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REDOX ALTERATIONS IN INFLAMMATORY DISEASES;DIABETES,KIDNEY FAILURE AND CANCER

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Redox Alterations in Inflammatory Diseases; Diabetes, Kidney Failure and Cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my Family and Friends

ABSTRACT

In order to combat oxidative stress and maintain the intracellular redox homeostasis, cells are equipped with antioxidant systems. The thioredoxin and glutaredoxin systems are the major thiol reducing systems and central antioxidant systems in mammalian cells. The fundamental functions of thioredoxins include redox regulation, transcription factor activation and regulation of intra and extracellular signaling. Glutaredoxins are crucial for catalyzing redox reactions, DNA synthesis and apoptosis. The thioredoxins as well as glutaredoxins are affected in chronic inflammatory diseases. The aim of this thesis was to study how these redox systems are altered in chronic inflammation and how they may be modulated by treatment of the underlying disease.

In **Paper I,** we investigated the effect of anti-cancer drug, histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) on thioredoxin 1 (Trx1). SAHA treatment of HeLa cells induced a shift in Trx1 oxidation state to more oxidized, leading to less reducing capacity. Additionally, SAHA treatment increased the expression of Trx1 inhibitor Txnip. Thus the cytotoxicity of SAHA may at least partially be explained by Trx1 inhibition and by multiple mechanisms, decreasing its capacity to scavenge reactive oxygen species.

In **Paper II,** the potential redox effects of antioxidant CoQ10 was studied in diabetes patients undergoing three months of CoQ10 or placebo treatment. CoQ10 treatment significantly decreased total antioxidant capacity and Grx activity in diabetes mellitus patients, correcting the disrupted redox balance in diabetes patients. We also demonstrated that Grx catalyzes the reduction of CoQ10 by GSH. In conclusion CoQ10 decreases serum Grx activity towards normalization.

In **Paper III,** we measured serum Grx levels in patients at different stages of CKD, at dialysis start and again after 2 years of dialysis. Grx levels were increased in CKD compared to control subject and correlated well with oxidative stress marker pentosidine. Grx levels were significantly higher in hemodialysis (HD) patients compared to peritoneal dialysis (PD) patients. In conclusion, CKD patients have oxidized extracellular environment and Grx may be a useful marker of the degree of oxidative stress in CKD during treatment.

In **Paper IV**, we investigated the effect selenium compounds on ovarian cancer cells and immune cells. At cytotoxic doses, selenium compounds (selenite and methylseleninic acid (MSA)) were cytotoxic to ovarian cancer cells but not to immune cells. Improved T cell function was observed after T cell incubation in preconditioned media from MSA treated tumor cells. MSA decreased tumor cell HIF 1-α, VEGF and PDL1 expression, suggesting that MSA enhances T cell mediated killing of ovarian cancer cells via decreasing PDL1 and VEGF expression.

In conclusion, both the intra- and extra-cellular redox balance is perturbed in chronic inflammation, and may be corrected by treatment of the underlying disease.

LIST OF SCIENTIFIC PAPERS

I. Johanna Ungerstedt* , Yatao Du*, Huihui Zhang , **Deepika Nair** , Arne Holmgren
In vivo redox state of human thioredoxin and redox shift by the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA).
Free Radic Biol Med 53 (11): 2002-2007, 2012.

II. Sergio J. Montano , Jacob Grünler , **Deepika Nair** , Michael Tekle , Aristi P. Fernandes Xiang Hua , Arne Holmgren , Kerstin Brismar , Johanna S.Ungerstedt
Glutaredoxin mediated redox effects of coenzyme Q10 treatment in type 1 and type 2 diabetes patients.
BBA Clinical 4: 14-20, 2015

III. Anna Levin* , **Deepika Nair*** , Abdul Rashid Qureshi , Peter Barany , Olof Heimburger , Björn Anderstam , Peter Stenvinkel , Annette Bruchfeld , Johanna S. Ungerstedt
Serum Glutaredoxin activity as a marker of oxidative stress in chronic kidney disease.
Manuscript in print, Nephron

IV. **Deepika Nair** , Emelie Rådestad , Prajakta Khalkar , Nuria Diaz-Argelich , Paula Codó , Axel Schröder , Johanna S. Ungerstedt Mikael Uhlin , Aristi P. Fernandes
Methylseleninic acid sensitizes ovarian cancer cells to T-cell mediated killing by decreasing PDL1 and VEGF levels.
Manuscript submitted

*Equal contribution

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LIST OF ABBREVIATIONS

AGEs	Advanced glycation end products
AOPP	Advanced oxidation protein products
AP-1	Activator protein-1
ARE	Antioxidant response element
ASK1	Apoptosis signal-regulating kinase 1
CKD	Chronic kidney disease
CoQ10	Coenzyme Q10
DM	Diabetes mellitus
EMT	Epithelial mesenchymal transition
ETC	Electron transport chain
ER	Endoplasmic reticulum
GFR	Glomerular filtration rate
GPX	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione
GSSG	Oxidized glutathione
HD	Hemo dialysis
HDACi	Histone deacetylase inhibitor
HIF-1 α	Hypoxia-inducible factor 1-alpha
IFNγ	Interfron gamma
IL-2	Interleukin-2
MDA	Malondialdehyde
MMP	Matrix metalloproteinase
MNC	Mononuclear cells
MSA	Methylseleninic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NF1	Nuclear factor 1
NFκB	Nuclear factor kappa-light-chain-enhancer of activated Bcells
NOS	Nitric oxide synthase

NOX	NADPH oxidase
Nrf2	Nuclear factor erythroid 2–related factor 2
PBMC	Peripheral blood mononuclear cells
PD	Peritoneal dialysis
PDL-1	Programmed death-ligand-1
PICOT	Protein kinase C interacting cousin of thioredoxin
PI3K/Akt	Phosphatidylinositol-3-Kinase and Protein Kinase B
Prx	Peroxiredoxin
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
SAHA	Suberoylanilide hydroxamic acid
Se	Selenium
SOD	Superoxide dismutase
TGR	Thioredoxin glutathione reductase
Trx	Thioredoxin
TrxR	Thioredoxin reductase
Txnip	Thioredoxin-interacting protein
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 REACTIVE OXYGEN SPECIES (ROS)

ROS are chemically reactive molecules formed by the partial/incomplete reduction of molecular oxygen [1]. ROS can be divided into radical (hydrogen peroxide (H_2O_2)) and non-radical (superoxide ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$)) class. ROS at low concentration is required for several cellular processes such as regulation of protein function, apoptosis, activation of transcription factors etc. [2] [3] [4]. Nevertheless at higher concentrations ROS can readily react and damage DNA, proteins and lipids [5]. At about 2% of electron has been reported to be leaked from respiratory chain and form ROS [6]. Some of the most known ROS molecules are the $OH^{\bullet}$, $O_2^{\bullet-}$ and H_2O_2. When oxygen gains an electron superoxide is formed. Superoxide is not very recactive by itself and lacks ability to penetrate membranes however, it is a precursur to other reactive species. Superoxide is then dismutated by superoxide dismutase (SOD) to form H_2O_2. H_2O_2 is later reduced by Cu^{+} or Fe^{+} (Fenton reaction) to form hydoxyl radical, and hydroxyl anion. This hydoxyl radical can take up an electron to form water (Figure 1) [7]. The hydoxyl radical is very rective and can be harmful to biomolecules. Therefore, it is very important to reduce H_2O_2 to water (either by peroxiredoxins (Prx), glutathione peroxidases (GPX), catalases etc.) to prevent Fenton reaction. The respiratory chain is not the only source of ROS, other sources which produces oxidants as a part of their normal enzymatic functions includes NADPH-dependent oxidases, xanthineoxidase, cyclooxygenases, cytochrome p450 enzymes, and lipoxygenases [8]. Other exogenous factors that can contribute to the production of ROS includes ultraviolet light (UV), ionizing radiations , environmental pollutants etc.

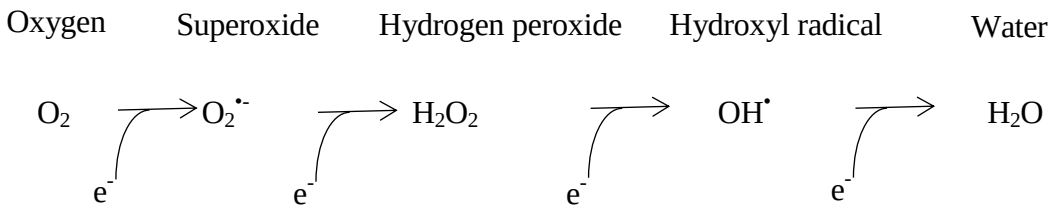


Figure 1: Formation of reactive oxygen species by the stepwise reduction of oxygen to water.

Intracellular redox homeostasis is obtained via the balance between antioxidant enzymes, by scavenging these ROS molecules and the ROS formation. Oxidative stress is caused by disrupted redox homeostasis, by either increased ROS or by decreased cellular antioxidant capacity [9].

1.2 OXIDATIVE STRESS

Oxidative stress can be defined as an imbalance between ROS and the cellular defense mechanisms adapted to eliminate ROS. Excessive oxidative stress results in ROS-mediated damage of nucleic acids, proteins, and lipids, and are related to many disorders including carcinogenesis [9].

Excessive oxidative stress has also been known to cause neuronal cell death leading to neurodegeneration [10]. Several conditions are triggered as a consequence of increased oxidative stress, for example atherosclerosis, diabetes, and aging [9]. In order to prevent cells from the harmful damage caused by excessive ROS, cells regulate a balance between ROS generation and elimination, where ROS elimination is achieved by several antioxidant systems involving both enzymatic and non-enzymatic systems [11].

1.3 ANTIOXIDANTS

Detoxification of ROS is carried out by antioxidant systems either enzymatically or non-enzymatically. The nonenzymatic systems include lipophilic and hydrophilic antioxidant whereas enzymatic system includes superoxide dismutases and hydroxyperoxidases, such as glutathione peroxidase, catalase, and other hemoprotein peroxidases [12].

1.3.1 Enzymatic antioxidants

1.3.1.1 Superoxide dismutases

Superoxide dismutases (SOD) are metal containing enzymes that catalyses the dismutation of superoxide anion into O_2 and H_2O_2.

Humans, possess three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD) [13].

1.3.1.2 Glutathione peroxidases

The GPX is a selenium (Se) containig enzyme that contains single selenocysteine (Sec) residue in each of the four identical subunits [14]. It catalyses the reduction of hydroperoxides using GSH. Five GPX isoenzymes are found in mammals.GPX1 (cytosolic and mitochondrial in most tissues), GPX2 (cytosolic most tissues except

gastrointestinal tract and kidneys), GPX3 (extracellular), GPX4 (cytosolic and membrane in most tissues), GPX5 (epididymis specific and is selenium-independent), GPX2 and GPX3 (cytosolic, extracellular in most tissues except gastrointestinal tract and kidneys) [15]. GPX can also react effectively with lipid and other hydroperoxides.

1.3.1.3 Peroxiredoxins (Prx)

Prx's are thiol dependent peroxidases that are efficient in reducing H_2O_2, peroxynitrite and other hydroperoxides [16]. Six different types of Prx are expressed in mammalian cells; Prx 1, 2 and 6 (cytosol and nucleus), Prx 3 (expressed in mitochondria), Prx 4 (endoplasmic reticulum (ER)), and Prx 5 (cytosol, mitochondria, and peroxisomes) [17].

1.3.1.4 Catalases

Catalase is a heme containing enzyme that catalyzes the reduction of H_2O_2 to form water and molecular oxygen [18].

1.3.2 Non enzymatic antioxidants

1.3.2.1 Vitamins

Carotenoids (vitamin A analogues) are fat soluble molecules required to inactivate excited molecules for e.g. Singlet molecular oxygen produced during lipid peroxidation of bio membranes [19]. They are found in dietary products such as brightly colored fruits and vegetables.

Ascorbate (vitamin C) most important antioxidant found in extra cellular fluids and have been shown to efficiently scavenge superoxide, H_2O_2, singlet oxygen etc. [20].

α-Tocopherol (vitamin E) is a lipid soluble antioxidant that protects polyunsaturated fatty acids in the membrane from lipid peroxidation.

1.3.2.2 Ubiquinone / CoQ10

CoQ10 is a lipid soluble antioxidant that exists in both oxidized (ubiquinone) and reduced (ubiquinol) form. It plays an essential role in mitochondrial oxidative phosphorylation. Because of its high solubility CoQ10 protects various lipoproteins and lipids from peroxidation and oxidative damage [21] .

1.3.2.3 Glutathione

Glutathione is an important antioxidant found in both oxidized (GSSG) and reduced (GSH) form and prevents damage caused by ROS. Besides being an antioxidant, glutathione is also involved in posttranslational modifications via S-glutathionylation (addition of glutathione to the cysteine residue of protein) that is crucial for cellular functions like metabolism, differentiation and cell growth [22].

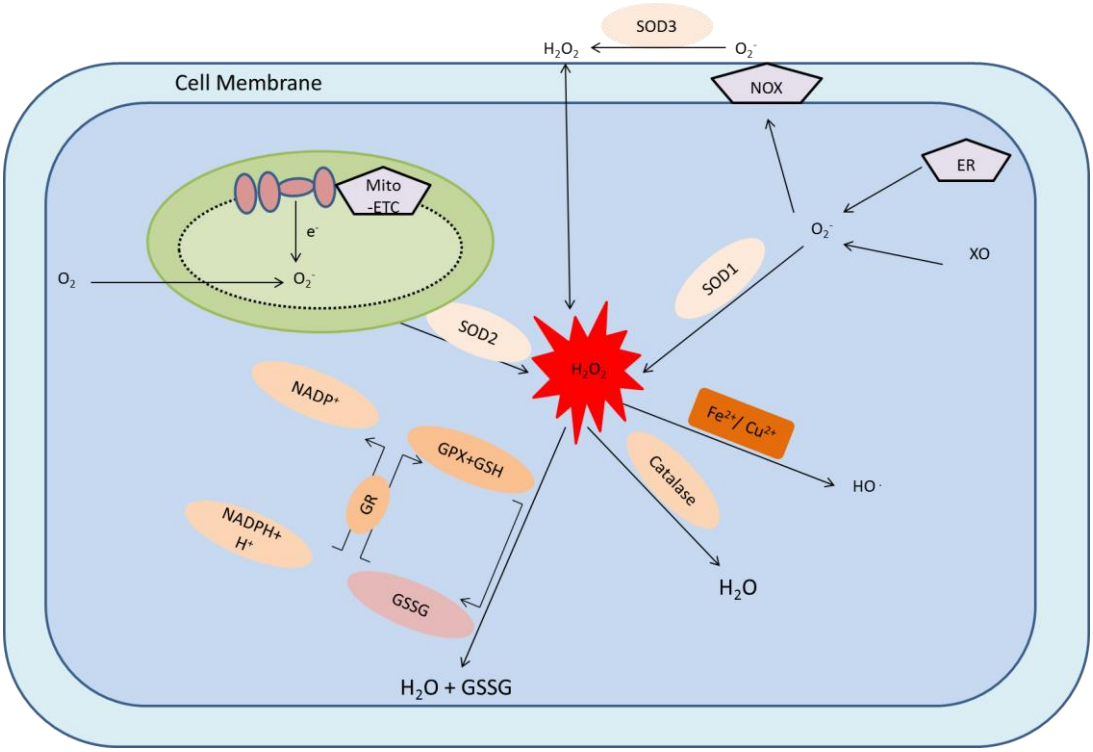


Figure 2: Schematic representation of ROS generation and elimanation process by antioxidants , figure adapted from [23]. Superoxide (O2-), generated from mitochondrial electron transport chain (ETC), endoplasmic reticulum (ER), and membrane NAD(P)H oxidase complex (NOX) can be converted to H_2O_2 by superoxide dismutase (SOD). H_2O_2 forms hydroxyl radicals (HO) in the presence of Fe^{2+}or Cu^{2+} ions. H_2O_2 can also be converted to $H_2O + O_2$ by catalase, peroxiredoxins or GPX.

1.4 THE THIOREDOXIN SYSTEM

The thioredoxin system comprises of NADPH, thioredoxin reductase (TrxR), and thioredoxin (Trx). Trx was first discovered in 1964 and characterized as the hydrogen donor of ribonucleotide reductase (RNR) enzyme involved in the formation of deoxy ribonucleotides from ribonucleotides in *E. coli* [24]. In the Trx system, TrxR catalyzes transport of electron from NADPH to the inactive thioredoxin (Trx-S_2) and transforms it to the active form (Trx-$(SH)_2$). Reduced Trx can then reduce many other proteins [24] [25]. Intracellularly the Trx

system protects cells from damage caused by oxidative stress. It scavenges ROS via thiol-dependent thiol-disulfide exchange reactions and helps in upholding a cellular redox balance [26]. Apart from this the Trx system plays an important role in regulation of cell growth [27], apoptosis [28] and activation of transcription factors [29].

1.4.1 Thioredoxin Reductase

TrxR is a homodimeric flavoprotein belonging to the pyridine nucleotide-disulfide oxidoreductase family which transfer electron from pyridine nucleotides to disulfides [30]. Mammalian TrxR's are known to contain selenium in the form of selenocysteine residue which is part of a conserved Gly-Cys-Sec-Gly motif that is crucial for TrxR function [31]. Targeted disruption of either TrxR1 or TrxR2 genes is known to be embryonically lethal [31] [32] Based on their molecular weight TrxR are divided into two classes, high molecular weight TrxR with 55 kDa for each subunit and low molecular weight TrxR with 35 kDa for each subunit [30]. Mammalian cells have three different isoforms of TrxR's, cytosolic TrxR1, mitochondrial TrxR2, and a testis-specific thioredoxin glutathione reductase (TGR) [33]. The structure of mammalian TrxR is similar to glutathione reductases (GR), which are composed of two subunits; each contains a flavine adenine dinucleotide (FAD) binding domain, a NADPH binding domain and an interface domain. An exception among the TrxR's is TGR which contains above mentioned domains and an extra Grx domain in the N-terminus [34]

1.4.2 Thioredoxin

Trx's were first identified in *E. coli* as an electron donor for ribonucleotide reductase (RNR), an essential enzyme for DNA synthesis. Trx's are a 12 KD protein that has a highly conserved active site (-Trp-Cys-Gly-Pro-Cys-Lys-) from bacteria to human. Trx is composed of five strands of β sheets, surrounded by four α-helices with an active site at N terminal containing Cys32 and Cys35 [35].

Oxidative stress primes the activation of different signaling pathways leading to activation of different transcription factors. These transcription factors binds DNA element known as antioxidant response element (ARE). Under normal conditions nuclear factor (erythroid-derived-2)-like 2 (Nrf2) is bound to an inhibitor called Kelch-like ECH-associated protein 1 (Keap1) which targets Nrf2 for degradation [36]. In case of oxidative stress Keap1 does not interact with Nrf2, leading to Nrf2 translocation into the nucleus, where Nrf2 binds to an ARE and starts the transcription of several antioxidants including TrxR [37] and Trx [38].

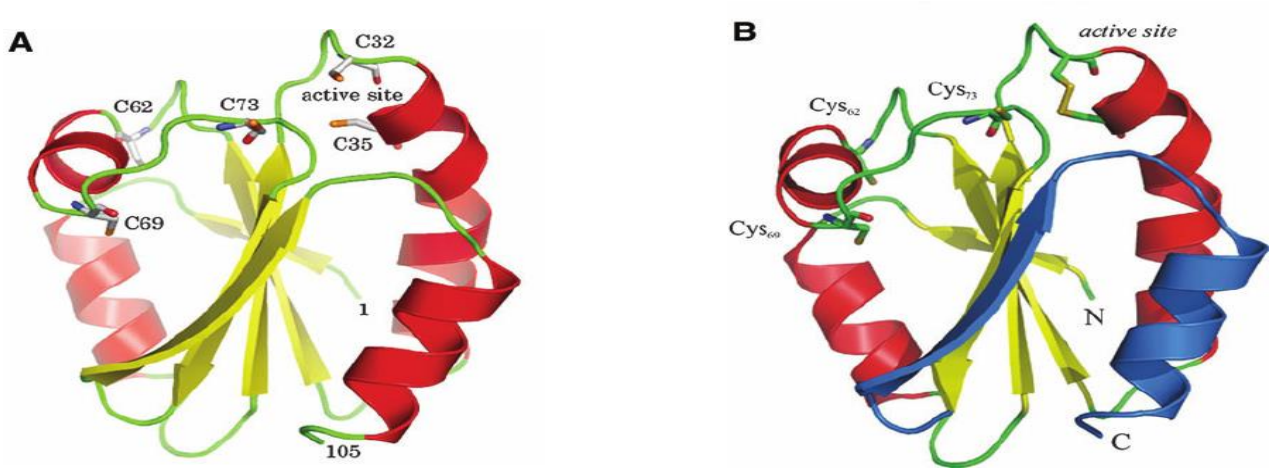
Trx's protects the cells against oxidative stress by redox regulation via mostly thiol-disulfide exchange reaction. Trx binds and reduces the Cys residues of target protein (Reaction 1).The oxidized Trx is then reduced by electrons from NADPH via a reaction catalyzed by TrxR (Reaction 2) [39].

$Trx\text{-}(SH)_2 + Protein\text{-}S_2 \longrightarrow Trx\text{-}S_2 + protein\text{-}(SH)_2$ (Reaction 1)

$Trx\text{-}S_2 + NADPH + H^+ \xrightarrow{TrxR} Trx\text{-}(SH)_2 + NADP^+$ (Reaction 2)

Mammals have two isoforms of Trx which includes cytosolic Trx1 and mitochondrial Trx2 both have highly conserved CGPC active site motif and are embryonically lethal [40].

Besides the two above mentioned active site cysteine, mammalian Trx1 contains three additional cysteine residues Cys62, Cys69, and Cys73 (Figure 3) [41].



Reduced hTrx1 Oxidized hTrx1

Figure 3: Structure of Trx1 adapted with permission from [41]

Even though Trx1 is a cytosolic protein it can be translocated into the nucleus in response to certain signaling events [42]. Upon treatment of cells with the anti-cancer drug cisplatin [43] H_2O_2 [44] and UV [45] Trx1 translocation has been detected.

Several cellular functions have been attribute to Trx1 which includes redox regulation of signaling molecules ,transcription factors, as a growth factors, antioxidant, co factor etc. [46].

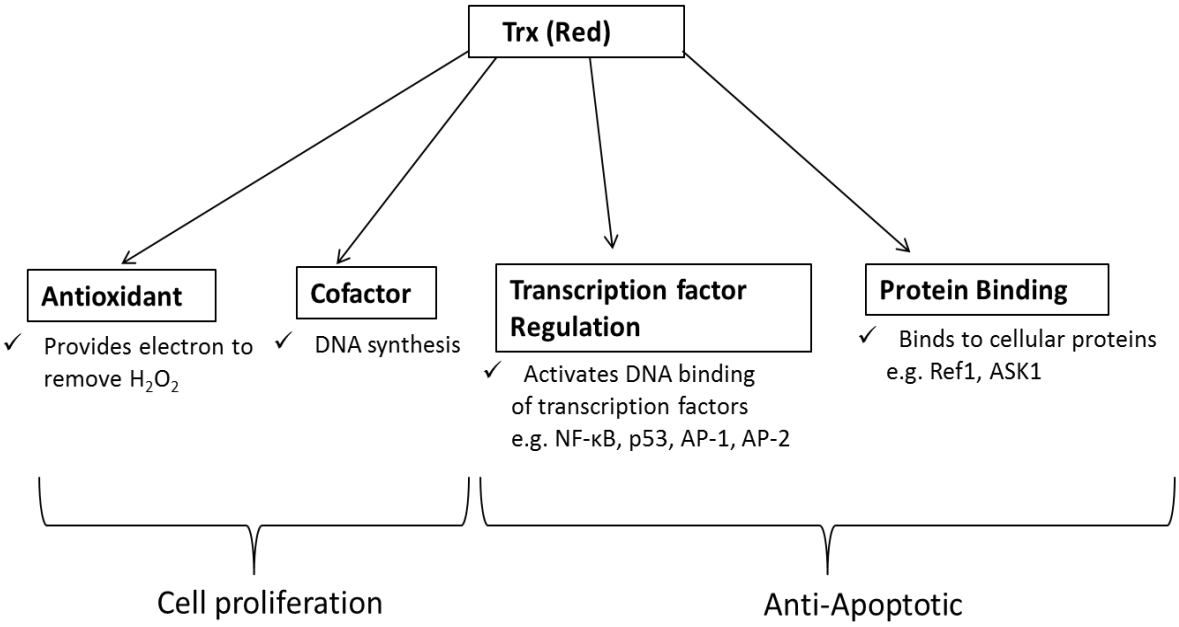


Figure 4: Functions of Trx1,figure adopted from [46]. Trx is reduced by TrxR, only the reduced Trx facilitates protein-protein interactions. Trx interacts with several redox-sensitive molecules by transferring electrons e.g. Prx thus carrying out cell survival and antioxidant function. Trx also mediates cellular signaling pathways by regulating post translational modification.

Transcription factors like NFκB is known to be regulated by Trx1, Trx1 reduces the Cys 62 residue of NFκB which promotes DNA binding and expression of NFκB regulated genes [47]. Other transcription factors that are regulated by Trx1 includes glucocorticoid receptor [44] , AP-1 [29], P53 [43] etc. Trx1 is also known to regulate variety of proteins via protein binding, Apoptosis signal-regulating kinase 1 (ASK1) is one among them. Binding of reduced Trx1 to N-terminal of ASK1 suppress the kinase activity of ASK 1 and the subsequent ASK1-dependent apoptosis [48].

Thioredoxin interacting protein (Txnip) also called as vitamin D_3 up-regulated protein-1 and is the only known endogenous inhibitor of Tx1. It binds and negatively regulates the biological function and expression of Trx1 [49]. By inhibiting Trx1 activity, Txnip induces cellular oxidative stress, affecting a wide variety of cellular processes cell proliferation and death [50]. Many other functions of Txnip includes tumor suppression [51], regulation of glucose metabolism [52] and controls gene expression by binding to the histone deacetylase (HDAC1 and HDAC3) proteins [53]. It has been shown that HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), can upregulate Txnip expression [54] and in

paper I we also show that SAHA increases Txnip levels, which decreases Trx1 activity and leads to increased ROS.

Trx2 is a mitochondrial protein which also has the conserved active site like Trx1 but lacks the structural cysteine residues. It has an N-terminal extension that plays a role in mitochondrial translocation signaling. Along with other proteins Trx2 helps in elimination of H_2O_2 thus maintaining reduced state within mitochondrial matrix [55]. Trx2 plays a vital role in controlling intrinsic apoptotic pathway by preventing release of cytochrome c, and inhibiting activation caspase 3 and caspase 9 [56] [57].

1.5 THE GLUTAREDOXIN SYSTEM

The glutaredoxin system was first discovered in mutant of *E.coli* lacking Trx1 in the year 1976, as a dithiol hydrogen donor system for ribonucleotide reductase [58]. The glutaredoxins system comprises of glutaredoxins (Grxs), glutathione reductase (GR), glutathione (GSH) and NADPH. The Grx system reduces protein disulfides via two different mechanisms i.e. dithiol and monothiol.

In dithiol mechanism, disulfide bond in the target protein is reduced at the expense of two electrons from active site cysteinyl thiolates of Grx (Reaction 3). The disulfide generated is reduced to dithiol by two molecules of GSH resulting in generation of glutathione disulfide (GSSG) (Reaction 4). GSSG is reduced back to GSH molecule by GR using electrons from NADPH (Reaction 5).

$$Grx\text{-}(SH)_2 + Protein\text{-}S_2 \longrightarrow Grx\text{-}S_2 + Protein\text{-}(SH)_2 \qquad \text{(Reaction 3)}$$

$$Grx\text{-}S_2 + 2GSH \longrightarrow Grx\text{-}(SH)_2 + GSSG \qquad \text{(Reaction 4)}$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \qquad \text{(Reaction 5)}$$

In monothiol mechanism the protein-GSH disulfide is attacked by the N- terminal cysteine residue, leading to the formation of a mixed disulfide between Grx and GSH (Reaction 6). The mixed disulfide is then reduced by another molecule of GSH (Reaction 7). The GSSG produced, is reduced to two molecules of GSH by GR.

$$Grx\text{-}(SH)_2 + Protein\text{-}S\text{-}SG \longrightarrow Grx\text{-}S\text{-}SG + Protein\text{-}(SH) \qquad \text{(Reaction 6)}$$

$$Grx\text{-}S\text{-}SG + GSH \xrightarrow{GR} Grx(SH)_2 + GSSG \qquad \text{(Reaction 7)}$$

1.5.1 Glutathione Reductase

GR is a pyridine nucleotide-disulfide oxidoreductases family of protein with homology to TrxR, and has a highly conserved N-terminal active site. GR functions to maintain the supply of reduced GSH in the cell by reducing GSSH using electrons from NADPH. GR is found in both the cytosol and mitochondria and are encoded by a single gene [59].

1.5.2 Glutaredoxin

Four types of Grxs have been discovered, the classical dithiol Grxs cytosolic Grx1, mitochondrial Grx2 and monothiol Grxs, Grx3 and Grx5.

Grx1 is 12 kDa protein, whose structure comprises a Trx fold and highly is conserved among vertebrates. Grx1 is mainly found in cytosol and it can also be found in nucleus [60], mitochondrial intermembrane space [61] and in plasma [62]. Grx1 is involved in the regulation of transcription factors such as nuclear factor1 (NF1) [63], nuclear factor kappa B (NF- κB) [64] and activator protein (AP1) [64]. Knocking out of Grx1 shows no abnormalities [65] however, Grx1 have been reported to be crucial in preventing inflammation [66], cardiovascular hypertrophy [67] and airway hyper responsiveness [68].

Grx2 has three isoforms Grx2a, Grx2b and Grx2c, which are encoded by the same gene. Grx2a is found in mitochondrial matrix [69] whereas Grx2b and Grx2c are located in the cytosol and are restricted to testes, immortalized cell lines and tumors [70] .

Grx2 can receive electrons from both GSH and thioredoxin reductase thus supporting monothiol and dithiol reactions [71]. Grx2 efficiently regulates superoxide production in mitochondrial complex I via glutathionylation and deglutathionylation [72] [73]. Grx2 is known to form iron–sulfur clusters and the presence of this cluster on the active site of two Grx2a with GSH as a ligand renders it inactive. Under stress conditions the cluster is degraded and Grx2 is activated meaning the iron–sulfur cluster serves as redox sensor for the activation of Grx2 [74].

Grx3 also known thioredoxin-like 2 (Txnl2) or protein kinase C interacting cousin of thioredoxin (PICOT), is a multidomain monothiol located in cytosol [75]. Several regulatory functions have been attributed to Grx3 such as knocking down of Grx3 leads to embryonic death [76], immune response [77] and cardiac hypertrophy [78].

Grx5 is a mitochondrial protein that is evolutionarily conserved in eukaryotes [79]. It plays a significant role in the biogenesis of mitochondrial iron-sulfur cluster and regulation of iron sulfur enzyme activity [80] and Grx5 deficiency has been described to cause anemia in a case report [81].

1.6 ANTIOXIDANT SYSTEMS IN INFLAMMATORY CONDITIONS

1.6.1 Cancer

Carcinogenesis, a process by which normal cell transforms into a cancer cells, and can be caused by the action of one or combination of chemical (benzopyrenes, heterocyclic amines), physical (UV, ionizing radiations), biologic (virus and bacteria) or genetic insults to cells. As a consequence of DNA alterations, the first step in carcinogenesis take place is called initiation. **Initiation** is the process of conversion of proto-oncogenes (often, genes that regulate cell division) to oncogenes thus transforming a normal cell to a malignant cell. The next step is **promotion** during which mutated cell divides and expands clonally, at this stage cells acquire additional mutations and form benign and malignant tumor. The final and irreversible stage is **progression** where tumor cells achieve properties like uncontrollable growth, metastasis and angiogenesis.

ROS plays an active role in all the three stages of carcinogenesis, oxidatively modified DNA lesions have been implicated in several cancers among which, 8-OH-G which is most commonly known DNA adduct and is formed by oxidation of guanine at C8 position [82]. Studies on mouse model of prostate cancer and others have shown ROS-induced Phosphatidylinositol-3-Kinase and Protein Kinase B (PI3K/Akt) [83, 84] which in turn activates Nrf2 to promote cell survival in cancer cells [85]. ROS levels have been reported to mediate and promote tumor progression via several pathways like MAPK, PI3K/Akt pathways, hypoxia inducible factors (HIFs) and the epithelial mesenchymal transition (EMT) and also other mechanisms like formation of micro domains [86].

Cancer cells grow and divide rapidly and possess high aerobic glycolytic rate, which produces high levels of ROS. These high ROS concentrations can trigger alterations in signaling pathways, affecting metabolism and growth.

To cope up with high ROS production, cancer cells develop an increased and maximized antioxidant capacity, as a compensatory mechanism to evade ROS-induced cell death e.g. by upregulating Nrf2 tumor cells withstand ROS levels and that promotes tumor survival [87] [85]. This however renders cancer cells extra vulnerable to an additional ROS induction. Some drug resistant leukemic cells are resistant to treatment induced ROS due to elevation of endogenous catalase to scavenge ROS [88]. High GSH levels have been shown to protect cancer cells from cell death [89]. Similarly high levels of Trx1 are found in many tumors and Trx1 levels are negatively correlated to apoptosis and promotes growth [90]. Both Trx and

Grx have been shown to be important for tumor progression as the combined inhibition of GSH and Trx antioxidant pathways leads to cancer cell death in vitro and in vivo [91].

Another vital mechanisms by which cancer cells survive includes the ability to escape immune surveillance, enabling replicative immortality, inducing angiogenesis, and activating invasion, metastasis and reprogramming of energy metabolism [92].

Histone deacetylase inhibitors (HDACi)

HDACi blocks activity of HDAC enzymes thus keeping histones acetylated. However HDACi's affect several non-histone target proteins as well and HDACi therapy affects around 10% of transcribed genes, apoptosis, cell-cycle arrest, necrosis, autophagy, differentiation, and migration via non-transcriptional mechanisms [93]. This suggests that HDACi's are potent drugs with several in the late stage clinical trials and some of them are approved for both solid and hematological tumors [94]. One of the HDACi's is suberanilohydroxamic acid (SAHA), which is known to induce ROS as one of the mechanism of cytotoxicity [95].

Selenium compounds

Selenium compounds are emerging as a potent anti-proliferative agent due to their selectively [96] and specificity [97] towards malignant cells. The effect of selenium compounds largely depend on their chemical form, dose, redox state and experimental model used [98]. The possible mechanisms by which they exert their anti-cancer properties are via effecting cell signaling pathways, generating ROS, inducing cell cycle arrest and apoptosis, chromatin modifications and immunomodulation [99].

1.6.2 Diabetes Mellitus (DM)

DM is a disease with abnormally high levels of glucose in the blood and too low glucose levels intracellularly. There are two distinct types of DM; Type 1 DM is initiated by low production of insulin, due to autoimmune destruction of insulin producing pancreatic beta cells. Type 2 DM is initiated over time by insulin resistance. Risk factors for type 2 DM are obesity, unhealthy diet and lifestyle. The increase in the blood sugar levels can lead to hyperglycemia and hypoglycemia intracellularly. Elevated blood glucose, (hyperglycemia) has been linked to morbidity and mortality associated with DM. Hyperglycemia reduces antioxidant levels and generates ROS [100] [101]. Possible mechanisms by which hyperglycemia contributes to the generation of ROS includes increased polyol pathway flux, increased intracellular formation of advanced glycation end products, activation of protein

kinase C and increased hexosamine pathway flux [102] [103]. Brief description of above mentioned pathways are as follows; Increased polyol pathway leads to increased enzymatic activity of aldose reductase resulting in increased production of sorbitol and decreased NADPH. This leads to redox imbalance in the cells as NADPH is required for the production of GSH [104]. Increased production of advanced glycated end products (AGEs) increases RAGE–ligand interaction leading to the activation of NADPH oxidase system thus generating ROS. As a consequence the Ras-MAPK pathway is activated leading to activation of NF-κB [103]. Hyperglycemia leads to vascular diacyl glycerol (DAG) accumulation thus PKC activation which in turn effect various signaling pathways e.g. NOX, NF-κB, Akt, etc. [103]. Hyperglycemia leads increased O-GlcNAcylation of the transcription factor specificity protein 1 (Sp1) which leads to increased TGFβ signaling [103].

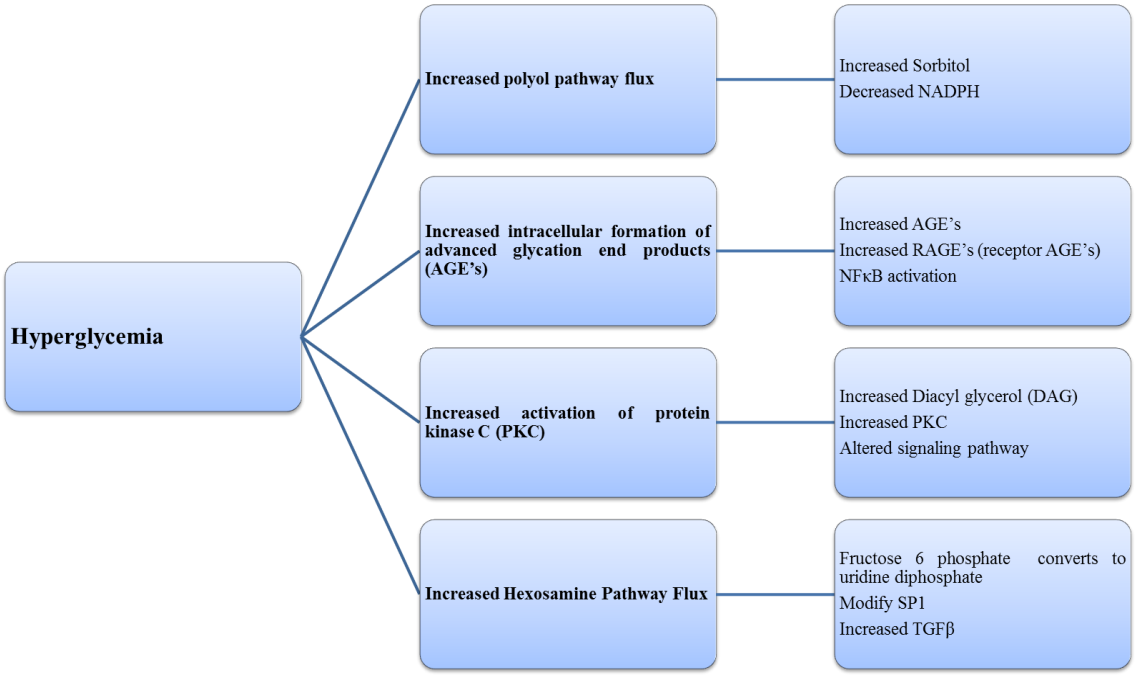


Figure 5: Mechanism by which hyperglycemia induces ROS leading to diabetes.

The treatment for type 1 DM is insulin administration. However, in type 2 DM; the therapeutic approach varies depending on the characteristics of disease, patient, and degree of insulin resistance, insulin or insulin analogs may be needed but there is a multitude of other available therapy, including primary prevention to avoid DM complications, with exercise, weight reduction and dietary restrictions, to prevent myocardial infarction, stroke, and kidney failure.

1.6.3 Chronic kidney disease (CKD)

Chronic kidney disease can be described as the gradual loss of kidney function over time. Oxidative stress plays a major role in the progression of CKD, causing glomerular damage and renal ischemia. Oxidative stress can also lead to inflammation, and as a consequence hypertension, and endothelial dysfunction [105].

Major causes of the increased ROS in CKD is activation and upregulation of ROS-producing enzymes, including NAD(P)H oxidase (NOX) isoforms, cycloxygenase-2, lipoxygenase, and nitric oxide synthase (NOS), mitochondrial dysfunction, and endoplasmic reticulum stress. [106]. Activation of NF-κB and accumulation of inflammatory cells can also induce oxidative stress in CKD[105].

In addition, dialysis, which is the treatment of the end stage CKD, can also induce oxidative stress by altering the extracellular fluid volume or by the dialysis devices triggering release of proinflammatory cytokines [107]. Therefore, it is important to monitor the degree of oxidative stress during CKD and a goal of nephrology to find clinical markers of oxidative stress, as well as treatments that can reduce the oxidative stress during CKD to prevent secondary events like myocardial infarction or stroke.

2 PRESENT INVESTIGATION

2.1 AIM OF THE THESIS

The overall aim of this thesis was to investigate how redox proteins are altered upon treatment of inflammatory disease and cancer, diseases where the redox balance is known to be impaired, and to study the effect of redox active compounds on immune cell function.

Paper I

To investigate the mechanism by which anticancer agent SAHA acts on Trx1, assessing Trx1 redox state and SAHA's effect on the endogenous Trx1 inhibitor (Txnip).

Paper II

To assess the effects of oral antioxidant CoQ10 treatment in DM patients on intracellular and extracellular levels of antioxidant Grx and total antioxidant capacity.

Paper III

To evaluate if serum Grx levels can be used as a predictive marker for the degree of oxidative stress in CKD, and whether serum Grx levels can be used to predict risk of CKD related complications; myocardial infarction and stroke.

Paper IV

To investigate if selenium compounds can be used to enhance immune cell mediated tumor killing in ovarian cancer.

2.2 METHODOLOGY

This section provides a brief description of specific methods used in paper I-IV. For more detailed information please see the materials and methods section of each paper.

Cell culture (paper I and IV)

a) Routine cell culture

In paper I HeLa cells were obtained from ATCC and cultured in Dulbeco's modified Eagle's medium (DMEM supplemented with 1g/ml glucose) containing 10% FBS, 100 units/ml penicillin,100 µg/ml streptomycin and 2mM-L glutamine. In paper IV CP70 and A2780 (ovarian cancer cell lines) cells were used, CP70 is cisplatin resistant variant of A2780 cells. Both the cell lines were cultured in RPMI 1640 media with ultraglutamine I and supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin,100 µg/ml streptomycin and 2 mM glutamine.

b) Isolation and maintainence of immune cells ovarian cancer patient donors and healthy donors.

In paper IV, mononuclear cells (MNC's) were isolated from ovarian cancer patient ascites using density gradient centrifugation. MNC's from healthy donor buffy coats were obtained by density gradient centrifugation (1200 RPM without brake) after which cell suspension was positively selected for NK cells (anti-human CD56 antibody) and T cells (anti-human CD45 RA) using biotinylated antibodies and Mojo sort Streptavidin Nanobeads. To ensure purity, the enriched T cell populations were further FACS sorted using CD3 antibody. The isolated cells were either used freshly for experiments or vital frozen (RPMI, 20% FBS, 10%DMSO) at -180^{o}C until further use.

For T cell stimulation experiments, we used 24 well plates where 1x 10^6 T cells were cultured in RPMI containing 30 IU of IL-2 and recommended amount of human T cell activator CD3/CD28 for 96 h, where after cells were used in experiments.

Activity Assays for Trx1 and Grx (paper I, II and III)

In paper I Trx1 activity was measured using a kinetic assay based on reduction of eosin labeled insulin in presence of TrxR and NADPH in a 96 well plate format. Whole cell lysates were prepared by lysis in ice cold RIPA buffer (50 mM Tris-HCl, 1 mM EDTA, 0.15M NaCl,1% NP-40 ,0.5% sodium deoxycholate,0.1%SDS) containing protease inhibitor cocktail. 20µg of whole cell lysate were mixed with assay buffer (containing 50 mM Tris-HCl pH 7.5, 1 mM EDTA, and 0.2 mg/ml bovine serum albumin), 1 µM TrxR and 0.2 mg/ml

NADPH. Background is corrected using a sample without TrxR. Reaction mixture along with sample was incubated at 37 °C for 30 minutes after which fluorescent insulin as substrate was added to each well. The emission at 545 nm was recorded after 520nm excitation for 60 minutes. Trx1 activity was calculated by comparing the increase in fluorescence intensity over time to a standard curve within linear range of the reaction.

In paper II and III, a kinetic Grx activity assay was used. It is based on reduction of fluorescent albumin in the presence of glutathione reductase (GR) and NADPH. In a 96 well plate 20 µl - 40µl serum/PBMC lysate protein were mixed with reaction mixture containing 100 mM phosphate buffer, 1mM EDTA buffer, 0.50 mM GSH, 0.2 mg/ml NADPH, 0.2 mg/ml alkylated bovine serum albumin, 50 nM baker yeast, and GR. Background is corrected using a sample without reaction mixture. Fluorescent substrate was added and emission at 545 nm was recorded after 520nm excitation for 60 minutes. Grx activity was calculated comparing the increase in fluorescence intensity over time to a standard curve within linear range of the reaction.

Detection of redox state of Trx1 (paper I)

Redox state of Trx1 was determined by using redox western blot [108]. In this method pure Trx1 (denatured in Urea, reduced with DTT) is incubated with varying molar ratios of iodoacetic acid (IAA) to iodoacetamide (IAM) to achieve six protein isoforms with given number of acidic carboxymethyl thiol adducts (-SA^-) and neutral amidomethyl thiol adducts (-SM). On urea-PAGE, depending upon the number charges the protein will migrate towards the anode. The ionized -SA^- group protein migrates faster thus separating the six isoforms, which are used as a mobility standard for representing the number of -SA^-.

Thus Trx1 oxidative state can be assessed in the sample, by comparing to the mobility standard produced above. Samples were prepared by lysis in TUE buffer (8 M urea in 50 mM Tris HCL, 1mM EDTA, pH 8.0) containing 30 mM IAA that will bind to free thiols thus preventing further Trx1 oxidation. Thereafter, excess IAA are washed away with 1M HCl, and resuspended in TUE buffer with 3.5mM DTT to reduce all remaining disulfides. Hereafter the sample was incubated with 10mM IAM to alkylate the free thiols. Then, standards with Trx1 and samples at the same protein concentration were subjected to Urea-PAGE, and western blot membranes were probed with anti-Trx1 antibody.

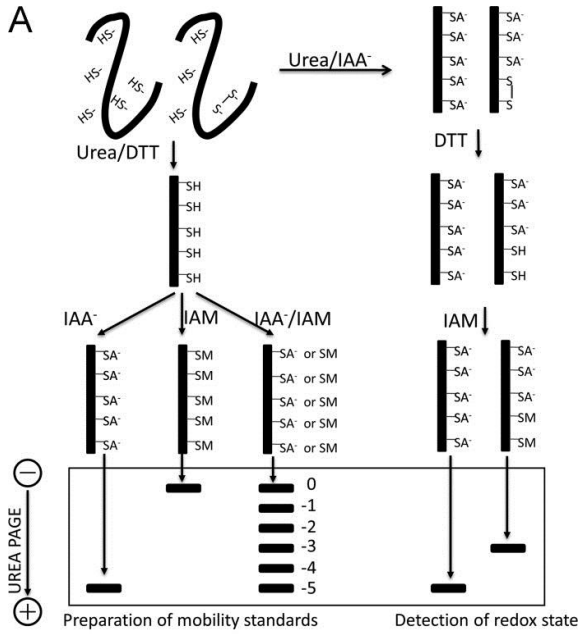


Figure 4: Principle of redox Western blot published with permission from [108]

Flow cytometry based cell toxicity assay (paper IV)

In order to assess the cytotoxicity of NK and T cells, a flow cytometry based assay was used which is comparable to standard ^{51}Cr release assay [109].

The method is described briefly in following steps;

1) Labelling of target cells

A2780 and CP70 (10×10^6) cells were washed well with serum-free medium before staining. The cells were then resuspended in 200 µl diluent C (an aqueous, osmolarity-regulating solution containing no Ca^{2+} or other physiological salts; Sigma). In a separate tube dye was prepared in 200 µl diluent C and 0.8µl (stock 1 mM in ethanol). To ensure a homogenous staining, the dye solution was added while pipetting the cells up and down. Cells were incubated for 4 minutes at room temperature, the staining was then stopped by addition of 400 µl FBS for 1 minute (helps to bind remaining lipophilic PKH-dye to serum proteins). Cells were washed three times with 8 ml RPMI-1640 medium containing 10% serum (to wash away all the unbound stains). The labeled target cells were then incubated with the effector cells (activated T cells and NK cells) at different *E*/*T* ratios for 3.5h.

2) Staining with propidium iodide (PI)

After co-incubation of labelled target cells with effector cells, the cells were washed with PBS and stained with PI (5µg/ml) for 10 minutes in dark at room temperature. The cells were

then fixed with 1% paraformaldehyde (PFA) and stored in FACS buffer (2% FBS in PBS) until further FACS analysis.

3 SUMMARY AND DISCUSSION

3.1 PAPER I

In vivo redox state of human thioredoxin and redox shift by the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA).

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Free Radical Biology and Medicine 53 (11): 2002-2007, 2012

*Equal Contribution

Background

Trx is a central disulfide reducing agent, required to maintain a reduced intracellular environment, which is important for activation of several transcription factors which are involved in cell growth, apoptosis, and inflammation. Reduced Trx1 is known to reduce a number of oxidized proteins like RNR and also transcription factors such as NFκB, AP1 etc. [40]. On reducing the oxidized proteins, Trx1 is oxidized in return and subsequently reduced by TrxR [51]. In this paper, we investigated the changes in oxidation state of thioredoxin (Trx1) in HeLa cells after treatment with HDACi SAHA, using the redox Western blot. We and others have shown that SAHA induces ROS formation, apoptosis and autophagic cell death, involving the Trx1 system [110] [111]. Txnip, a tumor suppressor and the only known endogenous inhibitor of Trx1, is downregulated in many tumors. It has been shown that *in vitro* treatment of tumor cells with SAHA increases mRNA levels of Txnip [54]. Here we also assessed the changes in expression levels of Txnip on treatment of tumor cells with SAHA.

Main findings

- <u>SAHA inhibits Trx1 activity and induces cell death</u>

  Treatment of HeLa cells with varying concentration of SAHA resulted in a dose dependent decrease in the cell viability. Consistently SAHA also inhibited Trx1 activity in HeLa cells.

- Upon SAHA treatment, there is a shift towards more oxidized Trx1

  Redox western blot on Trx1 after SAHA treatment revealed that under physiological conditions, about 67% of Trx1 is fully reduced and about 25 % is oxidized at only one thiol. There was a time dependent shift of Trx1 towards incresing oxidation after SAHA treatment of HeLa cells. Trx1 is even more oxidized in HeLa cells comitted to cell death after SAHA treatment.
- SAHA treatment increases Txnip levels

  On treatment of HeLa cells with SAHA increased the protein expression of Txnip,whereas protien levels of Trx1 were unchanged.

Discussion

SAHA is an anticancer agent which induces ROS, and is known to affect biological functions like cell cycle arrest, apoptosis, autophagy and invasion in tumor cells [112] [113]. Normal cells have been shown to upregulate Trx1 on treatment with SAHA. Several studies have been reported on the protein binding function of Trx1 and that the protein binding occurs only with reduced but not oxidized Trx1 [48, 114]. This indicates the importance of a tool to determine the oxidative state of Trx1. In the present study we show a method which allows differentiating all the six oxidation states of Trx1. We also have shown a time and dose dependent shift in the oxidation state of Trx1 on treatment with Trx1 oxidants, diamide and ebselen.

SAHA induces dose dependent decrease in HeLa cell viability with no changes observed in Trx1 protein levels. The susceptibility of tumor cells to SAHA induced cell death can be attributed to the inability of tumor cells to withstand extra ROS induced by SAHA [111]. As Trx1 is maximally upregulated in tumor cells they cannot further upregulate it in response to stress which here is induced by SAHA this could the reason for no change in protein levels of Trx1 in HeLa cells upon treatment with SAHA.

SAHA has been known to increase Txnip mRNA levels [54]. Here we reported an increase also in the protein levels of Txnip in HeLa cells after SAHA treatment. The inhibition in Trx1 oxidoreductase activity reported here can be a result of Txnip binding and inhibiting Trx1. Treatment of HeLa cells with SAHA shifted the oxidative state of Trx1 towards more oxidized state. This shift in oxidative state can be either due to the increased ROS or increase in Txnip levels leading to compromised Trx1 activity.

In conclusion, SAHA induces the expression of Tnixp, binding and inhibiting Trx1. Trx1 inhibition leads to reduced oxidoreductase function causing ROS accumulation and

eventually cancer cell death, as one of the many mechanisms of action of HDACi SAHA as anticancer drug.

3.2 PAPER II

Glutaredoxin mediated redox effects of coenzyme Q10 treatment in type 1 and type 2 diabetes patients

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BBA Clinical 4: 14-20, 2015

Background

Oxidized Grx is reduced by GSH and dysregulation of Grx/glutathionylation have been implicated in diabetes, cardiovascular diseases and cancer [115]. Hyperglycemia results in the production of ROS via several enzymatic reactions in mitochondria. Due to the presence of excessive ROS, the intracellular environment in DM is highly oxidized [116]. Our group has previously reported extracellular Grx to be elevated in type 2 DM patients [117]. Presence of Grx in serum has been noted in response to glucose stimulation suggesting that Grx can be used as a marker for oxidative stress status [117].

CoQ10 is an endogenous antioxidant that has been reported to protect endothelial cells from glucose induced oxidative stress [118]. CoQ10 has been reported to reduce cardiovascular-mortality and improve heart function in heart failure patients with diabetes [119]. There have been several studies on the beneficial outcome of CoQ10 intake in diseases, however meta-analysis results shows no favorable effect of CoQ10 in DM patients [120]

This study shows the possible beneficial effect of three months supplementation of CoQ10 tablets, in DM patients. We assess the intra and extracellular Grx levels, and study the possible interactions between CoQ10 and Grx.

Main findings

<u>Effect of CoQ10 treatment on clinical parameters</u>

Out of 22 patients, 13 had type 1 DM and 9 had type 2 DM, statin treatment was used by 67% of type 2 DM and about 31% of type 1 DM patients. The baseline lipid profile was disrupted in type 2 DM group. When subdividing the DM patients groups based on statin

treated /non-statin treated, serum CoQ10 levels increased significantly upon CoQ10 treatment in both the groups.

Decrease in serum Grx activity on CoQ10 treatment

Serum Grx activity decreased significantly after three months of CoQ10 treatment compared to baseline. When dividing patients based on the type of DM and statin treated or not, we found a significant decrease in Grx activity in the non-statin treated group. We confirmed this by measuring total antioxidant capacity (TAC), which also decreased upon CoQ10 treatment.

CoQ10 treatment increase intracellular Grx activity

Both Grx activity and Grx mRNA levels in peripheral blood mononuclear cells (PBMC) were significantly increased after three months of CoQ10 treatment.

Grx catalyzes the reduction of CoQ10 by glutathione (GSH)

This reaction was measured by incubating different concentrations of CoQ10 with GSH, GR, NADPH, with or without Grx. We found that GSH reduces CoQ10, which is comparable to TrxR, and the reduction reaction of CoQ10 is catalyzed by Grx. In order to compare the catalytic efficiency of Grx, an additional assay was performed as mentioned in [121] and we observed that the turnover number of Grx is equal to TrxR.

Discussion

In the current study, we show a decreased extracellular Grx activity with a decreased TAC, representing an improved extracellular redox environment, in response to oral CoQ10 treatment. When further subdividing DM patients between statin treated and non-treated we show a significantly decreased, oxidized LDL, fp-LDL and cholesterol levels also improved HbA1c levels upon CoQ10 treatment especially in the non-statin treated group. Statins work by inhibiting mevalonate metabolic pathway [122] and mevalonate is used to synthesize cholesterol and CoQ10 [123]. So, statin simultaneously lowers CoQ10 levels while decreasing cholesterol. Also long term statin treatment has been shown to reduce serum CoQ10 levels [124]. These studies explain the lack of positive effect of CoQ10 treatment in statin treated group could be due to reduced CoQ10.

Some previous studies have been reported on CoQ10 supplementation showing beneficial outcome in reducing cardiovascular events and mortality and the importance of higher serum CoQ10 levels in disease condition, however, a meta-analysis shows lack of evidence of any beneficial outcome of CoQ10 with regard to cardiovascular events [125]. Similarly an

another meta-analysis on the effect of CoQ10 supplementation in diabetes shows that CoQ10 had no effect on glycemic control, lipid profile or blood pressure in patients with diabetes, while reducing triglycerides levels [120]. CoQ10 is known increase fatty acid oxidation resulting in less mitochondrial fatty acids also increase in lipolysis of triglycerides [120]. Like many other possible positive affect of CoQ10 our study, also reports significantly reduced oxidized LDL, fp-LDL and cholesterol levels and improved Hb1Ac levels. In our study, a CoQ10 treatment resulted in a significant increase in serum CoQ10 levels in all patients; this may be due to increased levels of circulating p-LDL in the patients, as p-LDL binds to CoQ10 and thus keeps high levels of CoQ10 in the circulation. Even though there are several contradictory studies on the clinical effect of CoQ10, our results show an overall beneficial effect of CoQ10 on the assessed clinical parameters. However, our study is small and a large, prospective study over a long period of time, with solid biomarker evaluation over time, is needed to validate the results.

It is known that TrxR can reduce CoQ10 [121]. In the current study, we demonstrate for the first time that Grx catalyzes GSH dependent reduction of CoQ10, suggesting a new mechanism for *in vivo* CoQ10 reduction.

Taken together, we show a novel mechanism for *in vivo* CoQ10 reduction via GSH, catalyzed by Grx, and that the extracellular redox balance is improved by oral CoQ10 treatment.

3.3 PAPER III

Serum Glutaredoxin activity as a marker of oxidative stress in chronic kidney disease

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***** *Equal contribution*

Manuscript in print, Nephron.

Background

Oxidative stress causes vascular and tissue injuries as well as nucleic acid damage in CKD patients and promote progression of CKD [126]. Thus, there is a need for biomarkers measurable in blood or serum, to follow the degree of intra- and extracellular oxidative stress during the progression of CKD, for monitoring of redox state and possibly to initiate treatment to improve redox control.

Several biomarkers have been assessed and used in clinical studies as potential markers of oxidative balance, e.g., malondialdehyde (MDA/TBARS), advanced glycolsylation end products (AGE) including pentosidine, advanced oxidation protein products (AOPP), 8-hydroxy-2´doxyguanosine (8-OH-dG) [127] however none is routinely used in clinic. Grx is a major redox protein and it has been shown that upregulation of Grx decreases revascularization after limb ischemia (16), suggesting that high levels of Grx may contribute to atherosclerosis and other organ damage. The aim of our study was to assess Grx as a potential clinical marker of oxidative stress in CKD, by measuring serum Grx in patients with varying stages of CKD compared to healthy age matched controls, and CKD patients before and after 2 years of dialysis.

Main findings

<u>Higher Grx levels in CKD compare to control subjects</u>

Grx activity was significantly increased already in moderate CKD compared to the control subjects. Interestingly, Grx did not increase further with deterioration of kidney failure. Grx levels correlated to pentosidine levels and IL-6, but not to kidney function measured as creatinine, urea or glomerular filtration rate (GFR), or age.

Peritoneal dialysis (PD) is benefical over Hemo dialysis (PD)

Grx levels remained unchanged after 2 years of dialysis in PD patients whereas in HD patients, a significant increase in the Grx levels was observed after 2 years of dialysis, indicating an increased oxidative stress imbalance in HD patients. Interestingly, after censoring all dialysis patients that underwent kidney transplant, in the remaining 25 patients, 4 were alive and 21 dead, Grx levels increased more in the patients that later died than the 4 that remained alive, although not statistically significant, p=0.12. In patients suffering from stroke or myocardial infarction at any time after the initial sample, there was a borderline significant increase in Grx at 2 years compared to 0 years, p=0.052, despite a very small number of patients in each group. When comparing the difference in Grx between patients without ever any cardiovascular event (median difference -1.1 ng/ml, n=9) to patients with a cardiovascular event at any time (median increase 2.75 ng/ml serum, n=16), the difference in Grx increase was not significant p=0.09 however, a trend towards higher Grx increase in patients suffering from a cardiovascular event. Again, only few patients were included as all transplanted patients were censored for this analysis.

Discussion

Oxidative stress plays a critical role in the pathogenesis and progression of CKD [128].This study reports a significant increase in major redox protein Grx levels in patients with CKD compared to age matched controls. On comparing the levels of Grx in different stages of CKD, Grx was significantly increased in CKD 3 and did not increase further between CKD3 and CKD5, even though it is well known that the redox balance is worsened as CKD progresses. We believe that several factors may contribute to the actual Grx levels, and that a larger, prospective study with serial sampling of each patient is needed, to correct for variance in e.g. fluid volume, acute infection or inflammation, to understand if indeed Grx increases as CKD progresses, in a given individual patient. Indeed, the variable of dialysis-related factors also may contribute to the degree of oxidative stress. This we could in fact measure, as we noted a significant increase in Grx levels over the two years of dialysis, in HD patients but not in PD patients. There are a number of studies reporting NADPH oxidase activation and ROS generation in HD, by dialysis membranes, dialysis fluids, trace amounts of endotoxin in the system, and other factors [129]. No increase of Grx levels was seen in the PD patients, indicating a possible beneficial effect of PD on extracellular redox state, maintaining equilibrium and not further deteriorating the redox balance.

Grx levels in the whole cohort correlated well with pentosidine, another marker of oxidative stress, providing support that Grx may be used as a novel marker of oxidative stress in CKD.

When comparing the levels of Grx between diabetic and non-diabetic CKD patients, there was no significant difference between DM and non DM patients, nor was there any correlation between Grx levels and HbA1c levels. In previous studies, we have found that DM patients have higher Grx levels than healthy. Perhaps in the current cohort with careful CKD follow up, DM was well controlled, and in fact, their HbA1c levels were essentially normal. However, this is not easy to interpret or value, as in CKD HbA1c is lower due to an increased erythrocyte turnover, and HbA1c is known to be an unreliable marker of diabetes control in CKD patients.

There was a tendency to lower Grx difference at 2 years in the patient that survived compared to patients that later died, and in patient suffering from myocardial infarction or stroke, Grx levels were increased compared to patients who never experienced myocardial infarction or stroke, however differences were not statistically significant, although the trend was always towards worse Grx in patients with events, whether it was death, or myocardial infarction or stroke. In summary, Grx is increased in CKD patients compared to age matched controls. Grx correlates to pentosidine, and may be a marker of redox balance in CKD. We are now planning to set up a prospective analysis measuring Grx annually in a large cohort of CKD patients, and a prospective study of patients entering dialysis, to evaluate if Grx can be a predictor of cardiovascular events during dialysis.

3.4 PAPER IV

Methylseleninic acid sensitizes ovarian cancer cells to T-cell mediated killing by decreasing PDL1 and VEGF levels

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Manuscript submitted

Background

Selenium (Se) compounds are emerging as potential anti-cancer and apoptotic agents due to their effects on growth, cell cycle and apoptosis. The cytotoxic effects of Se compounds on cancer cells are highly dependent on the chemical form, dose, redox state and experimental model used [98]. Tumor selective killing property of selenium has been reported in many cancers including prostate cancer cells [96]. Several mechanisms have been reported by which Se compounds target tumors cells [99]. Among them, Se compounds have been shown to target tumor cells by decreasing the expression of inhibitory ligands thereby increasing their immune cell recognition [130] [131] , but the direct effect of Se compounds on immune cells has so far not been reported. Tumor cells evade immune attack for their survival by immune suppression, which can be achieved by secretion of factors like TGF-β and VEGF-A, secretion of which leads to dendritic cell suppression, and inhibition of T-cell function [132]. Thus, restoring the compromised immune system in cancer is an important anti-cancer strategy.

This study mainly focuses on the effect of selenite and MSA on immune cells at doses that are cytotoxic for the cancer cells but not to the immune cells. The aim was to study the possible immune modulatory mechanism of Se compounds on tumor cells to enhance immune cell recognition.

Main findings

<u>Se compounds do not affect immune cell viability at doses that are cytotoxic to ovarian cancer cells</u>

Sodium selenite had different effects on viability in different cell lines; A2780 cells were resistant to sodium selenite treatment, whereas their cisplatin resistant counterpart, the CP70 cell line, was more sensitive to selenite. The differences in sensitivity could be correlated to the uptake mechanism. MSA proved to be equally toxic for both CP70 and A2780 cells.

Neither selenite nor MSA had an effect on cell viability of natural killer (NK) cells or T cells at the doses significantly cytotoxic to the tumor cells.

MSA enhances the NK cell mediated killing of tumor cells

In our study selenite or MSA neither directly activated NK cell or T cell nor did they alter their population in the immune cell compartment. However, pretreatment of A2780 cancer cells with MSA significantly increased the lytic potential of IL-2 stimulated NK cells.

MSA decreases the expression of programmed death ligand 1 (PDL1)

Treatment of CP70 cell lines with selenite and MSA significantly decreases the expression of PDL1. The downregulation of PDL1 did not occur at the transcriptional level as the mRNA expression of PDL1 was increased upon MSA treatment. However matrix metalloproteineases (MMPs) that are known to degrade PDL1 were significantly upregulated (MMP-9, MM-13 and MMP-19) in response to MSA treatment.

MSA enhances the T cell mediated killing of tumor cells

MSA resulted in an enhanced cytolytic activity of T cell cultured in preconditioned media from A2780 and CP70 cells respectively treated with selenite or MSA. An increase in IFN-γ levels was observed which correlated with the enhanced lytic activity. These results suggest that treatment with MSA not only increases T cell function but also enhance the T cell mediated killing of tumor cells.

MSA reduces HIF-1 α and VEGF levels in tumor cells

VEGF is known to be regulated by HIF-1α, and also affects T cell function. Selenite did not alter the levels of VEGF, a significant decrease in VEGF levels were observed after MSA treatment in both the cell lines. MSA also significantly reduced HIF-1α levels. This correlated with an increased T cell mediated tumor cell killing, which could be revoked with the addition of VEGF (1pg/ml) in the tumor condition media

Discussion

We show that MSA mediates enhanced lytic activity of T cell on ovarian cancer cell lines, via inhibition of VEGF. Selenite and MSA have been reported to induce tumor specific killing, especially to drug resistant tumor cells [99, 133]. This is consistent with our result that selenite and MSA is toxic to tumor cells but the T cells and NK cells are not affected by the treatments. It is also in alignment with our findings that selenite is more cytotoxic to CP70

(cisplatin resistant) compared to A2780 ovarian cancer cell lines. We also show that the resistance of immune cells to selenite could be via upregulation of Nrf2 targeted genes. Selenite treatment is known to induce ROS and Nrf2 has been shown to be activated upon oxidative stress [134]

Selenite has been shown to induce posttranscriptional inhibition of HLA-E leading to increased sensitivity of tumor cells to CD94/NKG2A positive NK cells and HLA-E antigen is expressed in ovarian carcinoma [130]. In addition, MSA regulates the expression of ligands that trigger immune activation through the lymphocyte receptor NKG2D [131]. We report a trend in increased NK cell lysis with selenite, and significant increase in NK cell mediated tumor cell lysis upon MSA pretreatment of ovarian cancer cells.

MSA and selenite downregulated the PDL1 protein levels in CP70 cells, however we could not detect any change in mRNA levels. MSA has previously been reported to upregulate mRNA levels of MMP, [135] and these MMPs have recently been shown to degrade PDL1 [136]. The degradation of PDL1 by MMPs may therefore explain the mechanism behind the decrease in PDL1, as the expression of three MMPs were found to be increased, upon MSA treatment, in the ovarian cancer cell line CP70. VEGF suppresses T cell activation (via VEGF2 receptor) and function (via prostaglandin E2) [137] [138] . As mentioned earlier MSA have also been shown to decrease the expression of VEFG [139] [140] . Consistently we also show decrease in the expression of HIF-1α and the levels of VEGF on treatment with MSA. As VEGF has been shown to be immunosuppressive, the decrease in VEGF levels on treatment with MSA may improve the immune cell response. This study indicated that incubation of activated T cells in tumor conditioned media with selenite or MSA indeed enhanced the T cells mediated killing of ovarian cancer cells which also supported by the increased IFN-γ and Granzyme B levels. The enhanced tumor killing activity is therefore at least partly explained via the inhibition of VEGF. VEGF expression has been reported to affect the expression of PD 1 on the T cell surface (immune inhibitory receptor) [141]. PD1 interacts with PDL1 (expressed on tumors and some other immune cells) and inhibits T cell activation and cytokine production [142].

To conclude, we show that selenite and MSA does not affect immune cell viability at the doses used, but are cytotoxic to tumor cells. MSA was found to decrease the levels of PDL1, HIF-1 α and VEGF, thereby rendering the tumor cells sensitive to T cells. This study shows a potential benefit of selenium compounds in anticancer treatment in enhancing immune function while at the same time targeting the tumor cells. However, these results need to be confirmed in animal models before moving into a clinical trial setting.

4 CONCLUSION AND FUTURE PERSPECTIVE

The importance of Trx and Grx systems in maintenance of the redox homeostasis is well established and accumulating evidence support that these proteins are deregulated in several disease conditions associated with chronic inflammation.

In **Paper I**, we demonstrate a mechanism of action for the cytotoxicity of SAHA by decreasing Trx1 activity by multiple mechanisms. Decreased Trx1 activity reduces the cancer cells ability to cope with the high levels of oxidative stress associated with high cell turnover rate in cancer cells. No increase in Trx1 was found, likely due to already maximally increased protein levels of Trx1 as is frequently found in tumor cells.

Future perspectives: These findings suggest one of the many mechanisms by which SAHA exerts its cytotoxicity on tumor cells. However, the cytotoxicity of SAHA may vary depending on cell types thus for this to be regarded as a general mechanism of action, other cell types e.g. suspension cells, as well as primary tumor cells, must be assessed. Generally, the field of targeting redox state with anticancer drugs is expanding as increased ROS in cancer cells makes transformed cells vulnerable to selective cell death. Some of the treatments are selenium compounds, TrxR inhibitors and GSH inhibitors.

In **Paper II**, we studied the effects of CoQ10 treatment on Grx in type 1 and type 2 DM patients. CoQ10 is an endogenous antioxidant. In the present study analyzing samples from a small clinical trial, we found that the effect of CoQ10 on certain clinical parameters varies between DM patients treated with/without statin. CoQ10 has significantly reduced the levels of oxidative stress markers in each treatment groups indicating its antioxidant function. Decrease in serum Grx activity and TAC levels upon CoQ10 treatment shows that the extracellular environment has been reduced by CoQ10. Additionally we show that CoQ10 is a substrate of GSH in a reaction catalyzed by Grx.

Future perspectives: The effect of CoQ10 has been evaluated in several studies where it is beneficial in some while contradictory in many others. One reason for this variation among the studies could be due to enrollment of patients with diverse baseline levels of clinical parameters e.g. Hb1Ac. Other factors that can be a cause for the existing conflict of positive aspects of CoQ10 are dosage, treatment term, interference of other medication (like statin) and the control population. A well designed and well randomized study considering above mentioned aspects might provide a satisfying study on the effect of CoQ10 in DM or in any other disease condition. It also will be interesting to investigate why CoQ10 treatment decreased serum Grx activity but increased intracellular levels of Grx.

In **Paper III**, we measured the serum levels of Grx in CKD patients. We found an increase in the serum Grx activity in CKD patients compared to the controls which was clear indication of oxidized extracellular environment in patients, and a tendency to higher increase in Grx during dialysis in patients experiencing a myocardial infarction or stroke, compared to patients that did not, however due to small sample size, these differences were not statistically significant.

Future Perspective: Grx may be a good clinical marker of oxidative stresses in CKD patients and possibly, also a predictive marker of future cardiovascular events. This remains to be assessed in a larger prospective patient cohort.

In **Paper IV,** the aim was to find the effect of selenium compounds on immune cells compared to cancer cells. Selenium when used at lower doses act as an indirect antioxidant and at higher doses it is cytotoxic. Each selenium compounds (organic and inorganic) metabolizes differently so follow distinct mechanism for inducing cellular toxicity. Several mechanisms by which Se compounds induce cell death have been proposed and one among them being improving immune cell response. In this paper we found that immune cells are resistant to cell death at the doses that are sensitive to ovarian cancer cells this shows that treatment Se compounds will not harm the immune cells. Also treatment of immune cells with selenite / MSA does not activate them directly. However pretreatment of tumor cells with MSA shows an increase in the NK cell mediated lysis. MSA / selenite pretreatment of tumor cells also decreased the protein expression of PDL1. Incubation of T cells with tumor preconditioned media increased the T cell mediated killing of tumor cells; this enhanced T cells activity was supported by increase in Granzyme B and IFN-γ secretion. MSA was found to decrease the expression of HIF-1 α which leads to decrease in the VEGF levels. Additional VEGF in the cell culture was found to decrease the lytic activity of T cells.

Future perspectives: Taken together, this study shows ability of selenium compounds to boost immune function in anticancer treatment although; it needs further animal studies before any conclusion. This study was mostly done on ovarian cancer cell line but addition of patient sample will support the finding. It will be really interesting to study molecular alterations of MSA on T cells level even though we report T cells being not activated upon MSA treatment.

In summary: The aim of this thesis was to investigate alterations in redox proteins in chronic inflammatory conditions and to study the effect of redox active selenium compound on immune cells. We found that several treatment modalities used for anticancer treatment affect

redox protein balance, and that selenium compounds at chemotherapeutic doses improve immune cell functions targeting the tumor cells.

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