitrosylate NO$^\circ$ synthase (NOS) to inhibit its NO$^\circ$ synthase activity by interaction with dithiols in the regulatory domain of NO$^\circ$ synthase (Patel et al., 1996).

ONOO$^-$ is known to induce membrane oxidation, especially at lower pH (Radi et al., 1991b). ONOO$^-$ is also a potent oxidant that mediates oxidation of both nonprotein and protein sulfhydryls: the rate of oxidation has been shown to be 3 orders of magnitude greater than the corresponding rate constants for the reaction of hydrogen peroxide with sulfhydryls (Radi et al., 1991a). reviewed by (Bolanos et al., 1997a). DNA bases are also susceptible to oxidation by ONOO$^-$ (Epe et al., 1996).

The above examples of damaging effects of different ROS and RNS in biological systems indicate the importance of free radical scavenging systems in the cells.

1.3. **Scavengers of Reactive Oxygen Species in Biological systems:**

A wide variety of molecules are involved in ROS scavenging to prevent the oxidizing effects of ROS. These anti-oxidants consist of enzymatic and non enzymatic systems. Catalase, glutathione peroxidase, thioredoxin reductase and
thioredoxin peroxidase (peroxiredoxins) and superoxide dismutase comprise the major enzymatic systems. Vitamins C (ascorbate) and E (tocopherol) as well as small molecules such as glutathione and thioredoxin are important nonenzymatic antioxidants. There are evidences of anti-oxidative function of other molecules such as carotenoids as a singlet oxygen quencher (Cooper et al., 1999), and urate a protector against ozone as well as an iron chelator (Ames et al., 1981; Cross et al., 1992). Albumin-bound bilirubin is also an endogenous antioxidant in newborns. It contains sulfhydryl groups which enable it to bind to copper ions and therefore inhibit hydroxyl radical formation. Flavonoids are dietary derived plant compounds with pharmacological properties (Havsteen, 1983). They show antioxidant activities such as singlet oxygen quenching capacity, inhibition of superoxide formation by decreasing xanthine oxidase activity and even iron chelating (reviewed in Juurlink and Paterson, 1998). Amongst all the above mentioned antioxidant compounds, superoxide and peroxide scavengers are of special importance.

Superoxide anions are the main source of ROS formation in living cells. Once they are generated they are removed through an efficient group of superoxide dismutase (SOD) enzymes to prevent further production of RNS as well as OH$^-$ (Fig. 1.1). There are three isoforms of SOD found in mammalian cells: Cytosolic copper-zinc (Cu/Zn) SOD (32 kDa), mitochondrial or manganese (Mn) SOD (40 kDa), and extracellular (Cu/Zn) SOD (Fridovich, 1995). MnSOD knockout mice die soon after birth, due to lung damage and neurodegeneration (Lebovitz et al., 1996). Mice lacking cytosolic and extracellular SODs develop and survive quite well but after stress, such as ischemia and reperfusion, they show increased neurologic and histologic damages (Carlsson et al., 1995; Reaume et al., 1996). The extracellular SOD is found on the
surface of only few cell types, such as fibroblasts and endothelial cells (Karlsson et al. 1993).

\[ 2 \, \text{O}_2^{-} + 2 \, \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]  
(SOD)

Hydrogen peroxide is produced in mitochondria or during SOD activity. \( \text{H}_2\text{O}_2 \) must be catalyzed to water to prevent its reaction with transition metals and production of \( \text{OH}^- \). The hydroxyl radical, as was mentioned earlier, can start self propagating chain reactions that result in protein/lipid oxidation. These chain reactions will continue to propagate until two radicals combine to form a stable product, or a chain breaking antioxidant react with the radicals. Vitamin E is an example of a chain-breaking antioxidant and protects polyunsaturated fatty acids in membranes and lipoprotein particles.

\[ \text{LOO}^- + \text{TOH (Vitamin E)} \rightarrow \text{LOOH} + \text{TO}^- \]

\[ \text{AscH}_2 (\text{Vitamin C}) + \text{TO}^- \rightarrow \text{TOH} + \text{AscH}^- \]

\[ 2 \, \text{AscH}^o \rightarrow \text{AscH}_2 - \text{Asc (dehydroascorbate)} \]

\[ \text{Asc} + 2\text{GSH} \rightarrow \text{GSSG} + \text{AscH}_2 \]

Tocopheroyl radical is relatively innocuous and is scavenged by vitamin C, and glutathione is necessary to restore the cellular vitamin C pool (Juurlink and Paterson. 1998). Thioredoxin (Trx(SH)2) system is also involved in vitamin C reduction during oxidative stress. This system will be discussed later in this chapter.

The lipid hydroperoxide can react with transition metal ions, therefore resulting in formation of lipid radicals (reviewed in Juurlink, 1999).

\[ \text{LOOH} + \text{Fe}^{2+} \rightarrow \text{LO}^o + \text{Fe}^{3+} + \text{OH}^- \]

\[ \text{LOOH} + \text{Fe}^{3+} \rightarrow \text{LOO}^o + \text{Fe}^{2+} + \text{H}^+ \]
The essential function of vitamin E is to trap peroxyl radicals and break the chain reaction of lipid peroxidation (Burton et al., 1986), but it does not put the cell out of direct danger of lipid peroxidation since a lipid hydroperoxide is formed (Juurink, 1999). Therefore proper peroxide scavenging seems to be the essential way to protect cells from oxidative stress-mediated damages.

1.4. Peroxide Scavenging Systems:

Peroxide scavenging systems are composed of several different enzyme systems. These are responsible for catalyzing the reduction of peroxides to water or related alcohol, therefore minimizing their oxidative properties. Several enzymes are discussed here: catalase, glutathione dependent peroxidase, glutathione reductase, glutathione transferase, and thioredoxin related enzymes such as thioredoxin reductase and thioredoxin peroxidases.

1.4.1 Catalase was the first enzyme to be characterized. It is a 56-kDa cytosolic haemoprotein homotetramer that can act without a cofactor to convert \( \text{H}_2\text{O}_2 \) to water and \( \text{O}_2 \) (Kirkman et al., 1987).

\[
2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2 \text{ (Catalase)}
\]

This enzyme has very high capacity \((10^{7} \text{ M/sec})\), implying that it is virtually impossible to saturate the enzyme (Young and Woodside, 2001). Catalase can be irreversibly inactivated by oxidation and demonstrates decreased activity after ischemia-reperfusion (Clarke and Sokoloff, 1999). Using microinjection of catalase-encoding cDNA, it was shown that upregulation of catalase increases the peroxide
scavenging ability of neurons (Herpers et al., 1999). Herpers et al., have shown that such transfected motoneurons better stand H₂O₂ induced cell damages.

1.4.2. Phase 2 Enzymes: H₂O₂ scavenging is also catalyzed by glutathione S-transferase enzymes (GSTs) (Prohaska, 1980), which are part of a group of detoxifying enzymes known as phase 2 enzymes. GSTs play a minimal role in peroxide scavenging system of the cell and are responsible for the conjugation of a broad spectrum of xenobiotics to the sulfur atom of glutathione, which results in water solubility of the adducts and their excretion from the cell (Pickett and Lu, 1989). Phase 2 enzymes are coordinately regulated by similar enhancer sequences in their promoter regions that are designated as the antioxidant response element (ARE) in rat and electrophile responsive elements (EpRE) in mouse (Prestera et al., 1993). Important enzymes such as L-γ-glutamyl-L-cysteine synthase, quinone reductase, epoxide hydrolase, UDP-glucuronosyltransferase, and peroxiredoxins are other known members of phase 2 enzymes family (Prestera et al., 1993).

Xenobiotics are detoxified by two phases of enzymatic reactions: phase 1, that is performed by cytochrome P-450 and results in reduction or oxidation of these reactive substances to often harmful secondary products, and phase 2 enzymes solublize these secondary products by conjugation with endogenous ligands such as glutathione and glucuronic acid. (Prestera et al., 1993). A wide variety of chemicals (reviewed in Prestera et al., 1993) can induce the induction of phase 2 enzymes, as it has been shown for quinone reductase: 1) diphenols, phenylenediamines and quinones, such as 2(3)-tert-butyl-4-hydroxyanisole (BHA) which is needed to be converted to
tert-butylhydroquinone (tBHQ). 2) Michael reaction acceptors, such as olefins conjugated with aldehydes and ketones. 3) isothiocyanates that are found in plants. 4) hydroperoxides and high concentration of \( \text{H}_2\text{O}_2 \). 5) mercaptans. 6) arsenicals. and 7) heavy metals. Many of these inducers such as quinones and \( \text{H}_2\text{O}_2 \) are strong oxidants that change the redox balance of the cell, therefore induce transcription of ARE-related genes (Maher and Schubert, 2000).

1.4.2.1. ARE-mediated gene regulation

The nucleotide sequences of ARE have been characterized. The human and rat quinone reductase ARE contain two or more AP1 or AP1-like elements in a short stretch of the DNA (40-45 nucleotides) (Jaiswal, 1994). The AP1 element (TGACTCA) is found in the upstream regions of many genes such as human metallothionine and human collagenase (Hai and Curran, 1991; Herschman, 1991). It is also termed as TRE (TPA/PMA response element) and increases gene regulation in response to phorbol 12-myristate 13-acetate. The AP1 binding site is followed by the nucleotides GCA, known as "GC" box. The AP1-like sites flank this site on each side and the first AP1-like is found in inverse direction (Li and Jaiswal, 1992). AP1 binding site is not essential for tBHQ-mediated gene ARE expression, but the GC box and the flanking sequences are essential for maximal inducibility (Moehlenkamp and Johnson, 1999).

AREs bind to a complex of several nuclear transcription factors that may be comprised of c-Jun, Jun-B, Jun-D, Nrf1, Nrf2, c-Fos, Fra1 (Venugopal and Jaiswal, 1996; Venugopal and Jaiswal, 1998), and Maf proteins (Dhakshinamoorthy and Jaiswal, 2000; Itoh et al., 1997). The mechanism of ARE-mediated gene expression is
not quite known. It has been shown that the Nrf family of transcription factors heterodimerize with other nuclear transcription factors, such as c-Jun (Venugopal and Jaiswal, 1998) which upregulates gene regulation, or c-Fos (Venugopal and Jaiswal, 1996), which represses the ARE-mediated gene expression. The role of Maf small proteins is still controversial, as it has been demonstrated that Maf-Nrf2 stimulates gene regulation (Itoh et al., 1997), while another report showed this heterodimer might negatively regulate ARE-mediated gene expression (Dhakshinamoorthy and Jaiswal, 2000). In these heterodimers Maf, c-Jun, c-Fos are responsible for DNA binding, while Nrf2 induces the transcriptional activity. Nrf2 plays the key role in gene regulation, as it has been shown that Nrf2-deficient mice failed to induce phase 2 enzymes (Itoh et al., 1999a). In unstimulated cells Nrf2 is mainly kept in the cytoplasm bound to Keap1 (Itoh et al., 1999b). Upon stimulation, Nrf2 is released from Keap1 and translocates to the nucleus, where it binds to Maf2 (nuclear transcription factor) and induces ARE transcription (Itoh et al., 1999a).

It seems that different signaling mechanisms result in Nrf2 activation and translocation to the nucleus. Activation of Nrf2 may be mediated by ROS signaling (Itoh et al., 1999b) or induction of oxidative stress (Dhakshinamoorthy and Jaiswal, 2000). Lee et al. (2001) have recently shown that in IMR-32 human neuroblastoma cells, tBHQ-induction of ARE-mediated gene expression is independent of oxidative stress as they found that tBHQ can induce ARE activation in the presence of exogenous antioxidants. But when cells are pretreated with antioxidants, the DEM-induced activation of quinone reductase is decreased (Lee et al., 2001). These authors have concluded that Nrf2 induction in tBHQ treatment is not dependent on oxidative stress. The possibility of PKC involvement in tBHQ-induced activation of Nrf2 has
been ruled out (Moehlenkamp and Johnson, 1999). as PKC inhibition by PMA does not affect ARE activation by tBHQ. Phosphorylation of MAPKs (ERK and p38 MAPK) seems to be an upstream event of Nrf2 binding to the antioxidant element in mouse (Zipper and Mulcahy, 2000). The phosphatidylinositol 3-kinase might also be involved in tBHQ-induced Nrf2 activation (Kang and Eum, 2000).

Since the natural phase 2 enzyme inducers are found in plants (Jaiswal et al., 1997; Khan et al., 1992) it may be possible that upregulation of phase 2 enzymes can increase the ability of the cells to withstand the oxidative stress conditions. In fact, induction of phase 2 enzymes using tBHQ has been shown to prevent the glutamate toxicity in N18-RE-105 (Murphy et al., 1991). These cells were also more resistant to H₂O₂ following phase 2 enzyme induction (Duffy et al., 1998). Overexpression of quinone reductase and GST, two members of the phase 2 enzyme family, did not mimic the protective effects of phase 2 inducers (Duffy et al., 1998). This observation suggests the possibility of involvement of other genes and systems that are upregulated by phase 2 inducers and results in better peroxidase scavenging abilities in neural cells.

1.4.3 Glutathione dependent enzymes: The system consists of glutathione (GSH), glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-R). The role of GSH and GSH-Px in scavenging hydrogen as well as organic peroxides is very well documented (Juurink and Sweeney, 1997).

\[ H(R)OOH + 2 \text{GSH} \rightarrow \text{GSSG} + \text{H}_2\text{O}/(\text{ROH}) \quad \text{(catalyzed by GSH-Px)} \]

The oxidized glutathione is then reduced by electrons from NADPH via a reaction catalyzed by GSSG-R.
GSSG + 2 NADPH + H⁺ → 2GSH + 2 NADP⁺ (catalyzed by GSSG-R)

Since the glutathione dependent systems play important roles in management of oxidative stress, especially peroxide scavenging, this system is further described here.

1.4.3.1. Glutathione: This small tripeptide (GSH: γ-L-glutamyl-L-cysteinyl-glycine) is the most abundant thiol present in mammalian tissues (up to 12 mM) (Dringen et al., 2000). Since its discovery over 100 years ago, glutathione has attracted a lot of interest, resulting in a vast knowledge of the importance of this small tripeptide (γ-L-glutamul-L-cysteinyl-glycine) in many aspects of life (reviewed in Cooper and Kristal, 1997). Some of its critical roles are listed here:

1) Cysteine storage compartment: sulfhydryl group of cysteine is toxic to the cells due to its ability to auto-oxidize and interact with aldehydes thereby can inhibit some enzymes, e.g., glutamate decarboxylase. The sulfhydryl group of GSH is less reactive and allows GSH concentrations to be 100 times higher than those of cysteine.

2) GSH as an enzyme cofactor: a number of enzymes such as glyoxylase, maleylacetoacetate isomerase and dehydrochlorinase, are examples enzymes that need GSH in their catalytic functions (Meister, 1989).

3) GSH as a transport means for amino acids: GSH and it derivatives, such as cysteinylglycine (CysGly) might be important in cysteine transfer across membranes, as it is an excellent substrate of γ-glutamyltranspeptidase (Meister et al., 1986).

4) GSH as an antioxidant, redox sensor and central player against ROS toxicity: It is also involved in regulation of the redox status of the cell, therefore regulating cell proliferation or cell death (reviewed in Cotgreave and Gerdes, 1998).
GSH in mitochondria plays an important role in ROS detoxification and depletion of GSH in this organelle renders the cell more susceptible to oxidative stress originating in mitochondria. A small fraction (~15-20%) of the total cellular GSH pool is sequestered in mitochondria by the action of a carrier that transports GSH from the cytosol to the mitochondrial matrix. Chronic ethanol-fed hepatocytes are selectively depleted of GSH in mitochondria due to a defective operation of the carrier responsible for transport of GSH from the cytosol into the mitochondrial matrix (Fernandez Checa et al., 1997).

A key mechanism that accounts for all these metabolic and cellular regulatory properties of GSH is the thiol-disulfide equilibrium. The sulfhydryl group of the cysteinyl residue of GSH is responsible for the versatility of GSH. The GSH:GSSG ratio may be important in modulation of certain transcription factors and signaling pathways e.g., lower GSH levels may decrease cell proliferation (Cotgreave and Gerdes. 1998). Protein folding is one of the post-synthesis modifications, which is performed in the endoplasmic reticulum for plasmalemmal and secretory proteins. This is accomplished by formation of disulfide bonds and the oxidizing conditions required for that is provided by release of GSH and small thiols (cysteine) from this organelle (Carelli et al., 1997).

1.4.3.2 Glutathione peroxidase (GSH-Px) GSH-Px catalyzes the reduction of hydrogen peroxide as well as organic peroxide, using electrons from GSH. This enzyme is remarkable in having a covalently attached selenium atom (Se). Its active site contains the selenium analog of cysteine in which Se has replaced S. The selenoate form of this residue reduces the peroxide substrate to an alcohol and is in
turn oxidized to selenic acid (E-SeOH). Glutathione now comes into the action by forming a selenosulfide adduct (E-Se-S-G). A second glutathione then regenerates the active form of the enzyme by attacking the selenosulfide to form oxidized glutathione (Stryer, 1995). It has been shown that exposure of different oxidizing agents decrease the GSH-Px activity and viability of cells is reduced. This can be due to damaging effects of singlet oxygen on histidine (Tabatabaie and Floyd, 1994). The selenium in GSH-Px is situated in a pocket of histidine, phenylalanine and tryptophan residues. Similarly GSSG-R has a histidine residue in its active catalytic site. Damage to these residues and other essential amino acids may be responsible for GSH-Px inactivation (Tabatabaie and Floyd, 1994).

GSH-Px system consists of a set of selenocysteine enzymes, which work in different cell compartments in a mutually complementary way. Four isoforms of GSH-Px, all encoded by separate genes, are currently known: i) the classical isoform (GSH-Px1), it is situated to the cytosol and mitochondria. It is a scavenger of hydrogen and organic peroxides such as free fatty acid hydroperoxides. (Flohe, 1979) (Wendel, 1980), ii) Gastrointestinal GSH-Px which has been recently described, is present in the gastrointestinal tract: it has some similar properties to the classical isoform (Chu et al., 1993) iii) phospholipid hydroperoxide GSH-Px (PHGPx), the scavenger of membrane-bound phospholipid hydroperoxides and other organic peroxides as well as hydrogen peroxide (Maiorino et al., 1990; Ursini et al., 1985; Thomas et al., 1990), and iv) plasma GSH-Px (GSH-Px-P) that can scavenge hydrogen peroxide as well as organic peroxides (Maddipati and Marnett, 1987). In most tissues the major (90%) peroxides scavenger is generally GSH-Px 1 while PHGPx activity
comprises most of the remaining activity. The GSH concentration affects directly the scavenging efficiency for peroxides of all these forms of GSH-Px.

The critical role of GSH-Px in enabling the cell to cope with oxidative stress has been clearly shown in experiments where GSH-Px activity has been decreased using inhibitors and increased by micro-injection of GSH-Px (Michiels et al., 1994). It also has been reported that in human breast T47D cell transfectants, increasing of GSH-Px provides a remarkable protection against DNA damage induced by \( \text{O}_2^- \)/\( \text{H}_2\text{O}_2 \) (Mirault et al., 1994). These observations demonstrate clearly that up-regulation of glutathione peroxidase activity could be an ideal way for increasing of cell resistance during oxidative stress.

As was mentioned, GSH-Px family are members of selenoproteins that share general aspects of protein synthesis with the other proteins, but some features are quite singular. This unique group of proteins contains one or more selenocysteine residues, often at their active sites. The gene transcripts for these proteins encode an atypical amino acid by a UGA codon in their mRNA reading frames. This special codon normally acts as a stop signal in genetic code (Kollmus et al., 1996). In prokaryotes, this alternative interpretation of the UGA codon as a selenocysteine occurs if it is followed immediately in the reading frame by a region that has been shown to form a stem-loop structure (Hubert et al., 1996; Kollmus et al., 1996). Eukaryotes selenoprotein-encoding mRNAs contain an element with analogous function referred as selenoprotein insertion sequence (SECIS) or selenium translation element (Shen et al., 1995). This regulatory region exists in the 3’ untranslated region (3’-UTR) and forms a stem loop structure (Hubert et al., 1996). Comparison of the sequences of
SECIS in rat and human iodothyronin deiodinase, cellular glutathione peroxidase and selenoprotein P mRNA has revealed only three very short conserved sequences, but all share a common computer predicted secondary structure featuring a long stem, several bulges, and an apical loop with three short conserved sequences (Shen et al., 1995). It seems that topographic situation and size of SECIS element have important roles in its identification by the relevant binding proteins (Kollmus et al., 1996). In addition to SECIS, some other factors are necessary for selenocysteine insertion into these proteins (Hubert et al., 1996). First of all, a seryl-tRNA$^{\text{sec}}$ is synthesized by seryl-tRNA synthetase. Selenocysteine synthase catalyzes the conversion of seryl-tRNA to selenocysteinyl -tRNA$^{\text{sec}}$ (Sec-tRNA). Selenophosphate is the selenium donor, and selenophosphate synthetase controls its level. SELB is another required factors, which is a bifunctional protein in prokaryotes with an N-terminal Sec-tRNA-specific EF domain (elongation factor), and a C-terminal SECIS RNA-binding domain (Forchhammer et al., 1989; Kromayer et al., 1996). In mammals, the SELB role is played by two proteins: SECIS-binding protein (SBP2), and Sec-tRNA specific elongation factor, termed eEFsec, with specific binding to Sec-tRNA and not seryl-tRNA (Tujebajeva et al., 2000) which is responsible for delivering the tRNA to the ribosome. Presence of SBP2 (Low et al., 2000) as well as the configuration of the stem loop structure (Kollmus et al., 1996) influences the efficiency of selenocysteine incorporation.

1.4.3.3. **Glutathione Reductase:** Reduction of oxidized-glutathione is allowed by this enzyme. It is a dimer of 50-kDa subunits. Electrons from NADPH
are transferred to a flavin adenine dinucleotide (FAD), tightly bound to this enzyme and then to a disulfide bridge between two cysteine residue in the enzyme subunit, and finally to oxidized glutathione. This reaction is important for maintaining the cellular reductive environment as it regenerates GSH. Levels of GSH is a determining factor in NF-κB inhibition (Flohe et al., 1997). GSSG-R is also susceptible to oxidative damages (Tabatabaie and Floyd, 1994) and also by glycation which is non-enzymatic process of glycosylation that leads to protein inactivation (Blakytny and Harding, 1992).

1.4.4. Thioredoxin dependent system: Thioredoxin (Trx(SH)2) system is another protective system developed by cells against the destructive effects of ROS. This system is very similar to glutathione system and along with that protects the reducing cellular environment by controlling the oxidation state of thiols. The thioredoxin system consists of thioredoxin, thioredoxin reductase (Trx-R) and thioredoxin peroxidases or peroxiredoxins (Prx).

1.4.4.1. Thioredoxin: Thioredoxins are small (12kDa), globular proteins that are evolutionarily conserved between prokaryotes and eukaryotes. They participate in many different cellular functions that are dependent on thiol-disulfide reactions, catalyzed by a conserved catalytic site “Trp-Cys-Gly-Pro-Cys-Lys” in a protrusion of the three dimensional structure of the protein. Oxidation of the cysteine residues in this active site results in formation of the intermolecular disulfide bond. Trx-(SH)2 was first described as an interleukin-2 receptor inducing factor in human lymphotropic
virus 1 transformed cells from patients with adult T-cell leukemia (Teshigawara et al., 1985). ADF (adult T-cell leukemia derived factor) cDNA was later cloned and was reported to be the human counterpart of Trx(SH)2 (Tagaya et al., 1989). Cytosolic Trx(SH)2 in mammalian cells is called Trx1, and compared to yeast and bacteria, has extra cysteine residues (structural role) that can regulate its enzymatic activity by oxidation and dimer formation. Invitro preincubation with DTT is required for obtaining fully active Trx1. The structural cysteines are not present in mitochondrial Trx(SH)2 (Trx2). The Trx2 gene is located at chromosome 22q12.1-q13 (Miranda-Vizuete et al., 2000).

As part of its role in maintaining the reducing environment of the cell, Trx(SH)2 spontaneously react with oxidized proteins to reduce the disulfide groups of those proteins (Holmgren, 2000). Trx(SH)2 is the major dithiol reductant in the cytosol because it has a low reduction potential (-270mV) and depending on the redox potential of its substrate, thioredoxin may reduce or form disulfide. The rate of reduction of disulfide bonds in proteins by thioredoxin is faster than those for GSH and dithiothreitol (Arner and Holmgren, 2000). The oxidized thioredoxin (Trx-S2) is reduced by Trx-R using electrons from NADPH (Holmgren, 2000).

\[
\text{Trx(SH)2 + Protein-S2} \rightarrow \text{Protein-(SH)2 + Trx-S2}
\]

\[
\text{NADPH + Trx-S2 + H}^+ \rightarrow \text{NADP}^+ + \text{Thioredoxin-(SH)2}
\]

Trx(SH)2 administration is a potential therapy for modulating the redox status of the cell, as it inhibits the expression of AIDS virus in human macrophages by 71%; This is 30,000-fold more effective than N-acetylcysteine (NAC) (Newman et al., 1994). Macrophages cleave Trx(SH)2 to generate the inflammatory cytokine,
eosinophil cytotoxicity enhancing factor: although using this cytokine increases the production of HIV by 67%. AIDS patients with higher plasma Trx(SH)$_2$ levels tend to have lower CD4 cell counts. It has been recently shown that the high levels of Trx(SH)$_2$ at final stages of the disease inhibits neutrophil migration in response to lipopolysacharides, therefore eliminating the final barrier against establishment of other infectious diseases (Nakamura et al., 2001). These authors showed that NAC treatment in vitro blocks thioredoxin release from HTLV-I-transformed cell line.

Thioredoxin is involved in several other functions. In plants it plays a key role in coordinating the light and dark reactions of photosynthesis (Stryer, 1995). Trx(SH)$_2$ system is the electron carrier from NADPH to ribonucleotide reductase, so this enzyme can generate deoxyribonucleotides necessary for DNA synthesis (Spyrou and Holmgren, 1996). Trx(SH)$_2$ participates in H$_2$O$_2$ reduction by peroxiredoxins (Prx) (Chae et al., 1999; Kang et al., 1998b). Trx(SH)$_2$ also interacts with transcription factors and regulates their DNA binding properties, for example, AP-1 and NF-$\kappa$B (Hirota et al., 1997; Hirota et al., 2001). Role of Trx(SH)$_2$ in AP-1 DNA binding activity is mediated through direct interaction of Trx(SH)$_2$ with redox factor 1 (Ref-1), which is identical to a DNA repair enzyme, AP endonuclease (Hirota et al., 1997). These authors showed that Ref-1 is activated directly by binding to Trx(SH)$_2$ in the nucleus.

Trx(SH)$_2$'s binding to apoptosis signal kinase-1 (ASK-1) inhibits apoptosis due to prevention of the downstream signaling mechanisms (Saitoh et al., 1998). The extracellular Trx(SH)$_2$ might act as a chemokine and eosinophil stimulating factor (Silberstein et al., 1993). The truncated form of Trx(SH)$_2$ (~10 kDa) which lacks the
reducing activity is responsible for the cytokine and eosinophil stimulating abilities (Silberstein et al., 1993). Trx(SH)2 is sought to be involved in early pregnancy during fetus implantation to establish pregnancy, as it has been shown that trophoblast cells synthesize and release Trx(SH)2 (Di Trapani et al., 1998). Matsui and coworkers (Matsui et al., 1996) showed that targeted Trx(SH)2 gene disruption in mouse interferes with fetus implantation. This group demonstrated that heterozygote animals were viable, fertile, and appeared normal. In contrast, homozygous mutants died shortly after implantation, and the concepti were resorbed prior to gastrulation. When such homozygous knock-out preimplantation embryos were placed in culture, the inner cell mass cells of the homozygous embryos failed to proliferate (Matsui et al., 1996).

Trx(SH)2 has also been studied as a pathophysiological factor and a drug target (Becker et al., 2000), since Trx(SH)2 system is necessary for cellular DNA synthesis and therefore cell division, it is a potential target for cancer therapy. It also might be involved in the inability of T cells to kill tumors, since Trx(SH)2 reduces NK-lysin, a perforating protein secreted by natural killer cells. The reduced NK-lysin is unable to exert its fatal effects (Andersson et al., 1996). By reducing dihydroascorbate (May et al., 1997) as well as ascorbate free radicals (May et al., 1998). The Trx-R system, along with GSH system, regenerates cellular vitamin C. Most but not all of these functions depend on the disulfide reductase activity of Trx(SH)2, and availability of NADPH as electron source and the activity of Trx-R.
1.4.4.2. Thioredoxin reductase: Thioredoxin reductase is a member of a larger family of pyridine nucleotide disulfide oxidoreductases, that includes lipoamide dehydrogenase, mercuric reductase and glutathione reductase. Trx-R catalyses the electron transfer between pyridine nucleotides and disulfide/thiol compounds via its closely associated FAD, reviewed in (Williams et al., 2000). The mammalian Trx-R are homodimers, and its molecular weight has been estimated about 58 kDa/subunit. for rat liver and the bovine liver enzyme, and 65 kDa for human placental enzyme (Arner and Holmgren, 2000). Mitochondria have their own Trx-R (Trx-R2) with an amino-terminal extension with high content of positively charged residues and a potential α-helix followed by β-sheets. both features of mitochondrial targeting sequences (Gasdaska et al., 1999). The human Trx-R gene is located at chromosome 12q23-q24.1. and the mitochondrial enzyme (Trx-R2) gene is localized to chromosome 22q11.2 (Miranda-Vizuete et al., 1999).

Trx-R is a selenoenzyme with a similar synthetic machinery to GSH-Px. It has a hydroperoxide as well as lipid hydroperoxide (HPETE) scavenging capacity (Bjornstedt et al., 1995), although its functional importance as an antioxidant is completely unknown. Trx-R contains a terminal selenocysteine (SeCys) residue in a conserved sequence Gly-Cys-SeCys-Gly: and UGA is the stop codon that is translated into selenocysteine (Gasdaska et al., 1995). by a stem loop structure known as selenocysteine insertion sequence (SECIS) (Zhong et al., 1998). Selenocysteine is essential for catalytic activity of Trx-R (Zhong et al., 1998). Although long term Se supplementation increases Trx-R activity and protein levels. the specific enzymatic activity drops. It has been hypothesized that induction of stable disulfide bonds between Trx-R and Se metabolites. can inhibit Trx-R activity (Ganther, 1999).
It has been shown that Trx-R can directly reduce lipid hydroperoxides using NADPH as electron donor, which will result in oxidation of Trx. Trx-R also can reduce selenocystine into two molecules of selenocysteine (Bjornstedt et al., 1995). Selenocysteine has been reported to decrease single-strand DNA breaks by peroxynitrite scavenging (Roussyn et al., 1996) and as well has peroxidase activity (Bjornstedt et al., 1995). This reaction is catalyzed by Trx-R. Mammalian Trx-R has a wide range of substrates and can also interacts with 5,5'-dithiobis (2-nitrobenzoic acid) (Holmgren, 1977), selenodiglutathione (GS-Se-Gs) (Bjornstedt et al., 1992), selenite (Kumar et al., 1992), vitamin K (Luthman and Holmgren, 1982), alloxan (Holmgren and Lyckeberg, 1980) and is an important electron donor to selenocysteine residue in GSH-Px-P (plasma GSH-Px) (Bjornstedt et al., 1994). The effects of Trx-R system in detoxification of harmful agents, such a peroxides, and prevention of oxidative stress-induced protein damages has been reviewed recently (Arner and Holmgren, 2000). Enhanced thioredoxin reduction could have beneficial effects in oxidative stress, but adverse effects have been reported for malignant cells (Becker et al., 2000; Ganther, 1999) as Trx-R system provide the tumor cells with necessary materials for cell division and also protects the cells from the body immune system.

1.4.4.3 Thioredoxin peroxidases: These are a family of proteins that exhibit peroxidase activity in a Trx-dependent manner. A 25-kDa enzyme was originally purified from yeast (Kim et al., 1988) with anti-oxidative properties. This protein was called TSA, as it provides thiols protection against oxidation (Kim et al., 1989). Soon it become clear that TSA is a member of a family of proteins with peroxidase activity in the presence of Trx(SH)2. Trx-R and NADPH (Chae et al., 1994a). therefore, this
family was named thioredoxin peroxidase, or TPx. Yeast TPx homologs have been identified. They show high conservation of two cysteine residues, which correspond to yeast Cys47 and Cys170 (Chae and Rhee, 1994). Six sequences were shown to have peroxidase activity, but only five of them showed TPx activity, and the sixth one, which contains only one cysteine (the NH2 Cys terminal), does not exhibit thioredoxin peroxidase activity. The designation TPx was changed to peroxiredoxins (Prx) (Rhee, 1999). The 2-Cys and 1-Cys Prx enzymes exist as homodimers, in a head-to-tail manner (Chae et al., 1994b; Hirotsu et al., 1999). The NH2-terminal of these proteins is the site of oxidation by peroxides and the sulfhydryl group is oxidized to sulfenic acid (Cys-SOH), which immediately reacts with COOH-terminal Cys to form an intramolecular disulfide bond. This bond is reduced by Trx(SH)2. The reducing factor for 1-Cys-Prx enzymes is still unknown (Kang et al., 1998a).

Three types of Prx are known in mammalian CNS. Prx1 has heme-binding activity, and thus called HBP. This protein also acts as a natural killer cell-enhancing factor (NKEF-A). Prx1 is upregulated under oxidative stress conditions and electrophile agents in macrophages (Ishii et al., 1999). Prx 2 is also another natural killer cell-enhancing factor (NKEF-B) and originally named thiol-specific antioxidant (TSA). Prx2 is upregulated under stress conditions, such as radiation (Park et al., 2000). These authors noticed the increased expression of Prx2 in cancer tissues isolated from patients who did not respond to radiation therapy, whereas Prx2 expression was weak in tissues from the patients with regressed tumors. Induction of Prx2 was observed after γ-radiation of cancer cells; such induction correlated with decreased radiation-induced cell death. Application of Prx2 antisense to cells in
cultures increased radiation sensitivity and cell death. Prx 1 and Prx 2 are both cytosolic. Prx 3, or SP-22, is found in mitochondria (Watabe et al., 1997). Prx 4 has also been characterized recently (Matsumoto et al., 1999).

The antioxidant function of the Prxs includes:

1) The reduction of hydrogen peroxide and alkylhydroperoxide to water and corresponding alcohols by the overall reaction:

\[
\text{Trx(SH)}_2 + \text{H}_2\text{O}_2 (\text{ROOH}) \rightarrow (\text{Trx})\text{SS} + \text{H}_2\text{O} (\text{ROH})
\]

2) Detoxifying the thyl radical (RS\(\cdot\)) or oxidized thyl radical anions, generated during the course of antioxidant protection. In oxidative stress, thiols (RSH) react with free radicals to neutralize the radical. The thyl radicals have the potential of triggering oxidative damage to biological macromolecules (Chae and Rhee, 1994).

The role and distribution of peroxiredoxins as well as other members of Trx(SH)\(2\) dependent system in the CNS is not well known. The catalytic efficiency of mammalian peroxiredoxins indicated by \(k_{\text{cat}}/K_m\) is significantly lower than that of catalase or GSH-Px (Rhee et al., 1999). \(k_{\text{cat}}\) is defined as the maximal catalytic rate when substrate is saturating, and \(K_m\) is the substrate concentration at which the reaction rate is half its maximal value (Stryer, 1995). Considering the restricted localization of catalase in peroxisomes, as well as low levels of cytosolic GSH-Px, the high levels of Prx expression in all tissues (0.2-0.4% of total soluble protein) may indicate their functional importance as alternative peroxide scavengers.

In the following sections, the recent information on the peroxide scavengers of the brain is reviewed.
1. 5 Peroxide scavenging systems in CNS

Due to high energetic demands of the mammalian adult brain, high rate of ROS is produced in this organ. Although it comprises only 2% of the body weight, up to 20% of all the oxygen consumed in the body is used by the brain (Clarke and Sokoloff. 1999), therefore a large quantity of ROS are generated during oxidative phosphorylation in CNS mitochondria. Some areas of the brain have high iron content, which is capable of generation of the damaging OH\(^{-}\) from peroxides (Gerlach et al., 1994). The brain is also endowed with a high concentration of polyunsaturated fatty acids and therefore is very susceptible to lipid peroxidation (Juurlink and Paterson. 1998). Although one assumes that the brain must be well protected by antioxidant systems, in fact this is not the case and the brain is highly susceptible to oxidative damages by peroxides as well as other ROS and RNS. Catalase activity in CNS is relatively low (Cooper and Kristal. 1997; Mirault et al., 1994) and it is not sufficient to put the cell out of peroxide danger. It is localized only in peroxisomes, which restricts its access to the cytosolic peroxides (Moreno et al., 1995), as well as mitochondrial peroxides, although it is exceptionally found in the heart mitochondria (Radi et al., 1991c). Catalase is not an efficient H\(_2\)O\(_2\) scavenger at concentrations below 100 \(\mu\)M (Simmons and Jamall, 1988), and shows lower affinity towards H\(_2\)O\(_2\) than does GSH-Px (Mirault et al., 1994). In contrast to GSH-Px, catalase can not scavenge lipid peroxides or any other organic peroxides (Juurlink and Paterson, 1998). Although Desagher et al. (Desagher et al., 1996) reported that catalase is the main peroxide scavenger of astrocytes, the rapid GSH consumption after H\(_2\)O\(_2\) addition to
astrocyte cultures indicates the importance of GSH dependent system in these cells (Dringen and Hamprecht. 1997).

1.5.1 Glutathione dependent system in neural cells

Glutathione: The available data on the glutathione system in neural cells is based on in vitro as well as in vivo reports. The GSH levels in astrocyte cultures is higher than neuronal cultures. (Dringen and Hamprecht. 1997; Dringen et al., 1999b; Eftekharpour et al., 2000). Immunohistochemical studies confirmed those results in the brain tissue (Rice and Russo Menna. 1998: Slivka et al., 1987: Trenga et al., 1991). Microglia seem to have the highest levels of GSH in the brain (Chatterjee et al., 2000; Hirrlinger et al., 2000). and Juurlink et al (Juurlink et al., 1998) found lower levels in oligodendrocyte compared to astrocytes. The GSH level is a determining factor in cell fate during oxidizing conditions. as it has been shown for rat cortical neurons (Li et al., 1997); if GSH levels reaches below 20% of normal for more than a few hours. the programmed cell death is initiated. Although other requirements for inducing cell death such as protein and mRNA synthesis (Tan et al., 1998b). as well as caspase activation (Tan et al., 1998a; Tan et al., 1998b) and activation of 12-lipoxygenase (Li et al., 1997) must be met more or less simultaneously. The coupling between GSH depletion and these events show the responsiveness of the underlying mechanisms to redox status of the cell. The level of GSH depends on two factors: 1) reduction of oxidized glutathione 2) glutathione synthesis. (Juurlink, 1999) .

Astrocytes GSH levels following oxidative stress induction decreases sharply while GSSG is increased (Dringen and Hamprecht. 1997). GSSG is reduced by GSSG-R, as NADPH is used as electron source. GSSG-R is susceptible to free radicals
(Tabatabaie and Floyd, 1994) and is used as a marker of oxidative stress (Barker et al., 1996).

The brain has ~2-3 mM GSH, which is much greater than the blood GSH concentration (~15 μM) or CSF (~5 μM) (Cooper and Kristal, 1997). Although brain might be able to take up GSH from the blood (Kaplowitz et al., 1996), most of the GSH is synthesized de novo. Cysteine, glutamate and glycine are the necessary amino acids for GSH synthesis which is performed in two steps: In the first step, γ-glutamylcysteine synthase catalyzes the production of γ-glutamylcysteine. In the second step, GSH is formed by GSH synthase when γ-glutamylcysteine reacts with glycine (Meister, 1989). Although neurons contain GSH and the enzymes necessary for its synthesis (Makar, 1994), considering the number of astrocytes compared to neurons, neuronal GSH pool seems to be small (Cooper and Kristal, 1997). The astrocytes' cysteine and glutamate originate from plasma (Fig. 1.2). Cysteine is the rate limiting amino acid for GSH synthesis. Cysteine easily auto-oxidizes to cystine; hence in the plasma and extracellular fluid cystine is found at a concentration of 100μM, whereas cysteine concentration is about 10μM (Bannai, 1984). Astrocytes take up cystine by a cystine/glutamate antiporter (XDC) system in a sodium independent manner (Bannai, 1984). During glutamate toxicity, following increased levels of extracellular glutamate, this antiporter system can reverse (Bannai and Kitamura, 1981), which results in depletion of cellular cystine/cysteine, and therefore lowers GSH synthesis (Murphy et al., 1989). Cystine moieties may also enter the (Oldendorf and Szabo, 1976) brain as cystinylglycine and γ-glutamylcyst(e)ine (γ-GluCys) (Jain et al., 1991). Methionine is taken up into brain by L-type transporter (Oldendorf and

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Figure 1.2. GSH synthesis in astrocytes and neurons
Szabo. 1976) also might be used as an alternative source of cysteine sulfur by
cystathionine pathway (Meister et al.. 1986). Cysteine precursors such as N-Acetyl-
cysteine might be able to replace cysteine (Kranich et al.. 1998). Glutamine is
synthesized in both astrocytes and neurons. but the astrocytes pool is smaller with a
faster turn over rate than the neuronal pool. which is larger and shows slower turn over
(Cooper and Kristal. 1997). While both astrocytes and cortical neurons are able to
take up cysteine and glutamate to use for GSH synthesis. only astrocytes can use
cystine and glutamate, and neurons are dependent on astrocytes to reduce cystine to
cysteine or convert glutamate to glutamine (Kranich et al.. 1996). Astrocyte culture
can release GSH in health (Dringen et al.. 1997a; Wang and Cynader. 2000) or under
the ischemic insults (Juurlink et al.. 1996). This GSH serves as a substrate for
astrocyte \( \gamma \)-transpeptidase (Dringen et al.. 1997a). allowing release of CysGly
(cysteinylglycine). CysGly is taken up by the peptide transporter PepT2 (Dringen et
al.. 1998a) that releases cysteine and glycine into the cytosol. enabling them to be used
for GSH synthesis (Dringen et al.. 1997b). CysGly as well as \( \gamma \)-GluCys and N-
acetylcysteine can be used by neuronal cultures to synthesize GSH (Dringen. 2000).
Neurons are more efficient than astrocytes in using CysGly for GSH synthesis,
although the underlying mechanism is not still clear. it has been proposed that neurons
must contain a similar mechanism to astrocytes Pep T2 (Dringen. 2000).

The importance of GSH for management of oxidative stress has been well
documented. Kamencic et al (2001) have reported that GSH synthesis promotion by
administration of the procysteine compound L-2-oxothiazolidine-4-carboxylate
(OTC). after induction of spinal cord injury in rats. decreases the oxidative stress and
allows tissue preservation. thereby enabling otherwise paraplegic animals to locomote.
Upregulation of GSH levels in oligodendrocytes prevents oxidative stress when exposed to 140 mW/m² of blue light (Thorburne and Juurlink, 1996).

**Glutathione reductase:**

Little is known about the distribution of GSSG-R in the brain. In the studies by (Knollema et al., 1996), these authors showed a differential distribution of GSSG-R protein in the murine brain. The GSSG-R positive neurons were detected in most areas, and also glial positive cells were detectable in areas with no positive neurons. In their in vitro studies, Gutterer et al. (1999), reported no difference in GSSG-R activity for rat neurons and glial cultures, and using double staining, they observed higher GSSG-R protein for neuronal, microglial as well as oligodendrocytes, than astrocytes. Another study with chick neural cell cultures found no significant difference between astrocytes and neurons (Makar et al., 1994), while Eftekharpour et al. (2000), showed higher GSSG-R activities in mouse cortical astrocytes than cortical neurons. The importance of this enzyme is well appreciated, as Spina and colleagues (Spina et al., 1992) showed that BDNF protects dopaminergic neurons against 6-hydroxydopamine and N-methyl-4-phenylpyridinium ion (MPP⁺) toxicity by upregulating GSSG-R activity. Also Kojima et al., (1998) reported an increase in GSSG-R mRNA and enzymatic activity after low doses of γ-rays, which induces the antioxidant defense systems in the mouse brain. GSSG-R is inactivated during oxidative stress (Kamencic et al., 2001; Plateel et al., 1995; Starke et al., 1997).
**Glutathione peroxidase** seems to be the main peroxide scavenger in the nervous system. The GSH-Px1 is found both in cytosol as well as mitochondria (Esworthy et al., 1997). GSH-Px4 is also expressed in the brain (Zhang et al., 1989) within the membranes of the plasmalemma, and endoplasmic reticulum, as well as within the inner layer of mitochondrial membrane. Brain shows lower GSH-Px activities than other tissues (De Marchena et al., 1974; Zhang et al., 1989).

The distribution of GSH-Px in neural cells is not clear, as Takizawa et al. (1994) reported a weak signal throughout the brain in both neurons and glia, which is upregulated in activated glia in stroke-infarcted area. Another study (Damier et al., 1993) did not show any detectable GSH-Px protein in neurons. the signal was more intense in glia adjacent to dopaminergic neurons. Immunohistochemical studies of Trepanier et al. (Trepanier et al., 1996) also showed a low level of expression in both astrocytes and neurons, but found strong signals in some neurons, e.g., cortical layer 2. Lindenau et al. (1998) have reported that in normal rat brain, GSH-Px protein was predominantly found in microglia. Three days after exposure to the excitotoxin quinolinic acid, immuno-reactivity increased in activated microglia in the lesion core and GSH-Px-positive activated astrocytes were detected in the environment of the lesion. These authors did not observe any increase in neuronal GSH-Px, and it was generally low. Microglia seems to have the highest GSH-Px activity amongst different neural cells (Hirrlinger et al., 2000), followed by astrocytes (Dringen et al., 1999a), neurons and oligodendrocytes (Eftekharpour et al., 1998) and protein levels correlate with the enzymatic activity levels.

The question arises whether endogenous antioxidants of the brain can be upregulated. Growth factors are well known factors that can stimulate neuronal
survival. Since neurons express tyrosine kinase receptors for bFGF, BDNF and NGF (both in vivo and in vitro) (Cheng and Mattson, 1991; Klein et al., 1990; Wanaka et al., 1990), activation of these receptors may play a role in neuroprotection (Cheng and Mattson, 1991; Mattson et al., 1995). NGF has been shown to increase catalase and GSH-Px activity and mRNA in PC12 cells (Sampath et al., 1994; Sampath and Perez-Polo, 1997). Although both catalase and GSH-Px mRNA have unusually long half-lives (~42 and ~45 h, respectively), NGF increases catalase mRNA stability, while it does not affect the GSH-Px mRNA (Sampath and Perez-Polo, 1997). In hippocampal neurons, Mattson et al. (1995) showed an increased GSH-Px activity in response to BDNF. PDGFs have also been shown to increase GSH-Px activity in hippocampal cultures, and protect them against glucose deprivation by more effective peroxide scavenging (Cheng and Mattson, 1995). Melatonin has also been investigated widely, and the mechanism of its protective role is still controversial. While one report indicates that its protective effects are due to enhancement of GSH-Px and SOD gene expression (Rodrigue et al., 1998), other argues that melatonin performs its effects by directly scavenging free radical (Daniels et al., 1998).

Currently, there is no report on effects of growth factors on astrocytes GSH-Px activity.

1.5.2 Thioredoxin dependent system in neural cells:

Thioredoxins: Thioredoxin plays several important biologic roles both in intracellular and extracellular compartments with its redox-regulating and reactive oxygen intermediates scavenging activities. Most of the knowledge on Trx(SH)2 presence in the mammalian CNS comes from in vivo in situ hybridization and
immunohistochemical studies. The distribution of this protein in neural cells in culture is still unclear. Asahina et al. (1998) (Asahina et al., 1998) showed that in humans, astrocytes and schwann cells express Trx(SH)2 as it has been shown by immunocytochemical in situ hybridization and RT-PCR techniques. But in the rodent CNS, Trx(SH)2 seems to be mostly expressed in neurons, ependymal and endothelial cells, and not in glial cells (Lippoldt et al., 1995; Mansur et al., 1998; Stemme et al., 1985; Tomimoto et al., 1993). A single report of mitochondrial Trx(SH)2 (Trx-2) expression in the rat neurons has also been published (Rybnikova et al., 2000). Using immunocytochemistry as well as in situ hybridization, the authors have shown that Trx-2 mRNA and protein is found predominantly in neurons.

Expression of Trx(SH)2 in rat astrocytes has been observed after exposure to ischemia, or ROS. Using immunocytochemistry, it has been shown that only 24 h after induction of global ischemia in gerbil astrocytes began to express Trx(SH)2 protein, and this upregulation lasted for 7 days (Tomimoto et al., 1993). Astrocytoma cells release Trx(SH)2 in response to low doses of H2O2, thereby increase neuronal survival (Hori et al., 1994). After ischemia induction using MCA occlusion, the Trx signal (protein and mRNA) decreased in the ischemic core, but in penumbra, it appeared 4 h after MCA occlusion and continued for 24 hours (Takagi et al., 1998c). This group also reported protective effects of Trx overexpression in mouse. These authors reported that Trx overexpression decreased the infarct area and volume as well as the neurological deficits (Takagi et al., 1999). The protective effects of Trx(SH)2 overexpression for ischemic brain was also evident when carbonyl content as well as c-fos expression were compared in transgenic and wild type animals; this showed
higher level of oxidative stress induced changes in wild type mice than transgenic mice (Takagi et al., 1999). Overexpression of Trx(SH)2 also attenuates the kainic acid-induced excitotoxic hippocampal injury in transgenic mice (Takagi et al., 2000). Enhanced expression of Trx(SH)2 protein and mRNA has been shown in human endothelial cells as well as macrophages in atherosclerotic plaques (Takagi et al., 1998a). These authors showed that induction of endothelial injury, using balloon-induced injury in rat arteries, increased Trx(SH)2 protein and mRNA in the neointimal regenerating endothelial cells: this seemed to be caused by NO° and peroxynitrite. These reactive intermediates fail to induce Trx expression in Trx-transfected cells (Takagi et al., 1998a).

All these reports indicate that Trx(SH)2 is a stress-inducible protein. Trx(SH)2 might be involved in intracellular signaling pathways, as it has been shown to translocate into the cell nucleus after ischemia and ischemia-reperfusion (Takagi et al., 1998b). Trx(SH)2 is a direct inhibitor of apoptosis signal-regulating kinase (ASK)1, a MAPKKK, that is necessary for TNF-α induced apoptosis (Saitoh et al., 1998). The Trx-R is responsible for maintenance of Trx(SH)2 levels.

**Thioredoxin Reductase:** Trx-R has been shown in the mammalian CNS: In rat spinal cord neurons (Stemme et al., 1985), where it is synthesized in the soma, and rapidly transported in axons both in anterograde and retrograde directions; in neuronal cells of the rat brain (Rozell et al., 1985): in developing as well as in mature retina (Hansson et al., 1989). and in tissue cultures of astrocytes as well as neurons (Eftekharpour et al., 2000).
The protective effects of Trx-R in brain are not well known. The brain Trx-R enzymatic activity is upregulated in Alzheimer’s disease patients brain in response to increased oxidative stress (Lovell et al., 2000). Treatment of hippocampal cultures with Trx(SH)2 or Trx-R in combination with toxic doses of amyloid beta-peptide led to a statistically significant concentration-dependent enhancement in cell survival against amyloid beta-peptide-mediated toxicity (Lovell et al., 2000). Inhibition of Trx-R by 1-chloro-2,4-dinitrobenzene (DNCB) in PC12 cells as well as rat glial cultures causes cell death (Ishikawa et al., 1999). NO° and NO° donors inhibit DNCB-induced cell death in both types of cells. The functional role of Trx-R as a peroxide scavenger in neural cell biology remains to be investigated.

**Peroxiredoxins:** The distribution and functional importance of thioredoxin peroxidase / Prx enzymes in mammalian CNS is not quite clear.

In a set of experiments using northern and Western blotting, (Sarafian et al., 1998) investigated the levels of Prx1 and Prx2 mRNA and protein in human tissues and rat brain cell cultures. They showed high levels of Prx1 and Prx2 mRNA in astrocytes, while relatively low levels of Prx1 mRNA was detected in neuronal and oligodendroglial cultures. Microglial cultures also had high levels of Prx1. Besides astrocytes, Prx2 mRNA was high in neurons and oligodendrocytes and low in microglia. In another report, the same group (Sarafian et al., 1999) using immunohistochemical techniques, studied the Prx1 and Prx2 protein expression in post-mortem human brain. They reported exclusive expression of Prx1 in glial cells, specifically in astrocytes and ependymal cells. Except for large cortical neurons, Prx1 signal was not found in neurons. Prx2 was found in neurons as well as axons and infarct areas associated with
axonal swelling, while astrocytes did not express Prx2. The importance of differential expression is not known yet. The preferential expression of Prx-1 in glial cells in rat brain has also been shown by Mizusawa et al., (Mizusawa et al., 2000).

Prx 4 expression has been studied in the rat brain as well as other tissues (Matsumoto et al., 1999). These authors showed that Prx4 is found in plasma, lung, liver, spleen, pancreas as well as testis, but not in the brain and heart.

Differential Prx peroxide scavenging activities among neural cells has not been investigated. The catalytic efficiency of mammalian peroxiredoxins is significantly lower than that of catalase or GSH-Px (Rhee et al., 1999), but because of restricted localization of catalase in peroxisomes, as well as low levels of cytosolic GSH-Px, the high levels of Prx expression in all tissues (0.2-0.4% of total soluble protein) may indicate their functional importance as alternative ROS scavengers.
2. AIMS

A wealth of evidence indicates that in neural cells, the GSH-dependent system and Trx-dependent system, two major peroxide scavenging systems, play central roles in management of oxidative stress (see sections 1. 4. 3 and 1. 4. 4). During oxidative stress in the course of stroke and ischemia, cancer or normal aging, peroxides as well as other ROS can oxidize almost all macromolecules in the cell and change the reductive conditions of the cytosol. These oxidative events change the molecular mechanisms that can lead to cell injury or death. Members of GSH and Trx systems remove the oxidants and restore the redox balance of the cell.

The susceptibility of neurons to oxidative stress and the astrocytes’ ability to resist the oxidative conditions is well known. Some aspects of the role of GSH dependent system in peroxide detoxification has been compared in oligodendroglial and astrocytes (Juurlink et al., 1998). and between rat neurons and astrocytes (Dringen et al., 1999a). The distribution and functional importance of Trx system in brain cells is not clear. It is possible that astrocytes ability to better resist the oxidative stress conditions. is due to having a better developed Trx system. Tissue culture is widely used to study the biology of isolated neural cells in different conditions, such as ischemia and oxidative stress or to address the effects of drugs, and the results seem to be reasonably applicable to in vivo condition (Juurlink et al., 1992).

In these studies I wanted to compare the distribution of GSH as well as Trx dependent systems in astrocytes and neurons. My other aim was to examine the effect
of the upregulating the peroxide scavenging systems in these cells, to determine whether they could better withstand the oxidative stress conditions.

2.1 Hypotheses.

2.1.1 The ability of astrocytes to better withstand the oxidative stress than neuronal cultures is due to a) Higher levels of the GSH dependent system, and b) Higher levels of the Trx dependent system.

2.1.2 Upregulation of GSH and Trx systems in neural cells better enables them to withstand the cellular oxidative conditions.

2.2 Objectives.

The following research objectives are addressed.

2.2.1 To examine the distribution of GSH, GSSG-R activity and GSH-Px activity and protein in astrocytes and neurons.

Although astrocytes are known to have higher GSH and GSH-Px activity than neurons, the relative distribution of GSSG-R activity and correlation of GSH-Px activity and protein in these two cells are yet to be determined.

Question 1.1. What is the GSH-Px activity of neural cells and what is the correlation of activity and GSH-Px protein content.

Expectations: I expected that GSH-Px activity would be higher in astrocytes than cortical neurons, and GSH-Px protein contents correlate with the enzymatic activities.
**Approach:** GSH-Px activity is measured according to (Prohaska and Ganther, 1976), and GSH-Px protein levels will be measured using Western blotting.

**Question 1. 2.** Is there a correlation between GSH-Px activity and GSH content?

**Expectations:** I expected that GSH levels would correlate with GSH-Px activity. If high levels of GSH were seen in cells with low GSH-Px activity, this would show other potential roles for GSH. It is also possible that other peroxide scavenging systems such as Trx dependent system could be active in such cells.

**Approach:** GSH content is measured as has been shown by (Fernandez-Checa and Kaplowitz, 1990).

**Question 1. 3.** Is there a correlation between GSH level and GSH reductase (GSSG-R)?

**Expectation:** I expected that GSH levels would correlate with GSSG-R activities.

**Approach:** Since GSH levels depend on GSH synthesis as well as reduction of oxidized glutathione, both mechanisms can be tested as was shown by (Juurlink et al., 1998). For measuring GSH synthesis activity, one could deplete the cellular GSH in both cells and compare their ability to regenerate their original levels of GSH. and the GSSG-R activity is to be measured by the method of (Eklow et al., 1984).

2. 2. 2. To determine whether GSH-Px activity in astrocytes is upregulated by growth factors
Tyrosine kinase receptors for a variety of growth factors have been shown on neuronal cells in vivo and in vitro. NGF, PDGF and BNDF have been shown to upregulate GSH-Px in neurons and neuronal cell lines (Jackson et al., 1994; Mattson et al., 1995; Sampath et al., 1994; Spina et al., 1992). The astrocytes response to growth factors is not known.

**Question 2. 1.** Do growth factors upregulate GSH-Px and/or Prx activity in astrocytes?

**Expectation:** It is expected that GSH-Px and Prx activity in astrocytes should be upregulated by growth factors.

**Approach:** GSH-Px and Prx activity (Chae et al., 1999) activity is measured in growth factor-treated astrocytes.

2. 2. 3. **To determine the distribution of Trx-related system in neuronal cells (cortical neurons), and glial cells (astrocytes).** The Trx-related system is composed of Trx, Trx-R and Prx.

Although there is one study on Prx 1 and 2 proteins in tissue cultured rat brain cells (Sarafian et al., 1998), the distribution of the other proteins are not known.

**Question 3. 1.** Do astrocytes have higher Trx, Prx(s), Trx-R protein and enzymatic activities than neurons?

**Expectation:** I expect that the Trx dependent system is more pronounced in astrocytes than neurons.
**Approach:** Western blot analysis to determine the protein levels, using polyclonal antibodies for Trx (Luthman and Holmgren, 1982). Prx (Kang et al., 1998a), and Trx-R (Rozell et al., 1985). The functional Trx is measured according to (Holmgren and Bjornstedt, 1995; Luthman and Holmgren, 1982). Trx-R is measured according to (Arner et al., 1999) and the total Prx activity is assayed according to (Chae et al., 1999).

2. 2. 4. To determine whether components of Trx-related peroxidase system (Prx/Trx-R) or GSH-related system behave as phase 2 enzymes in neural or glial cells.

Members of phase 2 enzyme family are upregulated by electrophile agents. Phase 2 enzymes scavenge the oxidants or increase reducing equivalents such as GSH. tertiary-butylhydroquinone (tBHQ) is a classical phase 2 enzyme inducer that could be used to address this objective.

**Question 4.** Are GSH and Trx related enzymes upregulated by phase 2 enzymes inducers?

**Expectation:** It is expected that phase 2 enzyme induction would increase these enzymes in astrocytes but not in neurons, since GST as well as quinone reductase activity is upregulated in rat cerebellar glial cultures but not in cerebellar neurons (Ahlgren-Beckendorf et al., 1999).

**Approach:** Enzymatic activities and protein levels will be measured according to the above mentioned techniques (Question 3).
2.2.5. To examine the effect of phase 2 enzyme induction on astrocytes

GSH, cysteine and cysteinyl-glycine (CysGly).

The rate limiting enzyme for GSH synthesis, gamma-glutamylcysteine synthase is upregulated in response to the phase 2 enzyme.

**Question 5.** Does tBHQ treatment have any effects on GSH, cysteine and CysGly in astrocytes?

If these thiol containing components are increased, would it be because of increase in the total (GSX) GSH+GSSG, or (cysteine+Cystine) or due to increased reduction of cystine/ GSSG? Since both GSSG-R as well as Trx and Trx-R can increase the reducing ability of the cell.

**Expectation:** It is expected that GSH levels are increased in tBHQ treated cells.

**Approach:** High performance liquid chromatography (HPLC) performed in our laboratory routinely according to the method of (Kamencic et al., 2000), is used to measure GSH, cysteine and CysGly. Reduced GSH also can be measured as has been shown by (Fernandez-Checa and Kaplowitz, 1990).

2.2.6. To examine the functional importance of phase 2 enzyme induction on the astrocytes' ability to cope with the oxidative stress

GSSG-R and Trx-R have been shown to reduce the oxidized proteins during oxidative stress (Wudarczyk et al., 1996) and inhibit opening of the permeability transition pore in mitochondria.
**Question 6.1** Does tBHQ treatment increase the ability of astrocytes to better withstand the oxidative stress condition?

**Expectation:** I expect that the ability of astrocytes to resist the oxidative stress is upregulated.

**Approach.** An in vitro model (Robb and Connor, 1998) is employed to induce the oxidative stress in astrocytes. Cells are exposed to high dose of tertiary-butylhydroperoxide for increasing amounts of time. Cell death is assayed by measuring lactate dehydrogenase activity in the medium. Oxidative stress levels also can be measured by 5-(and -6)-carboxy-2',7'-dichlorodihydrofluorescein (DCFH), an intracellular probe for oxidative stress (Husain and Juurlink, 1995).

Cell viability can be measured by mitochondrial membrane potential measurement in astrocytes under the oxidative stress conditions. by using Rhodamine 123 as has been shown by (Juurlink and Hertz, 1993).

**Question 6.2** If GSH was upregulated by phase 2 inducers, would glutathione depletion affect the protective effect of tBHQ treatment in astrocytes?

**Expectation:** GSH depletion should decrease cellular ability to stand the oxidative stress.

**Approach.** GSH can be depleted in astrocytes using a variety of drugs such as diethylmaleate and BSO. tBHQ treated, and control astrocytes as well as DEM treated of both groups, would be exposed to oxidative stress and cell death would be measured.

This can show if DEM treated controls and DEM + tBHQ treated astrocytes behave differently from non-DEM treated counterparts. If the DEM + tBHQ treated
astrocytes survived better than the control group or DEM treated astrocytes, then I would like to see if Trx-R inhibition can affect the oxidative stress levels/cell death in astrocytes.

**Question 6. 3.** Does inhibition of Trx-R by chemical inhibitors change the behavior of astrocytes under oxidative stress conditions?

**Expectation:** It is expected that Trx-R increases oxidative stress level and decreases the cell viability in astrocytes.

**Approach.** Trx-R will be inactivated by cis-diaminedichloroplatinum (II) (CDDP) (Sasada et al., 1996). Astrocytes will then be exposed to oxidative stress conditions and the level of cell death, cell viability and oxidative stress will be examined.

2.2.7. **To delineate the beneficial effects of phase 2 induction in astrocytes for neurons in transient cocultures.**

Better peroxide scavenging in neurons cocultured with astrocytes has been reported (Desagher et al., 1996). It has been proposed that astrocyte protect neurons by removing H$_2$O$_2$ from extracellular space and not by any other protective mechanism. Astrocytes are also known to release GSH precursors in the medium that can increase GSH content in neurons (Dringen et al., 1999b).

**Question 7. 1.** Does transient astrocyte-neuron coculture increases peroxide scavenging abilities of neurons and /or increase their GSH content?
**Expectation:** In these experiments, I expect to see a faster H$_2$O$_2$ removal from the incubation medium. Also, GSH level should be increased in neurons.

**Approach.** A model of transient coculture is used as has been used by Dringen et al., (1999) (Dringen et al., 1999b). H$_2$O$_2$ scavenging in control cortical neurons as well as in cortical neurons cocultured for 24 h in the presence of astrocytes (cultured on tissue culture inserts) is checked. After 24 or 72 h, astrocytes are removed from cortical neuron cultures, and neuronal medium is washed twice with Pucks solution (37° C). Then cells are incubated (5 min. 37° C) with a HEPES-incubation buffer. Cells are then switched to HEPES-incubation buffer (37 °C) containing 100 μM hydrogen peroxide. At different time intervals after exposure to H$_2$O$_2$, 50 μl of the incubation buffer is taken for further measurement of remaining H$_2$O$_2$ in the buffer using a method according to (Dringen and Hamprecht, 1997). GSH levels can be determined by monochlorobimane assay.

**Question 7. 2.** Does tBHQ treatment in astrocytes prior to incubation with neurons affect neuronal peroxide scavenging capability and/or neuronal GSH content?

**Expectation:** Since GSH levels in neurons have been shown to upregulate in astrocyte-neuron cocultures (Dringen and Hamprecht, 1997) I will expect to see an improved peroxide scavenging ability in neurons incubated with tBHQ-treated astrocytes.

**Approach.** Astrocytes are treated with tBHQ for 24 h. The medium is then removed and fresh growth medium is added to astrocytes. Astrocytes are then incubated with neurons and after 24 hours, the H$_2$O$_2$ scavenging ability of control
neurons. Neurons cocultured with astrocytes with and without tBHQ is measured. GSH levels of the all these groups is also measured.
Chapter 3

DIFFERENTIAL EXPRESSION OF GLUTATHIONE AND THIOREDOXIN
DEPENDENT PEROXIDE SCAVENGING SYSTEMS IN MOUSE
CORTICAL ASTROCYTES AND CORTICAL NEURONS

Adapted from:
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3.1. Introduction

Since the production of oxygen in the atmosphere, cells have been exposed to oxidative damage. Different byproducts of oxygen such as superoxide radical anion, hydroxyl radical, hydrogen peroxide, which are known as reactive oxygen species (ROS), are produced during the normal physiologic and pathologic conditions (for review see Juurlink (1999). In a healthy cell the levels of ROS and antioxidant mechanisms are balanced. As a result of increased production of ROS and/or decreased antioxidant capacity during the course of aging or neurodegenerative diseases, oxidative stress develops and disturbs critical cellular systems by damaging macromolecules and by altering the redox balance of the cell. The cells of the adult brain are highly susceptible to oxidative stress. This organ comprises only 2% of the body weight, but about 20% of total oxygen used by body is utilized by the brain (Dringen. 2000). Mitochondria are the major source of ROS (Chance et al., 1979); superoxide anion produced in this organ is reduced by superoxide dismutase (SOD) to hydrogen peroxide. (Fridovich. 1996). Although hydrogen peroxide is relatively innocuous, it is highly diffusible and can affect neighboring cells. Hydrogen peroxide can interact with transition metal ions and generate the hydroxyl radical, a strong oxidant, via the Haber-Weiss reaction. Hydroxyl radicals can initiate a chain of self-propagating oxidation reactions that lead to lipid peroxidation and targets the polyunsaturated fatty acids of the extensive membranous system of the brain cells (Juurlink. 1998). Proper scavenging of peroxides is an important way to prevent hydroxyl radical formation and interrupt the lipid peroxidation cascade. Brain cells are
equipped with enzymatic and nonenzymatic systems such as catalase, glutathione peroxidase (GSH-Px), vitamin E and C and the most abundant thiol of the body, glutathione (GSH) to protect against peroxides. The brain exhibits low catalase activity (Mirault et al., 1994); furthermore, this enzyme is mainly restricted to peroxisomes (Moreno et al., 1995). Catalase also has a low affinity, although high capacity, for hydrogen peroxide (Simmons and Jamall, 1988); in addition, catalase cannot scavenge organic peroxides. Although vitamins E and C are involved in the scavenging of lipid peroxyl radicals, they cannot efficiently terminate the lipid peroxidation cascade due to the inability to scavenge the resulting lipid hydroperoxides formed that can be converted by transition metal ions to lipid peroxyl and alkyl radicals and thereby initiate new cascades of lipid peroxidation (Juurlink, 1999). Therefore, the brain must rely on other peroxide scavengers such as GSH-Px or members of the thioredoxin (Trx(SH)₂)-dependent peroxidases (peroxiredoxins). GSH-Px is more efficient than catalase and SOD in decreasing cellular oxidative stress (Michiels et al., 1994). GSH-Px peroxidase activity requires GSH as the electron donor to scavenge peroxides, during which GSH is oxidized to GSSG and glutathione reductase (GSSG-R) is responsible for regeneration of GSH. Low levels of GSH is known to be a major cause of susceptibility of oligodendroglial to oxidative stress and upregulation of GSH levels increases the resistance of oligodendroglial to oxidative insults (Thorburne and Juurlink, 1996).

The Trx(SH)₂ family is another system with peroxidase scavenging abilities (Björnstedt et al., 1995; Sen, 1998). Trx(SH)₂ is a 12 kDa dithiol protein that is present at a much lower concentration (~10 μM) than the monothiol GSH (Björnstedt et al., 1997) but has a low redox potential (Holmgren, 1985: Arner and Holmgren, 1985).
and catalyzes reduction of disulfides many orders of magnitude faster than GSH. Trx(SH)$_2$ is a stress inducible protein whose expression is enhanced by viral infection and oxidative stress (Ohira et al., 1994; Bertini et al., 1999). During exposure of astrocytoma cells to mild concentration of hydrogen peroxide, Trx(SH)$_2$ is secreted and it is suggested that such secretion by glia may have neuroprotective functions (Hori et al., 1994). In a rat model of ischemia, Trx(SH)$_2$ expression in the penumbra was increased suggesting its involvement in ROS removal or regulation of cell signaling during ischemia (Takagi et al., 1998). The disulfide in oxidized-thioredoxin (TrxS$_2$) is reduced by TrxS$_2$ reductase (Trx-R) using NADPH as the electron donor (Arner and Holmgren, 2000). Mammalian Trx-R is a dimeric selenoprotein with a molecular weight of 55 kDa or higher. The active site of the enzyme is a selenenyl sulfide/selenol thiol (Zhong et al., 2000) and the enzyme has inherent hydrogen peroxide reducing activity (Zhong and Holmgren, 2000). We have previously shown that both cytosolic and mitochondrial Trx-R can be induced by a phase 2 enzyme inducer (Eftekharpour et al., 2000). Trx-R, itself, and particularly with selenocystine as a cofactor, can directly reduce lipid hydroperoxides using NADPH as an electron donor (Björnstedt et al., 1995).

The peroxiredoxins (Prx’s) I, II and III have demonstrated peroxide-scavenging capabilities (Chae et al., 1999) with the electron donor being Trx(SH)$_2$. Prx III is found only in mitochondria while PrxI and PrxII are cytosolic and comprise up to 0.4% of the total soluble proteins (Rhee et al., 1999). The functional importance of this family of peroxide scavengers in the CNS is not known.

In this study we have investigated the differential distribution of GSH and Trx(SH)$_2$ systems in mouse cortical astrocytes and cortical neurons, we also have
addressed the effects of growth factors on GSH and Trx(SH)$_2$-dependent peroxide scavengers in astrocytes.

3.2. MATERIALS AND METHODS

Cell Cultures:

Mouse astrocytes and cortical neurons were cultured as previously described (Juurlink and Walz, 1999). Briefly, for astrocyte cultures CD1 mouse newborns were killed using an overdose of the anaesthetic Metophane as approved by Canadian Council on Animal Care. Neopallial tissue was isolated and disassociated into a single cell suspension, planted at a low cell density (3 × 10$^5$ cells) into 60 mm Falcon tissue culture dishes. The growth medium was comprised of Dulbecco’s minimal essential medium (DMEM, from Gibco-BRL) supplemented with 7.5 mM glucose, 15 mM NaHCO$_3$, 2 mM glutamine and 10% (v/v) horse serum (Summit Biotechnology, Fort Collins, CO). Cultures were fed 3 times per week. Confluent cultures were used when 17-21 days old.

Cortical neurons were cultured from CD1 E15 mouse embryos. Neopallia were isolated, washed with Puck’s solution, trypsinized with 0.2% trypsin (1:250 trypsin obtained from Gibco-BRL) for 2 min at room temperature, and triturated with a glass Pasteur pipette. Cells were counted and plated on Falcon 60 mm poly-D-lysine-coated 35 mm Falcon tissue culture dishes at 3 × 10$^6$ per dish. Primary growth medium was DMEM supplemented with 2 mM glutamine, 14 mM NaHCO$_3$, 15 mM HEPES, 30 mM glucose, and 5% horse serum. The antimitotic agent, fluorodeoxyuridine (10 μM), and uridine (40 μM) were added to the cultures on day 3; after 24 h, the growth medium was changed to secondary medium: DMEM supplemented with 27.5 mM glucose, 15 mM NaHCO$_3$, 0.3 mM glutamine, 2 mM
alpha-ketoglutarate, and 5% (v/v) horse serum. Cultures were used on days 5-6.

**Biochemical Assays:**

*Preparation of cultures for biochemical assays:*

Astrocytes or cortical neurons were washed twice with ice-cold phosphate-buffered saline (PBS). Total cellular protein was extracted using a tissue extraction buffer [0.1 M KCl, 0.02 M K2HPO4, 0.001 M EDTA, 0.5% (v/v) Triton X-100, adjusted to pH 7.0 with HCl]. Cells were sonicated on ice for 3 x 5 seconds with 1 minute intervals. Centrifuged at 15,000 g for 10 min at 4° C, the supernatants were used for the western blotting and enzymatic assays. A bicinchoninic acid protein assay (Smith et al., 1985) was used to determine the total protein content with bovine serum albumin as the protein reference standard.

*Glutathione Peroxidase Assay:*

Total GSH-Px activity assay was measured according to Prohaska and Ganther (1976) with cumen hydroperoxide as substrate. Cumen hydroperoxide was added (final concentration of 0.15 mM) to a reaction mixture containing 0.1 M phosphate buffer (pH 7.00), 1 mM EDTA, 100 μl of sample, 1 mM GSH, 0.24 U GSSG-R, and 0.11 mM NADPH. Absorbance was measured at 340 nm. The GSH-Px activity was calculated based on a molar absorption coefficient of NADPH of 6270 M⁻¹ cm⁻¹ (Mizuno, 1984)

*Peroxiredoxin Assay:*

Prx activity was assayed essentially according to Chae et al. (1999). In brief, the reaction mixture contained 50 mM HEPES-NaOH pH 7.00, 250 μM NADPH, 50 nM mammalian TrxR, 5 μM E. coli Trx(SH)₂ and 20 μM H₂O₂. After 5 min
incubation at 30° C, the reaction was started by adding 50 μl of the samples. The disappearance of NADPH was monitored by a spectrophotometer at 340 nm. Calculation of Prx activity was based on a molar absorption coefficient of NADPH of 6270 M⁻¹ cm⁻¹.

*Functional Trx(SH) content:*

Functional Trx(SH)₂ was assayed enzymatically as previously described (Luthann and Holmgren. 1982; Holmgren and Björnstedt and Holmgren. 1985). In brief, for each sample, 20 μg of total protein was incubated at 70° C for 10 min to inactivate endogenous Trx-R. An activation mixture (2 μl) containing 50 mM HEPES, 1 mM EDTA, 1 mg/ml BSA and 2 mM dithiothreitol was added to heat-inactivated samples and incubated at 37° C for 15 minutes. The reaction mixture containing HEPES, EDTA, NADPH and insulin was added to each sample. The reaction was started by adding 1 unit of Trx-R for each sample and incubating at 37° C for a further 20 min. The reaction was stopped by adding 0.5 ml of 0.4 mg dithionitrobenzoic acid (DTNB)/6 M guanidine hydrochloride in Tris-HCl, pH 8.0. Functional Trx(SH)₂ activity was calculated based on the difference of absorbance between the sample and the relevant Trx-R-absent control at 412 nm using an extinction coefficient of 13600 M⁻¹ cm⁻¹ for the formed 5'-thionitrobenzoic acid. One unit of activity is defined as 1 nmole of 5'-thionitrobenzoic acid that was formed. A standard curve of known concentration of Trx(SH)₂ was used as a reference.

*Western Blots*

A standard western blotting technique was performed. A total of 40 μg of protein for GSH-Px, 20 μg for Trx(SH)₂ and 2.5 μg protein for Prx per lane was separated on 12% polyacrylamide gels, using the BioRad mini gel electrophoresis.
system. Using a semi-dry transfer system, proteins were transferred to PVDF membrane. GSH-Px1 protein was detected using a polyclonal rabbit antibody (Mirault et al., 1991; Trepanier et al., 1996) at a dilution of 1 in 10,000. Expression of Prx proteins was assessed by use of rabbit polyclonal antibodies as described by Kang et al. (1998) at a dilution of 1: 5,000. Trx(SH)$_2$ protein was detected by using a Trx(SH)$_2$ antibody described previously (Luthman and Holmgren, 1982; Rozell et al., 1985). A secondary peroxidase conjugated antirabbit antibody (Bio Rad) was used at a dilution of 1: 10,000. Proteins were detected using DuPont NEN Renaissance chemiluminescence reagents (Mandel Scientific, Ltd. Guelph, ON) according to the manufacturer’s instructions. Densitometric analyses of protein levels were done using NIH image analyzing software.

3.3 RESULTS

For description of results, astrocytes were used as the reference. Astrocytes have a higher (~ 5 folds) GSH-Px activity than neurons (Fig. 3.1); this correlates well with the relative GSH-Px protein levels in the two cell types (Fig. 3.2). Astrocytes showed higher (1.5 ± 0.13 x) functional Trx(SH)$_2$ content than cortical neurons (Fig. 3.3). This correlates well with lower (0.51 ± 0.12 x) Trx(SH)$_2$ protein level (Fig. 3.4) in cortical neurons. Prx activity is also lower (0.60 ± 0.05 x) in cortical neurons than in astrocytes (Fig. 3.5). Western blotting analyses showed abundant amounts of peroxiredoxin in neural cells, with Prx I and III more abundant in astrocytes and with cortical neurons having higher Prx II protein levels (Fig. 3.6). When compared to a series of increasing amounts of purified Prx I, Prx1 protein forms 0.8-1% of all extractable protein in astrocytes. A number of growth factors were examined for their ability to increase GSH-PX and Prx activities in astrocytes. GSH-Px activity was

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upregulated in astrocytes using NGF and EGF (Fig. 3.7). These growth factors had no effect on total Prx activity in astrocytes nor in neurons (results not presented).
Figure 3.1. GSH-Px activity in astrocytes and neurons.

Represented are the means ± SD (n=6) from two separate culture batches of neurons and astrocytes. GSH-Px activity in astrocytes is significantly greater than in neurons (P< 0.0001, two-tailed Student’s t-test).
Figure 3.2. GSH-Px protein expression in astrocytes and neurons.

Representative Western blot (four culture batches were examined) showing GSH-Px 1 protein in astrocytes and cortical neurons. 40 μg of total protein was used for electrophoresis. Densitometric analysis showed that cortical neurons have very low GSH-Px 1 protein level. (<0.2 x that of astrocytes). In some cases cortical neuron GSH-Px1 was not detectable. The left lane contains 10 μg of bovine GSH-Px protein. (P<0.0001, Welch’s t-test).
Figure 3.3. Functional Trx(SH)$_2$ content of astrocytes and neurons.

Due to variability between cultures, the results were normalized to astrocytes functional Trx(SH)$_2$. This graph represents the means ± SD from two separate sets of astrocytes and cortical neuron cultures (n=6). Astrocytes have significantly higher Trx activities than cortical neurons (P<0.0001, two-tailed Student's t-test).
Figure 3.4. **Trx(SH)$_2$** protein expression in astrocytes and neurons.

Representative Western blot (three culture batches were examined) data demonstrating Trx(SH)$_2$ protein level in astrocytes and neurons. 20 µg of total protein was loaded in each lane. Densitometric analysis of Western blots showed that Trx(SH)$_2$ protein of cortical neurons was 0.51± 0.12 of astrocytes (n=3, P=0.0044, two-tailed Student’s $t$-test).
Figure 3.5. **Total Prx activity in astrocytes and neurons.**

Results are represented from two different batches of cultured neurons (n=6) and three batches of astrocytes (n=9). Results are means ± SD normalized to astrocyte activities. Astrocyte values are significantly higher (P=0.0485) than neuronal values (two-tailed Student's t-test).
Figure 3.6. Differential expression of Prx proteins in astrocytes and neurons.

Representative Western blots showing Prx1, 2 and 3 proteins in brain (A), cultured astrocytes (B) and cultured neurons (C). A total of 2.5 µg total protein was loaded per lane. The bands were analyzed using NIH densitometric software, normalized to astrocyte Prx content and results presented as histograms. Neurons have higher Prx2 levels (n=6) while abundant Prx1 and 3 are found in astrocytes (n=5). Two-tailed Student’s t-test showed significant difference between astrocytes and neurons for all three Prx isoforms, P <0.0001.
**Figure 3.7. Effect of Growth factors on GSH-Px activity of astrocytes.**

NGF and EGF at 10 ng/ml increased GSH-Px activity in astrocytes. Results from two separate sets of astrocyte cultures are represented as mean ± SD (n=6). One-tailed Student’s t-test was performed, and the differences between basal and growth factor-induced GSH-Px activities are significant (P<0.0001).
3.4. DISCUSSION

Neurons have a high rate of oxidative metabolism and an extensive surface area of membranes with an abundance of polyunsaturated fatty acids. Metabolic disturbances result in extensive lipid peroxidation (Hall, 1997); therefore, neurons should have an efficient lipid peroxide scavenging system. There are two major mechanisms that can scavenge organic peroxides: the GSH and the Trx(SH)$_2$-dependent systems. Here we demonstrate that cortical neurons have approximately 20% of the GSH-Px activity and protein as have astrocytes. This is in agreement with the findings of Dringen et al. (1999) that astrocytes had a greater (by a factor of 3) ability to clear organic peroxides than did neurons in cultures prepared from embryonic rat brains. Our previous studies (Eftekharpour et al., 2000) showed that astrocytes have greater GSH content as well as greater GSH-R activity than neurons. Dringen et al. (1999) et al. have also demonstrated lower GSH levels in neurons than in astrocytes. We also examined the Trx(SH)$_2$-dependent peroxide scavenging system. We had previously shown (Eftekharpour et al., 2000) that astrocytes have significantly more Trx-R activity and protein than do cortical neurons. In the present investigation we demonstrate that astrocytes contain more Trx(SH)$_2$ protein and that this protein is functional as it can act as an electron donor in insulin disulfide reduction. When the peroxiredoxin proteins were examined by Western blotting Prx I was present in high amounts in astrocytes (forming ~ 0.8% of extractable proteins) and low in neurons (forming ~ 0.3% of extractable proteins). This is in agreement with previous
immunocytochemical findings (Sarafian et al., 1999; Mizusawa et al., 2000) reporting low expression of this protein in neurons and abundant presence in glial cells. In contrast, there was modestly more (20%) Prx II in neurons compared to astrocytes. This is in contrast to the immunocytochemical findings of Sarafian et al. (1999) who demonstrated that Prx II could only be found immunocytochemically in neurons and not in glia. This discrepancy between our present findings and that of Sarafian and colleagues may be due to fixative-dependent denaturation of antigenic sites recognized by the antibody used by Sarafian and colleagues. We also demonstrate that Prx III, the mitochondrial isoform (Chae et al., 1999), is also present in slightly higher amounts in astrocytes than in neurons. Overall, astrocytes contain more Prx protein than neurons. This increase in Prx protein correlated well with an almost 70% greater Prx activity in astrocytes compared to neurons. The presence of considerable amounts of Prx II and III in neuronal cells likely reflects the importance of these proteins in the CNS. Neurons have a higher rate of oxidative metabolism than astrocytes and therefore are exposed to more ROS produced in their mitochondria where Prx III is relatively abundant. Higher amounts of Prx II in neurons than astrocytes may also reflect another protective mechanism of these cells in response to elevated ROS levels. It has been shown that Prx II overexpression decreases ROS levels following oxidative stress in PC12 cells (Simzar et al., 2000). It may be that Prx plays a more important role in ROS scavenging than is apparent with the functional peroxiredoxin assay used in the present investigation. Overall, in neural cells Prx proteins appear to constitute 2-3% of the total extractable proteins in neurons and astrocytes; this high proportion suggests that Prx proteins are critical to cellular function.

It is intriguing that neurons, which produce strong oxidants more vigorously
than astrocytes and are especially vulnerable to lipid peroxidation, appear to have a less efficient peroxide scavenging capacity than astrocytes. GSH levels are approximately 2/3 of those found in astrocytes while GSH-Px activity is approximately 20% of that found in astrocytes. Similarly, Trx-R (Eftekharpour et al., 2000), enzymatically active Trx(SH)$_2$, and Prx in neurons is about 2/3 of that found in astrocytes. We therefore considered the possibility that the peroxide scavenging systems could be upregulated by growth factors. Indeed, Mattson and colleagues (1995) have previously shown that brain derived growth factor could increase GSH-Px activity in hippocampal neurons and thereby enabling the neurons to cope better with peroxide exposure. In the present investigation we examined the ability of epidermal growth factor (EGF), nerve growth factor (NGF), insulin-like growth factor 1 (IGF1) and fibroblast growth factor 2 (FGF2) to affect GSH-Px and Prx activity in neural cells. Of these growth factors, only EGF and NGF significantly increased GSH-Px activity in astrocytes but had no effect on Prx activity.

There are several other members of the Prx family described in mammalian cells: it may well be that some of these isoforms are present in higher amounts in neurons than in astrocytes: however, we did assess total Prx peroxidase activity and demonstrated that neurons have lower total Prx activity. Since both Trx(SH)$_2$ (Hori et al., 1994) and Prx IV (Okado-Matsumoto et al., 2000) are secreted proteins, it is possible that neurons are reliant upon Trx(SH)$_2$ and Prx IV secreted from astrocytes; however, it is not yet known whether astrocytes express Prx IV. Glutaredoxin, which catalyzes GSH-disulfide oxireductions, has been shown to act as an electron donor to human plasma glutathione peroxidase as well as to cellular GSH-GPx1 (Björnstedt et al., 1994). There may well be differences in glutaredoxin content between the two cell
types. Another enzyme family that can scavenge peroxides and other lipid oxidants such as 4-hydroxynonenal forming glutathiol adducts is glutathione S-transferase (Goon et al., 1993). To our knowledge, no quantitative examination of the enzymatic activities of this family has been performed in neural cells. It is very possible that these glutathione S-transferases play a more important role in neurons than in astrocytes; however, these enzymes cannot reduce hydrogen peroxide and require GSH, the levels of which are lower in neurons.
3.5. REFERENCES


Chapter 4
Thioredoxin Reductase And Glutathione Synthesis Is Upregulated By t-Butylhydroquinone In Cortical Astrocytes But Not In Cortical Neurons

Adapted from the original publication:
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4.1. INTRODUCTION

Peroxides, whether inorganic or organic, give rise to strong oxidants (Halliwell and Gutteridge, 1989); hence, peroxide scavenging is central to the ability of a cell to minimize oxidative stress. Peroxide scavenging is especially important in the CNS since it is particularly vulnerable to oxidative stress. The reasons for this include a high rate of oxidative metabolism that results in high rates of strong oxidant formation (Sokoloff, 1989), the large surface area comprised of cell membranes (Peters et al., 1991) which contain an abundance of polyunsaturated fatty acids (Agranoff et al., 1999) that are liable to lipid peroxidation (Braughler and Hall, 1989) and the ease with which oxidative insults result in initiation of excitotoxic cascades (Dugan and Choi, 1994). There are two major peroxide-scavenging systems that are defined by the electron donor used: the glutathione (GSH)-dependent (Ursini et al., 1995) and the thioredoxin (Trx-(SH)2)-dependent (Chae et al., 1999) peroxidases. There are marked differences in the cellular concentrations of the two electron donors. Cellular GSH concentration ranges from ~2 to 10 mM, depending upon the cell type (Uhlig and Wendel, 1992) while Trx-(SH)2 tissue concentrations tend to be around 10 μM or less (Björnstedt et al., 1997; Das and White, 1998).

Because of the sequential reactions of two GSH molecules with glutathione peroxidase in the scavenging of peroxide, increasing GSH concentrations markedly increases the peroxide scavenging efficiency (Carsol et al., 1997) (as an example note Thorburne and Juurlink, 1996). The cellular concentration of GSH is related to how readily glutathione reductase (GSSG-Red) reduces oxidized-glutathione (GSSG) and how quickly GSH is synthesized de novo (Meister, 1983). Under conditions of severe
oxidative stress much of the tissue GSH forms glutathione-protein mixed disulfides (Ravindranath and Reed, 1990; Schuppe et al., 1992; Shivakumar et al., 1995) and such oxidized-glutathione is unavailable for reduction by GSSG-Red: hence, the ability to synthesize GSH de novo becomes critical.

Trx-(SH)$_2$ not only acts as an electron donor for Trx-dependent peroxide scavenging but it can act as an electron donor to reduce oxidized-glutaredoxin enabling glutaredoxin to reduce glutathione-protein mixed disulfides, thus releasing GSH (Jung and Thomas, 1996). The low concentrations of Trx-(SH)$_2$ in tissues necessitates an efficient mechanism by which oxidized-Trx (Trx-S$_2$) is reduced. Trx-S$_2$ is reduced by the selenoenzyme Trx reductase (TrxR) which uses NADPH as the electron donor (Björnstedt et al., 1997). There are two identified isoforms of TrxR, a cytosolic (Arner et al., 1999) and a mitochondrial one (Rigobello et al., 1998). Besides reducing Trx-S$_2$, TrxR has been shown to have a number of other important anti-oxidant functions that include: i) reduction of selinite to selenide (Björnstedt et al., 1996) ii) reduction of the ascorbyl radical (May et al., 1998) as well as oxidized-ascorbate (May et al., 1997) and lipoic acid (Arner et al., 1996), and iii) with selenocyst(e)ine as a cofactor. TrxR can also directly scavenge lipid peroxides (Björnstedt et al., 1995) and peroxynitrite (Arteel et al., 1999).

The rate-limiting enzyme for GSH synthesis is L-$\gamma$-glutamyl-cysteine synthase (GCS) (Meister, 1989). GCS is a heterodimer comprised of a catalytic and a regulatory subunit with both subunits being upregulated by phase II enzyme inducers. Electrophilic compounds known as phase II enzyme inducers (Prestera et al., 1993) activate transcription factor complexes that promote transcription of genes encoding phase II enzymes. The catalytic subunit is under the control of the anti-oxidant
response element (Galloway et al., 1997) while the regulatory subunit appears to be controlled by an AP1 binding element (Galloway and McLellan, 1998). Because of its important role in the anti-oxidant system of the cell, we hypothesized that TrxR would belong to the same synexpression group (Niehrs and Pollet, 1999) as GCS and that it would be upregulated by phase II enzyme inducers.

The objectives of the present experiments were to: i) determine whether the phase II enzyme inducer t-butylhydroquinone (tBHQ) (Prestera et al., 1993) would increase GSH content of neural cells, and ii) whether tBHQ would increase cytosolic and/or mitochondrial TrxR of neural cells. The neural cells examined were the astrocyte and the cortical neuron.

4.2. MATERIAL AND METHODS

Cell Cultures:

Mouse cortical astrocytes and neurons were cultured as previously described (Juurlink and Walz, 1998) with minor modifications. Briefly, for astrocyte cultures CD1 mouse newborns were killed using an overdose of the anaesthetic Metophane as approved by Canadian Council on Animal Care. Neopallial tissue was disassociated into a single cell suspension and planted at a low cell density (3 X 10^5 cells) into 60 mm Falcon tissue culture dishes. The growth medium was comprised of Dulbecco’s Minimal Essential Medium (DMEM from Gibco/BRL) containing 7.5 mM glucose, 15 mM NaHCO3, 2 mM glutamine and 10% (v/v) horse serum (Summit Biotechnology, Fort Collins, CO). Cultures were fed 3 times per week. Confluent cultures were used at the beginning of the third week.
Cortical neurons were cultured from CD1 E15 mouse embryos. Neopallia were isolated, washed with Puck's solution, treated with 0.2% trypsin (1:250 trypsin obtained from Gibco-BRL) for 2 min at room temperature, and trituated with a glass Pasteur pipette. Cells were counted and plated on Falcon 60 mm poly-D-lysine-coated 35 mm Falcon tissue culture dishes at $3 \times 10^6$ per dish. Primary growth medium was comprised of DMEM that contained 2 mM glutamine, 14 mM NaHCO$_3$, 15 mM HEPES, 30 mM glucose, and 5% horse serum. The antimitotic agent, fluorodeoxyuridine (10 μM), was added together with uridine (40 μM) to the cultures on day 3; after 24 h, the growth medium was changed to secondary medium containing DMEM, 27.5 mM Glucose, 15 mM NaHCO$_3$, 0.3 mM glutamine, 2m M alpha-ketoglutarate, and 5% horse serum. Cultures were used on days 5-6.

tBHQ was dissolved in dimethylsulfoxide (DMSO) and added to the cultures at final concentrations of 0, 10 and 20 μM. The final DMSO concentration in control and experimental groups was 0.1%. Cultures were harvested after either 12 or 24 hr.

Biochemical Assays:

Tissue and Cell Homogenization. Astrocytes or cortical neurons were washed twice with phosphate-buffered saline (PBS). Total cellular protein was extracted using a tissue extraction buffer [0.1 M KCl, 0.02 M K$_2$HPO$_4$, 1 mM EDTA, 0.5% (v/v) Triton X-100, adjusted to pH 7.0 with HCl]. Cells were sonicated on ice, centrifuged at 15,000 g for 10 min at 4°C, the supernatants were used in the enzymatic assays.

For separation of mitochondrial fraction, cells were harvested in PBS and homogenized (50 strokes) on ice with a Potter-Elvehjem homogenizer. To remove nuclei, the samples were centrifuged at 180 g for 10 min, the supernatants were
removed and centrifuged at 15,000 g for 10 min at 4° C. The supernatants were kept for cytosolic studies while the pellets were retained as the mitochondrial fraction. The pellets were then sonicated on ice, centrifuged at 15,000 g for 10 min and the supernatants retained for enzymatic assays.

Cerebral hemispheres were homogenized in 9 volumes of the above mentioned buffer using a Potter-Elvehjem homogenizer. Samples were centrifuged at 15,000 g for 10 min at 4° C. Supernatants were used for enzyme activity assay and western blotting. A bicinchoninic acid protein assay (Smith et al., 1985) was performed to determine the total protein content with bovine serum albumin serving as protein reference standard.

*Thioredoxin Reductase:*

Trx-R was measured according to two enzymatic assays described in Arner et al. (1999) that use either 5, 5'-dithiobis-(2-nitrobenzoic acid) (DNTB) or insulin as substrates (Arner et al., 1999).

For the DNTB assay, the reaction mixture contains 2 mM DNTB, 200 μM NADPH, 1% ethanol, 0.2 mg bovine serum albumin, 1 mM ethylenediaminetetra-acetic acid (EDTA) and 100 μl of sample in 0.1 M phosphate buffer (pH 7.0). TrxR activity was determined by the increase of absorbance at 412 nm, using an extinction coefficient of 13.600 M⁻¹ cm⁻¹ for the formed 5'-thionitrobenzoic acid. One unit of activity is defined as 1 nmole of 5'-thionitrobenzoic acid that was formed.

Insulin Reduction Assay: 100 μL of sample was added to a reaction mixture containing final concentrations of 150 μM NADPH, 160 μM insulin, 1 mM EDTA,
and 3 µM *E. coli* Trx in 50 mM Phosphate buffer, pH 7.0. The oxidation of NADPH at 340 nm (extinction coefficient 6270 M\(^{-1}\) cm\(^{-1}\)) was monitored and TrxR activity was expressed as nmols NADPH oxidized per min per mg protein. One unit of activity is defined as 1 nmole of NADPH oxidized.

*Glutathione reductase.* GSSG-Red activity was measured as according to the method described in Eklow et al. (1984) as we have done previously (Juurlink et al., 1998). The reaction mixture contained 1 mM oxidized-glutathione (GSSG) and 100 µL of sample in phosphate buffer (pH 7.0) containing 1 mM EDTA. The reaction started with the addition of NADPH (final concentration of 0.11 mM) and the decrease in absorbance of NADPH at 340 nm was measured. One unit of activity is defined as 1 nmole of NADPH oxidized.

*Glutathione.* GSH was measured by adding monochlorobimane to the culture medium to a final concentration of 100 µM, harvesting cells and measuring the formed monochlorobimane adduct in the sonicates using the procedure of Fernández-Checa and Kaplowitz (1990) as we have done previously (Juurlink et al., 1998).

*Western blots.* A standard Western blotting technique was performed. A total of 15 µg of protein was separated on 12% polyacrylamide gels, using the BioRad mini gel electrophoresis system. Using semi-dry transfer system, proteins were transferred to PVDF membrane. A rabbit polyclonal antibody previously described (Rozell et al., 1985) was used at a dilution of 1:8000. Proteins were detected using Du Pont NEN
Renaissance chemiluminescence reagents (Mandel Scientific Co., Ltd, Guelph, ON) according to the manufacturer’s instructions. Duplicate gels were always run and stained with Coomassie brilliant blue: this demonstrated that equal protein amounts were loaded in each lane. Densities of the Western blot bands were determined using NIH Image.

4.3. RESULTS

The TrxR activity in cerebral cortex was determined to be 62.4 ± 8.9 units/min/mg protein (n=3). Astrocytic activity was somewhat lower (~50 units/min/mg protein) and neuronal activity lower yet at ~40 units/min/mg protein (Fig. 4.1B). The difference in TrxR activity between neurons and astrocytes was mirrored by the amount of TrxR protein present in the cells as determined by Western blotting (Fig. 4.2). The addition of tBHQ increased TrxR activity of astrocytes as determined by DTNB reduction with little difference seen between the addition of 10 and 20 μM tBHQ (Fig. 4.1). Maximal activity was seen 24 hr following addition of tBHQ (Fig. 4.1) with half-maximal activity seen at 12 hr (data not presented). The second assay of TrxR using disulfide bonds of insulin as substrate gave a similar doubling of activity following tBHQ addition (Fig. 4.3). In contrast, the addition of tBHQ had no effect on neuronal TrxR activity (Fig. 4.1). In the astrocytes, both cytosolic and mitochondrial fractions exhibited increased TrxR activity following addition of tBHQ (Fig. 4.4). The increase in TrxR activity in astrocytes following tBHQ exposure was also reflected in increased protein in the Western blots (Fig. 4.5).

Basal GSSG-Red activity in astrocytes was ~55 units/min/mg protein and 20 units/min/mg protein in neurons (Fig. 4.6). No significant change in GSSG-R
activity occurred following tBHQ addition to neuronal cultures but a borderline
significant increase was seen when tBHQ was added to astrocyte cultures (Fig. 4.6).

The addition of tBHQ to astrocyte cultures resulted in a doubling of GSH
levels over 24 hr with half-maximal induction after 12 hr (Fig. 4.7B). In contrast
to astrocytes, neuronal GSH levels did not increase following addition of tBHQ
(Fig. 4.7).
Trx-R activity, as measured by reduction of DTNB, in astrocytes and neurons 24 hr after addition of 0, 10 or 20 μM tBHQ. The data represent the means ± SEM (n=6) from two separate batches of cultures. Basal astrocyte values are significantly greater than basal neuronal values (P<0.001, Welch’s t-test). The addition of tBHQ significantly increases Trx-R activity in astrocyte cultures (P<0.001, Welch’s t-test), with no differences seen between 10 and 20 μM tBHQ.
Figure. 4.1-B. Dose response of astrocytes Trx-R activity to tBHQ after 24 h incubation.

Results are mean ± SD of two independent experiments. (n=6, P<0.001) analyzed by Student’s t-test).

Figure. 4.1-C. Time course of the Trx-R activity of astrocyte cultures during incubation with 20 μM tBHQ.

Results are mean ± SD of two independent experiments. (n=6, * P<0.05, ** P< 0.0001), analyzed by Student’s t-test).
Figure 4.2. Differential expression of Trx-R protein in astrocytes and neurons

Representative Western blot (3 culture batches examined) showing Trx-R (15 μg protein loaded) from astrocytic (Ast) and cortical neuronal (CN) cultures. Densitometric analyses demonstrated that astrocytes expressed 1.72 ± 0.3 times more Trx-R protein/mg cell protein than did neurons.

Figure 4.3. Effect of tBHQ on Trx-dependent insulin reduction activity in astrocytes

Trx-R activity, as measured by insulin reductase activity, in astrocytes 24 hr after addition of 0 or 20 μM tBHQ. Represented are the means ± SEM from two culture batches. There was some variability in basal control values between the two culture batches (4.38 ± 0.77 and 7.43 ± 0.84 nmoles NADPH oxidized/min/mg protein respectively, with errors being SDs); hence, values are normalized to the control values of each culture batch. Addition of tBHQ results in significantly higher (P=0.0066, Welch’s t-test) insulin reductase activity.
Figure 4.4. Effect of tBHQ on astrocyte mitochondrial and cytosolic Trx-R

Trx-R activity, as measured by DTNB reduction, in the mitochondrial and cytosolic fractions of the same cultures 24 hr following exposure to either 0 or 20 μM tBHQ. Represented are means ± SEM (n=5) from 2 culture batches. The addition of tBHQ significantly increases Trx-R activity of both the cytosolic and mitochondrial fractions.
**Figure 4.5.** tBHQ upregulates Trx-R protein in astrocyte cytosolic and mitochondrial fractions.

Representative Western blot (2 culture batches examined) showing Trx-R (15 µg protein loaded) from cytosolic and mitochondrial fractions of astrocyte cultures 24 hr after addition of 0 or 20 µM tBHQ. Densitometric analyses demonstrated that tBHQ increased Trx-R protein 1.84 ± 0.57 and 1.74 ± 0.16 in cytosolic and mitochondrial fractions respectively.
Figure 4.6. Effect of tBHQ on GSSG-R activity of astrocytes and neurons

GSSG-Red activity in astrocyte and neuronal cultures 24 hr following addition of 0 or 20 µM tBHQ. For neurons, represented are means ± SEM (n=6) from two different culture batches while for astrocytes represented are means ± SEM (n=9) from 3 culture batches. tBHQ does not significantly increase GSSG-R activity in astrocytes nor neurons. Astrocyte GSSG-Red activity is significantly different from neuronal GSSG-R activity (P<0.001, Student’s t-test) and astrocytes exposed to tBHQ have significantly higher GSSG-R activity (P=0.0442) than control cultures.
Figure 4.7. Effect of tBHQ on GSH level in astrocytes and neurons

GSH content in astrocyte and neuronal cultures 24 hr following addition of either 0 or 20 μM tBHQ. Represented are the means ± SEM (n=7 to 9) from 2 separate culture batches of neurons and 3 batches of astrocytes. There is no significant difference between basal GSH levels in astrocytes and neurons with a two-tailed Student's t-test but a significant difference with a one-tailed Student's t-test (P=0.0309). The addition of tBHQ significantly increased the GSH content of astrocytes (P<0.0001, Welch's two-tailed t-test) but had no effect on neuronal GSH content.
Figure 4.7-B. Time course of the GSH content of astrocyte cultures during incubation with 20 μM tBHQ.

Results are mean ± SD of two independent experiments. (n=6, P<0.0001, analyzed by Student’s t-test).
4.4. DISCUSSION

Neuronal GSH content (~20 nmoles/mg protein) was found to be less than astrocyte GSH content (~28 nmoles/mg protein). These differences between the neural cell types are similar to those previously reported by Sagara et al. (1993) and Kranich et al. (1996). The addition of tBHQ to the culture medium caused a significant increase in GSH content only in astrocytes. This is similar to the findings of Iwata-Ichikawa et al. (1999) who demonstrated that when cultures of mesencephalic neurons and astrocytes are exposed to oxidants such as hydrogen peroxide or 6-hydroxydopamine, only astrocytes increased their GSH content. In the studies of Iwata-Ichikawa and colleagues this increase in GSH was correlated with an increase in the activity of GCS: hence, we interpret our results to indicate that GCS expression is upregulated in astrocytes but not in neurons. tBHQ has also been shown to induce the phase II enzymes quinone reductase and glutathione S-transferase in Bergmann glia but not in cerebellar granule cell neurons (Ahlgren-Beckendorf et al., 1999). These findings collectively suggest that neurons cannot upregulate expression of phase II enzymes in general. It is not clear why astrocytes can upregulate phase II enzyme gene expression but not neurons. One possibility is suggested by the findings of Iwata-Ichikawa et al. (1999) that oxidative stress increases API activity in astrocytes but not in neurons. Since API is a redox-sensitive transcriptional factor complex that is required for phase II enzyme induction, one possibility is that redox differences between astrocytes and neurons may account for such differences in ability to induce phase II enzyme expression. An upregulation of GCS in astrocytes and not in neurons can, nevertheless, be
neuronoprotective since astrocytes can facilitate neuronal synthesis of GSH (Sagara et al., 1993) by supplying the rate-limiting amino acid cysteine in the form of the dipeptide cysteinyl-glycine (Dringen et al., 1999).

The GSSG-Red activity of cerebral cortical neurons is very similar to the activity previously reported in rat cerebellar granule cell neurons (Huang and Philbert, 1995). The astrocytic GSSG-Red activity is somewhat lower than that reported both by (Huang and Philbert, 1995) and ourselves (Juurlink et al., 1998) for rat astrocytes. Such differences may reflect species differences. The ~22% upregulation of GSSG-Red following exposure of astrocytes to tBHQ is so modest that it likely does not represent an anti-oxidant response. Since GSSG-Red is very susceptible to oxidative inactivation (Barker et al., 1996; Tabatabaie and Floyd, 1994), it is possible that this modest increase in activity represents a decreased oxidative-stress-induced inactivation because of the higher cellular GSH content. The very modest increase in GSSG-Red induced by tBHQ is in agreement with our hypothesis that under conditions of oxidative stress, de novo synthesis of GSH is more important than reduction of GSSG, likely because GSSG tends to form mixed disulfides with protein sulphydryl groups.

Previously, only immunocytochemical studies have been reported for TrxR in the nervous system (Hansson et al., 1989; Rozell et al., 1985) with both reports suggesting more TrxR protein in neurons than glia. This is the first quantitative report comparing TrxR activity and protein content in neurons and astrocytes. Astrocytes have a greater TrxR activity and protein per mg cellular protein than neurons. What is especially intriguing is that in response to tBHQ, TrxR activity doubled in astrocytes but not in neurons. Two different assays for TrxR were used.
The first depended upon the reduction of Ellman's reagent. This reaction gave basal values in neurons and astrocytes ranging from 20-25 nmoles DTNB reduced/min/mg protein. The second assay, which is more specific, used the disulfide bonds in insulin as the substrate using a non-saturating concentration of E. Coli Trx (Luthman and Holmgren. 1982): here the basal activity was about 6 nmoles disulfide bond reduced/min/mg protein. The addition of tBHQ to the culture medium resulted in a doubling of TrxR activity, whether examined using DTNB as substrate or insulin as substrate and a 186 ± 13 % increase in protein (Western blots not presented). GSSG-Red, in contrast, was only modestly (22%) increased by the addition of tBHQ to the culture medium indicating that the effect of tBHQ was not a general effect on cell metabolism. Both the mitochondrial and the cytosolic isoforms of TrxR were increased in astrocytes following addition of tBHQ to the culture medium. The increase in activity was correlated with an increase in protein content.

Little is known about the regulation of TrxR. As with other selenoproteins, TrxR mRNA has a 3' selenocysteine insertion sequence (Zhong et al., 1998) and thus translation into protein is directly regulated by selenium availability (Berggren et al., 1999; Björnstedt et al., 1997; Gasdaska et al., 1999). Activation of protein kinase C with phorbol ester causes a downregulation of TrxR in human umbilical vein endothelial cells (Anema et al., 1999) but upregulation in mouse skin (Kumar and Holmgren, 1999). Estrogens have been demonstrated to upregulate TrxR in bovine aortic endothelial cells grown in culture (Ejima et al., 1999), although the response element involved is not yet known. Of relevance to this study is that oxidative stress as represented by oxygenation of lung tissue has been
demonstrated to induce TrxR (Das et al., 1999); this suggests that there may be an anti-oxidant response element (ARE) governing TrxR transcription. The promoter region of TrxR has not yet been described so it is not known whether there is an ARE element in the promoter region.

Are there possible functional consequences for neurons with an upregulation of TrxR in astrocytes? It would appear that the high rate of aerobic metabolism that neurons have results in most of the electrons temporarily stored in the form of NAD(P)H being used for metabolic purposes such as reducing oxygen to water rather than being used for developing redox buffers such as GSH or Trx-(SH)₂. Coupled with this poor intrinsic ability for generating redox buffers there is a high rate of superoxide anion production because of the high rate of aerobic metabolism and, thus, a great need for redox buffering. Astrocytes, on the other hand, have a lower aerobic metabolic rate and have more of the electrons temporarily stored in NAD(P)H available for redox buffering. There is considerable evidence for redox coupling between neurons and astrocytes. One example already considered is the likely role that astrocytes have in providing neurons cysteine in the form of cysteinyl-glycine (Dringen et al., 1999). The shuttling of lactate from astrocytes to neurons is another example of a redox buffer for which there is good evidence (Robinson et al., 1998); this provides neurons not only another source pyruvate with the potential of the formation of ~18 moles of ATP for every mole of pyruvate but also electrons for reducing NAD⁺ to NADH.

TrxR activity can also indirectly provide the redox active cysteine to neurons, thereby facilitating GSH synthesis. Cysteine is the rate-limiting amino acid in GSH synthesis. Cysteine readily auto-oxidizes to cystine; hence in the
plasma and extracellular fluids cystine is normally present at a concentration of 100 μM (half-cystine equivalents) whereas the concentration of cysteine is about 10 μM (Bannai, 1984). Although both neurons and astrocytes efficiently take up cysteine, only astrocytes and not neurons, can take up cystine (Sagara et al., 1993). TrxR reduces lipoic acid to dihydrolipoate (Arner et al., 1996). Dihydrolipoate can reduce cystine to cysteine (Han et al., 1997). Lipoic acid is efficiently taken up by a variety of cell types such as human diploid fibroblasts and C6 glioma cells, reduced to dihydrolipoate and released back into the culture medium where it can reduce cystine to cysteine (Han et al., 1997; Handelman et al., 1994). There is evidence that addition of dihydrolipoate to the medium of cultured neurons minimizes GSH loss and cell death in neuronal cultures (Tirosh et al., 1999). Thus, one possible consequence of increased TrxR activity in astrocytes is to indirectly facilitate reduction of cystine to cysteine in the intercellular space.

The most important role of TrxR is to reduce Trx-S$_2$. Exogenous Trx-(SH)$_2$ can attenuate oxidative damage in a variety of cells (Isoya et al., 2000; Spector et al., 1988) including neurons (Hori et al., 1994). One of the neuronoprotective factors released by macrophages (Schwartz et al., 1999) has been demonstrated to be Trx-(SH)$_2$ (Endoh et al., 1993). Trx-(SH)$_2$ is secreted through a leaderless secretary pathway by a number of different cell types (Ericson et al., 1992; Rubartelli et al., 1992; Rubartelli et al., 1995) including astroglial cells (Hori et al., 1994). One possibility is that astrocytes release Trx-(SH)$_2$ and then take up the Trx-S$_2$ which can be reduced again by TrxR. This, of course is speculation and requires experimental verification.
In summary, we have demonstrated that a classical phase II enzyme inducer increases GSH and TrxR in astrocytes but not in neurons.
4.5. REFERENCES


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Chapter 5

Phase 2 Protein Induction in Astrocytes Protects Both Astrocytes and Neurons From Oxidative Stress

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5.1. Introduction

ROS as well as exogenous or endogenously generated electrophiles can interact with vital cellular macromolecules to cause cell injury or death. Enzymes responsible for detoxification of xenobiotics by converting them to water-soluble relatively inert products are placed into two groups: 1) phase 1 enzymes (mainly the cytochrome P-450 mono-oxygenase system) that functionalizes the xenobiotic by reduction/oxidation, and 2) phase 2 enzymes that conjugate such functionalized components with endogenous ligands such as glucuronic acid and glutathione (Prestera et al., 1993). Classical phase 2 enzymes include NADPH: quinone oxidoreductase (QR), glutathione S-transferase (GST), epoxide hydrolase and UDP-glucuronosyltransferase. The expression of phase 2 enzyme genes is regulated by the anti-oxidant or electrophilic response element (ARER/EpRE) (Jaiswal. 2000; Prestera et al., 1993).

More recently, it has been recognized that activation of the ARE promotes expression of the rate-limiting enzyme for glutathione (GSH) synthesis, γ-glutamylcysteine synthase (Wild and Mulcahy, 2000), thioredoxin reductases (Eftekharpour et al., 2000), and thioredoxin-dependent peroxidase I (peroxiredoxin I), heme oxygenase 1 and the cystine/glutamate antiporter (Ishii et al., 1999). Induction of phase 2 protein genes has been demonstrated to decrease cellular oxidative stress in cell types such as smooth muscle cells (Wu and Juurlink, 2001), and in principle should do so in other cell types (reviewed in Juurlink, 2001).
The thiol GSH, whose rate-limiting enzyme is γ-glutamyl cysteine synthase, plays a very important role in the management of oxidative stress in cells (Thorburne and Juurlink. 1996). particularly playing critical roles in scavenging peroxides. 4-hydroxyalkenals. α-oxo-aldehydes. as well as involved in the ultimate regeneration of vitamin E from the vitamin E radical (Juurlink. 2001). GSH is present in cells at concentrations ranging from 1-10 mM (Uhlig and Wendel. 1992). depending on cell type. When used as an electron donor. GSH is oxidized to GSSG. GSSG is reduced to GSH by glutathione reductase (GSSG-R) (Schirmer et al.. 1989). However. if GSSG is formed at rates greater than can be immediately reduced by GSSG-R then it forms mixed protein disulfides (Bellomo et al.. 1987: Kamencic et al.. 2001: Shivakumar et al.. 1995). In many situations. therefore. de novo synthesis of GSH is critical for a cell’s ability to cope with oxidative stress.

The small (12kD) protein thiol thioredoxin (Trx) also plays a role in the management of oxidative stress (Holmgren. 2000b: Sen. 1998). The reduced form of Trx, Trx(SH)₂ can act as an electron donor for the thioredoxin-dependent peroxide scavengers. peroxiredoxins (Prx’s) (Chae et al.. 1999). Oxidized-Trx is reduced by the selenoenzyme Trx reductase (Trx-R) (Zhong et al.. 2000). In the presence of selenocystine and NADPH. Trx-R can also reduce lipid hydroperoxides (Björnstedt et al.. 1995). Since Trx concentration in cells is ~ 10 μM (Björnstedt et al.. 1997). the ability of cells to reduce oxidized-Trx dictates the efficacy of the Trx-dependent redox-managing systems.

We have previously shown that Trx-R can be induced by the phase 2 enzyme inducer tertiary-butylhydroquinone (tBHQ) in astrocytes but not in neurons (Eftekharpour and Juurlink. 2000). A number of other studies have shown that phase
2 enzymes can be induced in astrocytes but not neurons (Ahlgren-Beckendorf et al., 1999; Eftekharpour et al., 2000; Murphy et al., 2001).

In the present study we addressed several objectives: 1) To determine whether phase 2 enzyme induction in astrocytes better enables these cells to cope with oxidative stress. 2) to delineate the relative roles of the GSH and Trx-dependent systems in enabling astrocytes to cope with oxidative stress. 3) to determine whether phase 2 enzyme induction in astrocytes better enables neurons to scavenge peroxides.

5.2. MATERIALS AND METHODS

Chemical reagents. All chemicals were purchased from Sigma-Aldrich Canada (Oakville, Ont), unless otherwise stated.

Mouse primary astrocyte cultures. Mouse astrocytes and cortical neurons were cultured as previously described in (Juurlink and Walz, 1998). Briefly, for astrocyte cultures CD1 mouse newborns were killed using an overdose of the anaesthetic Metophane as approved by Canadian Council on Animal Care. Neopallial tissue was disassociated into a single cell suspension and planted at a low cell density (9 X 10^5 cells) into 100 mm Falcon tissue culture dishes. The growth medium was comprised of Dulbecco's Minimal Essential Medium (DMEM from Gibco-BRL) containing 7.5 mM glucose, 15 mM NaHCO3, 2 mM glutamine and 10% (v/v) horse serum (Summit Biotechnology, Fort Collins, CO). Cultures were fed 3 times per week. Confluent cultures were used at the beginning of the third week.
Induction of phase 2 enzymes in astrocytes. Confluent astrocyte cultures were switched to serum free growth medium for 24 hours, and then were treated with 20 μM tBHQ or the vehicle dimethyl sulfoxide (DMSO) for control for 24 h. The final concentration of DMSO in cultures was 0.1%.

Cortical neurons. Cortical neurons were cultured from CD1 E15 mouse embryos. Neopallia were isolated, trypsinized (0.2 % crude trypsin) for 2 min at room temperature, and triturated with a glass Pasteur pipette. Cells were counted and plated on (Falcon) poly-D-lysine-coated 6-well plates (companion dishes) at a cell density of 1 x 10^6 per dish. Primary growth medium was DMEM that contained 2 mM glutamine, 14 mM NaHCO3, 15 mM HEPES, 30 mM glucose, and 5% horse serum. The antimitotic agent fluorodeoxyuridine (10 μM) together with uridine (40 μM) were added to the cultures on day 3; after 24 h, the growth medium was changed to secondary medium consisting of DMEM, 27.5 mM Glucose, 15 mM NaHCO3, 0.3 mM glutamine, umm alpha-ketoglutarate, and 5% horse serum. Cultures were used on days 5-6.

Astrocyte-cortical neurons transient co-cultures. Transient co-cultures were prepared as outlined by Dringen (Dringen et al., 1999). In brief, astrocytes were cultured on PET membrane 25 mm tissue culture inserts (Falcon) with a pore size of 0.4 μm (pore density 1.6 x 10^6 / cm²) and fed with astrocyte growth medium. At 3 weeks, confluent astrocyte cultures were used for phase 2 induction as described above. tBHQ treated astrocytes and vehicle-treated controls were transferred to the 6-well companion plates.
containing cortical neurons (5-6 d). Co-cultures were maintained for 24h or 72 h and neurons used for determination of GSH and peroxide scavenging ability.

**Biochemical Assays:**

*Preparation of cultures for biochemical assays.* Astrocytes or cortical neurons were washed twice with phosphate-buffered saline (PBS). Cellular protein was extracted using a tissue extraction buffer. (0.1 M KCl, 0.02 M K₂HPO₄, 1 mM EDTA, 0.5% (v/v) Triton X-100, adjusted to pH 7.0 with HCl). Cells were sonicated on ice 3 x 5 seconds with 1 minute intervals, and centrifuged at 15,000 g for 10 min at 4°C. The supernatants were used for the western blotting analyses and enzymatic assays. A bicinchoninic acid protein assay (Smith et al., 1985) was used to determine the total protein content with bovine serum albumin as the protein reference standard.

*Induction of oxidative stress in primary astrocyte cultures.* An in vitro model for induction of oxidative stress in mouse astrocytes was employed as described by Robb and Connor (1998). Astrocytes (3 weeks old) in 100 mm dishes were exposed to 1.5 mM tertiary butylhydroperoxide (tBOOH). After 0, 2, 4 and 6 h, 200 µl aliquots of medium was taken and stored at -80°C for cytotoxicity assay. Cells were washed twice with Pucks’ solution (a Ca²⁺-Mg²⁺-free balanced salt solution) 37°C and then used for cell viability assays.

We also used 10 µM thimerosal for 2-4 h to induce oxidation of sulphydryl groups that causes mitochondrial swelling (Wudarczyk et al., 1996).
Oxidative stress measurement. 5-(and -6)-carboxy-2', 7'- dichlorodihydrofluorescein (DCFH: Molecular probes, Eugene, OR, U.S.A.) was used as an intracellular probe of oxidative stress (Thorburne and Juurlink, 1996). Prior to oxidative stress induction, cells were loaded with DCFH ester (final medium concentration of 5 μM) for 1 h (37 °C) and then exposed to 1.5 mM tBOOH. After 0, 20, 45 or 90 min cells were washed with Pucks' solution (2 x 5 min at 37° C) and harvested (see preparation of cells for biochemical assays). The level of oxidative stress level was measured fluorometrically using the Fluoroskan II (Labsystems, Finland) with excitation set at 490 nm and emission set at 525 nm. The results were normalized to protein content.

Cytotoxicity measurement. Cell death was determined by measuring the released lactate dehydrogenase (LDH) activity in the medium (Frandsen and Schousboe, 1987) as we have done before (Juurlink and Hertz, 1993). Medium was collected at 0, 2, 4 or 6 h exposure to treatment conditions and centrifuged for 2 min at 10,000 g. The supernatant was used for measuring LDH activity according to Wahlfeld (Wahlfeld, 1983).

Cell viability assay. Cell viability was measured by assessment of mitochondrial membrane potential (MMP) as described previously (Juurlink and Hertz, 1993). Cells were washed twice with Pucks' solution at 37° C. growth medium was then added containing Rhodamine 123 (Rh123: Molecular Probes, Eugene, OR) at a final concentration of 50 μg/ml. After 1 hour incubation at 37° C. cells were washed twice with Pucks' solution and harvested in PBS, sonicated (3 x 5 sec) and centrifuged at 15,000g. The fluorescence in the supernatant was measured using the Fluoroskan II
with excitation set at 490 nm and emission set at 525 nm. The results were expressed as ng Rh123/mg protein.

**Neuronal hydrogen peroxide scavenging capacity assessment.** The procedure used was that described by Dringen and Hamprecht (1997). After 24 or 72 hr. the astrocyte inserts were removed from cortical neuron cultures, and neuronal medium was washed twice with warm Pucks’ solution. Neuronal cultures were then incubated for 5 min at 37° C in a HEPES-buffered incubation salt solution (20 mM HEPES, 145 mM NaCl, 1.8 mM CaCl₂, 9.4 mM KCl, 1 mM MgCl₂, 0.8 mM Na₂HPO₄, and 5 mM glucose, pH 7.4). Cells were then switched to HEPES-incubation solution containing 100 μM hydrogen peroxide. At 0, 5, 10, 20, 30 and 40 minutes after exposure to H₂O₂, 50 μl of the incubation solution was taken and stored at -80° C for later measurement of H₂O₂.

**H₂O₂ assay.** The H₂O₂ assay was performed according to Jiang et al. (1990). In brief, 10 μl of peroxide-containing solution was added to wells of a 96-well plate, then the following solutions were added: 140 μl of 25 mM H₂SO₄, 150 μl of reaction mixture comprised of 200 mM Sorbitol, 0.5 mM (NH₄)₂ Fe(SO₄)₂ and 200 mM xylenol orange in 25 mM H₂SO₄. After 30 min incubation at room temperature, the absorbance at 550 nm was measured. All samples were done in triplicates.

**GSH measurement:** GSH was measured according to the procedure of Fernandez-Checa and Kaplowitz (1990) and as we have done before (Eftekharpour et al., 2000)
by adding monochlorobimane to the culture medium to a final concentration of 100 μM. harvesting cells and measuring the formed monochlorobimane adduct in the sonicates using the Fluoroskan II with excitation set at 355 nm and emission set at 460 nm.

_HPLC_. Analysis of total GSH. CysGly and cysteine levels was done by reverse-phase HPLC using ultraviolet detection and precolumn derivatization with 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB) by a method modified from that of Komuro et al. (1985) as described in Kamencic et al. (2000). In brief, 300 μl of the cell supernatant or growth medium from harvested astrocytes were deproteinized by adding an equal volume of 10% 5-sulfosalicylic acid containing 0.2 mM EDTA. Macromolecules were pelleted by centrifugation at 10,000 g for 15 min and supernatants collected for the protein assay. The rest of the supernatant/medium was used for protein content assay. The reaction mixture for the HPLC analysis of free reduced sulfhydryl groups contained 0.5 ml 50 mM Tris-HCl buffer (pH 8.9), 0.13 ml sample or standard, 0.02 ml internal standard (400 μM D(-) penicillamine in cold 5% sulfosalicylic acid containing 0.1 mM EDTA), and 0.35 ml 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) made up in 0.5 mM K₂HPO₄ (pH 7.2), with or without dithiothreitol (DTT). After the derivatization reaction, the mixture was acidified with 0.05 ml 7 M H₃PO₄ and 50 μl was injected into the HPLC system. The HPLC system consisted of a Shimadzu high performance liquid chromatography system composed of a SCL-10A system controller, an SPD-10A variable wavelength spectrophotometric detector, SIL-10A automatic sample injector and LC-10AT solvent delivery system. Chromatography
was accomplished using isocratic elution on a Supelcosil™ LC-18-T column (150 x 4.6 mm, 3 μm) at 37 °C preceded by a Supelguard™ LC-18-T precolumn. The mobile phase consisted of 12.5% methanol (v/v) and 100 mM KH₂PO₄ (pH 3.85) at a flow rate of 1.1 mL/min. Sulphydryl-DTNB derivatives were detected by ultraviolet absorbance at 330 nm. After 9 minutes of isocratic elution, the methanol concentration was increased to 40% over a 1 min period and pumped for 7 min to elute excess DTNB reagent from the column. The methanol concentration was then decreased to 12.5% over a 1 min period, and pumped for 12 min before the next sample injection. Data were collected digitally with Shimadzu EzChrom Version 3.2 chromatography software, and peak areas were quantified. Standards contained L-cysteine, cysteinyil-glycine, glutathione, and DL-homocysteine. A standard curve was run in duplicate daily with samples.

_GSH depletion, Trx-R inhibition and sulphydryl oxidation._ Diethylmalate (DEM) was added to astrocytes (0.2 mM) for 24 hours. Cells were then washed in warm Puck’s solution and used for experiments. Trx-R activity was inhibited with 20 μM 1-Chloro-2,4-dinitrobenzene (DNCB) (Arner et al., 1995; Nordberg et al., 1998) or CDDP (40 μM) (Sasada et al., 1999). Reduction in activity was determined with the dithionitrobenzoic acid (DTNB) and/or insulin reduction assay as we have previously done (Eftekharpour et al., 2000). To examine the role of TrxR in coping with sulphydryl oxidation, astrocyte cultures were exposed to the thiol reagent thimerosal (Elferink, 1999).
5.3. RESULTS AND DISCUSSION

Role of GSH System in the Anti-Oxidant Mechanisms of Astrocytes. Our previous findings demonstrated that exposing astrocytes to tBHQ increased cellular GSH (Eftekharpour et al., 2000). The increase in cellular GSH levels was confirmed in the present study (Fig. 5.1). Another indicator that tBHQ increased the reducing capacity of astrocytes was that cysteine levels increased to 2.31 ± 0.37 nmoles/mg protein whereas in controls it was 1.63 ± 0.29 nmoles/mg protein. This difference between the 2 groups was also reflected in medium cysteine content where vehicle control astrocyte medium contained 15.36 ± 2.17 nmoles/mg astrocyte protein while the value for the astrocytes where phase 2 enzymes had been induced was 26.30 ± 2.25. Both cellular and medium cysteine contents were significantly different between the 2 groups (P<0.05. Student’s t-test). However, there was no difference between the vehicle and tBHQ-treated cells and the associated medium when total cysteine/cystine levels were examined. This indicates that the ability of astrocytes to reduce cystine to cysteine is increased after induction of phase 2 proteins.

To examine the rates of GSH synthesis in astrocytes exposed to DMSO vehicle or tBHQ, GSH levels were depleted with DEM and GSH repletion followed. GSH was reduced to 20% of basal levels in vehicle control cultures (Fig. 5.1); this is similar to what we have seen before (Juurink et al., 1998). In astrocytes previously treated with tBHQ for 24 hr. DEM reduced GSH levels to 47% of the basal level, a cellular concentration that is equivalent to basal GSH levels in the vehicle control astrocyte cultures. Over the next 6 hr. the rate of GSH repletion was 11.4 ± 3.5 nmoles/hr/mg protein in vehicle control cultures and 24.2 ± 5.3 in tBHQ-treated cultures. This
Figure 5.1. Induction of phase 2 enzymes increases the rate of GSH synthesis in astrocytes.

GSH was depleted using diethyl malate (DEM), and repletion of GSH was compared in vehicle and tBHQ treated astrocytes at indicated time intervals. (One-way ANOVA, followed by Bonferroni's test)

(Cnt. vs Cnt + DEM at 0 min. P<0.001)
(Cnt. vs Cnt + DEM, 3h , P<0.05)
(tBHQ vs tBHQ 0 h P< 0.001)
(tBHQ vs tBHQ, 6h P< 0.05)
difference in rates of GSH synthesis likely reflects the levels of \(\gamma\)-glutamylcysteine synthase activities and the greater reducing capacity of the cells as reflected in the increased cysteine:cystine ratios.

We posed the questions whether decreases or increases in GSH were related to the ability of astrocytes to cope with oxidative stress. In the first approach we examined astrocyte cell death following exposure to tBOOH as determined by release of LDH into the culture medium. Fig. 5.2 illustrates that GSH-depleted cultures exhibited the most rapid release of LDH into the medium while cultures exposed to tBHQ for 24 hr prior to exposure to tBOOH fared the best. Cultures exposed to tBHQ followed by GSH depletion had intermediate values of LDH release similar to that seen with the DMSO vehicle-treated control cultures: these latter 2 experimental groups had the same cellular GSH levels. This indicates that the glutathione-dependent anti-oxidant system plays a major role in coping with oxidative stress induced by tBOOH. This is in agreement with the findings of Dringen et al. (1998) who, by using inhibitors of catalase or GSH-Px, concluded that the \(H_2O_2\) scavenging of astrocytes is GSH-dependent.

We also examined the effect of tBOOH on maintenance of mitochondrial Rh123 accumulation in these 4 experimental groups. Rh123 is taken up into mitochondria in proportion to mitochondrial membrane potential (Chen. 1988). We have previously shown that Rh123 accumulation is inversely related to LDH release (Juurlink and Hertz. 1993). Fig. 5.3 illustrates the results showing that astrocytes previously exposed to tBHQ better maintained mitochondrial membrane potentials following tBOOH exposure. DEM-treated cultures fared the worst, while DMSO
vehicle-treated and tBHQ plus DEM-treated cultures were intermediate. Our experimental approach
Figure 5. 2. Phase 2 enzymes induction using 20 uM tBHQ better enables astrocytes to withstand oxidative stress.

Results are mean ± SEM of one experiment (n=3). Comparable results were obtained from two other independent experiments. At 4 and 6 h after induction of oxidative stress, tBHQ treated astrocytes had significant less cell death than the others. (p<0.01 One way ANOVA, followed by Dunnett’s multiple comparison’s test).
could not determine whether all cells experienced decreases in mitochondrial membrane potentials or whether the decline seen in mitochondrial membranes potentials indicated progressive cell death.

*Role of the Thioredoxin System in the Anti-Oxidant Mechanisms of Astrocytes*

To delineate the role of the Trx-dependent system on the ability of astrocytes to cope with peroxide-induced oxidative stress, we wished to selectively inhibit Trx-R and then expose astrocytes to tBOOH. At the concentration used in the present experiments, DNCB has been reported to be a specific inhibitor of Trx-R when examined on isolated Trx-R, with inhibition of GSSG-R requiring much higher DNCB concentrations (Arner et al., 1995; Nordberg et al., 1998). To verify such specificity in astrocyte cultures, we examined the effect of 20 μM DNCB on astrocyte Trx-R and GSH level. Fig. 5.4A demonstrates that DNCB does inhibit Trx-R activity; however, as seen in Fig. 5.4B, DNCB decreases GSH content in astrocytes. Hence, we also examined the ability of CDDP, which has also been shown to selectively inhibit Trx-R in cell-free systems, to selectively inhibit Trx-R in living astrocytes. As can be seen in Fig. 5.5, CDDP inhibits the activity of Trx-R but not GSSG-R, nor is cellular GSH affected. This inhibition of Trx-R does not affect the ability of astrocytes to cope with tBOOH-associated oxidative stress as determined by LDH release (Fig. 5.6). In contrast, as shown above, GSH depletion does decrease the ability of astrocytes to cope with tBOOH-induced oxidative stress. This suggests that the GSH-dependent system plays a more important role in countering peroxide-associated oxidative stress than the Trx-dependent system. This is in agreement with the findings of Holmgren
and Björnstedt (1995) that Trx-R ability to scavenge tBOOH is an order of magnitude less than GSH-Px.

Inhibition of Trx-R by CDDP in astrocytes followed by exposure to tBOOH does, however, cause a transient rise in cellular oxidative stress (Fig. 5.7) compared to the vehicle control cultures. It is possible that Prx's play an important role in the initial scavenging of peroxides and this initial increase in oxidative stress reflects the inactive state of this system. The initial increase in oxidative stress may also reflect the inactivation of the role of the Trx system in repairing the initial oxidative stress-related damage. The significance of this increase in oxidative stress associated with Trx-R inactivation is still to be elucidated.

To delineate the possible role of the Trx system in maintaining the sulfhydryl-disulfide equilibrium, astrocytes were exposed to the sulfhydryl oxidizing agent thimerosal. Oxidation of mitochondrial sulfhydryl groups has been shown to decrease the mitochondrial membrane potential, increase the membrane permeability transition and opening of unselective channels (Petronilli et al., 1994) resulting in decrease in the mitochondrial membrane potential. Hence, the response of the mitochondrial membrane potential to thimerosal was examined. In response to thimerosal, mitochondrial membrane potentials decreased (Fig. 5.8). This decrease was greater in CDDP-exposed astrocytes than vehicle-exposed control astrocytes (Fig. 5.8). Astrocytes where phase 2 protein genes were induced had the slowest rate of decrease in mitochondrial membrane potentials. Since there are both glutaredoxin and Trx-dependent thiol reductases (Holmgren, 2000a), and GSH is the electron donor for the reduction of oxidized-glutaredoxin (Holmgren, 2000a), this suggests that in
Figure 5.3. tBHQ treatment better conserves MMP in astrocytes during tBOOH-induced oxidative stress. Results are mean ± SEM from three independent experiment (n=6-9). MMP in tBHQ treated cells was significantly higher than other groups after 4 and 6 hours. GSH depletion affects MMP in tBHQ and in vehicle treated group (P<0.01), one-way ANOVA, followed by Dunnett's test.
Figure 5.4 Effect of 20 μM DNCB on Trx-R activity and GSH levels in astrocytes.

A) Treatment of astrocytes with 20 μM DNCB for 2 h, decreases the GSH level.
B) 20 μM DNCB decreases Trx-R activity as it was assayed by DTNB reduction assay. Results are mean ± SD from 3 independent experiments (n=9, P<0.0001, two-tailed Student's t-test)
Figure 5.5. Effects of 40 μM CDDP on Trx-R, GSH and GSSG-R activities of astrocytes

A) 24 h incubation of astrocytes with CDDP decreases Trx-R activity. Results are mean ± SD of four independent cultures, n=12, (P<0.0001, student's t-test).
B, and C) GSH content of astrocyte, as well as their GSSG-R activities are not affected by CDDP treatment. Results are mean ± SD from three independent experiments. P>0.05, two-tailed Student's t-test (n=9).
Figure 5.6. Role of Trx-R in cell survival during oxidative stress.

Astrocytes were exposed to 1.5 mM tBOOH, and cell death was measured using LDH activity. CDDP-treated astrocytes did not show any difference with the vehicle-treated group. Results are mean ± SD from one experiment, comparable results were obtained from another experiment (One-way ANOVA, followed by Dunnett’s test, n=6).
Figure 5.7. Effect of CDDP on oxidative stress levels following exposure to 1.5 mM tBOOH

Levels of oxidative stress is increased in astrocytes following 24 h incubation with 40 uM CDDP. Cells are then loaded with DCFH for 1 h, then washed and exposed to 1.5 mM tBOOH for 0-90 min. Results are mean ± SD from two different preparation (n=6). At 0 (before exposure to oxidative stress) and 20 min after induction of oxidative stress, CDDP treated cells showed higher levels of DCFH fluorescence. (P<0.01, P<0.0001, respectively, two tailed Student's t-test).
Figure 5.8. **Response of astrocytes MMP to thimerosal-induced oxidative stress**

After 2 h exposure to thimerosal, CDDP treated have significantly lower MMP levels than vehicle controls (P<0.01). The difference between controls and CDDP treated is still significant after 4 h (P<0.05). tBHQ treated cells can maintain the MMP levels better than the other groups throughout the experiment (P<0.001). Results are mean ± SD from two experiments performed in triplicates (Student's t-test, n= 6).
astrocytes the glutaredoxin thiol reductase system might play a more important role in reducing mitochondrial disulfides than the Trx-dependent system. The relative contributions of the glutaredoxin and thioedoxin systems to disulfide reduction has been shown to vary from cell type to cell type (Biaglow et al., 2000).

*Does phase 2 enzyme induction in astrocytes better enable neurons to scavenge peroxides?*

Incubation of astrocytes with cortical neurons in 24 h transient co-cultures increases the GSH content of neurons (Fig. 5.9), confirming the previous report by Dringen et al. (1999). These authors suggested that this increase of neuronal GSH was due to release of cysteine-glycine into the medium by the astrocytes. The basis of their conclusion were: i) cysteine-glycine added to the medium of cultured neurons increased neuronal GSH content and. ii) inhibition of γ-glutamyl transpeptidase, an ecto-enzyme found on astrocytes that forms cysteine-glycine from GSH prevented the astrocyte-associated increase in neuronal GSH. In the present study we examined the astrocyte medium for the presence of cysteine-glycine and found that astrocytes release cysteine-glycine into the medium. Over a period of 24 hr. on a per mg cell protein basis this was 4.84 ± 1.85 nmol for the vehicle controls and 7.42 ± 1.29 nmol for the tBHQ-treated cultures. Also, as indicated earlier, astrocytes release cysteine, the rate-limiting amino acid for GSH synthesis, into the medium.

The next question addressed was whether prior co-culture of neurons with astrocytes increased their ability to scavenge hydrogen peroxide. These results are
**Figure 5.9.** Effect of transient incubation of astrocytes with cortical neurons on the neuronal GSH levels.

Incubation of astrocytes with neurons for 24 h increases the GSH content in neurons (P<0.005, two tailed Student’s t-test). Pretreatment of astrocytes with tBHQ does not further increase the GSH content of neurons. (P>0.05, two tailed Student’s t-test). Results are means ± SD of two independent experiments, n=6)
Figure 5.10. Effect of transient coculture (24 h) with astrocytes on neuronal hydrogen peroxide scavenging ability.

Clearance of $\text{H}_2\text{O}_2$ by primary cultures of cortical neurons from the incubation after 24 h transient co-culture with vehicle treated astrocytes, or tBHQ treated astrocytes or no astrocytes. Neurons co-cultured with astrocytes display a faster rate of $\text{H}_2\text{O}_2$ scavenging from medium than neuronal cultures alone ($P<0.001$). However this ability is increased if astrocytes are treated with tBHQ 24 h prior to the experiment ($P<0.05$) (One-way ANOVA, followed by Dunnett's test. Results are mean $\pm$ SD from one experiment performed in triplicates. Comparable results were observed in one independent preparation.
Figure 5.11. Effect of transient coculture (72 h) with astrocytes on neuronal hydrogen peroxide scavenging ability.

Clearance of H$_2$O$_2$ from the incubation buffer of neurons primary cultures after 72 h incubation with vehicle-treated astrocytes, or tBHQ treated astrocytes or no astrocytes, was assayed. Results are mean ± SD from one experiment performed in triplicates. Comparable results were obtained from another experiment. At 5 and 10 min, the differences among the three groups is significant. (P<0.001, One-way ANOVA, followed by Dunnett’s test).
presented in figure 5.10. It is clear that prior culture with astrocytes enabled the neurons to more rapidly clear hydrogen peroxide. The protein content of the neuronal cultures did not differ in the 2 groups of neurons. Since both the astrocytes were removed during this assay and the growth medium had been replaced with a hydrogen peroxide-containing medium, the difference in scavenging capacity of the 2 groups of neurons is due to a change intrinsic to the neurons. This change in peroxide scavenging capacity appears to be due, in part at least, to the increased GSH content of neuronal cultures after 24 h. The increase in synthesis of GSH is likely related to the release of cysteine and cysteine-glycine into the medium by astrocytes.

Neurons that had been cultured with astrocytes in which phase 2 enzymes had been induced provided a slightly, but statistically significant, faster rate of hydrogen peroxide clearance during the first 10 min of exposure to hydrogen peroxide. It is not clear what the basis for this is. The longer the neurons were exposed to astrocytes, the greater the neuronal capacity to scavenge hydrogen peroxide (Fig. 5.11).

In conclusion, we have shown that induction of phase 2 protein gene expression increases the reducing capacity of astrocytes and their ability to synthesize GSH. This correlates with better capacity to scavenge peroxides. The peroxide scavenging capacity of astrocytes was GSH but not Trx(SH)2-dependent since inactivation of Trx-R did not affect the ability of astrocytes to cope with oxidative stress, whereas, increasing GSH promoted peroxide scavenging. The GSH-dependent system also appears to be more important than the Trx(SH)2-dependent system for reducing disulfides in astrocyte mitochondria. Transient incubation of astrocytes with neurons increases the intrinsic abilities of the neurons to scavenge hydrogen peroxide; this
appears to be due, in part, to the release of cysteine and cysteine-glycine by astrocytes
which facilitates neuronal GSH synthesis.
5.4. REFERENCES


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6. DISCUSSION

6.1. Overview:

Astrocytes take up about 40% of the brain volume. They constitute a structural syncytium as they are connected through gap junctions. Astrocytes are closely associated with neurons and are responsible for maintaining a stable environment necessary for neuronal functions. Neurons are mainly dependent on oxidative phosphorylation in mitochondria to generate ATP for their activities. The respiratory chain in mitochondria leads to superoxide formation, and considering the presence of polyunsaturated fatty acids as well as glutamate receptors and ion channels in neurons, these cells are very susceptible to oxidative stress. To reduce the oxidizing cytoplasm, cells need to maintain a large pool of NADPH to be used by the GSH and Trx dependent systems. Neurons tend to use a substantial portion of their reducing power to establishing the proton gradient across the inner mitochondrial membrane, therefore have a more oxidized cytoplasm than astrocytes. Neurons are dependent on astrocytes for reducing equivalents. One example is lactate which is generated in astrocytes and being used by neurons to supply electrons for generation of NAD(P)H, as well as a source for carbon chain. Cysteine is another example as neurons cannot use cystine for GSH production and rely on astrocytes to reduce cystine to cysteine.

Neuron vulnerability to oxidative stress is also due to lower level of antioxidant systems and redox buffers than astrocyte. Although there is some
information on the GSH system in neurons and astrocytes, there is very little information about the Trx dependent system in these cells. Primary cultures of neural cells were used in these studies. There is an extensive literature that the properties of astrocytes as well as neurons may differ from one brain region to another (Xu, et al., 2001). It has been shown that cell culture is a powerful approach to address the biological mysteries of cells. The most important advantage of the cell culture is that cells are isolated and studied. Although this can be considered as a weak point for cell culture, because the brain consists of a complicated network of various cell types that communicate, not of isolated cells. The in vivo cell function can be different from what is learnt by in vitro studies, therefore one must use considerable caution in interpreting data obtained from tissue culture (Juurlink and Hertz, 1985). But it is important to remember the words of Margaret Murray (1977), a pioneer in neural cell culture, that “anything a cell is seen to do in culture must be counted among its potentialities”.

In these studies I have addressed the distribution of GSH and Trx dependent systems in cultured cortical astrocytes and neurons. I also have explored the possible ways to upregulate the peroxide scavenging/redox buffering abilities of neural cells, and shown the importance of astrocytes in peroxide scavenging ability of neurons in a model of astrocyte-neuron co-culture.

6.2. Summary of results:

6.2.1. Differential distribution of GSH dependent and Trx dependent systems in neural cells
Peroxides are produced during normal and pathological conditions of the cell. In the presence of transition metal ions, peroxides generate hydroxyl radicals that can extract electrons (oxidize) from all classes of macromolecules. Sulphydryl groups of many proteins are especially sensitive to oxidation, which changes the proteins' roles in cell physiology. Oxidized thiols are being reduced by Trx and GSH, the redox buffers of the cells. The thiol redox status of intracellular and extracellular compartments is critical in the determination of protein structure, regulation of enzyme activity, and control of transcription factor activity and binding. Members of Trx and GSH dependent systems are important peroxide scavengers of the cells. The differential susceptibility of astrocytes and neurons to oxidative stress is well known. Although this is partly due to more efficient GSH dependent system in astrocytes than neurons (Dringen, et al., 1999), the differential expression of Trx(SH)2 dependent system in these cells is not clear. In the first part of this thesis, I hypothesized that astrocytes have also a better developed Trx(SH)2 system than neurons.

To address this hypothesis primary cultures of astrocytes and cortical neurons were used. The astrocyte cultures are mainly (~95%) composed of GFAP positive astrocytes, and although neuronal cultures were composed mostly of small GABAergic neurons it was estimated that up to 20% total protein of neuronal culture could be due to the astrocyte contamination (Juurlink and Hertz, 1993).

Using Western blotting and different biochemical assays (see section 3.2), I have shown that the GSH dependent system as well as Trx related system are better developed in cultured cortical astrocytes than cultured cortical neurons.
**GSH and Trx:** Cortical astrocytes have higher GSH as well as higher Trx(SH)$_2$ levels than cortical neurons. The sulfhydryl groups of GSH and Trx(SH)$_2$ are involved in many mechanisms, such as radical quenching and reduction of disulfide bonds in proteins (Denke, et al., 2000). Higher concentration of these molecules in astrocytes than neurons suggests the essential role for astrocytes to control the reducing environment of nervous system. This is the first study to compare the Trx(SH)$_2$ levels in neural cells and the distribution of Trx(SH)$_2$ *in vivo* has been discussed in section 1.5.2.

**GSSG-R and Trx-R:** GSH and Trx(SH)$_2$ are oxidized during the cellular activities. The GSSG and Trx(S)$_2$ are reduced by GSSG-R and Trx-R enzymes, respectively. These two enzymes are controlled by ARE, and show close structural and physiological similarities (see Introduction). My experiments showed that levels of these enzymes correlated well with their specific substrates, GSH and Trx. GSSG-R is especially important in reduction of GSSG during oxidative stress, and although the neuronal GSSG-R level is lower than astrocytes, it appears sufficient to quickly regenerate the GSH levels after oxidation by peroxides (Dringen et al., 1999a). Levels of Trx-R protein and activity in my neuronal cultures were lower than astrocytes (20-25% lower). This is the first report of Trx-R presence in neuronal cultures. The importance of Trx-R in neurons has not been addressed, but it is possible that lower Trx-R enzymatic activity and protein level in neurons is another reason for their vulnerability under oxidative stress. Since astrocyte secretion of Trx(SH)$_2$ during oxidative stress has been reported (Tomimoto et al., 1993), which enhances neuronal
viability (Hori et al., 1994). It seems possible that neurons may depend on astrocytes for reducing equivalents in the form of thioredoxin.

**GSH-Px:** GSH-Px activity was determined using cumene hydroperoxide, a specific substrate for GSH-Px (Kussmaul et al., 1999; Zhang et al., 1989). The level of GSH-Px activity was much higher in astrocytes than cortical neurons. The level of GSH-Px activity is correlated with the protein levels (GSH-Px 1 protein), as was confirmed using Western blotting. Neurons showed much lower GSH-Px protein so that in some Western blots, GSH-Px was undetectable. My findings regarding enzymatic activities are in agreement with findings of (Desagher et al., 1996; Dringen et al., 1999a), who measured H₂O₂ disappearance in neural cultures using specific enzyme inhibitors. The lower GSH-Px activity units reported here than other labs for astrocytes is probably due to use of cumene hydroperoxide, which is reduced at a significantly lower rate (3-5 fold) by GSH-Px (Dringen et al., 1999a).

My results are one of the few reports on GSH-Px activities in specific neural cell types, and the first report of correlation of GSH-Px activities with its protein level in astrocytes and neurons. Since up to 20% of total protein in these neuronal cultures can be composed of astrocytes, it is possible that GSH-Px protein and activity is even lower than in neurons that I have shown in these cultures. Dringen et al (1999a) have also reported that GSH dependent peroxide scavenging in neurons was less efficient than astrocyte and that the glutathione system could not functionally compensate for the loss of the catalase reaction in the clearance of H₂O₂.
Upregulation of GSH-Px by growth factors: Neurons and astrocytes express a variety of growth factor receptors. These receptors mediate the protective effects of growth factors on these cells (Sampath et al., 1994; Mattson et al., 1995; Cheng and Mattson, 1995). Although hipocampal neurons and PC12 cells can respond to NGF, PDGF or BNDF by increasing their peroxidase activity through upregulation of the GSH-Px or catalase activity (Sampath et al., 1994; Mattson et al., 1995; Cheng and Mattson, 1995), there is no report of growth factor effects on astrocyte cultures. Astrocytes as well as neuronal cultures were pretreated for 24 h with 10 ng of IGF, bFGF, NGF and EGF, and GSH-Px was measured. Astrocytes and cortical neurons express the specific receptors for all the mentioned growth factors: IGF (Han et al., 1987), bFGF (Clarke et al., 2001), EGF (Wang et al., 1987) and NGF. Only NGF and EGF could increase cellular GSH-Px enzymatic activities in astrocytes while no change was seen in neuronal enzymatic activity. This is the first report on GSH-Px upregulation in astrocytes. Upregulation of GSH-Px activity in astrocytes might be a possible mechanism to increase neuronal resistance to oxidative stress.

Peroxiredoxin: Prx proteins can also scavenge peroxides (Chae et al., 1999); hence they may form another peroxidase system in CNS. Prx enzymatic activity measurement showed that astrocytes contain higher Prx-dependent H_{2}O_{2} scavenging ability than cortical neurons. I have demonstrated that these proteins comprise a large portion of detergent-extractable proteins in both astrocytes and cortical neurons. Astrocytes contain higher levels of Prx1 and 3, while Prx2 is found in higher levels in neurons. My results indicate that the Prx proteins comprise 2-3% of Triton X-100 extractable cellular proteins. Considering the low GSH-Px activity and the abundance of Prx proteins in neuronal cultures, it is possible that Prx system comprises the major
peroxide scavenging system of the cultured cortical neurons. Trx(SH)2 is the electron donor for peroxides in this system. The epigenetic control for upregulation of Prx proteins seems to be different from GSH-Px regulation. as NGF and EGF did not change the Prx catalytic activity (Results not shown). This is the first comparative study of Prx enzymatic activity and Prx protein quantification in astrocyte and cortical neurons.

6.2.2. Effects of tBHQ on phase 2 enzymes in neural cells:

Upregulation of phase 2 proteins in astrocytes increases their ability to withstand oxidative stress (Duffy et al., 1998). To determine if members of Trx and GSH dependent systems are involved in such protective mechanisms, the effects of tBHQ on neural cell cultures were investigated.

I found that tBHQ increases phase 2 enzymes in astrocytes but not in neurons (Eftekharpour et al., 2000). Other groups have also shown that phase 2 induction in neural tissue is restricted to astrocytes (Ahlgren-Beckendorf et al., 1999; Murphy et al., 2001). tBHQ has been used extensively in phase 2 enzyme induction studies. It coordinately upregulates a group of enzymes such as quinone reductase (QR), GST glutathione S-transferase, γ-glutamylsysteine synthase (GCS) (Ahlgren-Beckendorf et al., 1999; Mulcahy et al., 1997). These enzymes increase cellular resistance to electrophile compounds through different mechanism (Duffy et al., 1998; Murphy et al., 1989). QR is responsible for reduction of quinones to hydroquinone which prevents redox cycling and production of free radicals (Lind et al., 1982). The GST enzyme transfers GSH to xenobiotics; such adducts are water soluble and, thus excretable (Pickett and Lu, 1989). GST also show some peroxidase activity (Prohaska, 1980) and GSH is oxidized during this reaction. GCS is the rate limiting
enzyme for GSH synthesis (Meister, 1989), and plays an important role in regulating cellular GSH levels.

**GSH levels**: tBHQ-treatment upregulated GSH level in astrocytes, but had no effect on neuronal GSH (Eftekharpour et al., 2000). GSH level was measured using monochlorobimane assay (Fernandez-Checa and Kaplowitz, 1990). The specificity of GSH upregulation in astrocytes was confirmed by an HPLC analytical approach.

The level of GSH in the cell depends principally on two factors: a) GSH synthesis, and b) reduction of GSSG. The robust increase of GSH in tBHQ-treated astrocytes can not merely be due to increased GSSG-R ability of the cell, as tBHQ-treatment only slightly (20%) increased GSSG-R activity. Since γ-glutatmylcysteine synthase (GCS), the rate-limiting enzyme for GSH synthesis, is a phase 2 enzyme (Mulcahy et al., 1997) and is controlled by ARE/EpRE, the observed increase in GSH must be due mainly to increased GCS activity. To compare the rate of GSH synthesis in tBHQ-treated astrocytes with controls, cellular GSH was depleted using 30 min incubation with diethyl maleate (200 μM). GSH formation was calculated to be 11.4 ± 3.5 nmol/hr/mg protein in vehicle control cultures and 24.2 ± 5.3 in tBHQ-treated cultures (Fig. 5.1). Thus tBHQ caused a doubling in the rate of GSH synthesis. Since GCS is the rate-limiting enzyme in GSH system and GCS is a phase 2 enzyme, my results suggest that GCS has been induced in the astrocyte cultures.

**Trx-R levels**: Total astrocytic Trx-R activity was increased in response to tBHQ treatment, as it was measured with the DTNB as well as the insulin reduction assay (Eftekharpour et al., 2000). There are cytosolic and mitochondrial isoforms of Trx-R that are coded by separate genes (Holmgren, 2000; Miranda-Vizuete et al., 2000). In my experiments upregulated Trx-R activity was detected in cytosolic as well
as mitochondrial fractions of astrocytes, while no change in Trx-R activity was observed for neurons. Increased Trx-R activity in astrocytes was shown to be due to increased levels of Trx-R protein in both cytosolic and mitochondrial Trx-R genes.

My results suggest that Trx-R belongs to the same synexpression gene group as QR, GST, CGS, etc. The tBHQ effect on these later enzymes is due to transcriptional activation of ARE/EpRE and coordinate upregulation of the phase 2 enzymes in astrocytes (Ahlgren-Beckendorf et al., 1999; Murphy et al., 2001). This suggests that there may well be an ARE element in the promoters of the Trx-R genes, however, the promoter region of these genes has not yet been described.

Phase 2 protein genes could not be induced in neurons as shown by myself and others (Ahlgren-Beckendorf et al., 1999; Murphy et al., 2001). This is due, at least in part, to low levels of Nrf2 in neurons compared to astrocytes (Jordan et al., 2000). Nrf2 is a necessary transcription factor for phase 2 protein gene transcription (Chan et al., 1996; Hayes et al., 2000; Itoh et al., 1997).

**Cysteine levels:** The levels of cellular and extracellular cysteine was also upregulated by tBHQ treatment in astrocytes, as it was determined using HPLC analysis. Total cyst(e)ine was measured using DTT to reduce the oxidized disulfide bonds. HPLC revealed that total cysteine levels did not change between tBHQ-treated and control astrocytes, therefore the observed increase in cysteine could be the result of improved reducing ability of astrocytes due to higher Trx-R. Cysteine easily auto-oxidizes to cystine; hence in the plasma and extracellular fluid cystine is found at a concentration of 100 μM, whereas cysteine concentration is about 10 μM (Bannai, 1984). The upregulated ability of astrocytes to reduce cystine by tBHQ may be mediated by Trx-R since Trx-R reduces lipoic acid to dihydrolipoate (Arner et al.,
1996) that, in turn, can reduce cystine to cysteine (Han et al., 1997). Lipoic acid has been reported to be taken up by human diploid fibroblasts and C6 glioma cells and is released as dihydrolipoate into the culture medium where it reduces cystine to cysteine (Han et al., 1997; Handelman et al., 1994. The rate limiting amino acid for GSH is cysteine. Neurons can take up cysteine but not cystine (Dringen and Hamprecht, 1996; Sagara et al., 1993). Astrocytes can take up cysteine, as well as cystine, the later of which is reduced to cysteine. The membrane cystine transport system (X_c^-) is regulated through a Nrf2 mediated ARE-activation (Ishii et al., 2000); therefore, upregulation of the cystine transport system can increase cystine uptake which can affect cellular cysteine as well as GSH synthesis in tBHQ-treated astrocytes.

**6.2.3. Functional importance of phase 2 protein induction in astrocytes.**

Astrocytes (tBHQ treated and vehicle-control) were exposed to an oxidative stress model according to Robb and Connor (1998) (see section 5.2). tBHQ treatment increases cellular resistance to oxidative stress, as it was determined by LDH release as well as mitochondrial membrane potential. tBOOH is a membrane soluble peroxide (Robb and Connor, 1998) which oxidizes thiol proteins (Petronilli, et al., 1994). Oxidation of mitochondrial thiols, will shift the gating potential of mitochondrial transition pore (MTP) to a higher voltage setting, making the pore opening more likely (Petronilli, et al., 1994). It appeared that tBHQ treatment in astrocytes increased tBOOH scavenging ability as well as thiol reducing properties of the cell.

Duffy et al. (1998) reported that tBHQ-mediated protective mechanisms against H_2O_2 were not mimicked by GST and QR overexpression in astrocytes. To
investigate the extent of GSH role in tBHQ-induced protective mechanisms in my experiments. GSH was depleted by DEM. GSH upregulation seems to be a major player in tBHQ-induced protective mechanisms as GSH depletion by DEM inhibited the protective effects of tBHQ-treatment (Figs. 5.2 and 5.3). tBOOH is a specific substrate for GSH-Px (Robb and Connor. 1998), which uses GSH as the electron source for peroxide scavenging.

To delineate the role of Trx-R, DNCB (Arner et al., 1995), and CDDP (Sasada et al., 1999) were used to inhibit Trx-R enzymatic activity. Application of 20 μM DNCB inhibited Trx-R by 75%, but GSH was also depleted in the astrocyte cultures (Fig. 5.4). This is in agreement with the findings of May et al. (1999) who showed that 10-20 μM DNCB decreased cellular GSH by 75-90% in U937 cells. Incubation of astrocytes with 40 μM CDDP for 24 h decreased the DTNB reduction activity by 70%, and GSH level was not affected (Fig. 5.5A, B). Although Trx-R and GSSG-R have structural similarity and both can reduce DTNB, CDDP did not change GSSG-R activity in astrocytes (Fig. 5.5C). This is in agreement with the findings of Milano et al. (1988) who showed that CDDP is nonreactive with GSSG-R. The exact mechanism of CDDP inhibition of Trx-R is still not clear. Whether the cysteine groups of the active site of this protein or the selenocysteine residue of Trx-R is the target of CDDP (Sasada et al., 1999). The selenocysteine group is essential for normal catalytic activity of selenoproteins (Berry et al., 1991; Berry et al., 1992). Inactivation of selenocysteine active site of Trx-R inhibits insulin reduction by Trx(SH)2 (Arner and Holmgren. 2000). The CDDP effects can be overcome by Trx over expression (Sasada et al., 1996). Increased Trx(SH)2 expression in some cancer cell lines is
responsible for their resistance to CDDP (Yamada et al., 1996). This mechanism ensures availability of Trx system for ribonucleotide reductase activity which is essential for cell proliferation (Becker et al., 2000).

Inhibition of Trx-R did not affect cell death in astrocytes (Fig.5.6). This experiment does not suggest any significant role for Trx-R in preventing tBOOH induced cell death, although this can be partly due to low tBOOH scavenging ability of Trx-R (Bjornstedt, et al., 1995). The level of cell viability (MMP measurement) was not addressed in this experiment. GSH-Px is another possible target for CDDP (Milano et al., 1988), but since cell death in CDDP-treated astrocytes was not different than vehicle-treated cells. I concluded that GSH-Px was not affected by 24h pre-incubation of astrocytes with 40 μM CDDP.

To address the possible involvement of Trx-R in management of oxidative stress level in astrocytes oxidative stress was measured in vehicle and CDDP-treated cell using 5-(and-6)-carboxy-2’7’-dichlorodihydro-fluorescein diacetate (DCFH-DA) (Wang and Joseph, 1999). The results showed that CDDP treatment increases basal levels of oxidative stress (0 min). These higher levels of oxidative stress in CDDP-treated astrocytes compared to control, which is detected at 0 min, must be due to Trx-R inactivation by CDDP. Oxidative stress levels were measured up to 90 min. No significant difference between CDDP-treated and the vehicle-treated cells was detected after 45 and 90 min (Fig. 5.7). This experiment showed that Trx(SH)2 dependent system might be the first line of defense against tBOOH-induced oxidative stress. I have shown that Prx proteins are abundant in astrocytes as well as neurons, since in CDDP-treated astrocytes Trx(SH)2 is not available for peroxide scavenging, the levels of oxidant is higher in this group than vehicle control treated astrocytes. The levels of
the strong oxidants returned to the levels in the vehicle control treated astrocyte after 45 min. It is possible that GSH-Px, which is more efficient than Prx system, reacts much slower than Prx system. This experiment showed the importance of Trx dependent system in management of oxidative stress during early phases of oxidative stress.

To investigate the Trx-R role in management of oxidative stress-induced thiol oxidation MMP was measured in CDDP-treated as well as vehicle-treated astrocytes during exposure to thimerosal, a thiol oxidizing agent (Petronilli et al., 1994). The retention of MMP has an inverse relationship to oxidation of sulfhydryl groups of mitochondrial membrane proteins and mitochondrial swelling (Petronilli, et al., 1994; Lenartowicz and Wudarczyk, 1995; Wudarczyk et al., 1996). This will result in cytochrome C release and activation of caspases which lead to apoptosis (Kirkland and Franklin, 2001; Lewen et al., 2000; Ueda et al., 1998). Since Trx-R/Trx system is responsible, in part, for reduction of oxidized proteins (Becker et al., 2000). I hypothesized that CDDP inhibition of Trx-R of astrocytes decreases the cellular ability to conserve MMP levels when exposed to oxidizing agents. When astrocytes were exposed to 10 μM thimerosal, which is a sulfhydryl oxidizing (Islam et al., 1993; Petronilli, et al., 1994; Jabr and Cole, 1995), the mitochondrial membrane potential decreased significantly (Fig. G.8). If astrocytes were treated with CDDP before exposure to thimerosal, the CDDP-treated cells had lower MMP than controls. This experiment showed that Trx-R inhibition increased the mitochondrial susceptibility to sulfhydryl oxidation. Prior tBHQ treatment of astrocytes confirmed the protective role of phase 2 proteins in this experiment.
This experiment confirmed the beneficial roles of phase 2 protein induction in astrocytes. As tBHQ application upregulates a series of protective genes, including Trx-R, and therefore better enables astrocytes to reduce the oxidized thiols in the cell.

6.2.4. Upregulation of Phase 2 enzymes in astrocytes increases H$_2$O$_2$ scavenging ability of cortical neurons co-cultured with astrocytes.

Cortical neurons can scavenge extracellular H$_2$O$_2$ at a slower rate than astrocytes Dringen et al. (1999a). Their experiments were conducted on separate neuronal rich and astrocyte rich cultures and did not examine the effect of astrocytes on neuronal endogenous peroxide scavenging abilities. In my experiments, neurons were co-cultured with primary astrocytes cultures only for 24 h prior to examining H$_2$O$_2$ scavenging ability of neurons, and astrocytes were removed when endogenous neuronal H$_2$O$_2$ scavenging ability was measured. The results showed that transient astrocyte-neuron co-cultures decreased the half time for H$_2$O$_2$ clearance by neurons. The half-life was even shorter when phase 2 enzymes were induced in astrocytes before co-culturing with neurons. When the incubation of astrocytes with neurons was prolonged from 24 h to 72 h, the rate of H$_2$O$_2$ disappearance from incubation buffer was increased significantly for both co-cultures with control astrocytes, and the one with tBHQ-treated astrocytes. Although the results showed statistically significant improved H$_2$O$_2$ scavenging ability for neurons co-cultured with tBHQ-induced astrocytes compared to neurons in co-cultures with untreated astrocytes, the biological benefits of this improvement in peroxide scavenging may not be important.
The beneficial role of astrocytes to the neurons ability to H$_2$O$_2$ in co-cultures has been shown before (Desagher et al., 1996; Drukarch et al., 1998), but in these studies, astrocytes were present when peroxide scavenging was measured. These investigators believed that astrocyte protective effects for neurons is due to higher catalase activity of astrocytes and not to any neuroprotective factor released by astrocytes. My results indicate that astrocytes may release factor(s) that enhance neuronal H$_2$O$_2$ scavenging capacity. Previously Dringen et al. (1999a) have suggested that astrocytes may release CysGly that promotes GSH synthesis in neurons. Indeed I have shown that astrocytes do release CysGly which correlates with neurons having a significantly higher level of GSH following transient co-cultures with astrocytes. This increased neuronal GSH synthesis may be mediated by neuronal uptake of cysteine and CysGly released by astrocytes. The increased level of GSH in co-cultured neurons explains the improved H$_2$O$_2$ scavenging capacity of these neurons. Although the difference in peroxide scavenging between neurons co-cultured with tBHQ-treated astrocytes and vehicle-treated astrocytes is significant, the GSH level was not significantly different in these two groups.

6.3. Conclusions:

My results give additional support to the concept of redox coupling between astrocytes and neurons. Neurons contain a more oxidizing cytoplasm than astrocytes. Since the NADH store of electrons are used for energy production in the mitochondrial electron transport chain rather than maintaining the redox buffers of the cell. I have
shown that astrocytes are better equipped with Trx-R as well as GSSG-R activities to reduce TrxS₂ and GSSG than neurons.

Previous research has shown that neurons in isolated cultures are more vulnerable to oxidative stress than astrocytes. My findings of lower GSH-Px, Prx, Trx-R and GSSG-R enzymatic activities, as well as lower GSH and functional Trx(SH)₂ content in neurons indicate that organic peroxide scavenging machinery of neuron is not as efficient as in astrocytes. Furthermore, no component of the peroxide scavenging systems in neurons could be upregulated by phase 2 inducers in neurons, whereas it could be in astrocytes.

Upregulation of phase 2 proteins in astrocytes increased cellular viability in response to peroxide challenge as demonstrated by better conservation of MMP in these cells. Trx-R seemed to be involved in reduction of oxidized thiol proteins which affects MMP. Cellular reducing ability was enhanced by upregulated phase 2 proteins, which increased levels of cysteine and GSH in cellular and extracellular compartments. Phase 2 protein induction seems to be a practical way to increase CNS reducing capacity, and possibly could be used for therapeutic purposes.

To seek the possible role of astrocytes in enabling neurons to better cope with oxidative stress, transient astrocyte-neuron co-cultures were used. I showed that neuronal peroxide scavenging ability was enhanced, in part, by promoting GSH levels of neurons, through astrocyte release of cysteine and CysGly. The neuronal H₂O₂ scavenging ability was further increased when astrocytes were pretreated with tBHQ, but GSH was not affected. It is possible that Trx(SH)₂ could be involved in upregulated peroxide scavenging ability of neurons. It has been shown that Trx(SH)₂
can be released from astrocytes under oxidative stress conditions. I did not observe any changes in cellular functional Trx(SH)$_2$ content of astrocytes following phase 2 protein induction (results not shown), but the Trx(SH)$_2$ content of medium should be measured.

My research also suggests that maintaining GSH is more important than maintaining Trx(SH)$_2$ in management of oxidative stress. This suggests that possibly glutaredoxin system play a very important role in maintenance of the sulphhydryl-disulfide equilibrium in astrocytes. This opens up an area of research that ought to be investigated.

In summary my results gives additional evidence for metabolic coupling between astrocytes and neurons that better enable neurons to cope with oxidative stress.
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