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REDOX PROCESSES AND OXIDATIVE STRESS IN CELL AND TISSUE DAMAGE

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Redox processes and oxidative stress in cell and tissue damage

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

Oxidative stress is described as an imbalance between oxidants and antioxidants, with a shift towards the oxidants. When the antioxidative systems are insufficient or the production of radicals is increased oxidative damage can occur, damaging DNA, lipids, and proteins. Oxidative stress has been implicated in several pathologies. This study has been focused on oxidative damage caused to the cysteine residues on proteins. The main function of the thioredoxin (Trx) and the glutaredoxin (Grx) systems is to reduce protein thiols and they therefore play an important role in the protection against oxidative stress.

Alzheimer's disease is a progressive disease with a high prevalence in an aging population. Oxidative stress has been implicated in the disease, as demonstrated by elevated levels of oxidized proteins, lipids, carbohydrates and nucleic acids. In our study we were able to show that Trx and Grx were secreted to CSF, and that the levels of the proteins correlated with the previously validated markers tau and p-tau in patients with different stages of AD. The secretion was not caused by cell death as the levels of lactate dehydrogenase did not change between the different stages. Furthermore, decreased axonal staining of Grx1 and Grx2, as well as decreased mitochondrial staining of Trx2 was observed in AD hippocampus.

Parkinson's disease (PD) is characterized by loss of dopaminergic neurons in the *substantia nigra*. The neurotransmitter dopamine has been implicated in the pathology, as rupture of the dopamine vesicles leads to increased cytosolic dopamine levels. Free in the cytosol, dopamine gets oxidized with a release of radicals in the process. We can show a direct interaction between the Trx system and the dopamine-quinone. Furthermore, a protective effect of TrxR against dopamine toxicity was observed in both the cell line SH-SY5Y and in the nematode *C. elegans*. In addition, decreased levels of Trx1 and TrxR1 were observed in *substantia nigra* from PD patients.

Ischemia causes a conversion of xanthine dehydrogenase into xanthine oxidase, upon reperfusion xanthine oxidase releases radicals, leading to an increased oxidative stress in the tissues. Ischemia/reperfusion was induced in human livers, and samples were taken before induction of ischemia, after ischemia and after reperfusion. We demonstrated that the major reperfusion damage was to the sinusoidal endothelial lining, where a retraction of the lining occurred after ischemia, but after 20 minutes of reperfusion the cell lining had partially recovered. No significant changes in mRNA levels of redox proteins could be observed, but likely redistribution of Trx occurred in the hepatocytes.

Cell lines are commonly used in medical research, but a consensus in how to culture the cells is lacking. In order to study the effect of media selection, three different cell lines were cultured in four commonly used cell culturing media. We could observe increased proliferation of cells grown in the high glucose containing DMEM. Increased expression for mesenchymal marker Vimentin in A549 cells cultured in DMEM, and decreased expression of epithelial marker CK18, which indicates a possible change in phenotype due to the selection of media. Furthermore, increased enzymatic activity of TrxR in cells cultured in

MEM compared to the other media was observed, and decreased selenite toxicity in cells cultured in DMEM compared to culture in RPMI or F12. This study highlights the importance of consistency in the choice of cell culturing media for the outcome of any cell experiment.

In conclusion, the thioredoxin family of proteins has been shown to be implicated in several pathological conditions where oxidative stress is believed to be important.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications and manuscripts which are referred to by their Roman numerical.

I. **Lisa Arodin**, Heidrun Lamparter, Håkan Karlsson, Inger Nennesmo, Mikael Björnstedt, Johannes Schröder, Aristi P. Fernandes.
Alteration of Thioredoxin and Glutaredoxin in the Progression of Alzheimer's Disease. *Journal of Alzheimer's Disease, 2014, 39, 787-797.*

II. **Lisa Arodin**, Antonio Miranda-Vizuete, Peter Swoboda, Aristi P. Fernandes.
Protective effects of the thioredoxin and glutaredoxin systems in dopamine-induced cell death. *Free Radical Biology and Medicine, 2014, 73, 328-336.*

III. Rim Jawad, Melroy D'souza, **Lisa Arodin Selenius**, Marita Wallenberg, Olof Danielsson, Greg Nowak, Mikael Björnstedt, Bengt Isaksson.
Morphological alterations and redox changes due to ischemia-reperfusion injury during liver surgery. *Manuscript.*

IV. Marita Wallenberg, **Lisa Arodin Selenius**, Olof Danielsson, Mikael Björnstedt.
The effect of media composition on cell growth, thiol status, redox proteins, and in selenium toxicity. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid beta
AD	Alzheimer's disease
APP	Amyloid Precursor Protein
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BCKDH	Branched chain 2-oxoacid dehydrogenase
CAT	Catalase
CTF	C-terminal fragment of Abeta
DOPAC	3,4-Dihydroxyphenylacetic
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ETC	Electron transport chain
$FAD/FADH_2$	Flavin adenine dinucleotide (oxidized/reduced)
Fe/S	Iron sulfur cluster
FMN	Flavin mononucleotide
GCS	glutamylcysteine synthase
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSK	Glycogen synthase kinase
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
IHC	Immunohistochemistry
IR	Ischemia/Reperfusion
Keap-1	Kelch like ECH-associated protein 1
$LOO^{\bullet}$	Lipidperoxyradical
MAO	Monoamine oxidase
$NAD^+/NADH$	Nicotinamide adenine dinucleotide (oxidized/reduced)
$NADP^+/NADPH$	Nicotinamide adenine dinucleotide phosphate (oxidized/reduced)
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
$^{\bullet}NO$	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
$O2^{-\bullet}$	Superoxide anion radical
OGDH	2-oxoglutarate dehydrogenase
$^{\bullet}OH$	Hydroxyl radical
OH^-	Hydroxide ion
PD	Parkinson's disease
PDH	Pyruvate dehydrogenase
PDI	Protein disulfide isomerase
Prx	Peroxyredoxin

sAPP	N-terminal soluble fragment of APP
SBP2	SECIS binding protein
SECIS	Selenocysteine insertion sequence
SN	Substantia nigra
Trx	Thioredoxin
TrxR	Thioredoxin reductase
xCT	Cystine/glutamate transporter
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

1 INTRODUCTION

1.1 POPULÄRVETENSKAPLIG SAMMANFATTNING

I litteraturen beskrivs oxidativ stress som ”En obalans mellan oxidanter och antioxidanter, med fördel för oxidanterna, vilket leder till störningar i redox signalering och kontroll och/eller molekylära skador”.

Varje cell i kroppen innehåller flera så kallade organeller, de största och viktigaste är kärnan, endoplamiska reticulumet (ER), och mitokondrien. Dessa har alla sina specifika uppgifter, i kärnan finns DNA vars kod läses av för att med hjälp av ER producera proteiner. Proteiner (tidigare kallade äggviteämnen) är funktionella molekyler som utför uppgifter åt cellerna. Mitokondrien är ansvarig för energiproduktionen och där omvandlas socker och syre till energiformen ATP. Den energi som bildas i mitokondrien används för att driva energikrävande processer i cellen.

I mitokondriens energiproduktion sker normalt ett läckage av elektroner på ca 1-2% av de elektroner som används för energiproduktionen. Dessa bidrar till bildandet av fria radikaler. En fri radikal är en extremt reaktiv molekyl som kan orsaka skada på de omkringliggande molekylerna. Som skydd mot fria radikaler använder sig cellen av så kallade antioxidanter, vissa tas upp ur födan och andra produceras av cellen själv.

I de fall när antingen mängden antioxidanter är för liten eller produktionen av fria radikaler är för stor uppstår en oxidativ stress i cellen. Fria radikaler kan orsaka skador på alla de väsentliga komponenterna i cellen, så som DNA, fetter och proteiner etc. I den här avhandligen undersöks de skador som uppstår i proteiner utav fria syre radikaler. Svavel är en väsentlig del i många proteiner, då den ofta är en del av den reaktiva mekanismen hos proteinet. Arbetena inkluderade i den här avhandlingen är fokuserade på två av dessa system, thioredoxin (Trx) och glutaredoxin (Grx) systemen. Dessa har som huvuduppgift att bryta de svavel-svavel bindningar som bildas i proteiner då de utsätts för oxidativ stress och därmed återställa deras funktion.

Mitokondriella förändringar har upptäckts i flera sjukdomar och i dessa fall så ökar läckaget av elektroner, varpå den oxidativa stressen ökar. Oxidativa förändringar har setts vid neurodegenerativa sjukdomar såsom Parkinsons sjukdom och Alzheimers sjukdom.

I det första arbetet har nivåerna av Trx och Grx bestämts i blodet och ryggmärgsvätskan från patienter med varierande grad av Alzheimers sjukdom. Vi kunde se att nivåerna av framförallt Trx korrelerade med andra, tidigare validerade, diagnostiska markörer. Vi föreslår att Trx bör mätas på patienter med misstänkt Alzheimer för att kunna diagnostisera sjukdomen i ett tidigare skede.

I det andra arbetet har vi studerat Trx och Grx roll i Parkinsons sjukdom. Vid Parkinsons sjukdom dör framförallt de nervceller som använder sig utav, och producerar, signalsubstansen dopamin. Normalt sett förvaras dopamin i små blåsor i cellen. Vid ökad

oxidativ stress går dessa blåsor sönder och dopamin läcker ut i cellen. Dopamin är extremt känsligt för oxidation och när det kommer i kontakt med syre förändras dess kemiska struktur och fria radikaler bildas. Vi har studerat Trx och Grx roll vid den cell död som orsakas av dopamin. Vi kan visa att då celler i odling behandlas med dopamin så skyddar Trx mot den toxiska effekten av dopamin. Vi kan även visa att patienter med Parkinsons sjukdom har minskade nivåer av Trx i de dopaminproducerande cellerna.

Ischemi är det tillstånd som uppstår då blodflödet temporärt stoppas till en vävnad. När blodflödet släpps på igen och syre strömmar in i vävnaden så uppstår en massiv oxidativ stress. I det tredje arbetet har vi studerat vad som händer med levern efter att den utsatts för ischemi. Vi har även studerat hur Trx kan vara involverat i denna process. Vi kunde visa att Trx nivåerna varierade mellan patienterna och att Trx troligen släpps ut från cellerna efter att blodflödet är återställt.

I det fjärde arbetet, som är ett metodarbete, har vi studerat hur olika cellodlingsmedium påverkar cellernas utseende, hur de klarar att hantera olika toxiska föreningar, samt hur nivåerna av Trx och Grx påverkas av att cellerna odlats i olika medium. Vi kan visa att val av cellodlings medium är av stor betydelse för att kunna jämföra data från olika studier.

Sammanfattningsvis så har vi har visat att Trx är involverat i flera olika sjukdomstillstånd där oxidativ stress tros vara en av de bakomliggande orsakerna.

1.2 OXIDATIVE STRESS

The field of oxidative stress has grown in recent years but the definition of oxidative stress is not always clear. Oxidative stress has, in a recent review on the concept of oxidative stress, been defined as follows: “An imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage” [1].

This study is focused on the role of thiol regulating protein systems during conditions of oxidative stress.

1.2.1 The complexity of redox regulation

Human diet contains antioxidants and prooxidants in varying proportions [2]. The beneficial effects of foods and drinks containing antioxidants have been extensively studied in the last years. For example, the risk of coronary heart infarction was shown to decrease in a dose dependent manner when the total antioxidant capacity of the diet increases [3]. Wine grape pomace flour was shown to improve blood pressure, fasting glucose and protein damage in a controlled study in human subjects [4]. Furthermore, in a study on primary human keratinocytes the antioxidant caffeine was shown to have wound healing effects [5], and in a human study on healthy males, dark roast coffee was shown to decrease the level of spontaneous DNA strand breaks [6]. Coffee was also shown to have a high peroxyl radical scavenging activity [7]. In contrast, in a study on coffee, tea, and cocoa, micromolar concentrations of H_2O_2 could be detected in coffee [8]. In addition, increased levels of H_2O_2 could be detected in the urine of healthy volunteers after drinking coffee [9]. This controversy highlights the fact that not all antioxidants are good, and not all oxidants are bad.

1.2.1.1 Oxidative stress produced by external stimuli

Throughout life we are subjected to several risks for an increased oxidative stress. Exogenous reactive oxygen species (ROS) production is derived from pollutants [10], tobacco smoke [11], iron salts, and UV [12] and ionizing radiation [13] etc. Furthermore, dependent on the dose, several dietary components has the ability to induce an increased production of reactive species. In addition, aerobic metabolism is constantly exposing the body to oxidizing insults where every breath increases the risk for oxidative modifications.

1.2.2 Cellular sources of ROS

The cellular sources of ROS during stressful and nonstressful conditions depend on the tissue type and the subcellular components. Usually mitochondria, the endoplasmic reticulum (ER) and peroxisomes are described as the main producers of cellular ROS, the levels produced by each organelle vary depending on the tissue and situation (Figure 1).

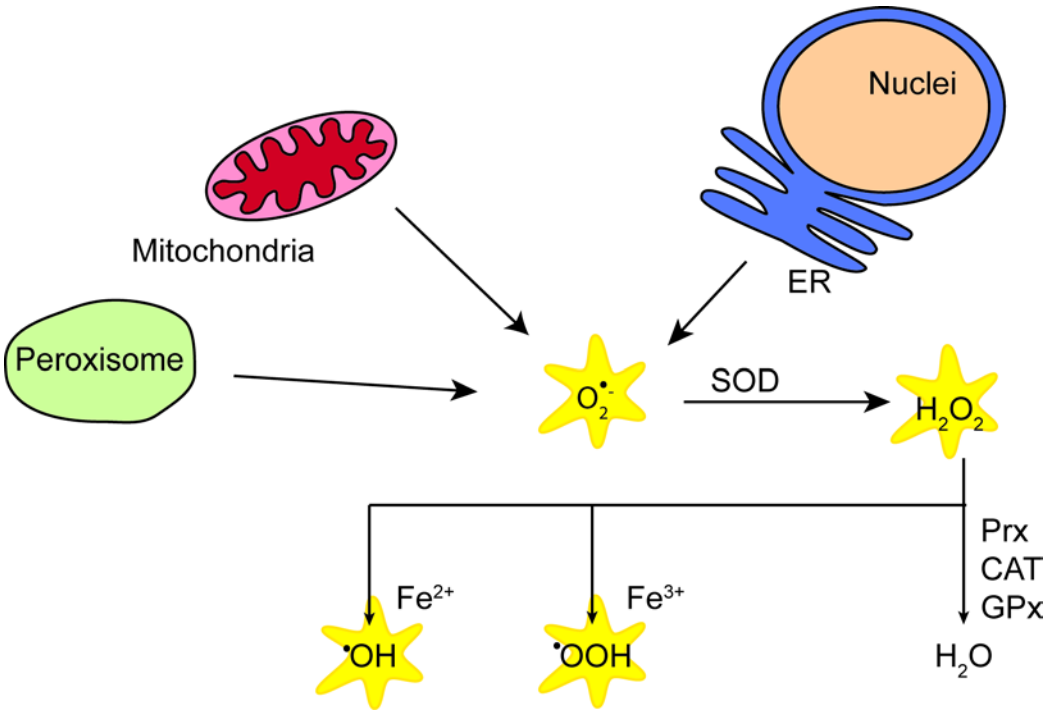

Figure 1. Formation and removal of radicals. *The first line of defense against oxygen radicals formed by redox processes in the cell. ER; Endoplasmic reticulum, SOD; Superoxide dismutase, Prx; Peroxyredoxin, CAT; Catalase, GPx; Glutathione peroxidase.*

1.2.2.1 Mitochondria

Oxygen is vital for all aerobic organisms in order to produce sufficient amounts of energy in the form of adenosine triphosphate (ATP) [14]. Molecular oxygen is used as an electron donor in the mitochondrial electron transport chain (ETC). In the Krebs cycle the reducing equivalents Nicotinamide adenine dinucleotide (NADH) and Flavin adenine dinucleotide ($FADH_2$) are formed. NADH + H^+ are used in complex I of the ETC, where it is oxidized to NAD. Furthermore, complex II is reducing FAD to $FADH_2$. The electrons released from reactions in complex I and II are then transferred, via a series of prosthetic Fe-S groups, to ubiquinone which is reduced by complex III, producing ubiquinol. In complex IV, the electrons from ubiquinol are used to reduce O_2, forming H_2O. Furthermore, during these processes, protons are pumped out into the intermembrane space of the mitochondria. The proton gradient created during the processes is used to drive the ATP production in complex V (The function of the ETC has been described in detail in [15]). During normal physiological conditions ~2% of the oxygen intake to the mitochondria is converted to superoxide radicals [16]. A leak of electrons can occur at several sites in the ETC, but the major physiological and pathological production of superoxide is at the Flavin mononucleotide (FMN) group in complex I [17]. The complexes of the electron transport chain is however not the only site in the mitochondria where ROS is produced [15]. ROS production from 2-oxoglutarate dehydrogenase (OGDH), Branched chain 2-oxoacid dehydrogenase (BCKDH), and Pyruvate dehydrogenase (PDH) have also been observed, and

depending on the substrates present, the dominant sites of superoxide/hydrogen peroxide production may be the OGDH and PDH complexes in the mitochondria, which previously could have been misattributed to complex I [18].

1.2.2.2 Endoplasmic reticulum

The endoplasmic reticulum (ER) is responsible for oxidative protein folding, and is rich in oxygenases and oxidases (e.g., cytochrome P450s, flavin-containing monooxygenases, prolyl and lysyl hydroxylases), which often produce ROS as a byproduct. For example, about one fourth of the ROS produced in a professional secretory cell comes from the oxidative protein folding in the ER [19].

1.2.2.3 Peroxisome

The primary function of the peroxisome is to participate in the cellular lipid metabolism and many peroxisomal enzymes catalyze redox reactions as part of their normal function. Peroxisomes also play a central role in the cellular metabolism of hydrogen peroxide, illustrated by the fact that these organelles harbor large amounts of enzymes that can produce the molecule. Peroxisomes also contain enzymes that generate superoxide (e.g. xanthine oxidase) as part of their normal catalytic activity, giving these organelles the potential to act as a source of radicals [20].

1.2.3 The benefits of radical formation

Normal metabolism yields free radicals which are used in cellular processes. At moderate or low levels ROS have beneficial effects and are involved in various physiological reactions. For example, free radicals are vital in the defense against pathogens. In phagocytes, such as macrophages and neutrophils, the plasma membrane contains nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase which reduces O_2 into superoxide ($O_2^{\bullet -}$), a mechanism called “oxidative burst” [21]. $O_2^{\bullet -}$ is relatively inactive towards microbial pathogens, and is therefore converted into hydrogen peroxide by superoxide dismutase (SOD) present in the pathogens ingested by the phagocyte, releasing the more reactive hydrogen peroxide [22]. In addition, signal transduction with the radical nitric oxide ($NO^{\bullet}$) is involved in a number of important physiological processes, such as synaptic plasticity, neuronal survival, vasodilation, vascular homeostasis, immune regulation [23]. During smooth muscle relaxation, $NO^{\bullet}$ is used as a signaling molecule in order to control the blood pressure in the body. $NO^{\bullet}$ is also involved in neuronal signaling but is, unlike other neurotransmitters, not stored in vesicles but instead produced when needed by nitric oxide synthase (NOS) [24]. Synthesis of $NO^{\bullet}$ is stimulated during transmission by a Ca^{2+}-dependent mechanism. Once $NO^{\bullet}$ has been synthesized, it can diffuse freely through membranes to the post junctional targets [25].

1.2.4 Reactive oxygen, free radicals and antioxidants

A free radical is an atom or a molecule with one or more unpaired electrons, for example superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($^{\bullet}NO$), or lipidperoxyl

radical ($LOO^{\bullet}$) [14]. The odd number of electron(s) of a free radical makes it unstable, short lived and highly reactive. Radicals will donate their unpaired electron to other molecules forming other types of radical species.

1.2.4.1 Superoxide ($O_2^{\bullet -}$)

The superoxide anion is the most widespread radical and is formed when an electron is transferred to molecular oxygen. The two unpaired electrons present in the outermost orbital of molecular oxygen can only accept one electron at a time when it is being reduced [26]. Superoxide is mainly produced in the mitochondria and can be formed by autooxidation and by nonenzymatic reactions where an electron is transferred to molecular oxygen. Furthermore, there are several enzymes that can produce superoxide, for example xanthine oxidase, lipoxygenase, cyclooxygenase, and NADPH dependent oxidase [27].

1.2.4.2 Superoxide dismutase

Dismutation of superoxide occurs enzymatically through superoxide dismutase, where superoxide is converted into hydrogen peroxide and molecular oxygen. There are three known isoforms of superoxide dismutase (SOD); SOD1 (CuZn–SOD) present in the cytosol, nucleus, and mitochondrial inner membrane, SOD2 (Mn–SOD) in the mitochondrial matrix, and SOD3 (Ni–SOD) extracellularly [28]. The dismutation by SOD keeps the concentration of superoxide in the low picomolar range [29].

1.2.4.3 Scavenging of hydrogen peroxide

Hydrogen peroxide reacts poorly or not at all with most biological molecules and low molecular weight antioxidants, reaction does however occur with selected biological targets with transition metal centers, selenoproteins, and a small selection of thiol proteins [30]. Hydrogen peroxide can be scavenged by three enzymatic systems; peroxiredoxin (Prx), catalase (CAT), and glutathione peroxidase (GPx), of which the most efficient is the Prx system.

Peroxiredoxins exist as dimers, with two critical cysteine residues per monomer; the peroxidatic cysteine and the resolving cysteine [31, 32]. Upon interaction with hydrogen peroxide, the peroxidatic cysteine is oxidized to a sulfenic acid, which generates an intermolecular disulfide with the resolving cysteine of the other monomer to release water. As neither SOD nor catalase has been found in ER, the Prx system is vital for dismutation of radicals in this organelle [19]. The kinetics behind the decomposition of hydrogen peroxide by catalase was first described in 1924 [33]. Catalase is primarily an intracellular enzyme; its highest concentrations in mammals are found in peroxisomes in erythrocytes and liver [34]. Glutathione peroxidase catalyzes the reduction of hydrogen peroxide concomitant with the oxidation of two molecules of glutathione (GSH) [35].

1.2.4.4 Hydroxyl radical ($^{\bullet}OH$)

Cleavage of the oxygen-oxygen bond in hydrogen peroxide (H_2O_2) leads to the formation of the highly reactive hydroxyl radical. The hydroxyl radical can be formed either by cleavage of H_2O_2 by UV irradiation [14], or in the presence of iron (or copper) according to the Fenton reaction (reviewed in [36]):

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$ (A)

$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + {}^{\bullet}OOH + H^+$ (B)

In reaction (A) one electron is donated from Fe^{2+} to hydrogen peroxide, which forms the hydroxyl radical and hydroxide ion (OH^-). The oxidized iron (Fe^{3+}) participates in reaction (B), where hydrogen peroxide is cleaved, forming the simplest form of peroxyl radical ($ROO^{\bullet}$); perhydroxyl radical ($^{\bullet}OOH$). Hydrogen peroxide has a half-life of milliseconds, is membrane permeable, and has a steady state concentration in the cell in the nanomolar to low micromolar range [29].

1.2.4.5 Antioxidants

In addition to enzymatic scavenging of radicals, there are several low molecular weight antioxidants present in the cell. The antioxidant effects *in vivo* are dependent on the bioavailability of antioxidants as determined by uptake, distribution, metabolism, and excretion. These low molecular weight antioxidants possess redox-active properties, and are capable of quenching radicals or diminish the oxidation of other molecules, playing a key role in cellular defense. Some antioxidants are ingested in the diet. For example, ascorbate (vitamin C), tocopherols (e.g., vitamin E), and napthoquinoids (e.g., vitamin K), are antioxidants that are not synthesized de novo in humans, but are acquired through dietary intake. Other antioxidants are produced by the cells, where glutathione (GSH) is probably the most important (further discussed in section 1.3.2.1). These non-enzymatic antioxidants are depleted or modified and must be replenished or restored after their interaction with ROS.

1.2.5 Selenium

Selenium is an essential trace element, present in group IV of the periodic table. As selenium and sulfur are present in the same group they share many chemical properties. In humans, selenium can have both antioxidant and prooxidant properties, dependent on the chemical species and the concentration. Selenium is absorbed from the diet, both in organic and inorganic forms. After absorption, selenium is transported via the blood to the liver which is supporting the rest of the body with selenium, mainly by the production and secretion of selenoprotein P [37]. Selenoprotein P, the major source of selenium in plasma, is further taken up into other organs by receptor mediated mechanisms [38].

Selenium is present in the 21st amino acid selenocysteine, and is crucial for the activity of several selenoproteins. Selenocysteine is incorporated into proteins through a unique and evolutionary conserved mechanism. The amino acid is encoded by the stop codon UGA and

incorporation of the amino acid requires a Selenocysteine Insertion Sequence (SECIS) element in the untranslated mRNA region [39]. Recruitment of a specific elongation factor by SECIS binding protein (SBP2) translates UGA as selenocysteine instead of termination [40]. Furthermore, selenocysteine is, in contrast to the other amino acids, synthesized on its specific tRNA (incorporation of selenium has been reviewed in [41]). So far, 25 human selenoproteins have been described, many of them are redox active (e.g. thioredoxin reductases and glutathione peroxidases), however the function of many selenoproteins are yet to be determined.

Selenium may be either an antioxidant or an oxidant. The function is strictly concentration dependent, where too low levels will lead to reduced levels of important selenoproteins, while a too high concentration leads to severe toxicity. Selenium deficiency has been shown to be implicated in numerous pathologies, for example cancer [42], cardio-vascular disease [43], and immunodeficiency [44]. In a recent study of selenium deficient elderly people a significantly increased risk of cardiovascular mortality (56%) was observed in persons with serum selenium in the lowest quartile compared to the quartile with the highest selenium level [45]. A previous study showed a significant reduction in cardiovascular mortality as a result of supplementation with selenium and coenzyme Q10 [46]. In contrast, high selenium intake may cause selenosis which might be lethal [47, 48].

1.2.6 Imbalance in the oxidative/antioxidative systems

An imbalance between prooxidants and antioxidants is causing an oxidative stress in cells. The reactive species that are not scavenged by antioxidants can cause oxidative damage to proteins, membranes, and DNA [49]. The impact on the organism depends on the type of ROS, the activity of the antioxidant systems, and the efficiency of the repair systems.

1.2.7 Oxidative modifications of protein thiols

Oxidative modifications to proteins can in some cases be beneficial, and occur in a controlled manner. For example in the ER, which has a highly oxidizing internal milieu, protein disulfide isomerase (PDI) is responsible for the folding of proteins and uses oxidation to shape them to their correct tertiary structure [50].

Oxidative modifications can also occur in a non-controlled manner where the cysteine residues play a vital role. The cysteine thiol is highly reactive, and is therefore sensitive to oxidation insults [29]. The thiol (-SH) moiety in the side chain of the amino acid cysteine is a nucleophilic functional group that is highly susceptible to oxidation, particularly by ROS. Oxidation of the -SH group in the side chain of cysteine can result in the formation of intramolecular and intermolecular disulfide bridges and the formation of sulfenic acid (S–OH), sulfinic acid (SO_2H), and sulfonic acid (SO_3H), all of which can alter the function or activity of proteins [51]. In addition, sulfenic acid is highly prone to glutathionylation (further discussed in 1.3.2.2) (Figure 2). These changes has for long been considered as negative, as many proteins become inactive due to the oxidative insult, but in recent years the role of oxidative modifications as redox switches has come into focus.

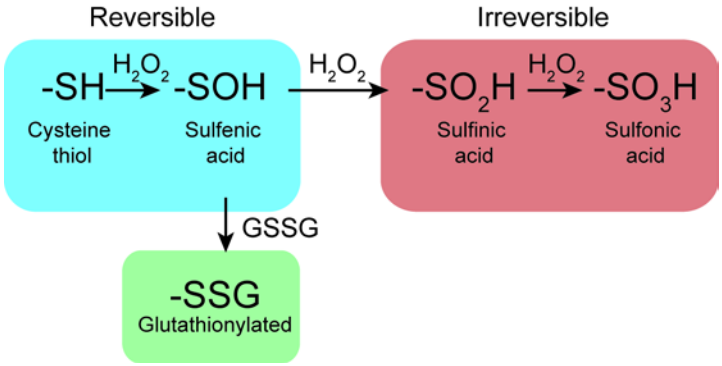

Figure 2. Oxidative modifications of cysteine residues in proteins.

1.2.8 Redox switches

Thiols can be readily modified in response to increased oxidative stress. A redox signal, such as hydrogen peroxide, can lead to a reversible redox alteration to thiols in a target protein. Once the initial redox signal has returned to basal levels, the alteration reverses, and the activity of the protein reverts to its initial level. This general scenario for redox signaling by thiol proteins has been found in a number of cytosolic signaling systems [52]. For example, several transcriptions factors are regulated by oxidation/reduction of cysteine residues (e.g. nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and nuclear factor erythroid 2-related factor 2 (Nrf2)/ Kelch like ECH-associated protein 1 (Keap1)) [53].

1.3 THIOREDOXIN SUPERFAMILY OF PROTEINS

The thioredoxin superfamily of proteins is a group of proteins with the main function to reduce disulfides formed by oxidation of cysteines. Several of the proteins share the same tertiary structure, named the thioredoxin fold, consisting of four β-sheets surrounded by three α-helixes [54]. The super family comprises the thioredoxin (Trx) and the glutaredoxin (Grx) systems, but also other groups of proteins, e.g. the protein disulfide isomerase (PDI) family.

1.3.1 Thioredoxin and thioredoxin reductase

Thioredoxin is a small (~12 kDa), ubiquitously expressed protein with two redox active cystine residues (-Cys-X-X-Cys-) in its active center [55]. Trx was first characterized in *E. coli* in 1964 as the hydrogen donor in the enzymatic synthesis of deoxyribonucleotides [56]. Shortly after, thioredoxin reductase (TrxR) was identified as the reductant of Trx [57].

The active thiol in Trx acts as a nucleophile to attack the target disulfide on the substrate, forming a disulfide bridge with the substrate, denoted a mixed thiol. The second cysteine residue in Trx, called the resolving thiol, is reducing the target proteins disulfide. The oxidized disulfide left in Trx will be recycled to its reduced state by TrxR, utilizing NADPH as the electron donor. Normal function of TrxR requires two subunits of TrxR arranged head-to-tail. Due to this homodimeric subunit configuration the enzyme has two active sites, and both subunits are needed for one catalytic cycle [58] (Figure 3).

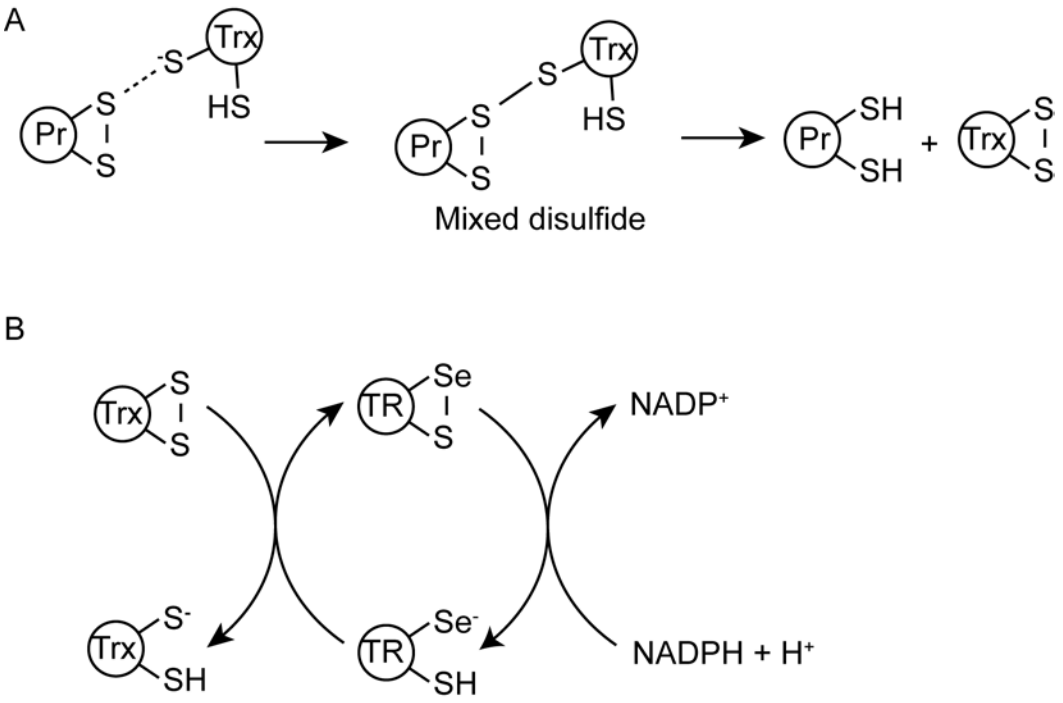

Figure 3. Trx/TrxR system. *(A). Reduction of an oxidized thiol on a protein. The active thiol on Trx binds to the target protein, forming a mixed thiol. The second thiol is further donating its thiol to the target protein leaving the target thiol reduced, at the expense of becoming oxidized itself. (B). Basic mechanism for reduction of Trx. Oxidized Trx is reduced by TrxR at the expense of NADPH and* H^+.

There are two human isoforms of Trx, the cytosolic Trx1 which also has the ability to translocate to the nucleus or to be secreted extracellularly [59, 60], and the mitochondrial Trx2 [61]. In addition, a truncated form of Trx1, Trx80, has been found in plasma. Trx80 is redox inactive, and is mainly produced by activated monocytes [62]. The biological function of Trx80 in plasma is not known, but it has been found to act as a cytokine [63, 64]. In addition to reduction of protein thiols, Trx has the ability to reduce Prx, and is for that reason indirectly involved in the scavenging of H_2O_2 [65].

Thioredoxin reductase (TrxR) is a selenoprotein with selenocysteine in its active site, and replacement of selenocysteine with a cysteine resulted in drastically decreased enzymatic activity [66]. TrxR is expressed in the cytosol (TrxR1) and in mitochondria (TrxR2), furthermore there is a testis specific TrxR3 [58]. Low level of TrxR has also been found in human plasma [67], and the level in plasma was increased in a mouse model treated with hepatotoxic compounds [68]. TrxR is the only enzyme known that can reduce Trx and is therefore an essential part of the thioredoxin system [69]. The main substrate for TrxR is Trx, but the list of substrates has grown long, and now includes *e.g.* selenium compounds [70, 71],

lipid hydroperoxides [72], ubiquinone [66], lipoic acid [73], and ascorbate [74]. Furthermore, TrxR has the ability to directly reduce disulfides in proteins using NADPH as its primary electron donor.

The expression of several antioxidant genes is regulated by the transcription factor Nrf2. Nrf2 binds to the DNA promotor region denoted the antioxidant response element (ARE) [75]. Nrf2 itself is a short lived protein and is rapidly subjected to proteasomal degradation, by either of its three negative regulators; Keap1, glycogen synthase kinase (GSK)3/β, or E3 ubiquitin ligase Hrd1. Cysteine modifications to Keap1 blocks its ability to bind to Nrf2, which hinders degradation of Nrf2 and allows it to translocate to the mitochondria and bind to ARE where transcription will be initiated [53]. Trx and TrxR are both expressed under ARE and are therefore, in addition to other transcriptions factors, regulated by Nrf2 [69].

1.3.2 Glutaredoxin

Glutaredoxin (Grx) has similar functions as Trx, with the main function to reduce disulfides. The glutaredoxins has three characteristic regions: the dithiol/disulfide active site, CSYC, the GSH binding site, and a hydrophobic surface area. There are two classes of Grx, divided according to their active site motifs. The first class has many similarities to Trx, with the active site Cys-X-X-Cys, and the second class has a monothiol active site [76, 77]. Due to the differences in the active site the Grx's has two different ways of exerting their function, a monothiol or a dithiol mechanism [78] (Figure 4). The glutaredoxins are kept in their reduced form by GSH which in turn utilizes NADPH dependent glutathione reductase (GR) for reduction.

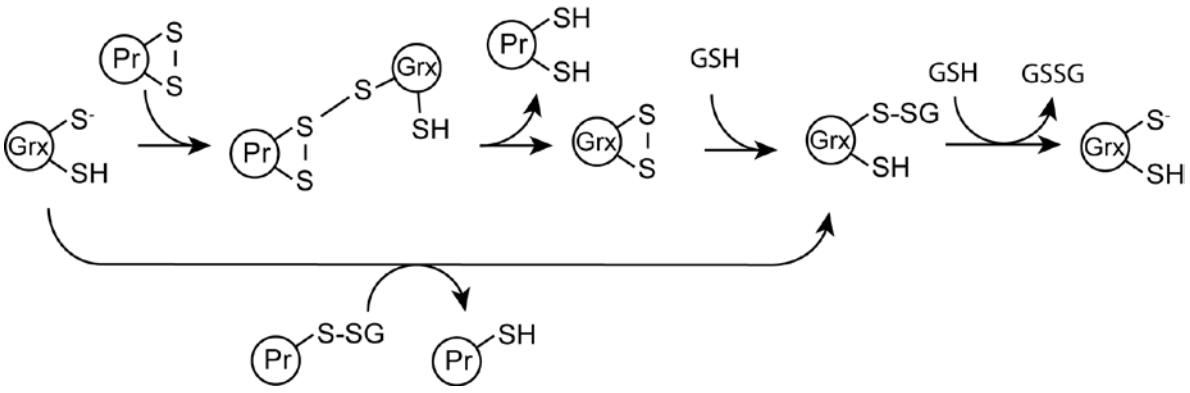

Figure 4. The Grx system and the deglutathionylation process. *The dithiol mechanism is described in the top part, and the monothiol/deglutathionylation mechanism below.*

There are four human Grx isoforms; Grx1 is cytosolic, Grx2 is expressed in three splice variants; mitochondrial Grx2a, nuclear Grx2b, and cytosolic Grx2c, Grx3 is expressed in the cytosol, and Grx5 is mitochondrial.

Grx1 is primarily a cytosolic protein, but it has been found to translocate into the nucleus where it regulates several transcription factors [79]. Human Grx2 was first described as a

mitochondrial protein [80], but in a second article published almost at the same time, Grx2 was found to have alternative splicing variants and a nuclear translocation signal [81]. Grx2 is an iron sulfur cluster (Fe/S) protein which in its inactive form is comprised by two Grx2 molecules and [2Fe-2S]. The Fe/S is released during oxidative stress and Grx2 becomes active [82]. Grx1 and Grx2 are dithiols and the mechanisms for reduction of a substrate are similar to the reaction for Trx1. Furthermore, both Grx1 and Grx2 has also been found to be secreted into plasma [83]. Grx2 catalyzes the reduction of glutathionylated substrates with a lower rate but higher affinity compared to Grx1, with a particular high efficiency for glutathionylated substrates including GSSG. In addition, it has been shown that Grx2a can be reduced by TrxR2 [84]. Grx3 (also known as PICOT) is also a Fe/S protein and has, in contrast to Grx1 and Grx2, only one thiol in its active center [85]. The less studied Grx5 is also a monothiol and has been shown to be important in the synthesis of Fe/S clusters within the mitochondria [86].

1.3.2.1 Glutathione and glutathione reductase

GSH is a tripeptide (γ-glutamyl-cysteinyl-glycine) synthesized in almost all eukaryotic cells, with a concentration of 0.5-10 mM in most cells [87, 88]. The major function of GSH is to serve as thiol redox buffer, thereby maintaining the redox balance and protect against oxidative stress [89, 90]. However, studies on GSH depleted cells showed that, unlike thioredoxin, GSH could not support by itself the redox duties of the cell [91].

The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed by two cytosolic enzymes, glutamylcysteine synthetase (GCS) and GSH synthetase. The production of GSH occurs in virtually all cell types, with the liver being the major producer and exporter [87]. The oxidized form of GSH is GSSG which is reduced by glutathione reductase (GR) at the expense of NADPH. Maintenance of optimal GSH/GSSG ratio in the cell is of importance for normal cell function and survival. In addition to several proteins in the Trx family of proteins, ARE regulates the cystine/glutamate antiporter (xCT) via Nrf2 which is essential for GSH synthesis.

1.3.2.2 Glutathionylation

There are many different types of reversible protein modifications, e.g. phosphorylation, acetylation, ubiquitination, and glutathionylation. Glutathionylation is a process where glutathione is bound to proteins as a way of either temporarily inhibiting their activity or as a way to protect them against irreversible oxidation [92]. Glutathionylation can, in contrast to e.g. phosphorylation which is enzymatically regulated, occur spontaneously, and any protein could be attacked by GSH if the protein sulfhydryl is in the form of sulfenic acid. However, the typical redox potential of protein-Cys moieties prevents formation of protein-SSG via thiol-disulfide exchange with GSSG unless the GSSG concentration is unusually high. Furthermore, in order for most Cys residues to be glutathionylated, there must first be an activated intermediate formed, such as sulfenic acid [92]. Glutathionylation may be a

mechanism to regulate the level of glutathionylated proteins in the cell, however in most studies glutathionylation has been found to occur during oxidative stress [93].

Grx can catalyze the deglutathionylation of glutathionylated proteins [94], and catalyzes the deglutathionylation of protein-GSH mixed disulfides far more efficiently than Trx or PDI [95]. The enzymatic mechanism of deglutathionylation with Grx involves two steps. First, one cysteine on Grx deglutathionylates protein-SSG via a thiol disulfide exchange reaction yielding protein-SH and a Grx-SSG intermediate. Secondly, Grx-SSG binds GSH and the glutathionyl moiety is removed, regenerating Grx1 and producing GSSG [96]. Protection of proteins by glutathionylation was shown in a study where cardiac mitochondria was exposed to hydrogen peroxide and α-ketoglutarate dehydrogenase was shown to be reversibly glutathionylated [97].

1.4 CELL AND TISSUE DAMAGE

Oxidative stress has been implicated in several pathologies. For example, increased oxidative stress has been observed in several types of cancer, where ROS has been associated with cancer development, metastasis, cancer progression and survival [98]. Atherosclerotic diseases such as coronary artery disease (CAD) and stroke has been associated to oxidative stress, where fruits and vegetables high in antioxidants has emerged as an alternative treatment strategy [99]. In addition, increased oxidative stress index was observed in patients suffering from ischemic stroke [100] and oxidative damage to DNA is one of the earliest detectable events in neurodegenerative diseases [101].

1.4.1 Neurodegeneration

Neurons possess properties that make them particularly vulnerable to oxidative stress, due to high energy and oxygen consumption and a membrane composed of high levels of unsaturated lipids. In addition, neurons contain high amounts of transition metals and their antioxidant defense is low compared to other types of cells [102]. Furthermore, the ATP turnover rate in the human brain is extremely high (4.7×10^9 ATPs/neuron/s), which means that a resting human brain utilizes approximately 5.7 kg ATP per day [103]. With this high oxygen turnover, even a small leakage of electrons from the ETC can lead to large changes in the redox balance.

1.4.2 Alzheimer’s disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease, characterized by impaired cognition and dementia. The major risk factor for AD is aging [104], but oxidative stress has been implicated in the pathogenesis. It is however debated whether oxidative stress is a cause or a consequence of the disease. AD is characterized by an irreversible loss of functional neurons primarily in the associative neocortex and hippocampus [105]. There are two major neuropathological signs for AD; extracellular accumulation of plaques (accumulation of β-amyloid (Aβ)), and the presence of intracellular neurofibrillary tangles

(aggregates of tau) [106]. The disease usually has a slow progression and major changes are likely to occur before any symptoms appear.

1.4.2.1 Tangles formed by hyperphosphorylated tau

In 1986, the paired helical filaments seen in AD brains were identified as the microtubule associated protein tau, a normal brain cytoskeletal protein which is stabilizing the axonal microtubule of the neuronal cells [107]. In AD, a phosphorylation of the protein occurs, causing the protein to aggregate and form intracellular tangles. Some degree of phosphorylated tau has been observed in patients without any clinical symptoms, suggesting that accumulation of the protein is an early event in the disease progression. An accumulation of phosphorylated tau impairs the neurons and decreases their functionality [108].

1.4.2.2 Amyloid precursor protein and β-Amyloid

Amyloid precursor protein (APP) is a protein expressed in the adult human brain. Increased expression of APP can be seen during neuronal differentiation and neurite outgrowth, suggesting a role for APP in the development of the nervous system (the functions of APP has been reviewed in [109]).

APP can be cleaved either by α-secretase or β-secretase [110], generating a secreted N-terminal soluble fragment (sAPPα or sAPPβ) and a C-terminal APP fragment (α-CTF or β-CTF). The CTF is further processed by γ-secretases whereby amyloid β (Aβ) peptides of various lengths are formed [111]. As α-secretase cuts APP within the Aβ region, Aβ generation is prohibited. However, cleavage of β-CTF leads to formation of Aβ peptides which can aggregate and form plaques (mainly $A\beta_{1\text{-}42}$). Furthermore, $A\beta_{1\text{-}42}$ has the ability to form oligomers that are soluble and highly neurotoxic, the basis for their potent toxicity is however not known [112]. Both Aβ oligomers and Aβ plaques are potent synaptotoxins that block proteasome function, inhibit mitochondrial activity, alter intracellular Ca^{2+} levels, and stimulate inflammatory processes [106]. All of these processes can further increase the formation of radicals, enhancing the oxidative stress in the affected neurons. Increasing evidence suggests that Aβ oligomers may be the primary cause of AD as they have a greater correlation with dementia than insoluble $A\beta_{1\text{-}42}$ [113].

1.4.2.3 Oxidative stress in AD pathology

In addition, analysis of Aβ plaques from human brains revealed accumulation of aluminum, iron, copper and zinc in the plaque [114, 115]. Furthermore, the levels of the iron-homeostatic peptide hepcidin, and the iron exporter ferroportin, were found to be reduced in hippocampal lysates from AD brains [116]. Aβ becomes a prooxidant when complexed to copper or iron generating hydrogen peroxide through the Fenton reaction. Due to the association of oxidative stress to AD, several studies with antioxidant treatment have been performed [117-120]. In these studies, the antioxidant vitamin E stands out as a promising treatment strategy in preventing memory loss in AD. It is however unclear whether the

positive effects seen early in the disease process will remain over longer periods of time or in more advanced stages of the disease.

1.4.3 Parkinson's disease

Parkinson's disease (PD) is characterized by a loss of the dopamine producing neurons primarily in the *substantia nigra* (SN) region of the human brain. The loss of these neurons leads to motor problems and manifests as involuntarily movements. The exact cause of the loss is still to be discovered, but oxidative stress has been implicated in the pathology [121], as shown by e.g. increased activity of SOD [122] and basal lipid peroxidation [123] in SN of PD patients. The major neuropathological sign of PD is inclusion bodies (Lewy bodies) which are constructed of α-synuclein [124, 125].

1.4.3.1 The role of oxidative stress in Parkinson's disease

Decreased levels of reduced GSH have been observed in the SN region of PD patients, the levels of GSSG did however not differ between PD and controls, suggesting an increased oxidative stress in the tissue [126, 127]. In contrast, increased levels of GSH and antioxidative enzymes were observed in the frontal cortex of PD patients compared to controls [128], which means that the major impact of oxidative stress probably occurs in the areas that are mainly affected in PD. Increased levels of iron has been observed in SN of PD patients, and no difference could be observed in the cerebellum [129]. Furthermore, decreased levels of selenium have been observed in patients with PD [130]. The redox imbalance in the affected neurons causes oxidative damage to the neurons and begins to alter the synthesis and metabolic pathway of dopamine, which leads to further increase in oxidative stress.

1.4.3.2 Dopamine

Dopamine is a signaling molecule in the motor neurons and is normally stored in vesicles intracellularly in the neurons and is released upon stimuli. In the presence of oxidative stress and α-synuclein the storage is disrupted leading to an increased dopamine concentration in the cytoplasm [124]. Once in the cytoplasm, dopamine will react with molecular oxygen forming 3,4-Dihydroxyphenylacetic acid (DOPAC) or Dopamine semi-quinone. The oxidation to DOPAC requires the enzyme monoamine oxidase (MAO) [131]. During the oxidation processes, hydrogen peroxide or superoxide is released [121]. (Figure 5)

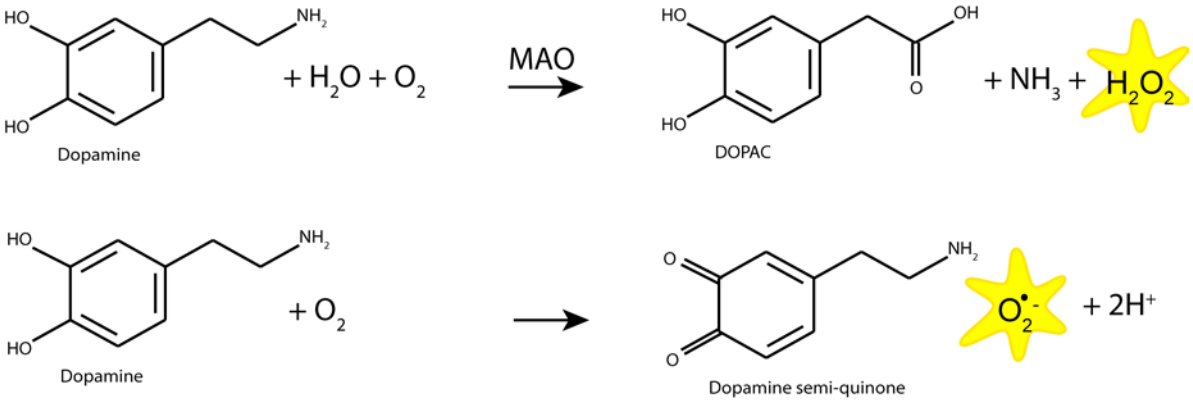

Figure 5. Dopamine and its metabolites.

1.4.4 Ischemia/Reperfusion

Ischemia is a phenomenon that occurs when the cells do not receive the amount of oxygen required for their normal metabolism due to restriction of blood flow, caused by e.g. stroke, heart infarction, or surgical procedures. When the blood flow returns, and oxygen reenters the tissue, reperfusion injury can occur. The major impact of reperfusion injury is attributed to oxidative stress. Increased production of radicals during reperfusion has been linked to xanthine oxidase, mitochondria, NADPH oxidase, and uncoupled nitric oxide synthase [132].

Hypoxanthine and xanthine are metabolites formed during ATP utilization. During normal oxygen levels, hypoxanthine is metabolized by xanthine dehydrogenase (XDH) to xanthine and ultimately to uric acid at the expense of NAD^+. During ischemia ATP is utilized, but cannot be resynthesized, and as a result of the mitochondrial oxidative phosphorylation its metabolites adenosine, inosine, hypoxanthine and xanthine accumulate. In addition, the levels of Ca^{2+} increases in the cytoplasm due to failure of the ATP dependent Ca^{2+} pumps. With the onset of ischemia, the rise in intracellular calcium results in a proteolytic conversion of XDH to xanthine oxidase (XO), which uses molecular oxygen instead of NAD as its electron acceptor. When the restriction of blood flow is removed, with a massive influx of oxygen into the tissue, XO will catalyze the production superoxide [133, 134] (Figure 6).

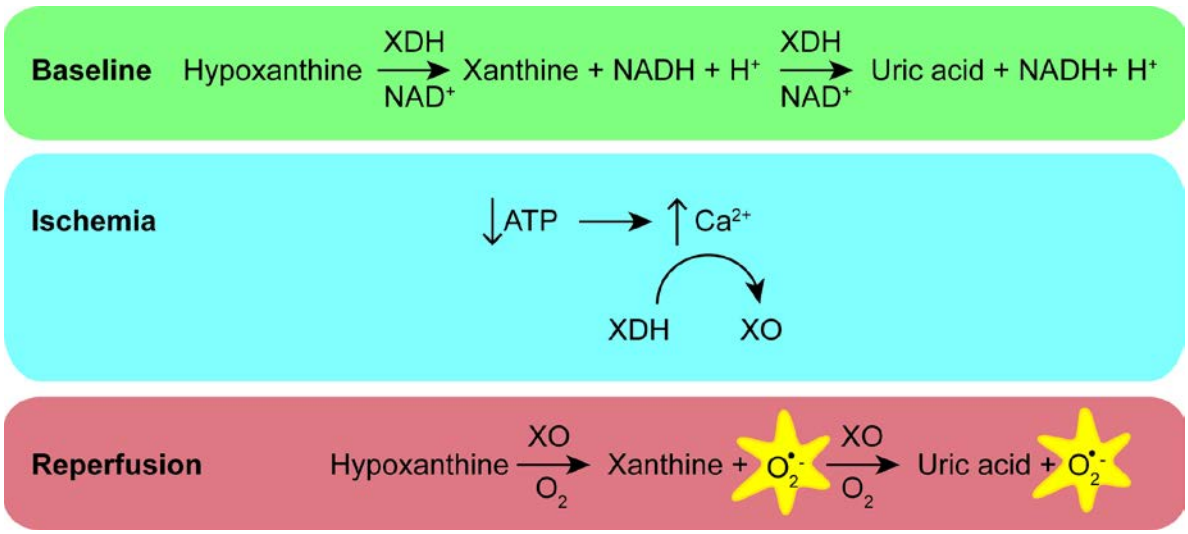

Figure 6. Conversion of XDH to XO during IR.

In addition to the production of ROS, reduced levels of antioxidants has been connected to injuries from IR, as the GSSG/GSH ratio increases when the liver is subjected to ischemia [135]. Furthermore, activation of the transcription factor Nrf-2 has been reported in studies of oxidative stress in relation to IR [136, 137].

Even though oxidative stress is usually considered as something negative, low levels of oxidative stress can be beneficial to the cells as the antioxidant systems are upregulated, preparing the cell for increased oxidation [138].

1.4.5 Effect of microenvironment

The microenvironment is of great importance in all systems including cell culturing. Minor changes in the microenvironment can lead to substantial changes in the response to toxic compounds. For example, dependent of the medium selected, the compound used may be oxidized in the culturing medium, leading to treatment by a different compound than intended [2]. The expression of membrane transporters may also affect the outcome of cellular experiments. The amount of extracellular thiols and the ability to sustain high levels of especially cysteine is one factor shown to determine the tumor specificity of selenium compounds [139]. Furthermore, the glucose level in the culturing medium was shown to induce epithelial to mesenchymal transition (EMT) in a recent publication using a breast cancer cell line [140]. Another recent study showed that high glucose induced EMT was caused by increased intracellular ROS production, and inhibition of Trx activity, which was prevented either by treatment with the antioxidant NAC or inhibition of thioredoxin interacting protein [141].

2 THE PRESENT INVESTIGATION

2.1 AIMS OF THE STUDY

Thioredoxin and glutaredoxin are important for protection against damage sustained by oxidative stress. This study aimed at evaluating the role of redox proteins in cell and tissue damage.

2.2 RESULTS AND DISCUSSION

2.2.1 Paper I

Alteration of Thioredoxin and Glutaredoxin in the Progression of Alzheimer's Disease

2.2.1.1 Background

Oxidative stress has been implicated in AD, and the Trx family of proteins plays an important role in protecting the cells against oxidative stress. Previous studies on the involvement of Trx and Grx in AD has been conflicting, and few human studies have been performed with respect to the redox proteins. In this study we were examining the role of Trx and Grx in AD. The levels in hippocampus in advanced stages of AD as well as the levels of the proteins in plasma and cerebrospinal fluid (CSF) from patients with different stage of AD were evaluated. Furthermore, before onset of clinical symptoms, AD is often mistaken for depression, and it is difficult to make a distinction between the two. A reliable panel of biomarkers would greatly improve in the diagnosis of the disease and help in determining which treatment strategy to pursuit.

2.2.1.2 Aim

The aim of the study was to evaluate the role of Trx and Grx at different stages of AD.

2.2.1.3 Methods and results

Sandwich enzyme linked immuno sorbent assay (ELISA) was used to study the levels of Trx1 and Grx1 in patients with different stages of AD. Patients with depression were used as a control group, as the early stages of the AD often resemble depression. Both Trx1 and Grx1 was shown to be released into CSF, furthermore the levels was shown to increase in early stages of the disease compared to mild cognitive impairment. As the levels of lactate dehydrogenase did not differ between the stages, we conclude that Trx and Grx are secreted rather than increased due to cell death. Trx and Grx levels were shown to correlate well with the levels of previously established biomarkers Tau and P-tau. When combining all the used diagnostic markers (MMSE-score, Tau and P-tau) with Trx and Grx levels we were able to distinguish between the different stages of the disease in 32 out of 33 patients. Trx was shown to be particularly valuable in the distinction between MCI sable and MCI converter.

Immunohistochemistry (IHC) was used to evaluate the expression of Trx and Grx isoforms in postmortem hippocampus sections of advanced stages of AD. Axonal staining for both Grx1

and Grx2 was observed in the control sections but not in the AD hippocampus. A nuclear localization of Trx1 was observed in the control sections while the staining was mainly cytosolic in the AD samples. Decreased intensity of mitochondrial staining for Trx2 was observed in AD compared to control.

2.2.1.4 Conclusion

Oxidative stress has been linked to early changes in AD. Including Trx in in the biomarker panel for AD could improve in the diagnosis, and especially improve in the distinction between mild cognitive impairment and early AD. Furthermore, a secretion of Grx and Trx into the CSF and plasma implicates that the proteins are important extracellularly for protection against ROS formation. Further studies are needed in order to fully understand the role of the redox proteins in AD.

2.2.2 Paper II

Protective effects of the thioredoxin and glutaredoxin systems in dopamine-induced cell death

2.2.2.1 Background

The exact cause of Parkinson disease is still unknown, but oxidative stress has been connected to early events in the disease progression. Increased oxidative stress in the dopaminergic cells can lead to the disruption of the storage of dopamine which is highly sensitive to oxidation. When dopamine is released in the cytosol, it will readily react with oxygen and form the toxic dopamine quinone. During this process, radicals will also be released. Since the Trx system is involved in protection against oxidative stress, the system is likely to be implicated in PD.

2.2.2.2 Aim

The aim of this study was to investigate the role of Trx and Grx in the defense against dopamine induced cell death.

2.2.2.3 Methods and results

IHC was used to determine the expression of Trx and Grx in *substantia nigra* of PD patients. Significant downregulation of Trx1 and TrxR1 were observed in PD patients compared to control.

The neuroblastoma cell line SH-SY5Y and the nematode *C. elegans* were used as model systems, and treatment with the dopamine metabolite 6-hydroxydopamine (6-OHDA) was performed. Pretreatment with low levels of selenite protected SH-SY5Y cells against 6-OHDA toxicity. No difference in mRNA level of the redox proteins could be observed by the pretreatment with selenite, but 6-OHDA treatment alone increased the mRNA levels of Trx1, TrxR1, TrxR2, Grx1 and Grx2. No change in the protein levels of Trx and Grx could be detected upon 6-OHDA treatment, the activity of TrxR was however shown to be increased

as determined by enzymatic assays. Furthermore, the toxicity of 6-OHDA was determined after knocking down the redox proteins with small interfering RNA, increased toxicity could be observed after a knock down of TrxR1, Grx1 and Grx2, but not in Trx1 siRNA. Knockdown experiments of *trxr-1* and *trx-5* in the nematode *c. elegans* were performed, and increased neuronal degradation could be observed in worms lacking TrxR1 upon treatment with 6-OHDA.

In addition, an enzymatic assay to determine if 6-OHDA could interact with the Trx and Grx systems was performed. We could demonstrate that TrxR had the capacity to reduce the 6-OHDA-quinone and that the reaction efficiency was increased in the presence of Trx. Even though not as efficient, GSH was shown to reduce the quinone, and the reaction rate increased in the presence of Grx.

2.2.2.4 Conclusion

This study demonstrate that the Trx and Grx system not only play an important role in dopamine induced cell death, but also exert critical protective effects by direct enzymatic reduction of the neurotoxic dopamine metabolite 6-OHDA-qouinone.

2.2.3 Paper III

Morphological alterations and redox changes due to ischemia-reperfusion injury during liver surgery

2.2.3.1 Background

Ischemia/reperfusion (IR) injury is a known cause for complications during liver resection and surgery, caused by oxygen deprivation followed by a burst of oxygen into the tissue. The deprivation of oxygen is leading to ATP depletion which causes an increase in the cytoplasmic Ca^{2+} levels which results in conversion of XDH to XO. When hypoxanthine is metabolized by XO superoxide will be produced as a side product. The increased oxidative stress in the cells during reperfusion is causing damage to the tissue. Only few studies have investigated the cellular and ultrastructural changes on human tissue due to IR.

2.2.3.2 Aim

The aim of this study was to evaluate the cellular and ultrastructural changes due to IR in human liver tissue. In addition, the impact on the Trx and Grx system was evaluated.

2.2.3.3 Methods and results

In this study liver surgery was used to model IR in a human tissue. During routine surgery of 11 patients, ischemia was induced by portal triad clamping (PTC), also known as the Pringle maneuver, which has been one of the most widely used methods to reduce blood loss during hepatic surgery. Liver samples were collected at three time points; before induction of ischemia, 20 minutes post-ischemia, and 20 minutes post-reperfusion. Electron microscopy (EM) was used to study the ultrastructure of the liver tissue. Post-ischemia a disruption of the

sinusoidal lining of the liver cells was observed in 10 out of the 11 patients. Post-reperfusion, the sinusoidal lining reappeared and a reactivation of the sinusoidal cells could be observed. In addition, paracrystalline inclusions could be observed in the mitochondria in seven of the 11 patients post-ischemia. These inclusions were still present post-reperfusion.

In order to study the involvement of redox proteins in IR, mRNA levels of the redox proteins were determined by qPCR, but no changes were detected. Immuno-gold electron microscopy was used to study Trx1 and Grx1 *in situ*. Even though there were no significant inter-individual differences in detected Trx1 levels, the intra-individual Trx1 levels differed between baseline, post-ischemia, and post-reperfusion.

2.2.3.4 Conclusion

This study was undertaken to evaluate the effect on liver morphology and expression of redox proteins during IR. We could show that the major burden of IR is borne by the endothelial cells lining the sinusoids in the liver, and the hepatocytes remained almost unaffected, deeming PTC a safe method to use in liver surgery. As Trx1 seems to change in localization we suggest a possible role for especially Trx in the protection against IR induced damage. Further studies are needed in order to fully understand the role of redox proteins in IR.

2.2.4 Paper IV

The effect of media composition on cell growth, thiol status, redox proteins, and in selenium toxicity

2.2.4.1 Background

Cell lines are extensively used in medical research, as they provide a cost effective and flexible experimental model. However, general guidelines of how to maintain a specific cell line with regard to culturing media are lacking. The composition of culturing media differs in terms of components and concentration, and the same media purchased from different suppliers may not have the same constituents.

2.2.4.2 Aim

The aim of this study was to evaluate whether different culturing conditions could influence the proliferation, morphology, differentiation, thiol levels, expression of redox proteins, and sensitivity to selenium toxicity.

2.2.4.3 Methods and results

The three cell lines used in this study (A549, Huh7, and HepG2) were cultured in four commonly used media (RPMI, DMEM, MEM, and F12). DMEM was shown to increase the proliferation rate in the cells, and culturing cells in MEM resulted in the lowest proliferation rate. Furthermore, cells were cultured on glass slides and stained for the epithelial marker cytokeratin (CK18) and the mesenchymal marker Vimentin. A549 cells had a low expression

of CK18 when cultured in MEM and DMEM, and the expression of Vimentin increased when cultured in DMEM.

The extracellular concentration of thiols was determined by enzymatic assay and was shown to be increased in Huh7 cells cultured in F12 compared to RPMI. The expression of the redox proteins Trx1, Grx1 and TrxR1 was studied using ELISA or western blot. No changes were observed in cells cultured in the different media. In contrast, increased activity of TrxR could be observed in A549 cells cultured in MEM compared to the other media.

To study the response to toxicity, the two selenium compounds, selenite and seleno-methylselenocysteine (MSC) were used and viability determined 48 hours after addition of the compound. The toxicity of selenite was shown to be decreased in A549 cells cultured in DMEM compared to culture in RPMI or F12.

2.2.4.4 Conclusion

The selection of culture medium is of outermost importance as changes in morphology, response to toxicity and activity of redox protein may differ widely depending on the particular medium selected. These results highlight that the culturing conditions are crucial, as changes in the micro environment may profoundly change the outcome of the study.

2.3 COMMENTS ON THE METHODOLOGIES

2.3.1 Evaluation and image analysis of immunohistochemical staining

Immunohistochemical staining is often used in order to show the localization and distribution of a particular protein. It is however difficult to correctly determine the level of staining. In order to make an unbiased evaluation of the level of staining image analysis software can be used. These programs are measuring the intensity or saturation of the staining. Technical pitfalls do however exist:

(1) In immunological staining, the final step is usually performed by enzyme based color development. This step is crucial as the reaction is stopped either at a specific time or when the optimal amount of color has appeared. Both ways are easily causing lack in consistency, especially if the staining is performed at different time points.

(2) Depending on the microscope and camera used, background color may differ, generating inconsistency in saturation levels.

2.3.2 The importance of the model system employed

Due to ethical considerations the possibilities to perform human studies are limited. Therefore, in order to study different aspects of disease, several model systems have been developed. Cell culture is usually used as a first set of tests. Cell lines are however far from a complex organism, and in order to fully understand the mechanism cell lines are usually not sufficient as an experimental model. When the researcher fails to acknowledge this, conclusions may be drawn that do not reflect the truth.

3 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Oxidative stress has been implicated as a major pathogenic mechanism in several diseases. The focus of this study was to investigate the role of the two redox proteins Trx and Grx in different pathological conditions.

The cause of most neurodegenerative diseases is still to be discovered. One likely contributor is oxidative stress which has been found to be elevated in both AD and PD. In **Paper I** the expression profiles of Trx and Grx were established in cerebrospinal fluid (CSF) and plasma at different stages of AD. We were able to show that the level of Trx in CSF differed in AD during disease progression. The level of especially Trx1 showed high correlation to the previously established and validated biomarkers tau and p-tau. There is a need for sensitive and specific markers in order to diagnose the disease also at an early stage and monitor the disease progression. In addition, we for the first time showed that Grx1 is released to the CSF. The levels of Trx1 and Grx1 in CSF did not correlate with the levels of lactate dehydrogenase, indicating that the proteins were secreted rather than released due to cell death. This finding suggests that Trx and Grx may play a role extracellularly in AD pathology. Further studies are however needed in order to elucidate their importance in the CSF. Furthermore, axonal staining for Grx1 and Grx2 was observed in the control brains, but not in the AD brains. A decreased mitochondrial staining for Trx2 was found in AD. However, this observation should be interpreted with caution due to the methodological limitations as a quantitative method.

Increased oxidative stress has been implicated in PD, which can lead to the disruption of dopamine storage. Dopamine is highly prone to oxidation and free radicals are released during the oxidation process. In **Paper II** the role of the Trx family of proteins in dopamine induced cell death was evaluated. We showed that Trx and TrxR could directly reduce the quinone of dopamine. In addition, in PD brains, decreased levels of both Trx1 and TrxR1 were detected. The effect of dopamine induced cell death was further evaluated in different model systems, the cell line SH-SY5Y and the nematode *C. elegans*. An increased toxicity of dopamine was observed in cells with reduced levels of TrxR1 or Grx1 (by siRNA), and increased neuronal degradation was observed in nematodes lacking expression of TrxR1. In conclusion, the Grx and particularly the Trx system play an important role in protecting neurons against dopamine induced cell death.

In IR, the major impact on the tissue is believed to occur during reperfusion, when for example the conversion of hypoxanthine to xanthine is producing radicals as a side product. In **Paper III**, we showed that the major changes in the liver occurred in sinusoidal endothelial cells and that changes observed after 20 minutes of ischemia were reversible and had partially reverted after 20 minutes of reperfusion. Furthermore, the role of Trx and Grx in ischemia and reperfusion was investigated. The mRNA levels of the proteins did not change during the study period. The lack of observed changes might be explained by the fact that the RNA was purified from whole lysate, and not from one specific cell type. We could not

detect any changes in protein levels of Grx1 and Trx1 in the hepatocytes when measured by immuno gold. However, in some patients lower levels of Trx were observed at reperfusion compared to the earlier time points. This effect might indicate a possible secretion of the protein and merits further investigation with a larger number of patients. In future studies, sampling of plasma would be important to confirm this hypothesis.

Paper IV is a methodological study where we studied the effect of cell culture media composition on cell growth, thiol status, redox proteins, and in selenium toxicity in three different cell lines. Depending solely on the media used we observed significant changes in TrxR activity, a possible change in phenotype, and differences in toxic effects of selenium compounds. A significant factor that may contribute to this observed culture media effect is the great variation in glucose concentrations in the different culture media used.

Our observed culture media effects rise serious concerns comparing experimental data from different cell culture studies when different media have been used and thus highlights the relevance and importance of this methodological study.

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"All we need is just a little patience"

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