International Doctorate Program in Molecular Oncology and Endocrinology
Doctorate School in Molecular Medicine

XXII cycle - 2006–2009

Coordinator: Prof. Giancarlo Vecchio

Signaling pathways and mechanism of action of the extracellular superoxide dismutase, SOD3

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LIST OF PUBLICATIONS

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I Laurila JP, Castellone MD, Curcio A, **Laatikainen LE**, Haaparanta-Solin M, Gronroos TJ, Marjamaki P, Martikainen S, Santoro M, Laukkanen MO: Extracellular superoxide dismutase is a growth regulatory mediator of tissue injury recovery.

Molecular therapy 2009; 17(3):448-54.

II Laurila JP, **Laatikainen LE**, Castellone MD, Laukkanen MO: SOD3 reduces inflammatory cell migration by regulating adhesion molecule and cytokine expression.

PLoS One 2009; 4(6):e5786.

III **Laatikainen LE**, et al.: Extracellular superoxide dismutase is a thyroid differentiation marker downregulated in cancer.

Manuscript.

ABBREVIATIONS

Akt/PKB	Akt/protein kinase B
AP-1	activating protein-1
cAMP	cyclic adenosine monophosphate
CREB	cAMP response element binding protein
DAG	diacyl glycerol
Duox	dual function oxidase
EGF	epidermal growth factor
Erk	extracellular signal regulated kinase
FGF	fibroblast growth factor
GPCR	G-protein coupled receptor
GPx	glutathione peroxidase
GTPase	guanine triphosphatase
H_2O_2	hydrogen peroxide
ICAM	intercellular cell adhesion molecule
IFN-γ	interferon-γ
IGF-1	insulin-like growth factor
IκB	inhibitor of κB
IL	interleukin
JNK	c-jun NH2-terminal kinase
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemotactic protein 1
MEK	MAPK/Erk kinase
MIP2	macrophage inhibitory protein 2
mTOR	mammalian target of rapamycin
NADPH	nicotinamide dinucleotide phosphate
NFκB	nuclear factor κB
Nox	NADPH oxidase
$O_2\bullet^-$	superoxide
PDGF	platelet-derived growth factor
PI3K	phosphoinositide 3-kinase
PIP	phosphatidyl-inositol phosphate
PKA	protein kinase A

PKC	protein kinase C
PLC	phospholipase C
Prx	peroxiredoxin
PTEN	phosphatase and tensin homolog
PTP	protein tyrosine phosphatase
Redox	reduction-oxidation
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
S6K1	p70 ribosomal protein S6 kinase 1
SOD	superoxide dismutase
T3	triiodotyronine
T4	tetraiodotyronine, thyroxine
Tg	thyroglobulin
TNF-α	tumor necrosis factor-α
TPO	thyroperoxidase
TSH	thyroid stimulating hormone, thyrotropin
TSH-R	TSH receptor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor

ABSTRACT

Reactive oxygen species (ROS) and especially hydrogen peroxide (H_2O_2) have been implicated in a multitude of cellular events ranging from pathogen eradication to finely tuned intracellular signal transduction under normal and pathological conditions. In some organs, such as thyroid gland, or in tissue injuries their expression is naturally high and requires special measures to keep it under control. Extracellular superoxide dismutase (SOD3) is an antioxidative enzyme, which converts superoxide into H_2O_2 thus reducing the harmful ROS burden of injured tissues. Recently, a more versatile role for SOD3 as an anti-inflammatory and signal modulating agent has begun to emerge. We studied the mechanism of action of SOD3 in tissue injury recovery and whether it has a role in the thyroid gland physiology and pathophysiology, which untill now have remained uncharacterized.

We performed an adenovirally mediated gene transfer of SOD3 into rat hind limb and mouse peritoneum to assess its effect on tissue recovery and leukocyte migration. Our rat hind limb ischemia model provided an acute ischemic injury to study the effects of therapeutic molecules on the initiation of inflammation. A mouse peritonitis model allowed a more specific analysis of the infiltrating inflammatory cell subtypes. We also determined the expression of SOD3 and its influence on thyrocyte function from rat and human thyroid tissues and cell lines.

SOD3 gene transfer markedly reduced the inflammatory cell accumulation into the rat hind limbs and the mouse peritonea as well as improved rat muscle tissue recovery. Interestingly, SOD3 seemed to have a pronounced effect on the macrophage population. Activation of the Ras-mitogen activated protein kinase (MAPK) pathway was upregulated whereas the expression of several inflammatory cytokines and cell adhesion molecules was downregulated. The SOD3 expression analysis and signal transduction studies on thyroid revealed high thyrotropin mediated SOD3 expression in normal rat thyroid and even higher in hyperproliferative goitrous tissue. Accordingly, silencing of *SOD3* by small interfering RNA reduced thyrocyte proliferation. However, thyroid cancer tissue from human patients and rat thyroid cancer cell lines had significantly lower SOD3 expression.

These studies describe a novel role for SOD3 in cell proliferation in injured tissue and in the thyroid gland physiology. Additionally, they elucidate the anti-inflammatory mechanism of SOD3. Thus, SOD3 could have therapeutic value in treatment of ischemic injuries, and in assessment of the degree of malignancy in thyroid hyperproliferative disorders.

1 INTRODUCTION

1.1. Hydrogen peroxide and other reactive oxygen species

Hydrogen peroxide (H_2O_2) is one of the reactive oxygen species (ROS) which is characterized by their high reactivity with numerous intra- and extracellular molecules. In redox balance the reactions of ROS for instance with reactive nitrogen species, such as nitric oxide (NO•), creates elaborate network where altering the expression of one component usually has a significant effect on the others. Some common oxygen and nitrogen radical species and reactive sulfur groups are presented in Table 1. $O_2•^-$ and H_2O_2 are involved in a multitude of signaling events, their over production can cause tissue damage, and they can act as precursors for other ROS such as peroxynitrite (ONOO-) and OH•. Accumulating knowledge of ROS has revealed a dual role for them as important signal tranduction molecules in the traditional immune responses and in cellular damage.

Table 1. Radical species and reactive groups.

Reactive oxygen species	
$O_2•^-$	superoxide radical
OH•	hydroxyl radical
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
Reactive nitrogen species	
NO•	nitric oxide
ONOO-	peroxynitrite
Reactive sulfur groups	
R-S-	thiolate anion
R-SH	sulfhydryl (thiol)
R-SOH	sulfenic acid
R-S-S-R	disulfide

R=organic

1.1.1. H_2O_2 concentration and oxidative stress

H_2O_2 is produced as a side-product of normal metabolism in e.g. mitochondria or peroxisomes, and scavenged quickly by antioxidative enzymes which maintain the normal reduction-oxidation (redox) balance of the cells. The intracellular H_2O_2 concentrations remain at a relatively similar level in mammalian cells ranging from 0.001 μM to a maximum of 0.5-0.7 μM in some cytotoxic immune cells (Oshino et al. 1973; reviewed by Chance et al. 1979). The optimal concentration varies strongly according to the cell type and the metabolic activity level of the cells. Actively proliferating and transformed

cells have been shown to produce more H_2O_2 as compared to cells in G_0-phase (Pani et al. 2000; Duval et al. 2003; Policastro et al. 2004). Consequently, the variable H_2O_2 concentrations, whether applied to tissue culture or generated endogenously, have a distinct biological effect on vertebrate cells (reviewed by Burdon 1995; reviewed by Stone and Yang 2006).

Extracellular H_2O_2 concentrations vary greatly according to the environment of the cell but, in general, there is a 7 to 10-fold difference between cell interior and exterior (Antunes and Cadenas 2000; Makino et al. 2004; Figure 1). Thus, the level of H_2O_2 under physiological conditions is usually in the range of 0.01-7 μM outside the cells. Alterations in the environment are rapidly reflected in the intracellular levels since H_2O_2 can diffuse across the plasma membrane, and additionally the cells are actively maintaining this gradient (reviewed by Chance et al. 1979; Makino et al. 2004). The phenomenon can be utilized in exposing the cell culture to H_2O_2 in order to reach a desired intracellular H_2O_2 concentration for research purposes.

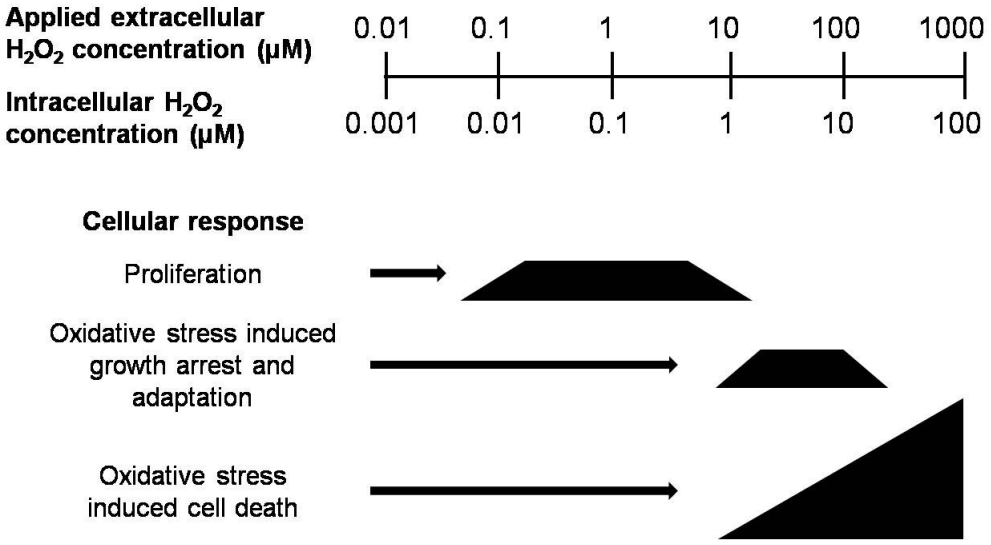

Figure 1. Effect of applied H_2O_2 concentration on cellular responses. A 10-fold difference quickly develops between cell exterior and interior after application of H_2O_2. Low H_2O_2 concentrations (0.1-7μM extracellular) stimulate cell proliferation. Higher concentrations (20-200μM) induce growth arrest but a later adaptation may occur allowing the cell to proliferate again. Concentrations exceeding 10 μM result in increasing cell death rate. The outcome of H_2O_2 application depends considerably on the cell type and culture conditions. (Modified from the review by Stone and Yang, 2006)

Excess H_2O_2 and other ROS production result in oxidative stress, proceeding from mild to severe according to the level of oxidation in the cell interior. Redox balance of the cells is altered during oxidative stress, and

normally leads to growth arrest and eventually to apoptosis (reviewed by Davies 1999; reviewed by Stone and Yang 2006; Figure 1). Oxidative stress is often associated with ischemia-reperfusion injuries and various inflammatory or metabolic diseases as well as with dysregulation of ROS generating and scavenging systems which are caused by genetic mutations in antioxidant enzymes or their regulators. For instance, hyperthyroidism, various cancers and cardiovascular injuries are known to involve excessive ROS production (reviewed by Ferrari 1995; reviewed by Venditti and Di Meo 2006; reviewed by Valko et al. 2006).

Mild stress caused by 10-200 μM extracellular H_2O_2 depending on the cell type, increases the activation of various peroxidases. Interestingly, it has been shown that mild stress can actually prime the cells for later more severe stress and enhance their survival by altering the cell metabolism and respiration (Wiese et al. 1995; reviewed by Davies 1999). More severe stress will deplete the cytosolic glutathione reductant pool and inhibit the peroxidases themselves thus impairing the antioxidant system of the cell (reviewed by Stone and Yang 2006). Consequently, this leads to more severe alteration of the redox balance and eventual activation of the apoptosis pathways (reviewed by Davies 1999; Figure 1).

1.1.2. ROS generating and scavenging systems

Cells, tissues and organisms are constantly exposed to chemical, physical, and environmental stress factors as well as microbial pathogens that induce expression of cytokines, growth factors, integrins, and hormones in order to maintain tissue homeostasis. Many of these agents stimulate the activity of ROS generating enzymes, the large family of various oxidases. In mammals the main radical generators, NADPH oxidases (Nox), produce superoxide which is then dismutated by superoxide dismutases (SOD) into H_2O_2 that is further neutralized into oxygen and water by e.g. catalase. Therefore SOD enzymes act in the first line in reducing oxidative stress.

1.1.2.1. H_2O_2 generators

Presently seven NADPH oxidases have been identified in human cells: Nox1, Nox2, Nox3, Nox4, Nox5, and two dual function Nox enzymes Duox1 and Duox2 (reviewed by Lambeth 2004). The functional enzyme is a complex formed by a catalytic subunit together several associated regulatory subunits and proteins as well as $Ca2^{+}$ which is needed by Nox5 and the Duox enzymes. The components reside in the cytosol in resting cells but upon activation, often phosphorylation by various kinases or binding to phosphatidylinositols, they translocate to the plasma membrane (reviewed by Geiszt 2006; Bissonnette et al. 2008). Nox enzymes are expressed throughout the body in numerous tissues

such as epithelia, gastrointestinal tract, inner ear, kidneys, vascular tissues, and in professional phagocytes (reviewed by Geiszt 2006). Activated phagocytic leukocytes, monocyte/macrophages and neutrophils, produce toxic amounts of Nox2 derived $O_2\bullet^-$ as the primary defense mechanism against invading pathogens in a process called oxidative burst (Cross and Segal 2004; Quinn and Gauss 2004). In the vasculature, Nox1, Nox2 and Nox4 have been found to mediate various important $O_2\bullet^-$ dependent signaling events related to e.g. endothelial cell proliferation and migration (Abid et al. 2000; Van Buul et al. 2005), cytokine production and endothelial permeability (Ali et al. 1999; Chen et al. 2004), and cell adhesion molecule expression to aid leukocyte transmigration (Fan et al. 2002; Chen et al. 2003). Nox enzymes are upregulated by a variety of growth factors, inflammatory cytokines and environmental factors such as hypoxia and shear stress (reviewed by Pani et al. 2001; reviewed by Li and Shah 2004).

Of particular interest in thyroid are the Duox (aka thyroid peroxidase, ThOX) enzymes which provide the H_2O_2 for thyroid hormone synthesis. The immature forms of Duox enzymes are post-translationally glycosylated at the endoplasmic reticulum before exportation of the mature enzymes with the help of DuoxA maturation factors (De Deken et al. 2000; De Deken et al. 2002; Grasberger and Refetoff 2006; Grasberger et al. 2007). However, majority of the enzyme remains stored in inactive form inside the cell and is matured and transported to the plasma membrane only when the thyrocytes are stimulated by thyroid stimulating hormone (TSH) (De Deken et al. 2002).

Mammalian superoxide dismutases 1, 2 and 3 convert the $O_2\bullet^-$ molecule into H_2O_2 using Cu and Zn or Mn cofactors at their catalytic sites. SOD1 (CuZn-SOD) and SOD2 (Mn-SOD) are expressed in a wide variety of cells but differ in their subcellular locations. SOD1 is found in the cytoplasm, nuclear compartments, and lysosomes, whereas SOD2 is restricted to mitochondria (Weisiger and Fridovich 1973; Geller and Winge 1982; Fridovich 1986; Chang et al. 1988). SOD3 is an extracellular isoform of the family with extensive homology to SOD1 including the CuZn core (Marklund 1984). The principal function of the SOD enzymes is the removal of $O_2\bullet^-$ in various conditions such as ischemia/reperfusion injuries, inflammation, or exposure to radiation, and thus attenuate damage to cellular macromolecules (Petrone et al. 1980; Wong 1995; Jones et al. 2003; Kim et al. 2007a).

Extracellular SOD3 is the most recently characterized SOD isoenzyme. It is expressed widely *in vivo* with highest expression in vascular smooth muscle cells, thyroid gland, aorta, lungs, epididymis, and kidney (Marklund 1984; Perry et al. 1993; Stralin et al. 1995). Additionally, skeletal muscles, heart,

liver, brain, and spleen show some SOD3 expression (Folz et al. 1997) suggesting a role in oxygen regulation in tissues susceptible to oxidative stress. However, based on the studies with SOD3 knockout mice which have a normal phenotype until exposed to oxidative stress (Carlsson et al. 1995; van Deel et al. 2008) suggest that in normal conditions SOD3 has a minor role which comes more prominent when the tissue redox balance is challenged. SOD3 is not freely diffusible but after secretion it attaches to extracellular matrix and cell surfaces through its binding sites for heparan sulphate, collagen and fibulin 5 (Nguyen et al. 2004; Petersen et al. 2004; Antonyuk et al. 2009). Thus, it is confined to the proximity of cell surface Nox enzymes and its substrate $O_2\bullet^-$. Bound SOD3 is most effective in eradicating the unwanted $O_2\bullet^-$as shown by studies where defective binding to extracellular matrix allows collagen proteolysis and higher tissue $O_2\bullet^-$ expression (Petersen et al. 2004; Nguyen et al. 2004).

Numerous studies have established a role for SOD3 as an anti-inflammatory antioxidant which can markedly attenuate tissue damage. Absence of SOD3 in knockout mice increased $O_2\bullet^-$ mediated damage and inflammatory injury after ischemia/reperfusion in brain or skeletal muscle and in arthritis (Sheng et al. 1999; Ross et al. 2004; Park et al. 2005). In contrast, gene transfer of SOD3 or overexpression in transgenic animals improves tissue recovery significantly in e.g. myocardium, vasculature, lungs, skin or liver (Laukkanen et al. 2001; Li et al. 2001; Durand et al. 2005; Rabbani et al. 2005; Ha et al. 2006). Possible mechanisms in addition to direct removal of excess $O_2\bullet^-$ have been suggested such as inhibition of leukocyte migration, and vascular tone regulation by simultaneous activation with nitric oxide synthase to reduce toxic ONOO- formation and thus increasing the availability of NO (Brady et al. 1997; Bowler et al. 2004; Na et al. 2007).

Interestingly, the expression of SOD3 shows variable pattern in different circumstances. In acute coronary ischemia patients the endogenous SOD3 expression was rapidly increased (Horiuchi et al. 2004), whereas in chronic cardiac disease as well as in systemic inflammation its expression and activity was downregulated (Landmesser et al. 2000; Tasaki et al. 2006; Ueda et al. 2008). This could be partly explained by the variable regulatory effect of inflammatory cytokines and growth factors on SOD3 expression: interferon (IFN)-γ, interleukins (IL-) 1α and 4, NO, and angiotensin II all promote SOD3 expression but tumor necrosis factor (TNF) α, and fibroblast, epidermal, and platelet derived growth factors (FGF, EGF and PDGF, respectively) reduce it (Stralin and Marklund 2000; reviewed by Zelko et al. 2002).

1.1.2.2. H_2O_2 scavengers

Hydroperoxidases comprise a large family of antioxidant enzymes involved in scavenging H_2O_2 and ONOO-. The most prominent mammalian members are peroxiredoxins (Prx), glutathione peroxidases (GPx), and catalase. Prx and GPx are more readily oxidized than catalase and act therefore mainly at physiological H_2O_2 concentrations whereas catalase remains functional at higher ROS concentrations occurring at its site of action in the lysosomes.

Prx enzymes exist in mammals as six isoforms divided into three subgroups with some variation in intracellular expression sites (reviewed by Rhee et al. 2005a). In their active sites all Prxs contain one or two essential Cys residues with a thiolate anion or a thiol group that is successively oxidized and reduced by H_2O_2 and thiols, respectively, during the catalytic cycle (see Table 1. for different oxidation states of the thiol group; reviewed by Rhee et al. 2005a). The mechanism involves hyperoxidation of the Cys residues which allows a local increase in H_2O_2 during signal transduction. Therefore Prx are recently suggested to have an important role in signal transduction modulation (reviewed by Fourquet et al. 2008).

The second large group of H_2O_2 scavengers are the GPx enzymes which differ from Prx by containing a selenocystein amino acid instead of normal Cys at their active site (reviewed by Papp et al. 2007). Otherwise their mode of action and catalytic cycle resembles that of Prx enzymes with reversible oxidation of selenium (instead of sulfur). In humans there are altogether seven GPx enzymes, although two of them have the conventional Cys residue (reviewed by Papp et al. 2007). They seem to have only a minor role in normal physiology, but instead constitute the primary antioxidant defense system of the body as they are more resistant to higher ROS levels than the Prx enzymes (reviewed by Papp et al. 2007; reviewed by Fourquet et al. 2008).

Catalase is one of the first antioxidant enzymes found. The majority of the mammalian catalase is in the peroxisomes even though minor expression can also be found in the mitochondria (Salvi et al. 2007). Catalase, which uses two molecules of H_2O_2 to generate water and molecular oxygen, is an efficient enzyme with very high enzymatic rate (reviewed by Kirkman and Gaetani 2007). Interestingly, deficiencies in catalase function or expression do not affect the normal phenotype and overexpression may even protect the tissues from oxidative damage but the absence of the enzyme becomes problematic when the tissue oxygen balance is compromised (Ho et al. 2004; Schriner et al. 2005). Catalase can inhibit cell proliferation by removing the growth promoting H_2O_2 signal and thus influences the regulation of cell growth (Policastro et al. 2004).

1.1.3. H_2O_2 in cell signaling

Nearly two decades ago it was hypothesized that oxygen radicals could act as second messengers as it became apparent that their generation was upregulated by proinflammatory cytokines TNF-α and IL-1, and that they activate the important stress-related transcription factor nuclear factor (NF) κB (reviewed by Schreck and Baeuerle 1991). More recently, especially H_2O_2 signaling has gained attention as it seems to be involved in great majority of autocrine and paracrine signaling pathways, both in normal physiology and in pathological conditions. Characteristic to this function is that at the global cellular level H_2O_2 concentration remains at low physiological level whereas the local rise in H_2O_2 concentration occurs at the site of stimulated receptor. The local generation of H_2O_2 is under strict regulation to avoid excess oxidative stress (reviewed by Rhee et al. 2005b).

Hydrogen peroxide has many features which render it a suitable signaling molecule. It is generated rapidly, it is relatively long-lasting compared to for instance superoxide or nitric oxide, and since it is not electrically charged it can diffuse long distances and cross lipid membranes (reviewed by Cai 2005). Additionally, H_2O_2 is capable of inducing its own expression thus amplifying the initial signal utilizing e.g. the mitochondria and cellular oxidases (reviewed by Cai 2005). However, this kind of continuous H_2O_2 production contributes to the development of oxidative stress in the surrounding tissues and further to e.g. vascular diseases and arthritis (Bauerova and Bezek 1999; reviewed by Cai 2005).

A general scheme of the signal transduction events involving ROS and leading to gene transcription has been proposed almost for 20 years ago (Schreck and Baeuerle 1991; Devary et al. 1993; Anderson et al. 1994). First, the extracellular stimulus, a cytokine, ROS, chemical compound or radiation, induces a common pathway leading to protein tyrosine phosphorylation. These phosphorylated factors then induce the activation of transcription factors, the most well-characterized of which is probably NFκB. Consequent changes in gene expression pattern eventually produce the specific cellular response to the stimulus in question. However, the detailed molecular mechanisms have been gradually discovered during the following years. Kamata et al. (2000) have suggested that several mechanisms become simultaneously activated to facilitate signal transduction e.g. oxidative protein tyrosine phosphatase (PTP) inhibition and intracellular ROS signal generation. Additionally, H_2O_2 can interfere signaling by oxidating important second messengers such as NFκB and diacyl glycerol (DAG) (Takekoshi et al. 1995; reviewed by Kabe et al. 2005).

Several growth factors (EGF, PDGF, vascular endothelial growth factor or VEGF), cytokines (tumor growth factor-β, TNF-α, interleukins), and G-protein coupled receptors (GPCR), and also biomechanical forces such as shear stress have been found downstream H_2O_2 signaling (reviewed by Thannickal and Fanburg 2000; reviewed by Stone and Collins 2002; reviewed by Rhee et al. 2003; reviewed by Li and Shah 2004). Additionally, various external stress factors such as UV light and hypoxia induce H_2O_2 signal generation (Masaki et al. 1995; Sato et al. 2005).

Nox or Duox activation occurs relatively early in ROS dependent signaling pathways as the regulatory subunits are recruited to the plasmamembrane e.g. through activated receptor binding scaffold proteins IQGAP1 (IQ motif containing GTPase activating protein) and TRAF (TNFα receptor associated factor), or through direct binding to the cytosolic domain of the receptor (Ikeda et al. 2005; Li et al. 2005; Li et al. 2006). Alternatively, the catalytic subunit itself may bind the receptor, e.g. the C-terminal region of Nox4 interacts with activated TLR (Toll-like receptor) 4 in vascular endothelial cells (Park et al. 2006). As stated above, the Nox enzymes then generate $O_2^{\bullet -}$ in a highly local manner which then either promotes directly or after conversion to H_2O_2 the signal transduction (reviewed by Ushio-Fukai 2006a).

1.1.3.1. Mechanisms of H_2O_2 mediated signal transduction

A well-studied mechanism for H_2O_2 regulation in signal transduction is based on the reversible oxidative inactivation of PTP and Prx enzymes by rapid increase in local H_2O_2 (Meng et al. 2002; Woo et al. 2003; reviewed by Rhee et al. 2005bh2o2; Salmeen and Barford 2005; Figure 2). PTPs inhibit the signal transduction of numerous protein tyrosine kinases by dephosphorylating their tyrosine residues (reviewed by Chiarugi 2005) whereas Prx enzymes remove excessive cytosolic H_2O_2 to nontoxic concentration (reviewed by Fourquet et al. 2008). All PTPs and Prx enzymes contain a Cys residue which at neutral pH is in thiolate anion form (Chae et al. 1994). It is readily oxidized by H_2O_2 and other oxidants into sulfhydryl and further sulfenic acid (see Table 1. for the oxidation states of sulfur) which inactivate the active site Cys and thus inhibit the enzyme (Denu and Tanner 1998). Inhibition of Prx results in prolonged H_2O_2 survival that in turn enables the inhibition of PTP and phosphorylation of the target signaling kinases (reviewed by Rhee et al. 2005b). Thus, the net increase in phosphorylated signaling proteins triggers the downstream signaling events. After removal of the original stimulus the amount of H_2O_2 decreases as the oxidation processes consume it, and the Prx and PTP enzymes are reactivated (reviewed by Rhee et al. 2005b).

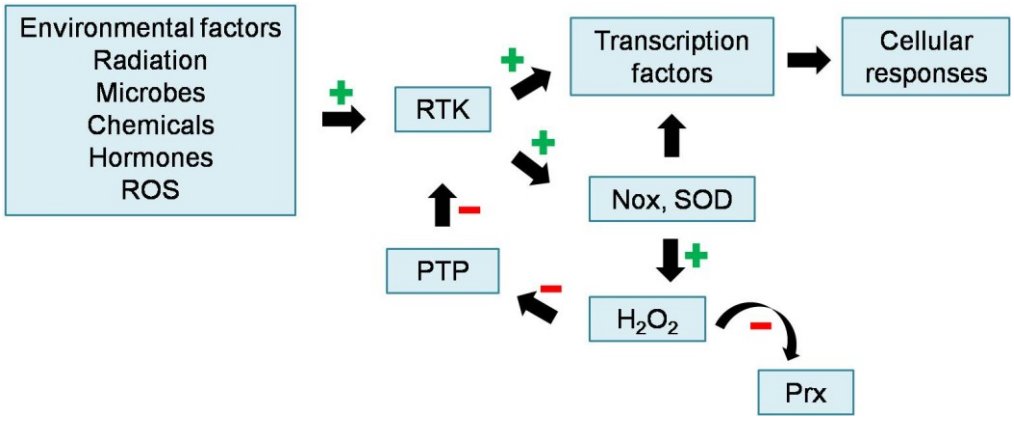

Figure 2. H2O2 signaling. Several factors can stimulate the H2O2 dependent signal transduction through RTKs. RTK activates gene transcription but is constantly inhibited by PTP mediated dephosphorylation. However, RTK also activates Nox and SOD resulting in increased local H2O2 concentration. H2O2 induces oxidative inactivation of Prx and PTP preventing its own removal and RTK inactivation, respectively. Thus, the signal intensity and duration are increased, and lead to enhanced gene expression and a change in cellular functions. In some cell types Nox may have direct influence on the transcription factors.

Another efficient mechanism of H_2O_2 to modulate signaling cascades is the activation of redox-sensitive transcription factors such as NF-κB, activating protein (AP-) 1 and p53 which are associated with a multitude of physiological and pathological functions (Abate et al. 1990; Anderson et al. 1994; Rainwater et al. 1995; reviewed by Thannickal and Fanburg 2000). NF-κB is an important factor in immune response and inflammation related gene expression. In the basal state it is inactivated by the inhibitor of κB (IκB) which in turn is subjected to degradation by multiple co-operating mechanisms, leading to NF-κB release, nuclear translocation, and DNA binding (reviewed by Kabe et al. 2005). H_2O_2 and $O_2^{\bullet -}$ participate in NF-κB regulation through receptor mediated Nox activation enhancing the c-jun NH_2-terminal kinase (JNK) activity that promotes IκB removal (reviewed by Kabe et al. 2005). It has also been suggested that the Prx enzymes, especially the cytosolic isoforms, have an active role in H_2O_2 signal regulation through modulation of intracellular H_2O_2 concentration and NFκB activation after e.g. growth factor or TNF-α stimulation (Jin et al. 1997; Kang et al. 1998). AP-1 is a Fos/Jun or Jun/Jun dimer whose Cys containing conserved regions have been suggested to mediate the redox sensitive regulation of DNA binding through reversible oxidation (Abate et al. 1990). A similar Cys-dependent mechanism has been found to regulate the tumor suppressor p53 activity (Rainwater et al. 1995).

Exogenous H_2O_2 is usually indicative of oxidative stress in cell cultures and within organisms under pathological conditions. Stress level of H_2O_2 can affect

the outcome of other signaling pathways by e.g. promoting p38 mitogen activated protein kinase (MAPK) activation but leaving extracellular signal regulated kinase (Erk) pathway intact after stimulation with the same growth factors leading to increased cell death (Zhang and Jope 1999b). H_2O_2 can also modulate cell growth and other events by inducing activation of cell surface receptor tyrosine kinases and their downstream kinases. For instance, EGF receptor has been found to become phosphorylated and to activate the Ras/MAPK pathway in a dose-dependent manner when the cells are exposed to H_2O_2 *in vitro* (Rao 1996; Kamata et al. 2000). Similarly, H_2O_2 incubation can activate PDGF receptor through PTP inhibition (Kappert et al. 2006).

1.1.4. H_2O_2 in ischemia and inflammation

Inflammation is the primary response of the tissues to injury, trauma or excessive stretching as well as to many pathological conditions ranging from cardiac disease to obesity (reviewed by Lambeth et al. 2008). Local hypoxia often develops in the tissue as blood circulation is hindered or the vessels are broken by e.g. blood clot or injury. Consequently, potentially harmful metabolites begin to accumulate and tissue cells become deprived of nutrients. Mitochondrial dysfunction is the main source of ROS in inflammation stimulating the cell to release inflammatory factors and to adapt to the new conditions (reviewed by Li and Shah 2004).

Acute inflammation, which develops immediately after the injury has occurred, is characterized by accumulation of fluid and inflammatory cells such as neutrophils and monocytes attracted to the site by chemokines. When the stimulus is removed the inflammation soon resolves. However, a controlled inflammatory reaction is necessary to launch the healing process. If the stimulus persists the inflammation becomes chronic as in e.g. tuberculosis, arthritis, and in many autoimmune disorders such as in thyroiditis. In chronic inflammation the persisting stimulus keeps attracting the inflammatory cells by inducing continuous production of inflammatory chemokines and cytokines from the tissue and previously infiltrated professional phagocytes. The neutrophils and macrophages get trapped at the site of inflammation where they continue to remove cell debris but also to produce ROS thus exacerbating the tissue damage and actually preventing the healing process.

Vascular endothelium has a major role in the inflammatory reaction due to its strategic location between the injured tissue and blood-borne leukocytes. Endothelial activation is a complex phenomenon occurring in response to a multitude of stimuli, and it is necessary to attract the leukocytes to the site where they then eradicate the pathogens and clear the necrotic tissue so that the healing process may begin (reviewed by Lum and Roebuck 2001; reviewed by

Alom-Ruiz et al. 2008). Hypoxic conditions launch the expression of a set of redox regulated signaling molecules e.g. prolyl hydroxylase domain-containing protein (PHD), hypoxia inducible factor (HIF), factor inhibiting HIF (FIH), and NFκB in tissues (reviewed by Fraisl et al. 2009). Inflammatory cytokines can also mediate hypoxia-independent HIF activation (Hellwig-Burgel et al. 1999; Gorlach et al. 2001). Among others, Nox enzymes have been shown to be upstream regulators of HIF in vascular smooth muscle cells (Gorlach et al. 2001). HIF controls many downstream cascades which modulate the cellular metabolism, initiate anaerobic respiration and angiogenesis as well as enhance macrophage and neutrophil activity and survival (reviewed by Frede et al. 2007; reviewed by Fraisl et al. 2009).

TNF-α, IFN-γ, interleukins 1 and 6, and VEGF are the most common mediators of inflammatory reactions (reviewed by Roy et al. 2008). These factors frequently activate Nox enzymes and utilize H_2O_2 signaling to further promote endothelial activation, subsequent cell adhesion molecule expression and cytoskeletal rearrangements in endothelial cells to facilitate leukocyte migration and angiogenesis (Chen et al. 2003; Wu et al. 2003; Park et al. 2006; reviewed by Roy et al. 2008). VEGF and endothelial Nox enzymes also drive the angiogenic process to restore the blood supply (reviewed by Ushio-Fukai 2006b). The most relevant adhesion molecules expressed by activated endothelial cells at the inflammation site are the immunoglobulin-family members intercellular, vascular and platelet/endothelial cell adhesion molecules (ICAM, VCAM, and PECAM, respectively), and P-, L- and E-selectins (reviewed by Salmi and Jalkanen 2005; reviewed by Galkina and Ley 2007). Activated leukocytes express ligands for these adhesion molecules and are thus able to attach and transmigrate through the rearranged endothelium into the tissues in a strictly regulated multistep process called extravasation or diapedesis (reviewed by Salmi and Jalkanen 2005; reviewed by Zarbock and Ley 2009; Figure 3).

Neutrophils and monocyte/macrophages appear in the tissues within few minutes to few hours from the first stimulus guided by hypoxia, cytokine induced macrophage inhibitory protein (MIP-2), and monocyte chemotactic protein (MCP-1) chemokine expression (Lakshminarayanan et al. 2001; Chen et al. 2004; reviewed by Bosco et al. 2008). The initial reaction of these phagocytes is oxidative burst, a massive and rapid production of $O_2\bullet^-$ secreted to the extracellular space or into phagosomes in order to destroy the invading pathogens (Babior et al. 2002; Quinn and Gauss 2004; Cross and Segal 2004). The primary phagocytic enzyme responsible for $O_2\bullet^-$ generation is the Nox2 (reviewed by Morel et al. 1991; Babior et al. 2002). However, other oxidases,

mainly myeloperoxidase, are also involved that produce more reactive ROS with wider scale of target molecules such as OH• and HOCl using $O_2•^-$ and H_2O_2 as precursors (reviewed by Leusen et al. 1996; Quinn and Gauss 2004; Cathcart 2004; reviewed by Lambeth 2004). However, an interesting alternative antimicrobial mechanism in neutrophils has been suggested in which Nox2 activation drives uptake of K+ that then induces activation of phagosome proteases thus contributing significantly to microbial killing (Reeves et al. 2002). The neutrophils and macrophages themselves express various antioxidant enzymes which allow them to stay functional at the site of oxidative burst (Yoshioka et al. 2006; Rakkola et al. 2007).

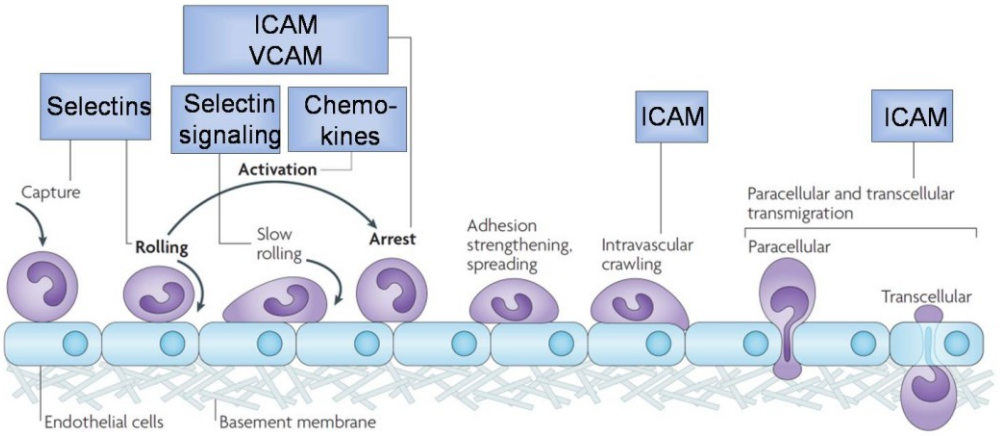

Figure 3. Leukocyte migration. Blood-borne leukocytes enter the tissues by transmigrating through the vascular wall guided by activated endothelium and chemokines. First they are captured and start rolling on endothelial selectins which slow them down. Chemokines activate the firm adhesion phase and the cells are arrested by cell adhesion molecules (CAMs) thus enabling the formation of suitable bonds between the leukocytes and the endothelium. The activated leukocytes are then able to crawl into the vascular wall and finally through it by para- or transcellular migration. (Modified from the review by Ley et al. 2007)

The healing process has not yet been thoroughly elucidated due to the vast number of players and interactions in the process. Inflammatory cells, especially macrophages, have been proposed to induce healing in muscle tissue since they can activate the satellite cells (muscle cell precursors), remove necrotic tissue, and influence muscle cell differentiation and proliferation (reviewed by Tidball 2005). They are also involved in angiogenesis through cytokine stimulated VEGF expression (Xiong et al. 1998; Ramanathan et al. 2003). However, neutrophils do not seem to have such a distinct effect and are therefore more detrimental to the tissue integrity (reviewed by Tidball 2005). Also restoration of NO availability by removing excess $O_2•^-$ could contribute to

healing by dilating the blood vessels and thus increasing the blood supply (Wilson and Kapoor 1993; Hirai et al. 1994; Hickner et al. 1997; Thomas et al. 1998). Additionally, NO has been found to be a negative regulator of inflammation in muscle tissue. In several *in vitro* and *in vivo* studies it has been shown to e.g. inhibit Nox enzymes, the expression of cell adhesion molecules, and to increase inflammatory cell apoptosis (reviewed by Tidball 2005). Recently, it has been shown that IFN-γ can also suppress VEGF and, thus, promote resolution of inflammation (Ray and Fox 2007).

Inflammation is characterized by massive apoptotic and necrotic cell death due to the high oxidative stress and consequent damage to DNA, proteins, and lipids. Additionally, macrophages and other inflicted cells produce high amounts of proapoptotic TNF-α (reviewed by Wajant et al. 2003). Currently, the mode of cell death is thought to be determined by various factors such as duration and severity of oxidative stress, cell types and ROS involved, and the particular signaling pathways activated (Gardner et al. 1997; Troyano et al. 2003; Dunning et al. 2009). The main death receptors mediating both apoptosis and necrosis are the TNF receptor (TNFR)1 and CD95 (Fas/Apo1) but the role of ROS in their direct activation remains controversial (reviewed by Shen and Pervaiz 2006). TNFR1 recruits the MAPK JNK pathway to increase mitochondrial ROS generation and inhibits NFκB regulated pro-survival gene transcription, thus initiating the death signaling cascade (reviewed by Shen and Pervaiz 2006).

In chronic inflammation the ROS production is continuous and affects the inflicted tissues substantially. ROS have been implicated in oxidative damage to DNA, lipids, proteins and other cellular and tissue molecules which may alter their properties and render them active or inactive, and thus affect the cellular metabolism and signaling events (reviewed by Valko et al. 2006). Persistent ROS expression induces chromatin mutations (Konat 2003) that often precede malignant transformation and lead to tumorigenesis when tumor suppressors such as p53 are inactivated or oncogenes such as Ras small GTPase become constitutively active (reviewed by Sun and Oberley 1996; reviewed by Vachtenheim 1997).

1.2. Thyroid physiology

1.2.1. Structure and function of the thyroid gland

The thyroid gland situates in front of the neck anterior to the trachea and is the largest endocrine gland of the human body. The thyroid gland consists of two pear-shaped lobes connected by an isthmus, weighing approximately 10-20 grams in adults depending on the body weight, age and iodine intake. It is an important regulator of development and growth on cellular and organismal

level. Additionally, it regulates multiple homeostatic functions in the body exerting its effect through the hormones triiodothyronine (T3), and tetraiodothyronine, or thyroxine, (T4). Its endocrinological function is dependent on the available iodine, mainly acquired from food and drink, since it is the crucial component of the hormones. Additionally, behind the thyroid gland there are two pairs of parathyroid glands which produce parathyroid hormone participating in the blood calcium ion and phosphate balance. The thyroid gland, and sometimes also the parathyroid glands, is enveloped in a capsule consisting of connective tissue.

At microscopic scale the thyroid gland has a follicular structure (Figure 4A). The follicular lumen contains colloid in which the main component is thyroglobulin (Tg), a protein used for storage and synthesis of the thyroid hormones. The follicles are lined by a single layer of follicular cells that are cuboidal epithelial cells of endodermal origin. They are responsible for the thyroid hormone production in response to thyroid stimulating hormone (TSH) by retaining the TSH receptor (TSH-R) and the necessary enzyme machinery. Follicular cells have numerous microvilli projecting into the lumen to increase the surface area available for hormone metabolism. Each follicle is surrounded by a basement membrane and connective tissue where are the parafollicular cells, or C cells, that produce calcitonin and thus regulate the calcium ion content of blood in cooperation with the parathyroid glands. Extensive capillary vasculature in the connective tissue provides surface area for the exchange of hormones, iodine, nutrients and oxygen.

The thyroid gland traps and concentrates iodine, synthesizes and stores the hormones, and releases them upon stimulation thus regulating the overall metabolic rate of the body. The thyroid gland function is mainly regulated by the hypothalamus which secretes TRH (thyrotropin-releasing hormone) and the anterior pituitary gland which secrete TSH causing increased thyroid gland activity (reviewed by Chiamolera and Wondisford 2009). These glands are, in turn, under finely tuned negative feedback regulation by T3 (reviewed by Chiamolera and Wondisford 2009; Figure 4A). However, other parts of the brain can also generate TRH in response to e.g. temperature and nutritional status (reviewed by Chiamolera and Wondisford 2009). Secondly, the thyroid gland is capable of autoregulation in response to available iodine so that low I-concentration stimulates thyroid function and an excess will inhibit its function (reviewed by Dumont et al. 1992). Finally, B lymphocyte-derived stimulating or blocking autoantibodies for the TSH-R can influence thyroid function in many disorders (reviewed by Dumont et al. 1992).

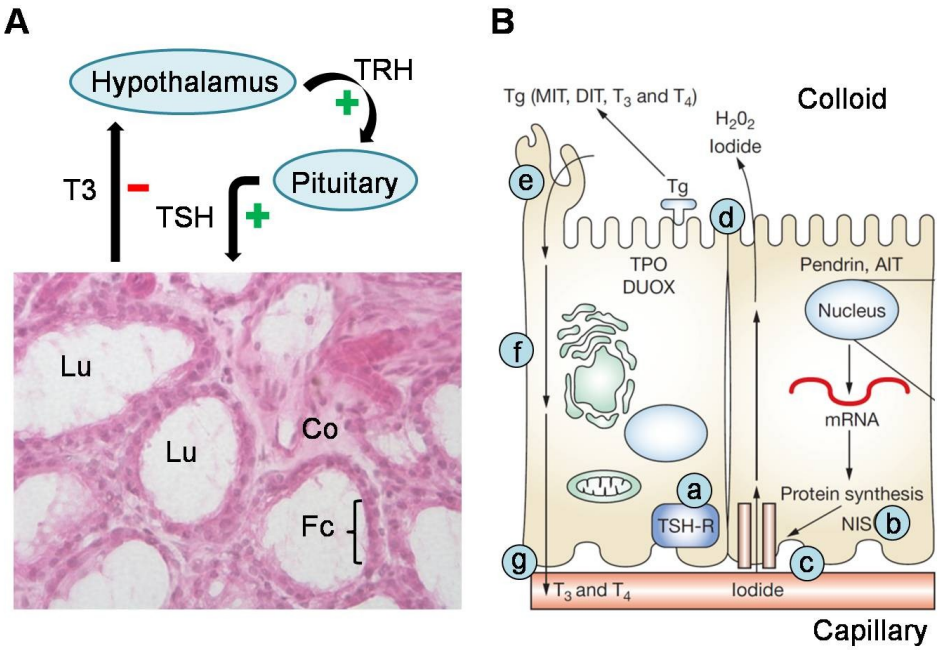

Figure 4. Thyroid gland structure and physiology. The negative feedback system of thyroid hormones and thyroid structure are presented in (A). Hypothalamus secretes TSH releasing hormone (TRH) which induces thyroid stimulating hormone (TSH) secretion from the anterior pituitary. TSH stimulates thyroid hormone synthesis in the follicular cells (Fc) lining the follicle lumen (Lu) filled with colloid. Connective tissue (Co) surrounds each follicle. Thyroid hormone T3 from the thyroid gland inhibits hypothalamic TRH secretion thus decreasing its own production. Overview of thyroid hormone synthesis is presented in (B). a) TSH activates the TSH receptor (-R) on follicular cell basolateral membrane. b) TSH controls thyroid specific gene expression and enhances iodine intake. c) Iodide is imported from the blood stream by Na/I+ symporter (NIS) and then transported across the apical membrane by pendrin and apical I+ transporters (AIT). d) Iodide organification and iodotyrosine (MIT, DIT) coupling by thyroid peroxidase (TPO) and dual oxidase (Duox) take place at the apical membrane. Iodotyrosines remain bound to thyroglobulin (Tg) and are stored in the lumen as colloid. e) Upon stimulation stored hormone-Tg complexes are endocytosed into the follicular cell. f) Tg is proteolytically degraded in the endosomes and the hormone is freed. g) Finally, T3 and T4 are secreted into the blood stream. (Modified from the review by Schlumberger et al. 2007)

1.2.2. Thyroid hormone synthesis

Thyroid hormone synthesis takes place in the follicular lumen on the apical surface of the follicular cells upon TSH stimulation (Strum and Karnovsky 1970; Tice and Wollman 1974; Ofverholm and Ericson 1984; Figure 4B). The location is optimal as the hormone can be readily stored in the lumen immediately after synthesis, and since the cell interior is protected from H_2O_2 produced in large amounts. Song and colleagues (Song et al. 2007) suggest the existence of a thyroxisome consisting of thyroid peroxidase (TPO) and Duox2, described as the iodination complex acting on the thyrocyte apical membrane.

In fact, the association between TPO and an NADPH oxidase, later identified as Duox2, and the change in subcellular location had been established earlier (Mizukami et al. 1985; Wang et al. 2005). The thyroxisome will then process both iodine and Tg to form the active precursors for hormone synthesis.

Iodide organification. The first step in thyroid hormone synthesis is the acquisition of I-. It is actively and effectively transported into the gland from blood stream with the Na+/I- symporter (Dai et al. 1996) on the basolateral membrane whose activity is enhanced by stimulation with TSH (Fenton et al. 2008). Next, I- is transported across the apical membrane into the follicular lumen through an anion transporter pendrin (Royaux et al. 2000) where it is oxidized by Duox2-derived H_2O_2 with TPO as catalyst (Taurog et al. 1996). Active iodide intermediate then detaches from TPO and attaches covalently to specific Tyr-residues on Tg to form mono- and diiodotyrosines (MIT and DIT, respectively) in a iodination process (Lamas et al. 1989; Dunn and Dunn 1999; Kessler et al. 2008).

Iodotyrosine coupling. According to the current understanding, the coupling process starts by a DIT radical formation catalyzed by TPO and involves oxidation of a second H_2O_2 molecule (Doerge et al. 1994; Taurog et al. 1994). The two activated DITs will then couple in a non-enzymatic condensation reaction, and rearrange so that T4 will remain attached to the acceptor site on Tg. The same scheme applies also to T3 formation from MIT and DIT.

Hormone secretion. The hormone-containing Tg remains stored in the follicular lumen bound to multimeric Tg (Berndorfer et al. 1996) until TSH stimulation. The Tg uptake occurs by micropinocytosis and it is packed into coated vesicles, which rapidly fuse with early endosomes (Bernier-Valentin et al. 1990; Bernier-Valentin et al. 1991). Thyroid hormones are released from Tg in the endosomes by selective proteolysis at the hormonogenic sites (Rousset et al. 1989b; lett. Molina et al. 1996) and transported to the blood stream. The remaining Tg is then transferred to late endosomes and finally to lysosomes where the Tg backbone is extensively degraded to small peptide fragments (Rousset et al. 1989a; Rousset et al. 1989b; Kostrouch et al. 1991). The thyroid gland produces primarily T4 which is then processed into more active T3 by deiodinases (reviewed by Bianco et al. 2002; Maia et al. 2005; reviewed by Gereben et al. 2008).

1.2.3. Thyroid hormone dissemination and function

T3 and T4 circulate in the body bound to one of the three types of carrier proteins so that over 99% of each hormone is unavailable for the tissues but these negligible amounts are exerting the hormonal activities of the gland

(reviewed by Sterling and Lazarus 1977). The amount of circulating T4 is markedly higher than that of T3 but the carriers, thyroxine binding globulin, thyroxine binding prealbumin and albumin, bind T4 more tightly than T3, and therefore the latter is more readily available for tissues (reviewed by Sterling and Lazarus 1977). Majority of the circulating T3 derives from the deiodination reactions in tissues, mainly liver and kidneys, and a carefully maintained equilibrium is kept between free and bound circulating hormones to ensure the correct dosage for tissue homeostasis (reviewed by Sterling and Lazarus 1977; reviewed by Bianco et al. 2002).

The thyroid hormones are actively transported into their target tissues by specific transporters such as monocarboxylate transporters (MCT) 8 and 10, and organic anion transporting polypeptide C1 (reviewed by Visser et al. 2008; reviewed by Heuer and Visser 2009). Once inside the cell, most T4 is deiodinated by the tissue specific deiodinases into T3 which is considered the active form of the hormone with several times higher potency than T4 (reviewed bys Bianco et al. 2002; Bianco and Kim 2006; Maia et al. 2005). T3 then binds one of the thyroid hormone receptors which act as transcription factors in the nucleus enhancing the target gene expression in many organs and tissues (reviewed by Yen 2001). In addition, non-genomic actions have been recognized as an important aspect of thyroid hormone signaling (reviewed by Davis et al. 2008). They may initiate multiple signaling pathways and nuclear events through activation of various signaling kinases, lipases and the well-known MAPK cascade, and further phosphorylation of several nuclear hormone receptors, as well as regulate plasmamembrane ion transporters and actin cytoskeleton (reviewed by Davis et al. 2005; Davis et al. 2008).

In general, the thyroid hormones have a stimulating effect on all organ systems in adults and, thus, induce cell proliferation, tissue turnover and increased metabolic rate. However, T3 actions usually need several hours or even days to take effect due to the long process of hormone internalization, translocation to nucleus and modulation of gene expression. During embryonic development T3 participates in e.g. tissue patterning and cell differentiation as well as brain and skeletal maturation (Butler-Browne et al. 1990; reviewed by Konig and Moura Neto 2002; reviewed by Morreale de Escobar et al. 2004; reviewed by Gereben et al. 2008). In adult body thyroid hormones keep the anabolic and catabolic reactions in balance, and control the many metabolic events such as bone formation, muscle function, heat production, activity of the nervous and vascular systems, and energy consumption (reviewed by Kerndt et al. 1982; Monzani et al. 1997; Lebon et al. 2001; reviewed by Bassett and Williams 2003; reviewed by Klein and Danzi 2007). Additionally, the

metabolism of other hormones and pharmacological agents can be enhanced by the thyroid hormones which may have influence on e.g. fertility, disease progression and medication. Interestingly, many of these effects are mediated through regulation of the deiodinases by other hormones and growth factors (reviewed by Bianco et al. 2002; reviewed by Gereben et al. 2008).

1.2.4. H_2O_2 and thyroid gland

As described in the section 1.2.2. the thyroid hormone synthesis depends on the available H_2O_2 as an oxidizer for I- and later for iodotyrosyl residue activation. This H_2O_2 is not derived from cell metabolism waste but rather generated on demand by the Duox2 enzyme after stimulation by e.g. iodide (Corvilain et al. 2000). The hormone generating system is relatively sensitive to oxidative stress causing a shutdown of I- uptake and organification at elevated H_2O_2 levels (Nadolnik et al. 2008). However, a certain amount of oxygen radicals are needed to maintain the thyrocyte function, confirmed by the finding that expression of TPO, Duox, Tg, and pendrin are downregulated on ROS depletion (Poncin et al. 2009).

The thyroid follicular cells in adult thyroid gland can live over 7 years (Coclet et al. 1989) after a period of rapid proliferation in childhood (Saad et al. 2006). During that time the thyrocytes are constantly exposed to heightened levels of H_2O_2 and other oxygen radicals due to the special characteristics of the thyroid hormone synthesis. Therefore, they utilize multiple different mechanisms to avoid oxidative stress derived damage; these have been recently reviewed by Song et al. (Song et al. 2007). Firstly, the exocytosis of vesicles containing the thyroxisome components is regulated. It is also activated only on the cell membrane forcing the H_2O_2 production to take place extracellularly. Secondly, the Duox2 enzyme requires post-translational modifications and Calsium ion binding for full activity besides the exocytosis. Lastly, the diffusion of H_2O_2 itself back to cells is restricted by special lipid raft-like membrane structures, and efficient intracellular H_2O_2 scavenging systems including the GPx, thioredoxin reductase, catalase, and Prx1 and 2 (Ekholm and Bjorkman 1997; Howie et al. 1998; Kim et al. 2000).

Due to their homeostatic effects the thyroid hormones can affect the oxidative status of the body. Hyperthyroid tissues suffering from excessive T3 exposure are known to be under increased oxidative stress as determined by higher levels of oxidized lipids and abnormal expression pattern of antioxidant enzymes (Venditti et al. 1997; Rybus-Kalinowska et al. 2008). Thyroid hormones can enhance tissue susceptibility to oxidative damage by increasing the expression of radical formation promoting cytokines and enzymes, impairing the global cellular antioxidant systems, affecting the lipid

composition of tissues enhancing their predisposition to oxidation, and accelerating mitochondrial functions (reviewed by Venditti and Di Meo 2006). Thus, hyperthyroidism has a distinct exacerbating effect on many hepatic, muscular and cardiovascular diseases which as such burden the tissues by heightened oxidative stress (reviewed by Venditti and Di Meo 2006).

1.2.5. Thyroid disorders

1.2.5.1. Hypothyroidism and hyperthyroidism

Hypothyroidism develops in the absence of thyroid hormones leading to overall slow metabolic rate of the organism, and it can vary from mild to severe according to the extent of hormone deficit. The endogenous disease can be classified into primary (thyroid failure), secondary (lack of pituitary TSH or hypothalamic thyrotropin releasing hormone), or peripheral tissue resistance induced hypothyroidism (reviewed by Topliss and Eastman 2004). Various genetic disorders producing e.g. defective hormonogenesis or thyroid response to TSH, or dysfunctional thyroid hormone receptors are often found in hypothyroid patients (reviewed by Gillam and Kopp 2001; reviewed by Kopp 2002). Hashimoto's thyroiditis is a common hypothyroid disorder characterized by expression of TSH-R blocking autoantibodies (reviewed by Bindra and Braunstein 2006). Additionally, there are numerous environmental factors such as exposure to irradiation, geographic location determining the iodine intake, age, microbial infections inducing thyroiditis, and pharmacological agents and treatments which may include radioisotope imaging or drugs affecting the hormone syntheses (reviewed by Topliss and Eastman 2004). However, dietary iodine deficiency remains as the main cause for hypothyroidism in the world although use of iodized salt in diet has reduced the occurrence of the disease tremendously in developed countries (reviewed by Topliss and Eastman 2004).

Hyperthyroidism is the hyperactive state of the thyroid gland and is characterized by overproduction of the thyroid hormones. Untreated hyperthyroidism leads to thyrotoxicosis which is a syndrome resulting from tissue exposure to large amounts of thyroid hormones by any cause. However, the terms are often used interchangeably. Common causes for thyrotoxicosis include the Grave's disease with stimulating autoreactive TSH-R antibodies, autonomous nodular adenomas, multinodular goiter and follicular carcinoma capable of hormone production, and different types of thyroiditis (reviewed by Topliss and Eastman 2004). Congenital non-autoimmune hyperthyroidism is generally characterized by activating mutations in the TSH-R or any of the components in the downstream proliferative cyclic AMP (cAMP) cascade (reviewed by Duprez et al. 1998; reviewed by Yen 2000). Of environmental

factors, excess iodine is capable of inducing hyperactivity in autonomously functioning thyrocytes e.g. within a nodule (reviewed by Roti and Uberti 2001).

Due to the wide-scale effects of the thyroid hormones their deficiency or excess will lead to systemic symptoms affecting the functionality of most organs. In newborns and children hypothyroidism causes mental and growth retardation, and nervous system abnormalities (reviewed by Gruters et al. 2003). In adult patients it causes, in addition to frequent goiter, e.g. lower cardiac output and respiratory rate, skin conditions, gastrointestinal dysfunction, slower renal filtration rate, anemia, neuromuscular complications and depression (Holdsworth and Besser 1968; Das et al. 1975; Duyff et al. 2000; reviewed by Yen 2001; reviewed by Guha et al. 2002; Almeida et al. 2007; reviewed by Klein and Danzi 2007; Artantas et al. 2009; reviewed by Iglesias and Diez 2009). In hyperthyroidism the symptoms are quite the opposite. The most evident are weight loss and sensitivity to heat due to increased metabolism, muscular and neuronal dysfunction such as weakness and numbness, accelerated gastrointestinal and renal function, heart arrhythmias and palpitations, and various psychiatric conditions such as restlessness and depression (Wegener et al. 1992; Duyff et al. 2000; reviewed by Yen 2001; reviewed by Silva 2003; reviewed by Klein and Danzi 2007; reviewed by Peiris et al. 2007; reviewed by Iglesias and Diez 2009). Additionally, goiter is a frequent finding especially in the presence of stimulating autoantibodies but is also present as hyperplastic nodules (reviewed by Topliss and Eastman 2004).

1.2.5.2. Thyroiditis

Thyroiditis comprises several inflammatory syndromes affecting the thyroid gland, and they can broadly be divided into painless and painful disorders (reviewed by Bindra and Braunstein 2006). Painless diseases are Hashimoto's, postpartum, drug-induced and Riedel's fibrotic thyroiditis which generally have an autoimmune origin. Hashimoto's thyroiditis is the most common form of thyroiditis, and like the other autoimmune thyroid disorders it is characterized by a high titer of antibodies mainly for TPO and Tg and infiltration of immune cells (reviewed by Pearce et al. 2003). Autoimmune types have a complex multifactorial origin including genetic and environmental factors (reviewed by Dayan and Daniels 1996; reviewed by Burek and Rose 2008). On the other hand, thyroiditis resulting from a microbial infection, tissue trauma or exposure to radiation often causes pain in the thyroid gland. Painful thyroiditis types are rather rare since the thyroid gland is well-protected from exogenous hazards (reviewed by Bindra and Braunstein 2006). As with

many other thyroid diseases, women are more susceptible to the autoimmune disease but the painful forms are equally presented in both sexes (reviewed by Pearce et al. 2003).

Common findings in the various types of thyroiditis are hypo- or hyperthyroidism, or possibly both. The inflammation or other destructive events cause a release of T3 and T4 from the gland and thus promotes hyperthyroidism until the stores are depleted and the patient becomes hypothyroid (reviewed by Pearce et al. 2003). Thyroiditis may destroy the thyroid gland permanently and eventually induce hypothyroidism for which suitable T4 administration is needed (reviewed by Pearce et al. 2003). Goiter is also involved in most types of thyroiditis with elevated TSH levels (reviewed by Bindra and Braunstein 2006). Long-term inflammation associated with e.g. Hashimoto's thyroiditis may predispose the patients to development of thyroid lymphomas (Holm et al. 1985; Kato et al. 1985).

Inflammatory reaction increases the oxidative stress in the tissues, and the main sources of ROS are dysfunctional mitochondria and the phagocytic immune cells. Elevated ROS has also been associated with thyroiditis, and may participate in the exacerbation of the inflammation by promoting ICAM expression and subsequent leukocyte infiltration (Sharma et al. 2008). Another factor increasing the ROS load of thyroid is iodine which is known to enhance thyroid hormone synthesis and thus H_2O_2 production. Excess dietary or drug-derived iodine can therefore induce thyroiditis and subsequent hypothyroidism in genetically susceptible persons (reviewed by Burek and Rose 2008).

1.2.5.3. Goiter

The most evident symptom in many hypo- and hyperthyroidism conditions is an enlarged thyroid gland, or goiter. They are present in multiple forms and differ in their function regarding the hormone generation and secretion. According to the appearance goiters can be classified into diffuse or nodular forms. Diffuse goiters are evenly enlarged producing a clearly visible swelling in front of the neck whereas nodular goiters have one or more nodules present in one or both lobes of the gland. Functionally goiters are broadly classified into toxic and nontoxic goiters. Toxic goiters produce excessive amounts of the thyroid hormones, and thus expose the patient to hyperthyroid state. Nontoxic goiters are characterized by normal or low thyroid hormone production instead.

Goiter is the most common thyroid disorder in populations all over the world. More than 1.5 billion people live in iodine-deficient areas, including many European regions, and are therefore at risk of developing the disorder (Delange 1995). Dietary iodine deficiency presents the most eminent cause for

the disorder worldwide (Delange 1994). Other dietary factors include certain goitrogenic plants, cobalt salt and excess iodine (reviewed by Dobyns 1969; Delange 1994). However, genetic factors seem to have a major role in predisposition to goiter in females and environmental factors have been suggested as the final switch determining the formation of goiter (Brix et al. 1999). The genetic factors include a number of mutated enzymes and other proteins in the thyroid hormone generating system such as Tg (Ieiri et al. 1991; Caputo et al. 2007), NIS (reviewed by Pohlenz and Refetoff 1999), pendrin (Everett et al. 1997; Coyle et al. 1998), TPO (Kotani et al. 1999; Santos et al. 1999), and Duox (Varela et al. 2006) which impair the normal hormonogenesis thus increasing TSH. Gender and age have been found to correlate with the development of goiter and further malignancies so that women and elderly people are at higher risk (Giles Senyurek et al. 2008; Morganti et al. 2005). Additionally, smoking and pregnancy appear to contribute to goiter if there is underlying iodine deficiency since thiocyanate in cigarette smoke inhibits normal iodine transport and organification, and the thyroid function changes during gestation increasing the need for iodine (reviewed by Glinoer 1997; Colzani et al. 1998; Knudsen et al. 2002). Additionally, microbial infections and thyroiditis may induce abnormal growth of the gland but this is rather rare (reviewed by Hamburger 1986; reviewed by Desailloud and Hober 2009).

Thyroid growth is regulated by the hypothalamic-hypophysial-thyroid axis which secretes the hormones thyrotropin releasing hormone and TSH, and elaborate negative feedback signaling system which keeps the thyroid hormone levels within narrow limits (reviewed by Chiamolera and Wondisford 2009). The ultimate cause of goiter differs in different thyroid disorders (reviewed by Dumont et al. 1992). In congenitally hypothyroid patients, defects in thyroid hormone production and I- metabolism remove the negative feedback effect of T3 on hypothalamus and pituitary and, thus, promote the release of thyrotropin releasing hormone and TSH which stimulate thyrocyte proliferation. Similar T3 downregulation is caused by antibodies against TPO or TSH-R in Hashimoto's thyroiditis. Additionally, thyroidal I- depletion removes the negative effect of I- on proliferation and sensitizes the gland for the stimulatory effect of TSH. The origin of sporadic goiter, i.e. occurring without evident congenital factors, remains elusive but iodine deficiency and genetic defects have been suggested (Agerbaek 1974; Brix et al. 1999). Finally, goiter associated with Grave's disease, the most common cause for hyperthyroidism, is induced by stimulating antibodies for the TSH-R which activates the proliferative pathway.

Goitrous thyroid gland with elevated T4 and T3 levels is usually subject to increased oxidative stress (Sugawara et al. 1988; Poncin et al. 2008a; Rybus-Kalinowska et al. 2008). However, in goiter patients with normal levels of thyroid hormones the oxidative stress does not seem to be elevated, unlike in cancerous thyroid tissue where lipid peroxidation indicating such stress was significantly higher (Sadani and Nadkarni 1996). Oxidative stress attracts leukocytes which respond to the inflammatory stimuli from thyroid tissue during gland involution but, interestingly, a non-growing goiter does not harbor inflammatory cells (Poncin et al. 2008a). Furthermore, thyrocytes inhibit their own functions by secreting the major inflammatory cytokines IL-1, IFN-γ and TNF-α and, thus, reducing their own hormone generation, growth and proliferation (Rasmussen et al. 1993; Tang et al. 1995; Gerard et al. 2006; Yu et al. 2006; Poncin et al. 2008b; Makay et al. 2009).

1.2.5.4. Toxic adenoma and thyroid cancers

Toxic thyroid adenomas are commonly benign, capsulated, and slow-growing tumors capable of autonomous thyroid hormone generation at variable levels (reviewed by Dumont et al. 1992; reviewed by Duprez et al. 1998). They present usually as single nodular neoplasia or goiter and are "hot" referring to their capability of metabolizing iodine as verified in radiographs after infusion of radioactive iodine isotope (reviewed by Dumont et al. 1992). As most thyroid diseases it is more prevalent in women and elderly people, especially in areas where dietary iodine is scarce (Giles Senyurek et al. 2008). Autonomously functioning adenomas suppress TSH production from the pituitary and therefore the normal thyroid tissue appears quiescent and atrophied (reviewed by Dumont et al. 1992; reviewed by Reid and Wheeler 2005). The suppressed thyroid tissue may harbor "cold" nodules incapable of iodine metabolism but with increased risk for later malignant transformation (Giles Senyurek et al. 2008). If the adenoma is large enough and the iodine supply is sufficient it may cause hyperthyroidism and thyrotoxicosis with the symptoms described above (Ermans and Camus 1972).

The origin of toxic adenomas has been under thorough investigation. The formation of monoclonal nodules is characteristic of toxic adenomas, and they arise commonly from various somatic mutations (Hicks et al. 1990; Namba et al. 1990a). Several studies have revealed mutations in the TSH-R which render it constitutively active and thus continuously stimulate thyrocyte proliferation (reviewed by Duprez et al. 1998; reviewed by Polak 1999). Some studies also suggest the activation of one of the TSH-R regulated G-proteins, Gαs, but it is likely to have lesser impact on the prevalence of toxic adenoma (Lyons et al. 1990; Parma et al. 1997; Trulzsch et al. 2001). Additionally, Ras oncogene

mutations have been indicated in some adenomas (Namba et al. 1990b). Ultimately, activation of either TSH-R or Gαs leads to increase in cytosolic cAMP which controls the signaling cascade promoting thyrocyte proliferation (reviewed by Vassart and Dumont 1992; Corvilain et al. 1994). It has been suggested that low-iodine induced initial increase in circulating TSH would select a subset of thyrocytes more sensitive to TSH stimulation due to the existing activating mutations (Laurberg et al. 1991; reviewed by Porcellini et al. 1997b; Trulzsch et al. 2001). This would also account for the high incidence of toxic adenomas in areas where iodine is scarce (Laurberg et al. 1991).

Several types of cancer are prevalent in the thyroid gland. They are broadly divided into papillary, follicular, medullary, and anaplastic carcinomas by appearance in histology. Papillary carcinoma (PTC) is the most prevalent (70%) followed by follicular (17,3%), medullary (3,2%), and anaplastic (2,3%) types (Correa and Chen 1995) and, interestingly, PTC incidence has grown rapidly over the past three decades as the others have stayed at the same level (Davies and Welch 2006). Nevertheless, typically only 5% of all thyroid nodule cases prove malignant (reviewed by Riesco-Eizaguirre and Santisteban 2007). The less aggressive forms, PTC and follicular carcinoma, have higher incidence in women but the more aggressive ones have nearly equal distribution among the sexes (Correa and Chen 1995). PTC is the least aggressive form with only rare occasions of metastasis and the patients have a very high survival rate whereas 40% of anaplastic thyroid carcinoma patients have metastasis with very poor survival rate (Correa and Chen 1995). Approximately 5-10% of PTC patients have familial predisposition (reviewed by Kouniavsky and Zeiger 2009).

Most thyroid cancers originate from single cells harboring mutations in the critical growth regulating genes. Characteristically, in thyroid tissue the misregulated genes are involved in the TSH-R downstream pathways and induce abnormal proliferation (reviewed by Rivas and Santisteban 2003). Among them are many growth and apoptosis regulating genes commonly mutated in other cancers such as Ras small GTPases, phosphoinositide 3-kinase (PI3K) and various protein kinases (reviewed by Rivas and Santisteban 2003). There are also a number of thyroid specific genes, e.g. NIS, TPO and pendrin, whose strong downregulation upon malignant transformation cause deficiencies in the most important thyroid functions, namely iodide uptake and organification (reviewed by Schlumberger et al. 2007). Therefore, malignant thyroid nodules are often “cold” and discharge radioiodine which renders them resistant to this therapeutic intervention (reviewed by Schlumberger et al. 2007). However, in addition to various genetic mutations and chromosomal

rearrangements, numerous other mechanisms have been associated with thyroid cancer initiation such as epigenetic silencing, alternative mRNA splicing and even microRNAs (reviewed by Kouniavsky and Zeiger 2009).

1.3. Signaling pathways in cell proliferation

1.3.1. Ras and PI3K mitogenic pathways

The Ras small GTPase is utilized by most receptor tyrosine kinases (RTK) to mediate the signal from cell exterior to extracellular signal-regulated kinases (Erk) which are the principal mitogenic regulators in many cell types (Figure 5). In a multitude of studies Ras has been shown to be activated by such factors as oxidative stress and H_2O_2, NO, growth hormones, the inflammatory cytokines TNF-α and IL-1, and growth factors (Lander et al. 1995; Lander et al. 1996; Aikawa et al. 1997; Auer et al. 1998; Page et al. 1999; Matsumoto et al. 1999; Palsson et al. 2000; Caraglia et al. 2003). Several receptors, such as G protein coupled receptors (GPCR) and tyrosine kinase receptors (RTK) activate Ras. The tyrosine kinase receptor activation and dimerization induces its autophosphorylation and binding of various enzymes, adapter proteins, and docking proteins which facilitate the propagation of sequential phosphorylations in the Ras downstream cascade (reviewed by McKay and Morrison 2007).

The classical Ras activation route initiates as inactive Grb2/Sos guanine nucleotide exchange factor (GEF) complex is recruited from cytosol to the phosphorylated receptor or associated docking proteins (Figure 5). Moreover, Ras activation occurs through different protein assemblies depending on the intracellular location e.g. Golgi, endoplasmic reticulum, or endosomes (Burke et al. 2001; Chiu et al. 2002; Jiang and Sorkin 2002). The GEF enzymes catalyze the GDP>GTP exchange thus activating Ras. Next, GTPase activating proteins (GAP) induce the Ras GTPase activity and GTP>GDP hydrolysis which enables the signal propagation forward from active Ras. They are in turn regulated by e.g. intracellular Ca2+ released on receptor activation or phosphatidyl-inositols (Lockyer et al. 1999; Walker et al. 2004; Kupzig et al. 2005).

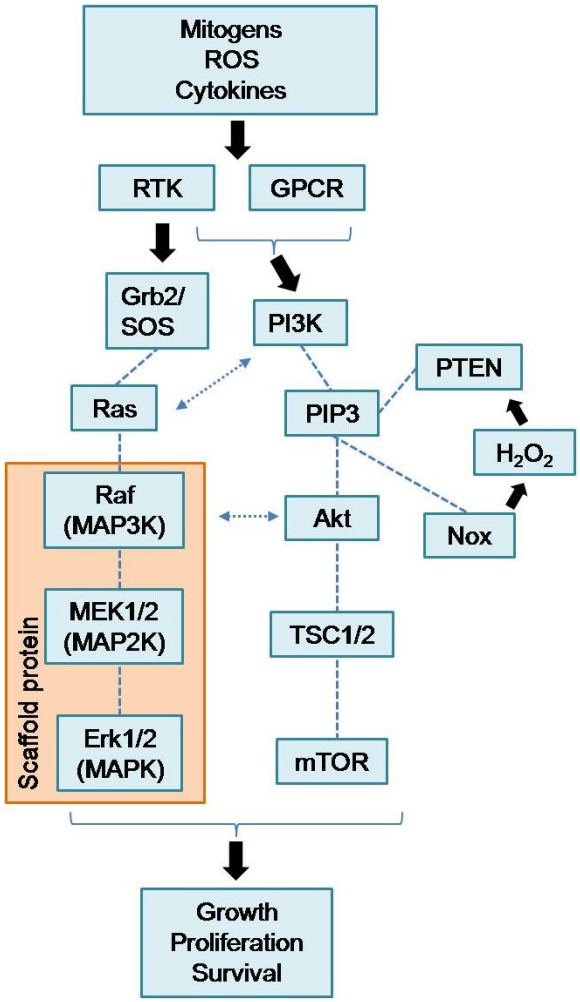

Figure 5. Ras-Erk1/2 and PI3K/Akt pathways. Various external factors can activate RTKs and GPCRs. Stimulated RTK (left) binds Grb2/SOS complex which induces Ras GTP binding. Ras initiates a phosphorylation cascade on Raf, MEK1/2 and Erk1/2 that ultimately results in gene expression and various cellular responses. A number of scaffold proteins bind some or all of the three kinases thus conferring specificity to the signaling event. Both RTKs and GPCRs (right) can induce PI3K activation and PIP3 second messenger generation which results in mTOR activation through Akt and TSC1/2, and changes in cell function. H_2O_2 derived from the Nox system inactivates PTEN, an inhibitor of PIP3 signal transduction, thus promoting signal intensity and duration. Additionally, the two pathways may interact at several points (blue arrows).

One of the most prominent and extensively studied signaling networks downstream of Ras is the extracellular signal regulated kinase (Erk) 1/2 MAPK pathway (Figure 5). Two other MAPK families include JNK 1, 2 and 3, and p38 α, β, γ and δ isoforms (reviewed by Chen et al. 2001; reviewed by Raman et al. 2007). The MAPKs lay at the end of a phosphorelay cascade starting from MAP kinase kinase kinases (MAP3K) such as MEKKs which phosphorylate their specific MAP kinase kinases (MAP2K), the MEKs. The multiple functions of the MAPK pathways are enabled by highly coordinated regulation of the numerous MAP3Ks and MAP2Ks, and by direct regulation of the MAPKs themselves. Erk1/2 are in general contributing to e.g. mitogenesis and cell division, differentiation, motility, and survival, while JNK and p38 are often activated by various stress factors and lead to cytokine production, inflammatory response, transformation, apoptosis, and necrosis (reviewed by Davis 2000; reviewed by Chen et al. 2001; Zarubin and Han 2005).

All Ras family members can bind and activate directly the Raf MAP3 serine/threonine kinases which are the principal MAP3 kinases on the Erk pathway (Figure 5). The intricate multistep process of assembling the functional Raf complex has been reviewed by McCubrey et al. 2006 and McKay and Morrison 2007. Ras induced heterodimerization of two Raf isoforms seems to be essential for their full activation and subsequent MEK phosphorylation (Weber et al. 2001; Garnett et al. 2005; Rushworth et al. 2006). Activated Raf and its downstream kinases MEK and Erk bind various scaffold proteins that regulate their interaction with other cascades and determine their subcellular localization, e.g. kinase suppressor of Ras (KSR) targets the complex to plasma membrane whereas paxillin is an integral part of focal adhesions (reviewed by Kolch 2005; Figure 5). In general, scaffold proteins are main determinants where in the cell the pathway is activated, and thus on the set of target genes expressed leading to specific changes in cellular properties. The scaffold proteins also enhance signal transduction as they bring the kinases in close contact and increase the signal duration (reviewed by Kolch 2005; reviewed by Dhanasekaran et al. 2007).

In contrast to the complex activation process of Raf, MEK and Erk activation by Raf and MEK, respectively, is a simple phosphorylation event of serine/threonine and tyrosine residues in their kinase domains (Payne et al. 1991; Zheng and Guan 1994). The MEK dual specificity kinase family consists of at least seven members named MEK1 through MEK7. MEK1 and 2 have specificity for Erk1/2, MEK2 being the most potent activator and MEK1 having a more modest effect (Zheng and Guan 1993). The other MEKs are associated with the activation of p38 and JNK in stress responses (reviewed by Kyriakis and Avruch 2001; Raman et al. 2007). In addition to Erk1/2 phosphorylation, MEK1/2 has been suggested to regulate nuclear localization of both active and inactive Erk1/2. The nuclear export signal (NES) on MEK1/2 and a similar sequence in Erk2 retain the MEK/inactive Erk complex in the cytosol until the upstream pathway is stimulated by mitogens leading to MEK-dependent Erk activation (Jaaro et al. 1997; Fukuda et al. 1997; Rubinfeld et al. 1999). In the nucleus MEK/Erk complex then dissociates, and MEK returns quickly back to cytosol driven by the NES. However, there is also evidence for constant unregulated MEK traffic in and out of the nucleus coexisting with the regulated traffic (Yao et al. 2001).

Active MEK1/2 phosphorylates conserved tyrosine and threonine residues on Erk1/2 inducing its full activation, dimerization and nuclear translocation (Canagarajah et al. 1997; Khokhlatchev et al. 1998; reviewed by Cobb and Goldsmith 2000; reviewed by Pearson et al. 2001). Active Erk1/2 generally

dissociates from the MEK1/2 and the scaffold, and is then either diffused freely or transported to the target site by an unknown mechanism (Gonzalez et al. 1993; Lenormand et al. 1998; Rubinfeld et al. 1999). The Erk binding to the target is mediated by binding motifs, two very common being the docking (D)-domain and docking site for Erk and FXFP (DEF)-domain (Biondi and Nebreda 2003; reviewed by Zhang and Dong 2007). Duration of the signal is crucial in determining the outcome of Erk1/2 signaling (Marshall 1995), and in addition to the persistence of the original stimulus, Erk1/2 itself regulates its own activity. For instance, a positive feedback loop to enhance Raf activation (Balan et al. 2006), and inactivation of specific phosphatases (Marchetti et al. 2005) are generally utilized methods. However, Erk can also exert negative feedback by phosphorylating many of its upstream Ras/MAPK cascade components, including MEK1/2 (Eblen et al. 2004), Raf (Dougherty et al. 2005), and SOS GEF (Corbalan-Garcia et al. 1996) or the nuclear MAPK phosphatase (MKP)1 (Brondello et al. 1997), thus inhibiting them, and finally terminating the original signal.

Erk1 and 2 are expressed ubiquitously in all cell types and throughout the cell. They are associated with a number of plasmamembrane receptors for growth factors, cytokines, and GPCR ligands, as well as integrins (Ly and Corbett 2005; reviewed by Raman et al. 2007; reviewed by Goldsmith and Dhanasekaran 2007). Erk1/2 may also regulate membrane transporters for Na+, K+, and glucose (Michlig et al. 2004; Izawa et al. 2005; Lin and Chai 2008) reflecting the wide range of cellular activities in which they participate. Interestingly, Erk1/2 substrate specificity and, thus, its modulation of the target proteins in the nucleus or in cytosol is regulated by the Ras subcellular localization and is mediated by the specific scaffold proteins to which it anchors along with Raf and MEK (Casar et al. 2009). Furthermore, substantial amounts of Erk1/2 is bound to microtubules, adherens junctions and focal adhesion sites through paxillin underlining their importance in cell motility and cell-cell and cell-matrix contacts (Reszka et al. 1995; Ishibe et al. 2004). Erk1/2 has also been found in various mitotic structures associating it mechanistically with cell division and cell cycle control (Wang et al. 1997; Guadagno and Ferrell 1998; Shapiro et al. 1998; Zecevic et al. 1998).

Activated Erk1/2 can induce phosphorylation of over 100 target proteins distributed in the cytosol and the nucleus (Yoon and Seger 2006). Nuclear targets of Erk1/2 are mostly transcription factors whose activity or expression or both are mediated by Erk1/2. For instance, the transcription factor AP-1 subunit c-Fos expression is induced by transcription factor Elk-1, and its activation is directly controlled by Erk1/2 phosphorylation (Whitmarsh et al.

1995; Chen et al. 1996; Hodge et al. 1998). Other important Erk1/2 regulated transcription factors are c-Myc and cAMP responsive element binding protein (CREB) resulting in the expression of growth factors, cell cycle regulators, cytokines, and apoptotic factors (Arany et al. 2005; reviewed by Meloche and Pouyssegur 2007). Outside nucleus Erk1/2 regulated targets include e.g. cytoplasmic phosphatases, other kinases and cytoskeletal proteins; at the plasmamembrane it controls proteins involved in survival, cell cycle progression, cell adhesion and migration (Aikawa et al. 1997; reviewed by Pearson et al. 2001; reviewed by Meloche and Pouyssegur 2007; reviewed by Ramos 2008; Figure 5).

The PI3K/Akt pathway carries signals from both RTK and GPCR after stimulation by e.g. EGF, PDGF and FGF, and is also induced by oxidative stress (Shaw et al. 1998; van Weering et al. 1998). Phosphoinositide 3-kinases (PI3K) belong to a family of lipid kinases which phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3), the active signal transducing second messenger. PIP3 signaling then activates the Nox1 or Nox4 enzymes and subsequent H_2O_2 generation by inducing Rac small GTPase phosphorylation (Bae et al. 2000; Park et al. 2004). H_2O_2 is needed for oxidative inactivation of PTEN which otherwise dephosphorylates PIP3 disabling the signaling cascade to Akt (Leslie et al. 2003; reviewed by Rhee et al. 2005b; Figure 5). Akt (aka protein kinase B, PKB), is a downstream effector of PI3K activated by binding to PIP3 and by following phosphorylations by PDK1 and PDK2 of which the latter was recently identified as mammalian target of rapamycin (mTOR) complex 2 (reviewed by Scheid and Woodgett 2003; reviewed by Yang and Guan 2007).

Akt activity is a crucially important survival signal in normal and stressed cells and has numerous downstream binding partners (reviewed by Brazil et al. 2002). A direct target of Akt is tuberous sclerosis complex (TSC) 1/2 which is inactivated on phosphorylation, and thus releases the mTOR complex 1 from inhibition allowing e.g. activation of p70 ribosomal protein S6 kinase 1 (S6K1) and inhibition of eukaryotic initiation factor 4E binding protein (4EBP) 1, thus contributing to translation initiation (reviewed by Yang and Guan 2007; reviewed by Huang and Manning 2009; Figure 5). Another Akt target is the glycogen synthase kinase (GSK) 3 which accounts for the regulation of e.g. glucose metabolism, the transcription factors AP-1 and CREB, and is involved in development (Cross et al. 1995; reviewed by Weston and Davis 2001). A feedback loop inhibits stimulus-activated PI3K/Akt cascade as mTOR/S6K1 inactivates the insulin receptor substrate (IRS) 1 which transfers the signal from RTK to PI3K (Zhang et al. 2003; Harrington et al. 2004; Shah et al.

2004). The mTOR complex 1 acts as a key integrator of growth signals as it is regulated, in addition to growth factors, by the growth promoting Wnt signaling, and the cellular energy levels (Inoki et al. 2003; Inoki et al. 2006).

There is established mutual modulation between the Ras downstream MAPK and PI3K/Akt pathways which confers them partially overlapping functions, e.g. in cell survival, but also result in differential cellular outcomes (Figure 5). Ras and PI3K have been shown to interact directly by binding each other which is essential for normal development as well as tumorigenesis (Rodriguez-Viciana et al. 2004; Gupta et al. 2007). Previously, it has been known that Akt activation inhibits Raf activation by direct phosphorylation and promotes vascular smooth muscle cell differentiation (Rommel et al. 1999). Recently, it was found out that constitutively active Raf can in turn inhibit Akt through MEK1 and thus induce growth arrest in an *in vitro* setting (Menges and McCance 2008). Both MAPK and Akt pathways have been shown to regulate TSC1/2 and subsequent mTOR complex 1 activation (Shaw and Cantley 2006; Ma et al. 2005), and thus contribute to e.g. proliferation, tumorigenesis, and senescence. Further downstream, the mTOR complex 1 inhibition by rapamycin upregulates the Erk1/2 pathway through a feedback loop involving the S6K1 and PI3K (Carracedo et al. 2008) implying that the Akt and MAPK pathways both contribute to cell survival (Figure 5).

1.3.2. TSH receptor pathway

Thyroid gland function is dependent on regulation by TSH which stimulates it to produce the required amounts of T3 and T4 and secrete them into the blood stream when necessary. The TSH signal is received and conveyed by the TSH receptor (TSH-R) on the follicular cells, and it is considered the most important receptor coordinating thyroid growth and function (Vassart and Dumont 1992; Szkudlinski et al. 2002). Also autoimmune antibodies may either stimulate or inhibit the receptor. The downstream pathway will then launch the hormone synthesis, secretion and follicular cell proliferation in a highly controlled manner to avoid excessive exposure of the organism to the thyroid hormones (Figure 6).

In addition to TSH, insulin and IGF-1 as well as other growth factors such as EGF, FGF and HGF are important inducers of thyrocyte proliferation (Medina and Santisteban 2000; reviewed by Kimura et al. 2001; Figure 6). There is large variance in responses to these growth factors between different cell lines and animal species; for instance, it has been suggested that TSH merely enhances the insulin/IGF-1 derived stimulation of DNA synthesis in rat PC clone 3 cell line whereas in human thyrocytes TSH/cAMP is the principal inducer (reviewed by Kimura et al. 2001). However, it appears that both TSH

and insulin/IGF-1 are required for thyrocyte growth *in vivo* since growth hormone treatment leading to normalized IGF level of patients with deficiency in both factors did not induce thyroid growth (Cheung et al. 1996). The requirement for both factors has been seen also *in vitro* according to several studies with human, rodent and dog thyroid cell lines (Tramontano et al. 1988; Takahashi et al. 1990). Additionally, TSH has been suggested to promote thyrocyte growth by inducing autocrine growth factors and growth factor receptor expression (Takahashi et al. 1990; Becks et al. 1994; Burikhanov et al. 1996; Cocks et al. 2000).

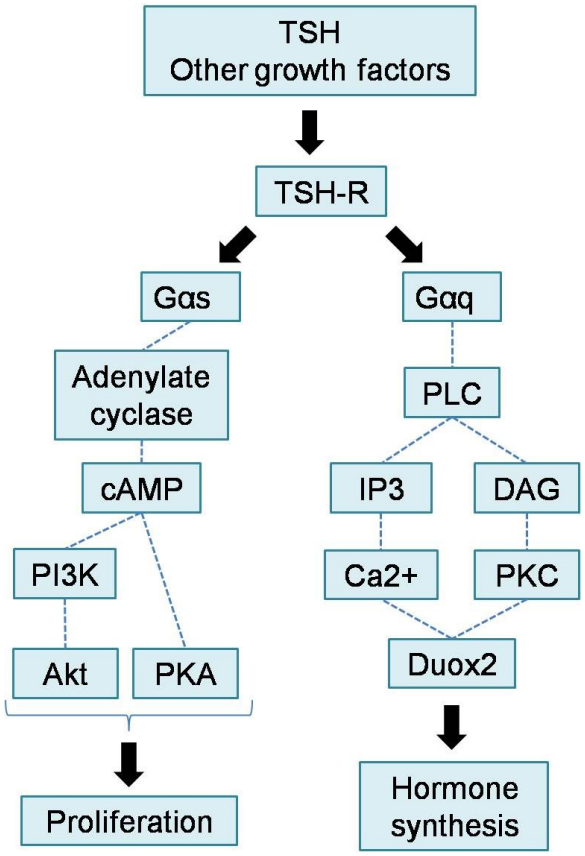

Figure 6. The TSH receptor pathways. TSH-R stimulation by various growth factors activates the α-subunits of Gs and Gq proteins. Gαs induces the cAMP dependent pathway leading to thyrocyte proliferation mainly through PI3K regulated Ras and Akt. Gαq mediates the activation of PLC dependent pathway which promotes Duox2 activity by Ca2+ release and PKC-induced phosphorylation, and thus contributes to thyroid hormone synthesis.

The TSH-R is a G-protein coupled membrane-spanning receptor located on the basolateral surface of thyrocytes (Parmentier et al. 1989; Nagayama et al. 1989; Loosfelt et al. 1992). Its post-translational modifications include early glycosylation and cleavage after insertion into the plasmamembrane (Misrahi et al. 1994). The cleavage process takes place only when the cells have established cell-cell contacts (Hai et al. 2008) and are properly glycosylated (Misrahi et al. 1994) to ensure that the hormonogenesis occurs only in mature follicles. The protease responsible for the cleavage has not been determined

but, for instance, the matrix metalloprotease family member ADAM10 has been suggested (Kaczur et al. 2007). Moreover, the receptor is internalized upon ligand binding and recycled back to the plasma membrane as the hormone ligand is degraded in lysosomes (Baratti-Elbaz et al. 1999).

The cleavage results in formation of two subunits named A and B which remain linked to each other by disulfide bridges (Loosfelt et al. 1992). The extracellular subunit A binds the TSH whereas the membrane-spanning subunit B is the candidate to interact with two G-proteins, Gs and Gq/11 (Nagayama et al. 1991; Allgeier et al. 1994). The G-proteins initiate separate downstream pathways referred to as the second messenger cAMP or phospholipase C (PLC) dependent pathway (Figure 6). Specific roles in thyroid hormone secretion and thyrocyte growth, or Tg iodination and hormone synthesis have been suggested for cAMP or PLC pathways, respectively (Corvilain et al. 1994). The decision on which pathway is activated appears to depend at least partly on TSH concentration so that low TSH can stimulate cAMP pathway but higher concentration is needed to induce also PLC pathway activation (Laurent et al. 1987; Allgeier et al. 1994; Corvilain et al. 1994).

In thyrocytes and other endocrine cells cAMP is a mitogenic inducer (reviewed by Iyengar 1996), and thus results in thyrocyte proliferation, thyroid specific gene expression and also I- organification. Additionally, it is required for the secretion of the hormone in response to TSH (Corvilain et al. 1994) implying that the cAMP and PLC pathways have partly overlapping roles. The early steps in the cAMP pathway follow the classical G-protein coupled signal transduction pathway in which the Gαs subunit mediates the TSH signal from the receptor to adenylate cyclase which then initiates a rapid intracellular cAMP production (Figure 6). The role of Gβγ subunit remains elusive in the thyroid follicular cell physiology. This branch of the pathway seems to be independent of the TSH-R cleavage (Hai et al. 2008). The main downstream effector kinases activated by cAMP are protein kinase A (PKA), and PI3K which induce further downstream signaling cascades (Cass et al. 1999; Figure 6) with the help of several modulators also activated by cAMP.

The regulation of PKA and PI3K is closely interconnected. Direct small G-protein Rap1 activation by cAMP induces a weak PI3K response which is markedly enhanced by the exchange protein activated by cAMP (Epac), and PKA-mediated Rap1 phosphorylation as shown by increased phospho-Akt formation and cell cycle progression (Tsygankova et al. 2001; Hochbaum et al. 2008). Simultaneously, Rap1 phosphorylation enables the small GTPase Ras to interact with its downstream target Raf (Hu et al. 1999), further stimulated by cAMP induced indirect Ras activation (Tsygankova et al. 2000). Additionally,

PKA can phosphorylate a PI3K regulatory subunit and promote its binding affinity to Ras (De Gregorio et al. 2007). However, the role of Ras in thyrocyte proliferation is ambiguous, although at least in rat cell lines it seems to be a requirement for cAMP induced proliferation (Ciullo et al. 2001). Studies on cAMP and PKA in a rat thyroid cell line and 3T3 fibroblasts revealed that they inhibit Raf activation (Hafner et al. 1994; reviewed by Burgering and Bos 1995), and therefore it seems that cAMP-induced Ras downstream signaling cascade is actively directed towards other substrates than Raf and MAPK, a strong candidate being guanine nucleotide dissociation stimulator for the Ras-related protein Ral (RalGDS) (al-Alawi et al. 1995; Miller et al. 1997; Miller et al. 1998). This is supported by an earlier finding that MAPK pathway can activate independently of cAMP (Saunier et al. 1995). Another study showed, however, how IGF-induced cAMP activated PI3K and MAPK pathways in parallel (Ariga et al. 2000).

The mitogenic pathway downstream from PKA and PI3K involves a number of transcription factors and signal regulators. The major route for cAMP to regulate mitogenic signaling in thyroid is the cAMP response element binding proteins (CREB) and modulators (CREM) which function as PKA-activated transcription factors for genes that contain the CRE in their promoters or enhancer regions (Woloshin et al. 1992; Uyttersprot et al. 1999). For instance, dominant negative CREB transgene caused marked growth retardation and hypothyroidism in mice, and the genes for TSH-R, TPO, Tg, and the thyroid specific transcription factors Pax-8, TTF-1 and TTF-2 were downregulated (Nguyen et al. 2000) causing deficient iodide metabolism and lack of thyroid hormones. Other PKA regulated factors are RalGDS and ribosomal protein p70s6 kinase (S6K) 1 which have a prominent role in DNA synthesis and mitogenesis (Miller et al. 1997; McManus and Alessi 2002).

Regulation of thyrocyte proliferation through PI3K is a complex network where different original stimuli can induce different downstream cascades. IGF-I and cAMP have been shown to regulate the cell cycle progression from G1 to S phase through distinct effects on the various PI3K-activated cyclins, cyclin dependent kinases and their inhibitors (Fukushima et al. 2008). In general, PI3K appears to be the main transducer of proliferative growth factor signals in thyrocytes (Coulonval et al. 2000; Saito et al. 2001). Its immediate downstream effector is Akt/PKB, and recently the Akt regulated mTOR/S6K1 pathway was shown to be a prominent route in thyroid follicular cell proliferation regulating e.g. the translation of cyclins D1 and D3 (Yeager et al. 2008). S6K1 activation by PI3K/Akt is apparently independent of PKA (Cass et al. 1999).

The second major pathway activated upon TSH-R stimulation relies on the Gq/11 protein, and results in the PLC-β pathway initiation, thyroid hormone synthesis, and also in enhancement of thyrocyte proliferation (Figure 6). In *in vitro* studies of human thyroid, TSH can activate the PLC pathway independently of the Gs/cAMP pathway but requires higher TSH concentration, and is a slower reaction (Laurent et al. 1987; Allgeier et al. 1994; Corvilain et al. 1994). The established classical pathway from GPCR to PLC applies also in the thyroid gland, and thus activated Gαq subunit directly stimulates PLC which then cleaves the phosphatidyl inositol anchored to the plasmamembrane and produces the two second messengers, inositol phosphates and DAG (reviewed by Ginsberg 1992; Figure 6). The inositol-tris-phosphate (IP3) then induces the release of Ca2+ from the endoplasmic reticulum to the cytosol, and DAG in turn activates the protein kinase C (PKC) (reviewed by Ginsberg 1992).

Production of IP3 and subsequent increase in intracellular Ca2+ concentration in response to TSH and various Ca2+ releasing chemicals in experimental settings have been found essential for H_2O_2 generation in thyroid hormonogenesis (Deme et al. 1985; Lippes and Spaulding 1986; Raspe et al. 1991; Figure 6). An important modulator of intracellular Ca2+ content is H_2O_2 itself which inhibits further Ca2+ release and thus protects the cell from excessive H_2O_2 production (Tornquist et al. 2000). Additionally, the phospholipase A2 and its product arachidonate have been suggested to act downstream of the PLC/Ca2+ pathway and promote H_2O_2 generation (Kimura et al. 1995). In addition to Ca2+ mobilization, the adenylyl cyclase function and cAMP generation need to be downregulated to avoid mitogenic activity during hormone synthesis, although a certain level of cAMP seems necessary for the process (Corvilain et al. 1994). Sphingosine 1-phosphate is a putative second messenger in a number of signaling pathways (reviewed by Kihara et al. 2007), and in thyroid it appears to enhance Ca2+ release and suppress adenylyl cyclase activity through different G proteins (Okajima et al. 1997). Once in the cytosol, the Ca2+ ions will then bind the EF-hand motifs in Duox1 and 2 enzymes as a cofactor, and thus promote H_2O_2 generation (Deme et al. 1985; Ameziane-El-Hassani et al. 2005).

The DAG/PKC branch is likewise regulated by multiple factors in synergy with the cAMP/PKA pathway and has several functions in thyroid physiology (Figure 6). Stimulation of thyroid hormone synthesis by PKC and PKA converge in phospholipase D mediated DAG generation to promote and increase the duration of PKC activation (Gupta et al. 1995; Ginsberg et al. 1997). PKC has recently been shown to be responsible for Duox2

phosphorylation and activation along with bound Ca2+ thus enabling efficient hormone synthesis upon TSH stimulation (Rigutto et al. 2009). Additionally, PKC in association with cAMP has been implicated in protection of the thyroid gland from hazardous autoimmune reactions through enhanced expression of the transcription factor signal transducer and activator of transcription (STAT) 3 which represses the IFN-γ mediated antigen expression (Park et al. 2002; Kim et al. 2003).

Duox 1 and 2 activation and subsequent H_2O_2 generation is the rate-limiting step in the thyroid hormone synthesis (Figure 6). However, in addition to the need for cytosolic Ca2+ and phosphorylation, not much is known about their regulation. They do not depend on the cytosolic regulatory subunits as the Nox enzymes but the respective dual oxidase maturation factors (DuoxA) 1 and 2 are necessary for their proper translocation from ER to the plasmamembrane (Grasberger and Refetoff 2006; Grasberger et al. 2007). A more recent study showed that these factors can also determine whether the Duox generates H_2O_2 or $O_2\bullet^-$ (Morand et al. 2009). Insulin was found to upregulate Duox2 mRNA expression (Morand et al. 2003) but TSH and TSH-R activation, on the other hand, seem to have only a modest effect on the expression level of the Duox enzymes in normal or goitrous thyroid gland implying that variations in the H_2O_2 production level in disease are brought on by modulating the enzyme activity (Milenkovic et al. 2007). Furthermore, whereas Duox2 is the main H_2O_2 producing enzyme in the human thyroid and is activated by the PLC/Ca2+ cascade, Duox1 has a minor role in the normal thyroid and is preferentially stimulated by the cAMP/PKA cascade (Rigutto et al. 2009).

2 AIMS OF THE STUDY

The aim of this study was to characterize the signal transduction routes and the role of the antioxidative SOD3 enzyme in the thyroid gland, as well as its anti-inflammatory and tissue healing promoting features in tissue injuries. Thyroid gland itself is dependent on oxygen radical generation for normal physiological function which renders it an interesting subject for redox biology studies. Ischemia, inflammation, and goiter, on the other hand, are associated with increased oxidative stress which may impede tissue healing and produce tissue damage at molecular level. Signaling networks around SOD3 are poorly known and therefore extensive signaling studies were conducted with the prospect of possible therapeutic intervention. More specifically the aims were:

I. Characterization of the role of SOD3 in tissue recovery from ischemic damage and inflammation.

II. Elucidation of the signaling cascades which are regulated by SOD3 expression and of the molecular mechanisms that mediate its effects in tissue healing and pathogenesis.

III. Characterization of SOD3 expression and function in normal and hyperproliferative thyroid gland.

3 MATERIALS AND METHODS

3.1. Adenovirus production (Manuscript (MS) I&II)

Complementary DNAs (cDNA) of rabbit *sod3* and bacterial *β-galactosidase* were cloned into replication deficient E1-partially-E3-deleted AdBglII virus vector and transfected into 293 cells for AdSOD3 and AdLacZ virus production in University of Turku Biotechnology Center (see manuscript I for references). The titer of the virus stock was $1x10^{10}$ pfu/ml.

3.2. Animal models

All animal experiments were conducted according to the European Union guidelines, and approved by the Experimental Animal Committee of the University of Turku. The animals had water and food ad libitum.

3.2.1. Rat hind limb ischemic injury (MS I&II)

Male Fisher 344 rats (age 4-6 weeks, weight 86-115 g; Harlan, Horst, the Netherlands) were divided randomly into groups and followed for 3, 7, 10 or 14 days after inducing the ischemia. The rats were first anesthetized by intraperitoneal injection of fentanyl-fluanisone (Janssen Pharmaceutica, Beerse, Belgium) and midazolame (Roche, Basel, Switzerland). Acute ischemia was induced by closing surgically proximal femoral artery, lateral circumflex femoral artery and distal femoral artery. The SOD3 or LacZ gene transfer was done immediately after ligation by intramuscular injection of $0.5x10^9$ pfu AdSOD3 or AdLacZ in 50 µl of PBS. Uninjured muscle tissue was collected for additional control.

3.2.2. Mouse peritonitis (MS II)

SOD3 or LacZ gene transfer was done to female Balb/C mice (age 8-10 weeks; local colony) by intraperitoneal (i.p) injection of $0.5x10^9$ pfu AdSOD3 or AdLacZ in 200 µl of PBS. After 72 hours peritoneal inflammation was induced by i.p. injection of 5% proteose peptone (BD Difco, Sparks, MD) in 1 ml of PBS complemented with 10 ng of IL-1β (R&D Systems, Minneapolis, MN). Control mice were injected i.p. with 50 mg/kg Dexamethasone (Oradexon, Organon, Oss, the Netherlands) 30 min before the peritonitis induction. Inflammatory cells were collected from the peritoneal cavity 18 hours after the induction of peritonitis by washing the cavity with 10 ml of RPMI complemented with 5 U/ml heparin (Løvens Kemiske Fabrik, Ballerup, Denmark). The cells in the lavage were counted, centrifuged onto glass slides at 1000 rpm for 5 min (Shandon cytospin 3, Shandon, Pittsburgh, PA), and stained with Reastain Diff-Quick (Reagena, Toivala, Finland) for leukocyte subtype analysis.

3.2.3. Rat PTU-induced goiter (MS III)

Male Sprague-Dawley rats (age 4-5 weeks; local colony) were given 0.25% 6-propyl-2-thiouracil (PTU; Sigma-Aldrich, St. Louis, MO) in deionized drinking water for 2 weeks *ad libitum*. The rats were then sacrificed and tissue samples from thyroid gland, aorta, heart, kidney and liver were collected, weighed, and prepared for histological examination and expression analysis with quantitative real-time PCR.

3.3. Analyses of the *in vivo* experiments

3.3.1. Immunohistochemistry

Muscle samples from rat hind limbs were snap frozen in 2-methylbutane (Riedel-de-Haën, Seelze, Germany) cooled with liquid nitrogen. The samples were then mounted in optimal cutting temperature compound (Tissue-Tek, San Francisco, CA, **MS I**; Sakura Finetek, Torrance, CA, **MS II&III**) and cut into 10 µm sections.

Frozen muscle tissue sections were stained with von Willebrand Factor (vWF; Abcam, Cambridge, UK), Ki67 (DakoCytomation, Glostrup, Denmark), 3-nitrotyrosine (Millipore, Billerica, MA), CD3 (Serotec, Oxford, UK), and CD68 (Serotec, Oxford, UK) antibodies, and counterstained with hematoxylin-eosin (Sigma-Aldrich, St. Louis, MO).

X-Gal (Promega, Madison, WI) staining for 6 h at 37C and hematoxylin-eosin counterstaining (Sigma-Aldrich, St. Louis, MO) were performed to assess the transduction efficiency of the adenoviral gene transfer into muscle tissue. Dihydroethidium bromide staining was performed as described previously (Zanetti et al. 2005) on freshly cut sections to detect the ROS, and the proportion of DHE-positive vs. total nuclei was determined. (**MS I**)

The size of the inflammatory area, i.e. the area with infiltrated CD68+ cells, and number of positively stained CD3 and CD68 cells were determined from total sections with Zeiss Axiovert 200M (Carl Zeiss, Oberkochen, Germany). (**MS II**)

Thyroid glands from PTU treated rats were similarly processed into 10 µm sections and stained with hematoxylin-eosin (Sigma-Aldrich, St. Louis, MO). (**MS III**)

3.3.2. PET imaging (MS I)

In vivo glucose metabolism in the injured hind limbs at 10-day time point was analyzed by positron emission tomography as described (see manuscript I for reference). Frozen muscle tissue samples were cut into sequential 30 µm sections onto glass slides, exposed to imaging plate (Fuji, Tokyo, Japan) and scanned with Fuji Analyzer (Fuji, Tokyo, Japan).

3.4. Cell cultures and *in vitro* experiments

3.4.1. Culture conditions and transfections

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, and 100U/ml penicillin-streptomycin. The thyroid follicular cell line PC Cl3 is derived from Fisher 344 rats, and is maintained in Coon's modified Ham F12 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% calf serum (Life Technologies Inc., Paisley, PA), and a hormone mix (Fusco et al. 1987) including 10 nmol/l thyrotropin (TSH), 10 nmol/l hydrocortisone, 100 nmol/l insulin, 5 µg/ml transferrin, 5 nmol/l somatostatin, and 20 µg/ml glycyl-histidyl-lysine (Sigma-Aldrich, St. Louis, MO). Plasmid transfections were made by Polyfect (Sigma-Aldrich, St. Louis, MO) or FUGENE-6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocols for each reagent.

3.4.2. HEK 293T and PC Cl3 transfections and incubations (MS I&III)

For signaling studies HEK 293T cells were transfected with 5 µg of SOD3, both SOD3 and catalase, Ras, BRaf, MEK1, Erk1, or Akt. Another set of transfected cells was incubated in serum free medium containing 20 µmol/l MEK inhibitor UO126 (Sigma-Aldrich, St. Louis, MO), 20 µmol/l diphenyleneiodonium (DPI; Sigma-Aldrich, St. Louis, MO), 50 µmol/l NADP (Sigma-Aldrich, St. Louis, MO) or 500 µmol/l H2O2 (Sigma-Aldrich, St. Louis, MO) for 6 hours. Samples were then collected for Ras-pulldown, western blotting, luciferase assay, SOD activity measurements, and quantitative real-time PCR.

To assess the effect of TSH-R pathway activation on SOD3 expression PC Cl3 cells were incubated in serum free medium with 1 mU/ml TSH or 40 µmol/l adenylate cyclase inhibitor forskolin (both Sigma-Aldrich, St. Louis, MO), 1 µmol/l PKA inhibitor N(2-((ρ-Bromocinnamyl)amino)ethyl)-5-isoquinoline-sulfonamide (H89) (both Calbiochem, San Diego, CA), or 0.5 µM thapsigargin (Research Biochemicals International, Natick, MA). TSH-R pathway signaling molecules Gαs-protein, Gαq-protein, and PKA in pCEF-expression vector were transfected into PC Cl3 cells using 5 µg of each plasmid. Samples were collected for quantitative real-time PCR and western blotting.

3.4.3. Ras-pulldown assay (MS I)

After transient transfection with 5 µg of SOD3, control vector, or signaling molecules the HEK 293T cells were lysed in the lysis buffer containing 50 mmol/l HEPES pH 7.5, 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 1

mmol/l EGTA 1.5 mmol/l MgCl, 10 mmol/l NaF, 10 mmol/l Na pyrophosphate, 1 mmol/l Na3VO4, 10 µg approtinin/ml, 10 µg leupeptin/ml (Sigma-Aldrich, St. Louis, MO). The supernatants were incubated with the GST-Raf1-RBD beads for 15 min at 4°C. The beads were then washed with the lysis buffer, and prepared for western blot analysis. Active Ras was detected with anti-Ras antibody (Millipore, Billerica, MA). Frozen muscle tissue samples were homogenized in the lysis buffer and treated similarly to determine *in vivo* Ras activation.

3.4.4. Luciferase reporter gene assay (MS I&II)

Luciferase reporter vectors pAP1-Luc, pCRE-Luc, pFA2-cJun-Luc, and pNFκB-Luc (all Stratagene, Cedar Creek, TX) were transfected into HEK 293T cells with SOD3 or control vector. The luciferase bioluminescence was detected with Tecan Ultra XFluor4 Fluorescence Reader (Tekan, Mannedorf, Switzerland).

3.4.5. SiRNA and BrdU labeling (MS III)

PC Cl3 cells were transfected with 20 nmol/l of *sod3* or control *gapdh* OnTargetplus SMART Pool oligos according to the manufacturer's protocol (Dharmacon, Lafayette, CO). Briefly, the oligos were resuspended in 100 µl of transfection buffer, mixed with the same volume of Optimem (Life Technologies, Carlsbad, CA) and incubated at room temperature for 5 minutes. For transfection solution, 5 µl of Dhermofect 4 reagent was added to 190 µl of Optimem and incubated at room temperature for 5 minutes. These two solutions were then combined, incubated at room temperature for 20 minutes and added dropwise to the cells for 48 hours, after which total RNA was isolated.

The 5'bromo-3'deoxyuridine (BrdU) Labeling and Detection Kit I (Boehringer Mannheim, Mannheim, Germany) was used to determine the DNA synthesis rate of the siRNA transfected cells. Briefly, the cells cultured on glass coverslips were starved for 16 hours in serum free medium and then stimulated with TSH for 6 hours after which BrdU (10µM) was added for 2 hours. The cells were then fixed with 3% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) and permeabilized with 0,2% Triton-X100 (Sigma-Aldrich, St. Louis, MO). BrDU was detected by labeling with FITC conjugated antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Lastly, the cell nuclei were labeled with Hoecst (1 µg/ml; Sigma-Aldrich, St. Louis, MO), and mounted on microscopic slides. The minimum of 300 cells were counted on different microscope fields and the percentage of BrdU stained nuclei was calculated.

3.4.6. Calcium uptake assay (MS III)

Intracellular Ca2+ concentration ([Ca2+]) was measured from PC Cl3 cells after thapsigargin treatment. Briefly, the cells were detached from culture plate using PBS/5 mmol/l EDTA, washed with HEPES-buffered medium (HBM; 137 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $CaCl_2$, 0.44 mmol/l KH_2PO_4, 4.2 mmol/l $NaHCO_3$, 10 mmol/l glucose, 20 mmol/l HEPES, and 1.2 mmol/l $MgCl_2$, pH 7.4), and loaded with 2 μmol/l fura-2 acetoxymethyl ester at 37 °C for 20 minutes. Then the cells were diluted with HBM without Ca2+ to final concentration of 0.3 mmol/l CaCl2 and stored at room temperature. Cellular Ca2+ content was monitored with a Hitachi F-2000 fluorescence spectrophotometer at 340 nm (excitation) and 505 nm (emission). For this measurement, an adequate amount of cells were spinned down, washed with and resuspended in HBM, and put in a thermostatted (37ºC) cuvette with magnetic stirring. Calibration was done with 60 μg of digitonin/ml (F_{max}) and 10 mmol/l EGTA (F_{min}). The intracellular [Ca^{2+}] was calculated from the fluorescence (F) using the equation $[Ca^{2+}] = (F-F_{min})/(F_{max}-F) \times 224$ nmol/l (Kd for fura-2), in which the extracellular fura-2 fluorescence was subtracted from F values.

3.4.7. Assays for O_2•- and H_2O_2 detection (MS III)

Cos-7 monkey kidney cells were first cotransfected with Duox2 and DuoxA1 or DuoxA2 maturation factors, or in combination with SOD1 or SOD3.

O_2•$^-$ release in extracellular space was determined by a chemiluminescence assay using the Diogenes reagent (National Diagnostics, Atlanta, GA) which is inhibited by superoxide dismutases. The cells were resuspended in HBSS + 10 mmol/l glucose, and 1 μmol/l ionomycin was added to stimulate Duox activity. The cells were then transferred to 96-well white plates ($1.5x10^5$ cells/250 μl well), and the measurements were taken automatically every 22 seconds during the total follow-up time of 10 minutes by a Microplate Luminometer at 37ºC. The curves were analyzed and the peak values of the curves are presented as relative light units (RLU)/second.

H_2O_2 release was determined by the homovanillic acid-based fluorimetric assay (Benard and Brault 1971). The cells were first transfected with human SOD3 (cDNA kindly provided by Professor Stefan L. Marklund from the University of Umeå, Sweden, and cloned into pcDNA3 vector (Life Technologies, Carlsbad, CA)), and after 48 hours incubated in Krebs-Ringer HEPES (KRH) medium pH 7.4 containing 0.1 μg/ml horseradish peroxidase type II, 440 μmol/l homovanillic acid and 1 μmol/l ionomycin at 37ºC for 2 hours 30 minutes. The fluorescence from oxidized homovanillic acid was

measured at 315 nm excitation and and 425 nm emission. The results are presented as ng of H_2O_2 per 6-well plate well, accumulated during the 2 h 30 min incubation.

3.5. SOD3 purification and activity assay (MS I)

SOD3 protein was purified from homogenated rat muscle tissue samples using Concanavalin A sepharose (GE Healthcare, Chalfont St. Giles, UK) as described previously (Marklund 1984). A volume of 500 µl of homogenized and centrifuged tissue sample was applied on the column and washed with phosphate buffer. SOD3 was then eluted with 50 mmol/l Na phosphate buffer containing 150 mmol/l α-methyl D-mannoside. Total SOD protein activity assay was performed as described (see manuscript I for reference) with control vector transfection and serum free medium as controls.

3.6. Quantitative real-time (q) PCR (MS I-III)

Total RNA (totRNA) was extracted from pooled tissue samples of 3-4 rats or cultured experimental cells by homogenizing them with Tri reagent (Sigma-Aldrich, St. Louis, MO) and using the standard phenol-chloroform extraction protocol. Messenger RNA (mRNA) was extracted from prepared totRNA using the GenElute mRNA Miniprep kit (Sigma-Aldrich, St. Louis, MO). Complementary (c)DNA synthesis was done with Revert-Aid M-MuLV (Fermentas, Burlington, Canada; **MS I&II**) or QuantiTect Reverse Transcription (Qiagen, Hilden, Germany; **MS III**), and PCR amplification with JumpStart RedTaq ReadyMix (Sigma-Aldrich, St. Louis, MO). QPCR was done using SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA). The used primers and running conditions for the qPCR are presented in Table 2.

3.7. Western blotting (MS I-III)

Tissue samples or cultured cells were homogenized in lysis buffer (50mmol/L HEPES pH 7.5, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1mmol/L EGTA, 1.5 mmol/L $MgCl_2$, 10 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 1mmol/L Na_3VO_4, 10 µg approtinin/ml, 10 µg leupeptin/ml) (Sigma-Aldrich, St. Louis, MO), and their protein concentration was determined by modified Bradford assay (Bio-Rad, Munich, Germany). Nitrocellulose membranes were stained with BRaf, IκB, SOD3 (all Santa Cruz Biotechnology, Santa Cruz, CA), MEK1/2, p-MEK1/2, 42/44 MAPK (Erk1/2), p-42/44 MAPK, Akt, and p-Akt (all Cell signaling, Danvers, MA), and α-tubulin (Sigma-Aldrich, St. Louis, MO) antibodies.

3.8. Statistical analyses

Statistical significance was analyzed by two-tailed t-test for means. Variation has been presented as ±SD (**MS I and III**) or ±SEM (**MS II**). The limit p-values for significance are $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Table 2. Primers and running conditions

Gene	Primer sequence	Tm	Manuscript
AdSOD3 for	GTT GCG TGA GCG GAA AGA TG	60	I
AdSOD3 rev	GTG AGC GCC TGC CAG ATC TC		I
huSOD3 for	CTT CGC CTC TGC TGA AGT CT	60	I, III
huSOD3 rev	GGG TGT TTC GGT ACA AAT GG		I, III
ratSOD3 for	GAC CTG GAG ATC TGG ATG GA	60	I, III
ratSOD3 rev	GTG GTT GGA GGT GTT CTG CT		I, III
ratCycD1 for	AAC GTC ACA CGG ACT ACA GG	55	I
ratCycD1 rev	TGT TCC ATG GCT GGG GCT CTT		I
huVEGF-A for	TCC GGG TTT TAT CCC TCT TC	55	I
huVEGF-A rev	TCT GCT GGT TTC CAA AAT CC		I
ratVEGF-A for	CAA TGA TGA AGC CCT GGA GT	50	I
ratVEGF-A rev	TTT CTT GCG CTT TCG TTT TT		I
ratTNF for	AGA TGT GGA ACT GGC AGA GG	60	II
ratTNF rev	CCC ATT TGG GAA CTT CTC CT		II
ratIL-1α for	TCG GGA GGA GAC GAC TCT AA	58	II
ratIL-1α rev	GAA AGC TGC GGA TGT GAA GT		II
ratIL-6 for	CCG GAG AGG AGA CTT CAC AG	55	II
ratIL-6 rev	ACA GTG CAT CAT CGC TGT TC		II
ratMCP-1 for	CTC ACC TGC TGC TAC TCA TTC ACT	55	II
ratMCP-1 rev	TGC TGC TGG TGA TTC TCT TGT AGT		II
ratMIP2 for	ATC CAG AGC TTG ACG GTG AC	55	II
ratMIP2 rev	GGA CTT GCC GCT CTT CAG TA		II
ratICAM for	AGG TAT CCA TCC ATC CCA CA	55	II
ratICAM rev	GCC ACA GTT CTC AAA GCA CA		II
ratVCAM for	TGA CAT CTC CCC TGG ATC TC	55	II
ratVCAM rev	CTC CAG TTT CCT TCG CTG AC		II
ratPSEL for	TTC CCA CAC TTC CTT CTG CT	57	II
ratPSEL rev	CAC GCT GTA GTC GGG GTA TT		II
ratESEL for	TTT TTG GCA CGG TAT GTG AA	57	II
ratESEL rev	AGG TTG CTG CCA CAG AGA GT		II
huβ-actin for	TGC GTG ACA TTA AGG AGA AG	55	I, II, III
huβ-actin rev	GCT CGT AGC TCT TCT CCA		I, II, III
ratβ-actin for	TCG TGC GTG ACA TTA AGG AG	55	I, II, III
ratβ-actin rev	GTC AGG CAG CTC GTA GCT CT		I, II, III

4 RESULTS

4.1. Analysis of the biological functions of SOD3 *in vivo*

4.1.1. Increased *SOD3* expression and attenuated oxidative stress after adenoviral gene transfer (MS I)

Adenovirus mediated gene transfer is a widely used method to introduce foreign gene expression in various tissues. We transferred AdSOD3 and AdLacZ as control into the rat hind limb muscle tissue immediately after inducing acute ischemia by femoral artery ligation and followed the animals for 3, 7, 10, or 14 days. Adenoviral LacZ expression was detected by X-Gal staining from 3-, 7-, and 10-day tissue sections (Figure 7). The virus transduction efficiency was in the range of 0.8-5% varying between the time points.

SOD3 mRNA expression was detected by reverse transcriptase (RT-)PCR from 3-day muscle tissue homogenate. The expected 547 bp *SOD3* sequence was amplified from the AdSOD3 treated muscle but not from the uninjured or AdLacZ treated muscle tissue (Figure 7). Control RT-PCR reaction with β-actin primers showed amplification from all tissue samples (Figure 7). SOD3 enzyme activity was increased 2-fold in the 3-day tissue sample purified by Concanavalin A sepharose further confirming the expression of functional SOD3 after gene transfer (see MSI).

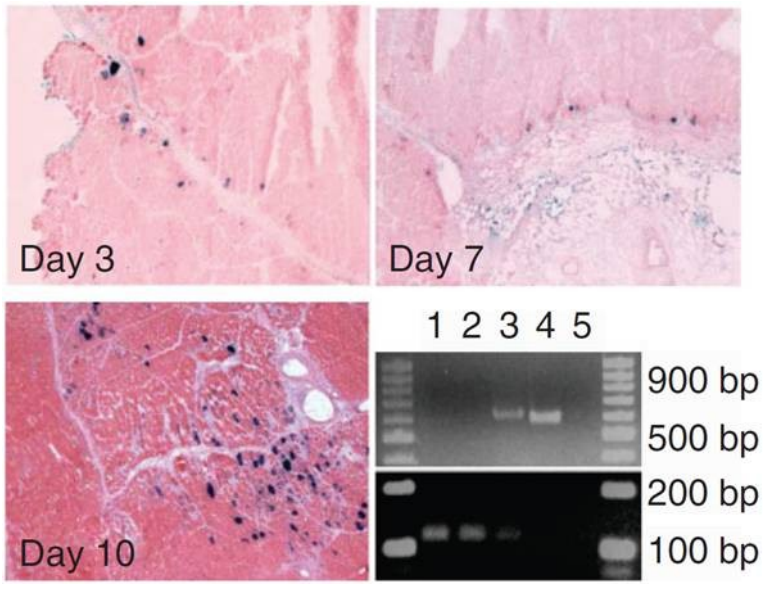

Figure 7. Expression of adenoviral transgenes. Photos show AdLacZ expression determined by β-galactosidase staining at 3, 7 and 10 days after transfection in rat hind limb muscle tissue. SOD3 expression was determined by RT-PCR. Upper panel shows the 574 bp SOD3 fragment and lower panel the 122 bp β-actin fragment. Lanes: 1, uninjured control muscle; 2, AdLacZ muscle; 3, AdSOD3 muscle; 4, SOD3 positive control; 5, water control. (Modified from MSI)

To assess the oxidative status of the muscle tissue we performed the DHE assay to detect ROS and 3-nitrotyrosine staining to detect reactive nitrogen species. DHE assay showed a significant 28% ($p<0.01$) decrease in DHE positive nuclei in AdSOD3 treated tissue sections as compared to AdLacZ animals (see MSI). The same trend was seen in the 3-nitrotyrosine stained sections, which showed less staining in AdSOD3 expressing tissues (see MSI). Thus, SOD3 had a clear antioxidative effect as shown in previous studies (Laukkanen et al. 2001; Laukkanen et al. 2002).

4.1.2. SOD3 improved tissue healing (MS I)

Tissue glucose uptake indicates the metabolic rate of the tissue (reviewed by Wasserman and Ayala 2005), and it can be measured with positron emission tomography (PET) imaging of radioactive [^{18}F] FDG glucose accumulation into tissues. The ratio of accumulated glucose between normal uninjured and operated hind limb was determined from 10-day AdSOD3 and AdLacZ treated animals after systemic injection of the PET marker. Injured AdLacZ legs vs. normal control legs in the same animal showed 46%, 64%, and 58% ($p<0.05$) higher glucose accumulation, whereas in the AdSOD3 legs it was closer to normal metabolic rate with only 24%, 27%, and 35% difference (see MSI).

Formation of new blood vessels and cell proliferation is characteristic to tissue healing and they are regulated by the oxidative status of the tissues (reviewed by Tidball 2005; reviewed by Ushio-Fukai 2006b). We determined the effect of SOD3 to angiogenesis and cell proliferation by von Willebrand Factor (vWF) and Ki67 staining, respectively, at 3-, 7-, 10-, and 14-day time points. The number of capillaries per mm^2 increased slightly in the course of the follow-up period, and peaked at 10 days (see MSI). However, there were no significant differences between the AdLacZ and AdSOD3 treated animals. Ki67 staining revealed significantly ($p<0.05$) increased cell proliferation at 3 and 7 days in the AdSOD3 group (see MSI). The overall number of proliferating cells in muscle tissue was low, about 0-8 cells per section which reflects the low muscle tissue turnover. These results indicate that *SOD3* gene transfer improves tissue recovery from ischemic damage by normalizing the tissue metabolism and inducing cell proliferation.

4.1.3. SOD3 reduced leukocyte accumulation in ischemia and inflammation (MS II)

Ischemia induces tissue inflammation by hypoxia derived tissue damage such as large amount of ROS and necrotic cell debris which activate a number signaling pathways leading to cytokine and chemokine expression, and leukocyte accumulation (reviewed by Alom-Ruiz et al. 2008; reviewed by Bosco et al. 2008). The first to appear are neutrophils and

monocyte/macrophages within hours or a few days (Paoni et al. 2002; reviewed by Tidball 2005), whereas lymphocytes appear later and fully activate only if they encounter microbial pathogens presented by antigen presenting cells.

To start elucidating the healing-promoting mechanism of SOD3 we first determined the inflammatory area of the tissue sections by calculating the ratio of CD68+ macrophage infiltrated area to the whole section area from 3-, 7-, and 10-day tissue sections. At all time points studied AdSOD3 treatment reduced the inflamed area significantly inducing a 3-fold difference ($p < 0.001$) already after 3 days, and growing to 12-fold at 10 days (see MSII).

The numbers of infiltrated CD68+ macrophages and CD3+ lymphocytes were determined from whole sections. For the whole 10-day period the amount of CD68+ macrophages remained 3-5 fold ($p < 0.05$) lower in AdSOD3 treated animals as compared to AdLacZ treated animals (Figure 8A). However, CD3+ cell numbers in both groups were at the same level at 3 days, and as the number of CD3+ cells remained at that level until 10-day time point in AdSOD3 animals the number increased gradually in AdLacZ animals rendering the difference significant ($p < 0.05$; Figure 8B). Thus, *SOD3* gene transfer has an anti-inflammatory effect in tissue damage as indicated by reduced inflammatory area and leukocyte accumulation.

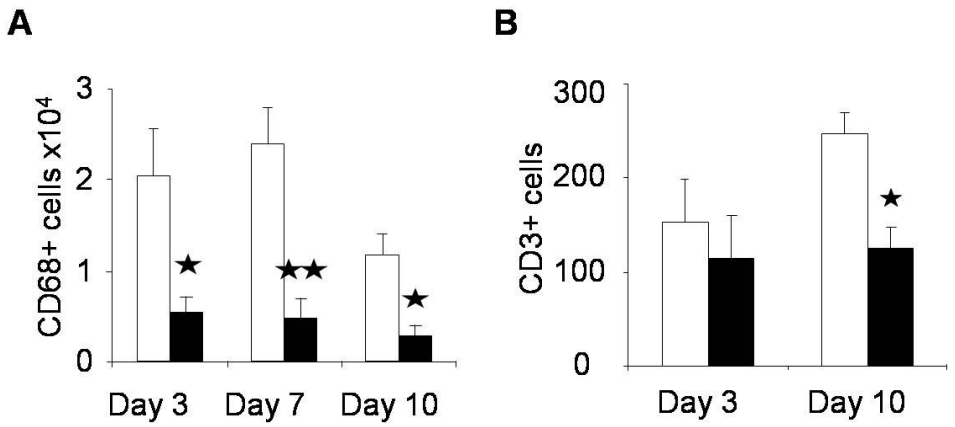

Figure 8. Inhibition of leukocyte migration in ischemic rat hind limb. (open bars: AdLacZ animals, black bars: AdSOD3 animals) Adenoviral SOD3 treatment of ischemic rat hind limb reduced efficiently the accumulation of CD68+ macrophages on a 10-day follow-up period (A). The number of CD3+ T lymphocytes remained at a steady low level throughout the same follow-up period in SOD3 treated animals (B). (Modified from MSII)

To further confirm and characterize the role of SOD3 in leukocyte trafficking in inflammation we induced mild acute peritonitis in mice and treated them with AdLacZ, AdSOD3, PBS, and the clinically approved anti-

inflammatory glucocorticoid drug Dexamethasone. The asset of this model is that the leukocyte subtypes can be readily analyzed from cytospinned peritoneal lavage containing the infiltrated cells. The adenoviruses were injected 3 days before the induction of peritonitis to ensure maximal *SOD3* or *LacZ* expression. AdSOD3 treatment reduced the total number of infiltrated cells by 30% ($p<0.01$), and had the most prominent effect on the monocyte/macrophage portion with 67% reduction ($p<0.001$; Figure 9A). Neutrophil and lymphocyte numbers were reduced by 20% (p= not significant) and 33% ($p<0.05$), respectively (Figure 9A).

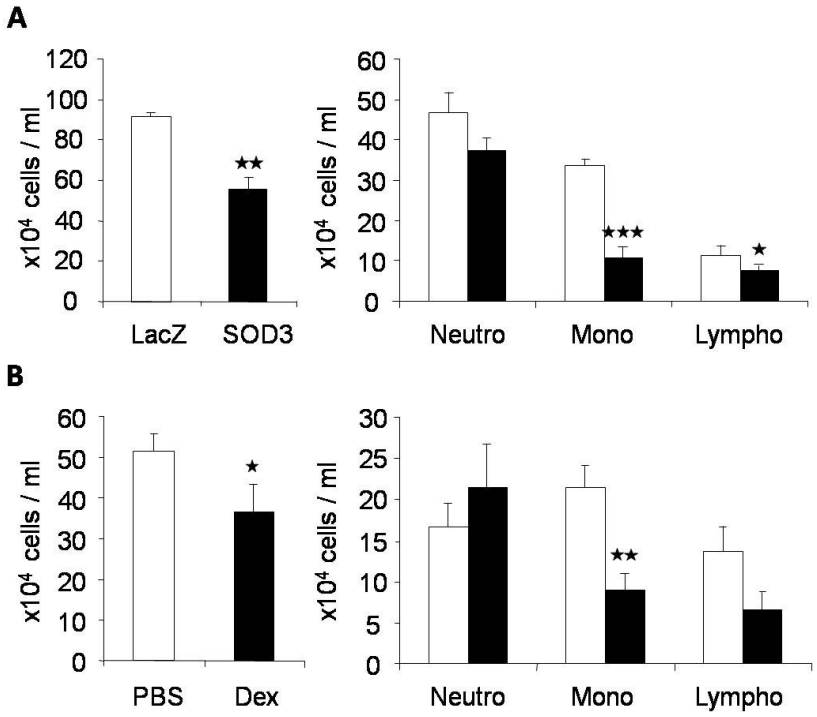

Figure 9. Inhibition of leukocyte migration in mouse peritonitis. (open bars: AdLacZ animals, black bars: AdSOD3 animals) AdSOD3 injection decreased the accumulation of leukocytes into peritoneum with the most notable effect on the monocyte (mono) subpopulation (A). A similar effect was seen in the Dexamethasone (Dex) treated animals (B). Either SOD3 or Dexamethasone did not affect significantly the neutrophil (neutro) migration, and only SOD3 could reduce the lymphocyte (lympho) infiltration. (Modified from MSII)

Treatment with Dexamethasone reduced the number of leukocytes by 20% ($p<0.05$) as compared to PBS control. Dexamethasone had no effect on the neutrophil subpopulation but monocyte/macrophages and lymphocyte counts were reduced by 60% ($p<0.01$) and 50% (p= not significant), respectively (Figure 9B). In these two groups the overall number of migrated cells was

markedly lower than in the virus-infected animals indicating an inflammation-enhancing effect for the virus, especially on the initial neutrophil accumulation. However, the AdSOD3 treatment was able to lower the monocyte/macrophage and lymphocyte numbers to the same level as Dexamethasone. Additionally, SOD3 showed a selective effect on the macrophage subpopulation suggesting a role in attenuation of early inflammatory reactions.

4.1.4. Enhanced expression of growth factors, cytokines and cell adhesion molecules by SOD3 (MS I,II)

In order to reveal the mechanisms by which SOD3 can mediate cell proliferation and regulate leukocyte migration we determined by qPCR the mRNA expression of several growth factors, cytokines, and cell adhesion molecules known to participate in these events. Vascular endothelial growth factor (VEGF-) A is an important vascular growth regulator but it has also a role in muscle regeneration (Rissanen et al. 2002), and cyclin D1 is the major factor determining the cell cycle propagation from G1 to S phase (reviewed by Tashiro et al. 2007). *VEGF-A* expression was increased 3-fold ($p<0.01$) in AdSOD3 treated tissue at 10-day time point as compared to both normal uninjured and AdLacZ controls (Figure 10A). At similar setting, the cyclin D1 expression in AdSOD3 animals was increased 2-fold ($p<0.05$) as compared to uninjured tissue but, interestingly, it was 6-fold ($p<0.01$) higher when compared to AdLacZ animals (Figure 10B). Indeed, SOD3 is capable of activating growth factor expression explaining its proliferative effect on muscle tissue.

Transcription factor NFκB is an important mediator of the inflammatory reaction and is responsible for the expression of a wide scale of cytokines and adhesion molecules (reviewed by Ghosh et al. 1998). We performed a luciferase assay for NFκB activation upon *SOD3* transfection into HEK 293T cells to see if SOD3 could affect this transcription factor. The experiment showed that *SOD3* gene expression reduced NFκB expression to approximately half of the original ($p<0.01$; see MSII). To further elucidate the mechanism behind this result we blotted a protein preparation from *SOD3* transfected cells and stained the membrane with an antibody for inhibitor of κB (IκB) which binds and restricts NFκB to cytosol (reviewed by Ghosh et al. 1998). As expected, *SOD3* induced increased IκB expression which is at least in part responsible for the reduced NFκB expression (see MSII).

We then studied the mRNA expressions of the cytokines tumor necrosis factor (TNF-) α, interleukin (IL-) 1α, IL-6, macrophage inhibitory protein (MIP-) 2, and monocyte chemoattractant protein (MCP-) 1 from the 3-day tissue samples. Their relative expressions were significantly decreased in

AdSOD3 treated animals to 10%-30% ($p<0.05$ or $p<0.01$) from the control AdLacZ animals set as 100% (Figure 10C).

The cell adhesion molecules (CAM) and selectins are in turn upregulated by the cytokines from leukocytes and tissue cells in order to facilitate leukocyte transmigration into the inflammation area. We therefore determined from the same samples the expression of E- and P-selectins, and intercellular (ICAM) and vascular (VCAM) cell adhesion molecules which mediate the leukocyte rolling and firm attachment to the vascular wall (reviewed by Ley et al. 2007). These results were in line with the cytokine study as the expressions of selectins and CAMs were significantly reduced after AdSOD3 treatment as compared to AdLacZ animals ($p<0.05$ or $p<0.01$; Figure 10D). Consequently, these results indicate that SOD3 is able to modulate the inflammatory reaction by inhibiting cytokine and adhesion molecule expression in an NFκB dependent manner, and thus preventing the inflammatory cells from accumulating in the inflicted tissues.

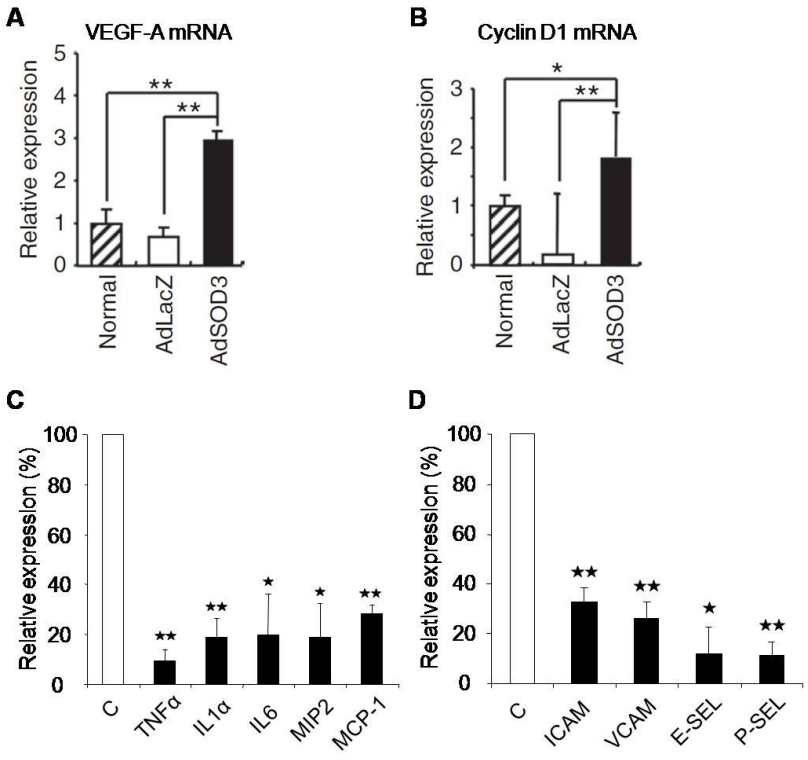

Figure 10. SOD3 effect on growth factor, inflammatory cytokine and cell adhesion molecule expression. Quantitative RT-PCR analysis showed significant increases in growth factor *VEGF-A* (A) and cell cycle regulator *cyclin D1* (B) expression after AdSOD3 treatment in rat hind limb as compared to normal uninjured or AdLacZ treated muscle tissue. AdSOD3 reduced significantly many major inflammatory cytokines and leukocyte chemokines (C) and cell adhesion molecules (D) as compared to AdLacZ treated rat hind limbs. (Modified from MSI and MSII)

4.1.4. High SOD3 expression was found in rat thyroid tissues (MS III)

The thyroid gland is naturally subject to high oxidative stress since its hormone production is dependent on H_2O_2 generation, and to prevent the harmful effects it also has extensive antioxidant defense systems (reviewed by Song et al. 2007). In order to characterize the role of SOD3 more specifically at organ level we studied its expression in the thyroid gland by isolating several tissues from untreated rats and running a qPCR with the extracted total mRNAs. When compared to heart, aorta had the highest level of expression with 4-fold increase ($p<0.05$; Figure 11) which is consistent with the major role of SOD3 as a vascular regulatory and protective enzyme (reviewed by Fukai et al. 2002). Thyroid gland compares well with aorta and kidneys with 2,5-fold higher *SOD3* expression ($p<0.05$) whereas liver, despite its high metabolic activity, was at the same level with heart (Figure 11). Thus, the high level of *SOD3* expression in the thyroid gland, also seen in a previous enzyme activity study (Marklund 1984), suggests a physiological role for it in this organ.

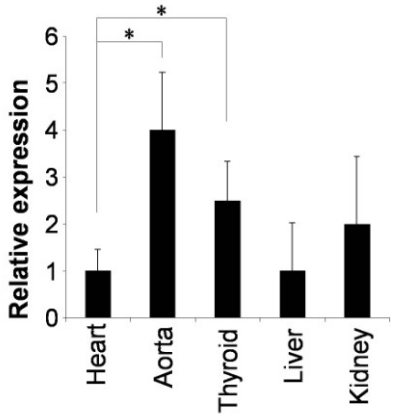

Figure 11. Abundant expression of SOD3 in rat thyroid. Thyroidal SOD3 expression was close to that in aorta which is one of the main expression sites of SOD3. Less expression was observed in liver and kidney. (Modified from MSIII)

4.2. Analysis of the role of SOD3 in cell signaling

4.2.1. SOD3 activated the mitogenic Ras/MAPK pathway (MS I)

Our studies with rat hind limb ischemia model suggested that SOD3 can induce cell proliferation through growth factor expression. One of the most prominent mitogenic signaling pathways is the Ras/mitogen activated protein kinase (MAPK) pathway consisting of Ras and its downstream Braf, MEK1/2, and Erk1/2 kinases. Akt/PKB is another crucial cell survival factor and an inducer of angiogenesis, and is also in part under Ras regulation (Kandel and Hay 1999). Therefore, we investigated the relationship between the Ras/MAPK pathway, Akt, and *SOD3* expression.

Ras-pulldown assay from the 7-day rat tissues revealed increased amount of Ras-GTP after *SOD3* gene transfer as compared to AdLacZ animals (Figure

12A). Similarly, the MAPK cascade components MEK1/2 and Erk1/2 were increasingly phosphorylated in AdSOD3 treated animals (Figure 12B). However, Akt phosphorylation was not affected by *SOD3* gene transfer *in vivo* at 7-day time point (Figure 12B). The inability of *SOD3* gene transfer to induce long-term Akt activation and thus angiogenesis could explain the similar capillary formation patterns in AdSOD3 and control groups (see MSI).

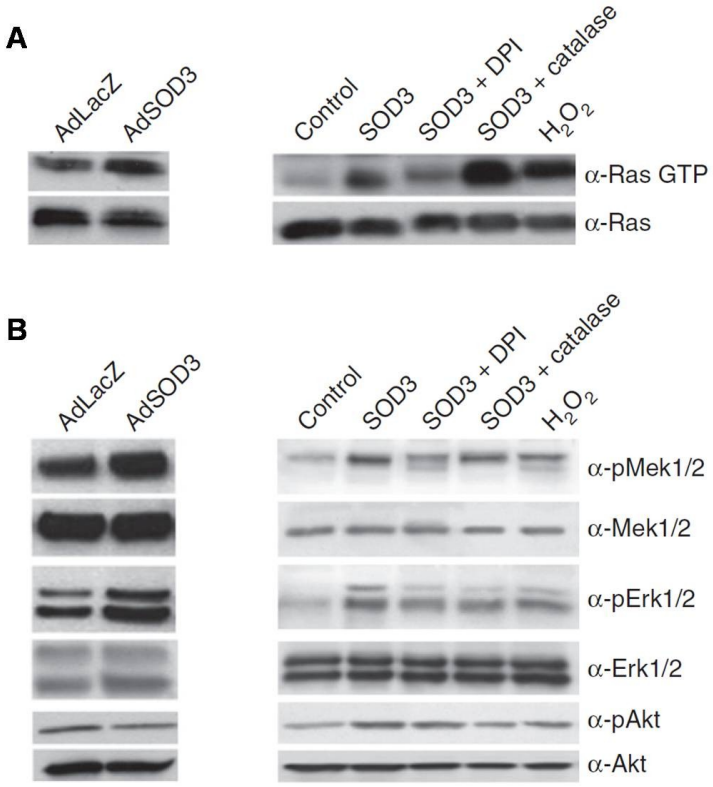

Figure 12. SOD3 activates the mitogenic Ras/Erk pathway. Ras-pulldown assay showed an increase in activated Ras (Ras-GTP) 7 days after AdSOD3 treatment *in vivo* (A, left panel) and after SOD3 transfection *in vitro* (A, right panel). Ras downstream signaling molecules MEK1/2 and Erk1/2 but not Akt were phosphorylated in 7-day tissues by SOD3 (B, left panel) and a similar expression pattern was seen *in vitro* with the exception that also Akt was activated (B, right panel). DPI diminished the activating effect of SOD3 but catalase did not have such a clear effect on MEK1/2 and Erk1/2 whereas H_2O_2 distinctly reproduced the effect of SOD3 on the signaling molecules (right panels). (Modified from MSI)

We also studied the Ras/MAPK pathway activation in a more controlled setting by transfecting *SOD3* into HEK 293T cells. Similar Ras-pulldown assay showed likewise increased Ras-GTP loading, and blotted protein samples from cell preparations contained more phosphorylated MEK1/2 and Erk1/2 as compared to control vector transfected cells (Figure 12). Furthermore, we exposed untransfected cells to H2O2 to mimic functional SOD3, and this

resulted again in Ras, MEK1/2, and Erk1/2 activation suggesting that the SOD3 end-product is needed for the stimulatory function (Figure 12). Incubation of the *SOD3* transfected cells in the NADPH oxidase (Nox) enzyme inhibitor DPI reduced Ras/MAPK pathway phosphorylation indicating that SOD3 mediated Ras/MAPK activation is dependent on the availability of its substrate, superoxide ($O_2^{\bullet -}$) (Figure 12). Treatment with catalase, the H2O2 scavenger, did not interrupt Ras, MEK1/2, or Erk1/2 activation (Figure 12A,B). However, Akt was phosphorylated upon *SOD3* transfection unlike in the *in vivo* setting, and its phosphorylation pattern was similar to those of MEK1/2 and Erk1/2 in all experimental conditions (Figure 12).

Erk1/2 has several downstream target genes whose functions are mediated by such transcription factors as AP-1, its component c-Jun, and CREB (Eriksson and Leppa 2002; Gelain et al. 2006). Their expression was studied by luciferase reporter gene assay. *SOD3* and *SOD3/catalase* transfections induced significant increase ($p<0.05$) in the expression of p*AP1*-, pFA2-*cJun*- and p*CRE*-luciferase reporter genes as compared to control vector, and H_2O_2 had similar effect on *AP-1* and *c-Jun* but actually decreased *CREB* expression. DPI prevented efficiently the SOD3 induced transcription factor expression (see MSI).

AP-1 and CREB target VEGF-A and cyclin D1 as their downstream effectors (Wu et al. 2007; Shen et al. 2008) providing a link between the SOD3 activated Ras/Erk pathway and growth factor or cell cycle regulator expression. We determined the effect of SOD3 transfection on *VEGF-A* and *cyclin D1* mRNA expression *in vitro* to confirm our *in vivo* results. Both molecules were significantly upregulated ($p<0.05$) upon *SOD3* transfection as well as upon *Ras*, *BRaf*, *MEK1*, and *Erk1* transfections supporting the role of SOD3 as a mitogenic signal molecule which exerts its effect through Ras/Erk pathway (see MSI).

4.2.2. SOD3 protein activity and expression were regulated through a positive feedback loop (MS I)

As described above SOD3 action is dependent on the substrate $O_2^{\bullet -}$ but it is likely regulated by other means as well. We studied whether SOD3 activation could be modified by a feedback signal from its downstream pathway by transfecting HEK 293T cells with the cascade kinases. Endogenous SOD3 protein activity was significantly increased upon *Ras*, *MEK1*, *Erk1* (all $p<0.05$), and *BRaf* ($p<0.001$) expression but after *Akt* transfection the activity remained at the same level with control (see MSI). Additionally, H_2O_2 incubation had a similar positive effect ($p<0.05$) as the signaling molecules

suggesting that the SOD3 end-product is necessary for both Ras/MAPK activation and stimulatory feedback signaling (see MSI).

Finally, to determine if increased SOD3 activity was due to enhanced gene expression we performed a qPCR for *SOD3*. The significant rise in *SOD3* mRNA after *Ras*, *BRaf*, *MEK1*, and *Erk1* transfections (all $p<0.001$), and H_2O_2 incubation ($p<0.05$) indicated that increased *SOD3* gene expression is indeed behind its higher activity (see MSI). As expected, Akt had no effect on *SOD3* expression (see MSI). Furthermore, we incubated untransfected cells with NADP which is an essential cofactor for the Nox enzymes, and detected again significantly increased *SOD3* mRNA expression ($p<0.001$) (see MSI). This effect was reversed by DPI and a MEK inhibitor UO126 (both $p<0.05$) confirming that the Nox derived $O_2^{\bullet -}$ and Ras/Erk pathway regulate *SOD3* expression (see MSI).

4.2.3. TSH-R pathway activation induced SOD3 expression (MS III)

Thyroid function mainly depends on the activation of TSH receptor pathway. It stimulates two G proteins, $G\alpha_s$ and $G\alpha_q$, which initiate follicular cell proliferation through cAMP dependent pathway and thyroid hormone synthesis through PLC dependent pathway, respectively (Allgeier et al. 1994; Corvilain et al. 1994). Interestingly, TSH stimulation of PC Cl3 cells induced a significant 3-fold ($p<0.01$) increase in *SOD3* expression (Figure 13A), and we therefore studied by qPCR whether these pathways could induce *SOD3* mRNA expression.

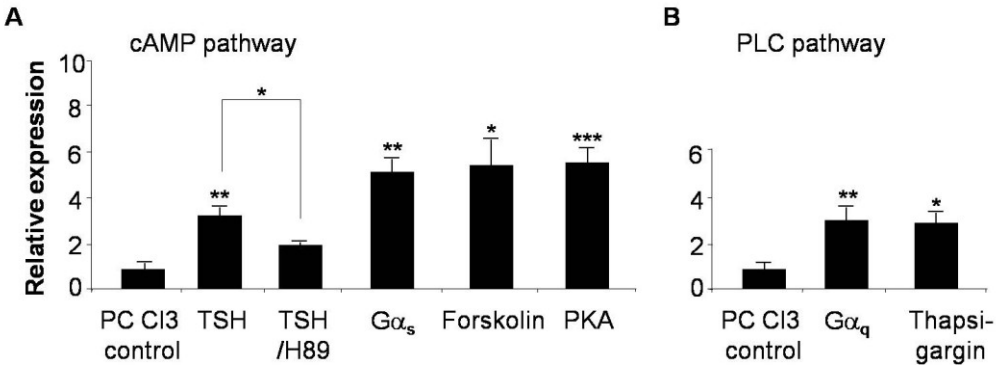

Figure 13. TSH-R activation induces SOD3 expression in PC Cl3 rat thyroid cell line. Stimulation of cells with TSH increased SOD3 expression, and was not abolished by the PKA inhibitor H89 (A). Activating the proliferative cAMP pathway by forskolin and transient transfections of Gαs and PKA also promoted SOD3 expression (A). The hormonogenic PLC pathway had similar effect as transient transfection of Gαq and treatment with Ca2+ releasing thapsigargin promoted SOD3 expression (B). (Modified from MSIII)

The $G\alpha_s$ signal is mediated through sequential activation of adenylate cyclase/cyclic AMP (cAMP) and protein kinase A (PKA). Overexpression of pCEF-*Gα$_s$* and pCEF-*PKA* by transient transfections induced significant 5,5-($p<0.01$) and 6-fold ($p<0.001$) increases in *SOD3* expression as compared to untreated control cells, respectively. Incubation with adenylate cyclase activator forskolin also resulted in 6-fold ($p<0.05$) increase, thereby confirming that the $G\alpha_s$/cAMP/PKA pathway is upstream of SOD3. However, the TSH induced *SOD3* expression was decreased ($p<0.05$) with simultaneous PKA inhibitor H89 incubation but was not completely abolished (Figure 13A) suggesting that *SOD3* expression is likely regulated by other mechanisms as well.

The second major TSH-R pathway starting from $G\alpha_q$ utilizes phospholipase C and subsequent Ca2+ release from the endoplasmic reticulum. *SOD3* mRNA was increased 3-fold ($p<0.01$) after pCEF-*Gα$_q$* transfection, and a similar increase (3-fold, $p<0.05$) was observed after treating the cells with the Ca2+ ATPase inhibitor thapsigargin, which induces Ca2+ accumulation into cytosol, as compared to untreated control (Figure 13B). Thus, both TSH-R signaling branches induce *SOD3* expression upon receptor activation implying a wider role for SOD3 in thyroid physiology.

4.2.4. SOD3 participated in thyrocyte proliferation but not in hormone synthesis (MS III)

These findings prompted us to investigate what role SOD3 could play in the thyroid gland. Since SOD3 had a clear growth stimulating effect in the muscle tissue (MS I) we studied whether it could function similarly in thyroid gland. The high endogenous *SOD3* expression compelled us to inhibit it by small interfering RNA (siRNA) since further upregulation of SOD3 by plasmid transfection could have proven difficult. We transfected rat PC Cl3 cells with *siSOD3* or *siGAPDH*, a general housekeeping gene commonly used in siRNA studies, and determined the *SOD3* expression by qPCR analysis. A significant 95% interference ($p<0.001$) in *SOD3* mRNA production was detected 48 hours later (Figure 14A). *In vitro* cell proliferation was then assessed by BrdU and Hoecst double labeling which stain the nuclei of proliferating and all cells, respectively. *SiSOD3* transfection reduced TSH-stimulated cell proliferation by 15% ($p<0.05$) as compared to *siGAPDH* transfected cells (Figure 14B). Thus, SOD3 seems to have a mitogenic effect also on thyrocytes.

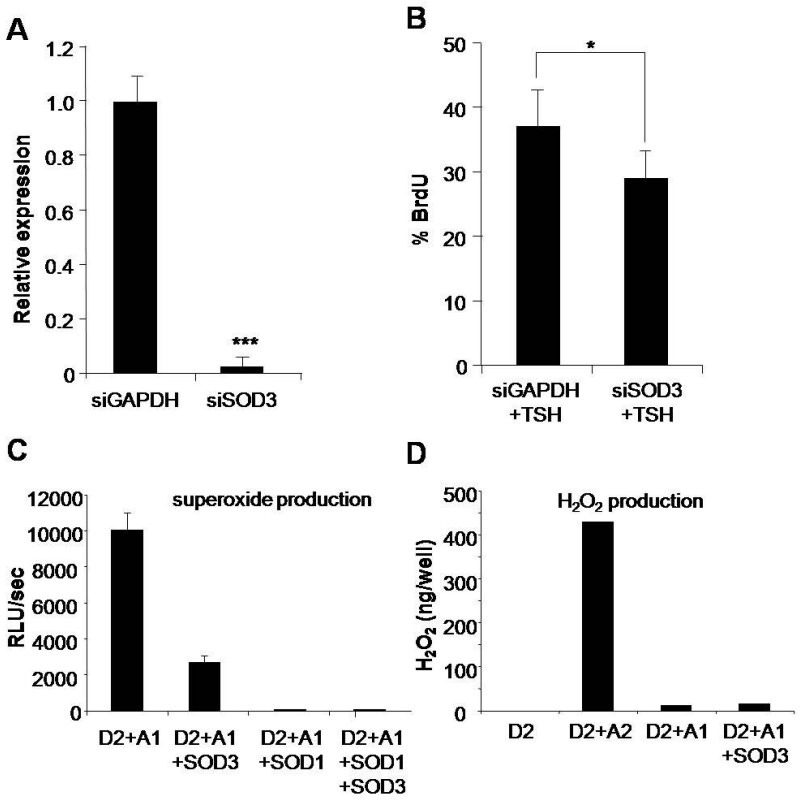

Figure 14. SOD3 participates in thyrocyte proliferation but not in hormone synthesis. SOD3 siRNA (siSOD3) transfection knocked down 95% of SOD3 expression in PC Cl3 cells as compared to control GAPDH siRNA (siGAPDH) (A). TSH stimulated proliferation was diminished by 15% in the SOD3 knock-down cells as determined by 5'-bromo-3'-deoxyuridine (BrdU) assay (B). Superoxide generation was reduced by SOD3 and SOD1 in Cos-7 cells cotransfected with Duox2 (D2) and the incompatible maturation factor DuoxA1 (A1) which cannot promote H2O2 conversion (C). Duox2 alone or with DuoxA1 cannot produce H_2O_2 but requires the compatible DuoxA2 (A2) maturation factor (D). Cotransfection of SOD3 and Duox2/DuoxA1 does not lead to significant H_2O_2 generation as compared to functional Duox2 (D). (Modified from MSIII)

We next compared the H2O2 production of SOD3 and Duox2, which *in vivo* is required for efficient thyroid hormone synthesis after $G\alpha_q$/PLC pathway activation, to see if SOD3 could contribute to this function. To confirm the functionality of the heterologous Cos-7 model we first made a set of transfections with Duox2 and DuoxA1 maturation factor with and without *SOD1* and *SOD3* co-transfections, and measured the $O_2\bullet^-$ production. The maturation factors DuoxA1 and DuoxA2 function with the Duox1 and Duox2 oxidases, respectively, and Duox2/DuoxA1 combination causes leakage of $O_2\bullet^-$ while Duox2/DuoxA2 complex produces H_2O_2 (Grasberger and Refetoff 2006; Morand et al. 2009). A chemiluminescence assay for $O_2\bullet^-$ showed highest

RLU/sec values when SODs were not present but when they were expressed simultaneously the peak values dropped dramatically (Figure 14C) indicating functional SOD activities in this *in vitro* model. H_2O_2 measurements with a fluorimetric assay showed a markedly 28-fold lower amount of H_2O_2 generation by SOD3 than by the functional Duox2 (Figure 14D). Therefore, it seems that SOD3 participates in thyrocyte proliferation as indicated by the siRNA experiment but is not required for $G\alpha_q$/PLC regulated hormonogenesis since the Duox2 can produce all the H_2O_2 necessary.

4.3. SOD3 in thyroid hyperproliferation

4.3.1. SOD3 expression was increased in goiter (MS III)

Goiter is one of the most common thyroid pathologies associated with nutritional iodine defects and disorders that compromise the balance of TSH and thyroid hormone secretion (reviewed by Dumont et al. 1992). Characteristic to goiter is the rapid proliferation of follicular cells stimulated by excess TSH or activating antibodies (reviewed by Dumont et al. 1992). Since SOD3 was upregulated upon TSH stimulation in normal rat thyroid cells we studied whether it participates in the goiter formation.

We induced goitrogenesis in rats by delivering 0.25% 6-propyl-2-thiouracil (PTU) in drinking water for two weeks and analyzed the isolated thyroid glands. PTU induced approximately 4-fold (p= not significant) increase in the thyroid weight as shown in Table 3. Additionally, typical morphological changes in thyroid histology were observed with diminished colloid in the follicular lumen and appearance of tall columnar follicular cells lining the lumen walls indicating hyperactive metabolism in goiter tissue (Figure 15A,B). These results were in line with a previous study in a similar setting (Laezza et al. 2006) confirming the typical signs of goiter formation in our experiment.

Table 3. Thyroid weight

Normal thyroid	12,2 mg
	15,6 mg
	15,1 mg
PTU treatment	17 mg
	46 mg
	51 mg
	130 mg

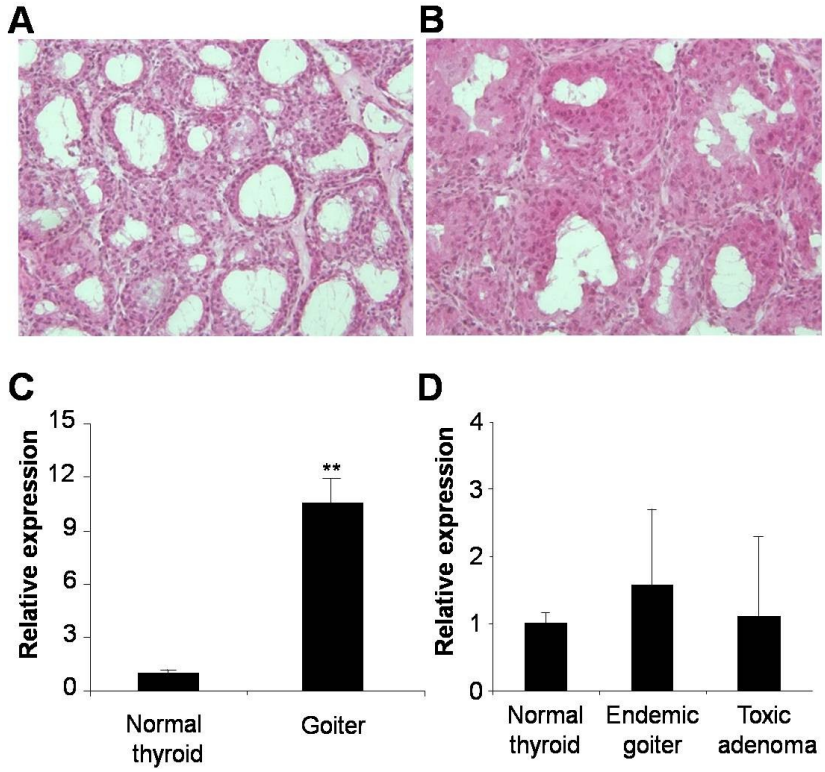

Figure 15. SOD3 expression increases in goiter formation. PTU administration to rats induced typical morphological changes in thyroid tissue such as shrinked follicles and hyperproliferation of follicular cells (A normal thyroid, B goiter). SOD3 expression was significantly higher in rat goiter tissue as compared to normal thyroid gland (C). However, in human patient samples of endemic goiter and toxic adenoma the SOD3 expression remained at the same level with normal tissue (D). (Modified from MSIII)

We then determined *SOD3* mRNA expression from normal and PTU treated rat thyroid glands by qPCR to see how *SOD3* expression pattern changes in this pathological condition. In rat goiter tissue the level of *SOD3* expression increased 10-fold ($p<0.01$) as compared to normal tissue (Figure 15C). However, further studies with human patient samples of endemic goiter and toxic adenoma did not reveal differences in *SOD3* expression as compared to normal thyroid tissue (Figure 15D) suggesting that chemically induced goiter in rat may not fully compare to natural human disease regarding the SOD3 regulation.

4.3.2. SOD3 expression was decreased in thyroid malignancies (MS III)

Malignant transformation of any cell type is characterized by abnormally high proliferation rate as the growth-promoting oncogenes are activated. We performed a qPCR on the rat PC Cl3 papillary thyroid carcinoma (PC PTC)

and PC E1A undifferentiated thyroid carcinoma cell lines to further characterize *SOD3* expression in hyperproliferation. In PC PTC cell line the *SOD3* expression was significantly (3-fold, $p<0.01$) lower when compared to normal PC Cl3 cells (Figure 16A). Interestingly, the more aggressive PC E1A cell line showed minimal *SOD3* expression ($p<0.001$; Figure 16A) as compared to normal cells or the less aggressive PTC cell line (Fusco et al. 1987; Berlingieri et al. 1993; Farid et al. 1994). This indicates that SOD3 is progressively downregulated as the cancer type becomes more aggressive.

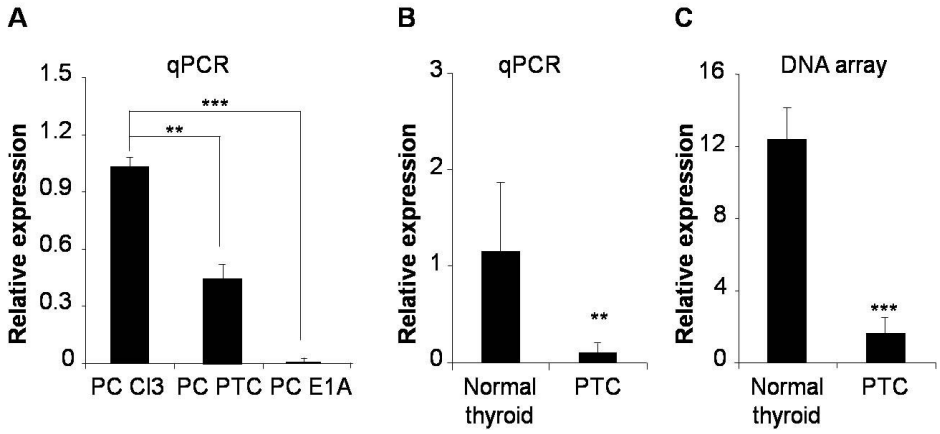

Figure 16. SOD3 is downregulated in thyroid cancer. SOD3 expression was significantly lower in rat PC papillary thyroid carcinoma (PTC) cell line and even lower in the more aggressive PC E1A cell line indiating a clear correlation with the degree of malignancy (A). Similar results were obtained from human PTC patient samples by quantitative (q)PCR (B) and DNA array (C). (Modified from MSIII)

PTC is the most common thyroid cancer comprising 70% of all cases, and is more prevalent in women (Correa and Chen 1995). To further characterize the role of SOD3 in human thyroid hyperproliferation we tested the expression of *SOD3* from clinical human PTC tumor samples by DNA array and qPCR. Quantitative PCR from the tumor samples revealed deceased ($p<0.01$) *SOD3* mRNA expression (Figure 16B) and, similarly, DNA array of the same samples gave significantly ($p<0.001$) decreased *SOD3* expression (Figure 16C) as compared to normal human thyroid tissue thereby establishing correlation between the rat model and the human disease. These opposing expression patterns of SOD3 in goiter and cancer establish SOD3 as a differentiation marker to estimate the stage of transformation in thyroid malignancies.

5 DISCUSSION

The extracellular SOD3 has been studied in various settings for its role in relieving oxidative stress under ischemic or inflammatory conditions such as myocardial and cerebral infarction, atherosclerosis, inflammatory bowel disease, lung injury and arthritis (Sheng et al. 1999; Landmesser et al. 2000; Bowler et al. 2001; Ross et al. 2004; Kruidenier et al. 2003; Tasaki et al. 2006). Interestingly, its expression level is often decreased in chronic pathological conditions resulting in reduced capacity to remove oxygen radicals from the inflicted tissues (Marklund et al. 1986; Kruidenier et al. 2003; Tasaki et al. 2006; Ueda et al. 2008), although it does not seem to be necessary under normal conditions since healthy SOD3 knock-out mice show normal phenotype (van Deel et al. 2008). However, in some acute ischemic events and in young hypercholesterolemic patients SOD3 may be upregulated (Landmesser et al. 2000; Horiuchi et al. 2004). Re-establishing SOD3 expression by e.g. adenoviral gene transfer has shown significant positive effects on tissue recovery by reducing oxidative stress (Li et al. 2001; Laukkanen et al. 2001; Brasen et al. 2007).

In our study, we observed reduced inflammatory reaction as well in both the rat hind limb ischemia model and the mouse peritonitis model after adenoviral *SOD3* gene transfer. Analysis of the gene transfer efficiency by histological staining and gene expression studies revealed sustained expression even 10 days after virus injection (MS I). Diminished oxidative stress, close to normal glucose metabolism, and higher cell proliferation rate indicated improved tissue functionality and recovery process in the SOD3 treated ischemic hind limbs (MS I). However, capillary formation in both hind limb ischemia groups followed the same course suggesting that exogenous SOD3 does not affect angiogenesis (MS I) although an important role has been suggested in another study where endogenous SOD3 was investigated (Kim et al. 2007b). Consistently, smaller inflammatory areas at all time points in rat tissues (MS II) and leukocyte counts from rat tissue sections and mouse peritoneal lavage confirmed that SOD3 can reduce tissue inflammation as determined by leukocyte accumulation (MS II).

Adenovirus gene transfer is an efficient method to deliver genes into *in vivo* systems but both adverse immunogenic effects and low toxicity of the adenoviruses have been reported and, thus, the safety of this method still remains under debate (Martin-Martinez et al. 2004; Li et al. 2008; reviewed by Hartman et al. 2008). Adenovirally induced gene expression peaks at three days after the infection in normal rats (Schulick et al. 1997 and our

observations, data not published), and therefore we chose the 3-day timepoint as the first one in our experiments to ensure that we see the early effect of SOD3 expression. Here, the cell counts from mouse peritoneum revealed higher total number of leukocytes in virus-injected animals as compared to animals injected with PBS or Dexamethasone (MS II) indicating that adenovirus injection itself enhances inflammation. However, no animals had to be sacrificed prematurely because of virus-related problems suggesting that in our models the adenoviral gene transfer is reasonably safe. Additionally, we used replication-deficient viruses to avoid systemic spreading from the original injection site. Determination of the long-term health effects of adenoviral vector gene transfer in our models would require further studies.

After determining the beneficial effects of *SOD3* gene transfer we started to investigate how SOD3 regulates and is regulated by different signaling pathways. The pathways upstream and downstream of SOD3 are poorly characterized but it has been associated with various cellular events such as survival, migration and proliferation (Turkseven et al. 2007; Ueda et al. 2008; Na et al. 2007; Auten et al. 2006). The observed improvement in tissue healing lead us to study the well-known Ras/MAPK and Akt signaling pathways which are known to be activated under ischemic and inflammatory conditions to promote cell proliferation and survival (Arany et al. 2005; Kwon et al. 2006; Sung et al. 2007). Our Ras pull down assay and western blots showed clearly how SOD3 gene transfer induces phosphorylation of Ras small GTPase and its downstream signaling kinases MEK1/2 and Erk1/2 both *in vivo* at 7 days and *in vitro* (MS I). Interestingly, Akt was not activated *in vivo* but showed phosphorylation *in vitro* (MS I) underlining the importance of the cellular environment.

Further analysis by luciferase assay of possible signaling events after Erk1/2 activation showed upregulation of the transcription factors AP-1/c-Jun and CREB (MS I) which mediate the Ras/Erk signals (Cook et al. 1999; Arany et al. 2005). Moreover, two target genes for these transcription factors, VEGF and cyclin D1 (Wu et al. 2007; Shen et al. 2008), were significantly upregulated both *in vivo* 10 days after SOD3 and *in vitro* after *SOD3*, *Ras*, *Braf*, *MEK1* and *Erk1* gene transfers (MS I) indicating their involvement in this signaling pathway. VEGF expression is upregulated in ischemic muscle tissue, and it has been suggested that it induces an autocrine effect on the inflicted tissue during recovery from an ischemic insult to promote healing (Rissanen et al. 2002; Germani et al. 2003). Together these results demonstrate that one mechanism for SOD3 mediated tissue healing is the induction of mitogenic Ras-Erk1/2 signaling, and subsequent growth factor expression and cell cycle progression.

The only substrate of the SOD3 enzyme is $O_2\bullet^-$ which is frequently converted to H_2O_2. Thus, the regulatory functions of SOD3 depend on its ability to modulate the extracellular ROS balance. Exposure of the cells to Nox inhibitor DPI which abolishes $O_2\bullet^-$ production reduced Ras/Erk phosphorylation, and transcription factor and target gene expression whereas H_2O_2 incubation increased them (MS I). It seems therefore that SOD3 regulatory function is determined by substrate availability and that the end-product H_2O_2 is the principal signal mediating molecule. Consistently, previous studies have found $O_2\bullet^-$ and H_2O_2 to be associated with Ras and Erk activation (Lander et al. 1995; Milligan et al. 1998; Sigaud et al. 2005; Novo et al. 2006). The H_2O_2 incubation (500 μM for 6 hrs) done here equals to transient oxidative stress, which can induce the activation of Erk pathway and increase cell survival (Arany et al. 2004; Arany et al. 2005).

In order to further characterize the relationship between Ras/Erk pathway and SOD3 expression we looked into the possibility of feedback mechanism regulating SOD3 function. We found that *Ras*, *Braf*, *MEK1*, and *Erk1* transfections increased significantly SOD3 protein activity whereas *Akt* had no effect (MS I) indicating that the Ras-Erk1/2 pathway can exert positive feedback to SOD3. The qPCR analysis confirmed that the increase in SOD3 activity was due to increased mRNA expression (MS I). Thus, the mitogenic Ras-Erk1/2 pathway can enhance its own H_2O_2-dependent activation by inducing SOD3 gene expression until the original signal from tissue is gradually terminated during the healing process. Our data did not reveal the exact molecular mechanism stimulating SOD3 but we found that NADP induced SOD3 expression while DPI and MEK inhibitor UO126 reduced it (MS I). Additionally, SOD3 gene is suggested to have binding sites for at least two Erk1/2 regulated transcription factors, AP-1 and CREB, and also for glucocorticoid response element (GRE) and antioxidant response element (ARE) binding proteins (Folz and Crapo 1994). Consequently, feedback stimulation for SOD3 expression appears to depend on the substrate $O_2\bullet^-$ generation as well as Erk1/2 mediated transcription factor activation.

Next, we set out to explain the observed anti-inflammatory function of SOD3 and analyzed its effect on leukocyte accumulation into the inflamed tissue. Neutrophils and macrophages are the first leukocyte populations to appear at the site (Fielding et al. 1993; Belcastro et al. 1996), and they are responsible for initial oxidative tissue damage brought about by the oxidative burst intended as the first-line defense against pathogens (reviewed by Jordan et al. 1999; reviewed by Fujiwara and Kobayashi 2005). High oxidative stress and tissue damage cause an inflammatory reaction which is nevertheless

required for the healing process initiation in muscle tissue (reviewed by Tidball 2005).

Our data showed that SOD3 inhibits leukocyte accumulation with the most prominent effect on the macrophage subpopulation, and minor effect on the neutrophils (MS II). The overall lymphocyte counts from both rat tissue sections at 3-day time point and mouse peritoneum remained low, probably because they characteristically migrate somewhat later; only 10 days after the gene transfer into rat hind limb was accumulation of CD3+ lymphocytes observed in LacZ control animals whereas in SOD3 treated animals the number of lymphocytes was still negligible (MS II). Lowered amount of $O_2\bullet^-$ due to SOD3 activity at the injury site could partly explain the diminished macrophage accumulation since $O_2\bullet^-$ has been found to enhance monocyte adhesion to endothelial cells and subsequent migration (Van der Goes et al. 2001).

Glucocorticoid drugs are potent inhibitors of leukocyte function and migration through various mechanisms, mainly inhibiting the expression of cytokines such as TNFα and MCP-1 (Steer et al. 2000; Zhou et al. 2007; Nehme and Edelman 2008), and are widely used in different clinical settings as anti-inflammatory and immunosuppressive drugs. We compared the effect of the clinically available glucocorticoid drug Dexamethasone and SOD3 on leukocyte accumulation into the mouse peritoneum, and found rather similar reduction in leukocyte counts in both groups with largest reduction again in the macrophage subpopulation and minor effect on the other subtypes (MS II). It seems therefore that SOD3 gene transfer has a potent anti-inflammatory effect similar to glucocorticoids regarding the leukocyte migration. However, direct functional comparisons cannot be done since the two molecules have different mechanisms of action.

Endothelial activation and leukocyte migration is a complex interplay of numerous signaling molecules, such as chemo- and cytokines and adhesion molecules, secreted by leukocytes and endothelial cells alike (reviewed by Ley et al. 2007). Many of these molecules are regulated by the ROS sensitive NFκB transcription factor which activates early under cellular stress (reviewed by Ghosh et al. 1998; Souza et al. 2000). We performed an *in vitro* luciferase assay to determine how SOD3 influences the expression of NFκB in muscle tissue. After SOD3 transfection NFκB expression was significantly reduced (MS II) which is in line with previous studies showing attenuation of NFκB expression by SOD3 in lung and liver injuries (Bowler et al. 2001; He et al. 2006). Interestingly, also the MEK-Erk pathway appears to inhibit NFκB (Janssen-Heininger et al. 1999), and therefore it would be interesting to further

study the Ras/Erk/SOD3 cascade effects on NFκB regulation, and its impact on the early inflammatory events. Additionally, we observed an increase in the expression of the main NFκB inhibitor, IκB, after *SOD3* transfection (MS II). IκB is known to be regulated by multiple stimuli such as inflammatory cytokines, microbial lipopolysaccharides and oxidative stress which induce its phosphorylation and subsequent degradation (reviewed by Kabe et al. 2005), and therefore the influence of SOD3 on its expression would require further studies.

Cytokines induce endothelial expression of several cell adhesion molecules which attach to their counterparts on the leukocytes and thus allow them to transmigrate through the vascular endothelium into the site of injury (reviewed by Ley et al. 2007). We therefore determined the expression of important NFκB regulated chemokines and cytokines three days after *SOD3* or *LacZ* gene transfer in rat hind limbs. We found significantly reduced expression of the cytokines TNFα, IL1α, IL6, MIP2 and MCP-1 in AdSOD3 treated animals (MS II). Since TNFα, IL1α and IL6 are known to enhance cell adhesion molecule expression we also checked the relative expressions of the major adhesion molecules ICAM, VCAM, E-selectin and P-selectin. They all were significantly reduced in AdSOD3 treated rats as compared to the LacZ animals (MS II) indicating that SOD3 can indeed regulate cytokine expression and subsequent leukocyte recruitment through adhesion molecule expression. Similar results have been acquired previously in *SOD3* knock-out and overexpressing mouse models (Bowler et al. 2004). These results also clarified the relationship between SOD3 and IκB. According to a study by Milligan and coworkers (1998) TNFα induces the proteolysis of IκB, and therefore SOD3 mediated downregulation of this cytokine could partly explain the observed increase in IκB expression.

The more pronounced effect of SOD3 on the macrophage population raised the question whether it has a specific effect on them. MCP-1 is a potent chemotactic factor for blood-borne monocytes but not tissue-resident macrophages (Sica et al. 1990; Lu et al. 1998), and its reduced expression could explain the marked reduction in the number of infiltrated macrophages which is supported by a previous study by Huang and coworkers (2001) (MS II). Additionally, MCP-1 does not affect the neutrophil subpopulation (Lu et al. 1998) and therefore it was not surprising that their accumulation was not drastically decreased (MS II). Reduced number of macrophages could further explain the decreased expression of MIP2 (MS II) since they start to secrete it once activated attracting more neutrophils to the site (Wolpe et al. 1989; reviewed by Driscoll 1994). This would prevent neutrophil accumulation after

the initial phase of the inflammation and, thus, reduce the overall severity of the inflammatory reaction. Furthermore, the lack of neutrophil and macrophage accumulation could partly explain the absent lymphocyte infiltration since their presence is required for lymphocyte chemotaxis (Taub et al. 1996; Huang et al. 2001; reviewed by Bosco et al. 2008). Consequently, we propose a second mechanism for SOD3 to enhance tissue healing in which SOD3 inhibits the early inflammatory reaction by downregulating cytokine and cell adhesion molecule expression thus reducing the oxidative damage induced by the accumulating neutrophils and macrophages. Souza and coworkers (2000) have previously suggested the dependence of early oxidative stress to the extent of tissue injury thus supporting our findings.

According to our studies presented this far SOD3 seems to have an important role in attenuating the oxidative stress and tissue injury after an ischemic insult in rat hind limb muscle tissue or an immunogenic challenge with proteose peptone in mouse peritoneal cavity. Under normal circumstances SOD3 does not seem to have a particular role in these tissues and is upregulated only when necessary (van Deel et al. 2008). In this respect, the thyroid gland is an interesting organ since its oxidative status is much higher due to the H_2O_2 needed for the thyroid hormone synthesis, and a certain basal level of ROS is actually required for normal thyroid function (reviewed by Song et al. 2007; Poncin et al. 2009). Thyroid is known to have efficient antioxidative defense systems based on the various peroxiredoxins, glutathione peroxidases, thioredoxin and its reductase as well as catalase (reviewed by Song et al. 2007). However, the role of SOD3 has been only little characterized in thyroid function under normal or disease conditions. Therefore, we investigated the *SOD3* expression in and its effect on normal thyroid gland and analyzed the changes in hyperproliferative glands.

We first determined the degree of *SOD3* expression in normal rat thyroid gland, and when compared to other tissues with higher than average oxidative status, namely aorta, kidney and liver, thyroid gland had expression levels close to those in aorta which is considered a major *SOD3* expression site (MS III; Laukkanen et al. 2002). This represents only the relative amount of *SOD3* mRNA, and it would be useful to measure also SOD3 enzyme activity in thyroid tissue to see how it compares to the other tissues. Conveniently, Marklund (1984) has presented extensive data on SOD3 enzyme activity in different tissues of several animal species, and indeed thyroid SOD3 activity was consistently higher as compared to heart or the average of all tissues, respectively, supporting our finding. The differences were smaller in rat than in human but, on the other hand, even rather little changes in tissue SOD3

concentration can induce marked physiological effects as shown in MS I and previous studies (Laukkanen et al. 2001; Laukkanen et al. 2002).

TSH, acting through the TSH-R, is the main regulatory factor of thyroid function being responsible for the thyrocyte proliferation as well as T3/T4 hormone synthesis. Interestingly, we found out that TSH stimulation of thyroid cells upregulated *SOD3* expression (MS III) which indicated some function for SOD3 in thyroid physiology. Proliferating cells *in vitro* increase their ROS production (Pani et al. 2000; Bello et al. 2003; Duval et al. 2003) and H_2O_2 is a crucial component in the process of thyroid hormone synthesis. Therefore, our next step was to assess whether the main thyroid signaling pathways downstream of TSH-R could stimulate SOD3 expression and whether SOD3 in turn could affect them. Indeed, stimulation of the cAMP dependent pathway leading to thyrocyte proliferation as well as the PLC dependent pathway leading to hormone synthesis both increased *SOD3* expression (MS III). Additionally, Ca2+ is an important cofactor for the Duox2 activation (Ameziane-El-Hassani et al. 2005), and therefore the observed increase in SOD3 expression after thapsigargin administration (MS III) would indicate that SOD3 expression is induced during hormonogenesis. However, the H_2O_2 generation capacity of Duox2 was considerably higher than that of SOD3 (MS III) and, thus, a role of SOD3 derived H_2O_2 in thyroid hormone synthesis seems very unlikely.

On the other hand, our siRNA data (MSIII) suggests that SOD3 could rather participate in thyrocyte proliferation instead. Considering a previous study in which THS was found to activate the MAPK/Erk in rat FRTL-5 thyroid cells in a cAMP-dependent manner (Iacovelli et al. 2001) and our data on Ras/Erk/SOD3 cascade (MSI) there seems to be a possibility that TSH could induce *SOD3* expression through Erk. However, later another study with dog and human thyrocytes suggested that the TSH effect is an artifact produced by proliferation stimulating contaminants in the hormone preparation (Vandeput et al. 2003). It was established, though, that Erk activation was still necessary for DNA synthesis in thyroid cells but the actual mechanism still remains uncertain.

Goiter formation is a result of thyrocyte hyperproliferation due to various causes such as thyroid hormone depletion, TSH-R stimulating autoantibodies as in Grave's disease or dietary iodine deficiency producing overt TSH release and subsequent endemic goiter. As an indication of heightened oxidative stress in goitrous thyroid gland the antioxidant enzymes peroxiredoxins and glutathione peroxidases as well as oxidized lipids are upregulated (Nadolnik and Valentyukevich 2007; Poncin et al. 2008a). However, usually the

antioxidant system can adequately process the increased ROS load during goitrogenesis and, thus, maintain the functionality of the gland (Poncin et al. 2008a; Poncin et al. 2009). On the other hand, elevated but subtoxic H_2O_2 level has been shown to be a potent mitogenic factor in cell cultures (reviewed by Burdon 1995), and also in this study SOD3 promoted cell proliferation through increased Ras-mitogenic pathway activation and likely through H_2O_2 generation (MS I).

To determine whether SOD3 was involved in thyroid hyperproliferation, we induced goiter growth in rats with a PTU treatment and the analyses revealed significantly higher *SOD3* expression in hyperproliferative goiter tissue (MS III), as has been previously shown in mouse models (Maier et al. 2007). However, the *SOD3* expression level in human endemic goiter or toxic adenoma did not show any differences when compared to normal thyroid tissue although the standard deviations were larger suggesting higher variation in the human disorders (MS III). In addition, some discrepancy was observed in the rat goiter wet weight (MS III) which implies that PTU administration in drinking water is not necessarily a secure way to induce goiter since it is difficult to assess whether the animals drink similar amounts. It is also possible that the rat with extremely large goiter had an undetected deficiency or illness making it more vulnerable to thyroid hormone depletion. Therefore, it is likely that there are several regulatory mechanisms involved which determine the final *SOD3* expression level in tissues. Furthermore, the results from rat and mouse models may not be fully comparable to the human disease which should be taken into account while interpreting and extrapolating the data.

Hyperproliferation is characteristic also to transformed cells and therefore we investigated if *SOD3* expression is further upregulated in cancerous thyroid cells *in vitro* and *in vivo*. To our surprise, a qPCR assay revealed a marked reduction of *SOD3* mRNA in rat thyroid cancer cell lines and also in human patient samples (MS III). This experiment brought out an interesting controversy in *SOD3* expression in goitrous and cancerous thyroid cells: *SOD3* expression increases in goiter and decreases in cancer. In many cases, cancer cells from e.g. ovary, pancreas, liver and breast are known to depend on high level of ROS generation which seems to aid in their proliferation (Szatrowski and Nathan 1991; Laurent et al. 2005; Teoh et al. 2007) since ROS and H_2O_2 induce several mechanisms which promote tumor growth such as VEGF expression, angiogenesis and activation of MAPK pathways (Lin et al. 2007; Ushio-Fukai and Nakamura 2008). Our finding could be explained by the differential effect of SOD mimetics on normal and cancer cells described by Laurent and coworkers (2005) who suggest that such factors raise the H_2O_2 to

toxic levels for cancer cells with often defective antioxidant defenses but do not present a threat to normal cells and, thus, SOD downregulation would be beneficial for cancer progression. Therefore, SOD3 expression could be a necessity for goitrous hyperplastic thyrocytes which respond to proliferative H_2O_2 signal as they are still able to keep its concentration at a subtoxic level but in thyroid cancer tissue and cells the downregulated SOD3 could not raise the H_2O_2 level high enough to prevent their proliferation.

Interestingly, the reduction in *SOD3* expression was clearly dependent on the degree of cancer malignancy. In the least aggressive form, rat PC PTC cells, the *SOD3* expression still remained at detectable level whereas in the very aggressive PC E1A cells, similar to human anaplastic carcinoma cells, it was almost completely missing (MS III). Similar tendency was seen in the PTC patient samples. Recent findings suggest that the increased number of Ras oncogene activating mutations correlate with the aggressiveness of thyroid tumor cells (Garcia-Rostan et al. 2003). Furthermore, excessive Ras activity could downregulate the traditional thyroid cancer differentiation markers such as Tg, TPO, NIS and some thyroid specific transcription factors possibly by epigenetic regulation (De Vita et al. 2005; Gazin et al. 2007). This model could also be applicable to our findings since *SOD3* is known to have a GpC island whose methylation is decreased in the proliferating cell population in atherosclerotic plaques (Laukkanen et al. 1999). Accordingly, *SOD3* expression could be considered as a differentiation marker for thyroid cancer indicating approximately the level of transformation, but the true connections between Ras, SOD3 and cancer aggressiveness as well as the molecular mechanism of SOD3 downregulation require further in-depth studies.

6 CONCLUSIONS

Our studies have elucidated the role of an antioxidative enzyme SOD3 in various tissues under normal and pathological conditions. Generally, SOD3 expression is activated when the tissues are confronted with oxidative stress, and therefore we studied the effect of ischemic injury and inflammation on SOD3 expression. Furthermore, since thyroid gland is naturally under high oxidative stress even in normal conditions we wanted to see if SOD3 has a functional role in this organ.

We found that SOD3 promoted tissue healing after an ischemic insult by at least two mechanisms. Firstly, SOD3 activated the Ras-Erk1/2 mitogenic pathway known to be a major regulator of cell proliferation. This pathway induced the pro-proliferative factors VEGF and cyclin D1 which resulted in myocyte proliferation. This study described a novel mechanism for SOD3 as a growth regulating molecule (MS I). Secondly, SOD3 prevented inflammatory leukocyte migration thereby reducing the oxidative stress induced tissue damage by neutrophils and macrophages. It had a marked inhibitory effect on the activation of the ROS sensitive transcription factor NFκB and the expression of many proinflammatory cytokines, chemokines and cell adhesion molecules which take part in leukocyte migration (MS II).

Studies with the thyroid gland revealed an important role for SOD3 related to our previous findings (MS III). In thyroid gland SOD3 expression was high, comparable even to the known high level in the vasculature, and markedly stimulated by TSH suggesting a role in thyroid physiology. Indeed, we found that thyrocyte proliferation was partly dependent on the SOD3 expression in normal thyroid cells and, analogously, SOD3 expression was increased in hyperproliferative goitrous thyroid tissue. However, we discovered an interesting exception in thyroid cancer tissue: SOD3 expression was increasingly downregulated in correlation with cancer aggressiveness and despite the fact that oxidative stress and inflammation are common in tumor microenvironment.

These studies have shown that SOD3 can act as a growth promoting factor in injured muscle tissue and in thyroid gland, as well as an anti-inflammatory factor regulating leukocyte migration. We have described new mechanisms of action for SOD3 at the molecular level, and started to characterize its previously poorly known role in the thyroid gland. According to our results SOD3 could be used as a therapeutic agent in treatment of ischemic injuries and as a differentiation marker in malignant thyroid transformation.

7 ACKNOWLEDGEMENTS

I want to express my gratitude to Prof. Giancarlo Vecchio, the head of Department of Cellular and Molecular Biology and Pathology 'L. Califano', for admitting me to the International Doctorate Program in Molecular Oncology and Endocrinology (DIOEM) and therefore providing me with a great opportunity to work abroad in a very enthusiastic scientific environment.

Many thanks to my thesis supervisor both in Finland and Italy, Dr. Mikko Laukkanen, who has given me the opportunity to work in his research group with several interesting projects.

I would also like to thank Prof. Massimo Santoro for having me to work in his laboratory, and all the people there for helping me out in everyday jobs as well as teaching me new things in the (sometimes mysterious) world of laboratory practises: Prof. Giuliana Salvatore, Dr. Mariolina Castellone, Dr. Anna Tamburrino, Dr. Donatella Vitagliano, Dr. Valentina de Falco, Dr. Annamaria Cirafici, Dr. Paolo Salerno, Dr. Tito Nappi, Dr. Aniello Cerrato, and the students Mara Cantisani, Roberto Bellelli, Magesh Muthu, Angela Di Giambattista, Simona Ventre and Manikandan Mayakannan. You all made my stay in Italy very pleasant and I will always carry the good memories with me!

Lastly, I want to thank my family and friends in Finland who have supported and encouraged me and lightened up my days far away from home with their calls and emails.

I also wish to acknowledge the following Finnish foundations for financial support during my studies: Ellen and Artturi Nyyssönen Foundation and Finnish Cultural Foundation, Varsinais-Suomi Regional Fund.

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APPENDED PUBLICATIONS

Extracellular Superoxide Dismutase Is a Growth Regulatory Mediator of Tissue Injury Recovery

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Extracellular superoxide dismutase (*SOD3*) gene therapy has been shown to attenuate tissue damages and to improve the recovery of the tissue injuries, but the cellular events delivering the therapeutic response of the enzyme are not well defined. In the current work, we overexpressed SOD3 in rat hindlimb ischemia model to study the signal transduction and injury healing following the *sod3* gene transfer. The data suggest a novel *sod3* gene transfer–derived signal transduction cascade through Ras-Mek-Erk mitogenic pathway leading to activation of AP1 and CRE transcription factors, increased vascular endothelial growth factor (VEGF)-A and cyclin D1 expression, increased cell proliferation, and consequently improved metabolic functionality of the injured tissue. Increased cell proliferation could explain the improved metabolic performance and the healing of the tissue damages after the *sod3* gene transfer. The present data is a novel description of the molecular mechanism of SOD3-mediated recovery of tissue injury and suggests a new physiological role for SOD3 as a Ras regulatory molecule in signal transduction.

Received 7 July 2008; accepted 20 November 2008; advance online publication 23 December 2008. doi:10.1038/mt.2008.282

INTRODUCTION

Acute ischemic injury and consequent healing process involve a cascade of cellular events including increased expression of growth factors and endothelial cell migration and proliferation. Reactive oxygen species (ROS) that are synthesized in injured tissues are important factors in injury response mediating a number of cellular functions.[1–5] It has been previously reported that ROS, e.g., NADPH oxidase-derived superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), are able to activate cell stress Ras-Erk1/2 signaling pathway leading to the induction of vascular endothelial growth factor (VEGF) production.[6,7] However, the extracellular sources of H_2O_2 causing induction of signal transduction are not thoroughly investigated yet.

Superoxide dismutases (SOD1, SOD2, and SOD3) are utilizing $O_2^{\bullet -}$ in dismutase reaction resulting in H_2O_2 formation, which is further metabolized to oxygen and water by catalase and glutathione peroxidase.[8–15] Extracellular superoxide dismutase, *sod3*, gene transfer has been shown to have a therapeutic response in various animal injury models *e.g.*, hamster ischemia model, mouse liver intoxication model, and rabbit restenosis model[16–19] indicating the importance of the enzyme for the tissue recovery. Commonly, SOD3 has been suggested to be an antioxidative enzyme[12] that has also an anti-inflammatory nature,[20] which could partially explain reduced injuries after *sod3* gene transfer, but the exact cellular mechanisms leading to improved healing have not been defined.

In the present work, we focused on the SOD3-driven healing process in rat hindlimb ischemia injury model and characterized the cellular events derived from *SOD3* gene transfer. According to our data, SOD3 caused at the biological level increased glucose metabolism and cell proliferation suggesting improved functionality of the tissue with consequent therapeutic response in the injured region. At the biochemical level, we identified a novel signal transduction pathway mediating the therapeutic response of adenovirus SOD3 (*AdSOD3*) gene transfer. SOD3 overexpression resulted in the activation of mitogenic Ras-Erk1/2 and PI3 kinase-Akt signaling pathways with consequently increased expression of transcription factors (like CRE and AP1) and VEGF-A. The effect of SOD3 on the Ras growth factor pathway may at least partially explain the improved healing reported in our previous publications.[17–19]

RESULTS

AdSOD3 gene transfer improves the functionality of the injured tissue

SOD3 (AdSOD3) and LacZ (AdLacZ) adenoviruses (0.5×10^9 pfu) were injected into rat hindlimb muscle tissue simultaneously with ligation of femoral artery, and detected by β-galactosidase staining, reverse transcription (RT)-PCR techniques, and SOD activity measurement from Concanavalin A–purified muscle homogenates at the end of the follow-up periods. The β-galactosidase staining showed *in vivo* expression of transferred gene in muscle and connective tissue cells at the injury site indicating that mostly these cells, and the cells having direct contact to them, mediated the effect of adenovirus gene transfer. The overall transduction efficiency in the injury region in different time points varied

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between 0.8 and 5% based on the X-gal staining. AdSOD3 mRNA expression in the injury was detected by RT-PCR amplification of the expected 574 bp sequence from the muscle preparation (**Figure 1a**). The increased SOD enzyme activity caused by adenovirus gene transfer was determined from Concanavalin A–purified 3-day muscle tissue homogenates.[21] The *AdSOD3* gene transfer increased the tissue SOD3 activity approximately twofold as compared to LacZ control muscles (2.6 U/mg ±0.09 and 1.5 U/mg ±0.31, $P < 0.01$, respectively). The overall effect of *AdSOD3* gene transfer at 3-daytime point on ROS and reactive nitrogen species by dihydroethidium and 3-nitrotyrosine is shown in **Figure 1b**, and is in line with the transgene expression as well as with the generally recognized antioxidative role of SOD3. We have demonstrated the *in vivo* antioxidative capacity of AdSOD3 in our previous reports using vascular wall as a model tissue,[18,19] and a recent article suggested a major role for SOD3 in ROS and reactive nitrogen species biology[22–24] confirming that SOD3 is able to reduce expression of superoxide and reactive nitrogen species.

To study the healing of the injured tissue, we monitored the *in vivo* functionality by determining the ratio of radioactive [^{18}F] FDG glucose accumulation into injured vs. healthy hindlimb after systemic delivery of the Positron emission tomography marker. The analysis showed significantly ($P < 0.05$) increased tissue metabolic performance at 10-daytime point in AdSOD3 animals as compared to AdLacZ-transduced animals, which yielded higher signal ratio intensity between injury and healthy leg (**Figure 1c**). The individual differences in glucose metabolism in AdLacZ rats between injured and healthy uninjured leg were 46, 64, and 58% (56% ± 9.17), whereas the corresponding differences in AdSOD3 rats were 24, 27, and 35% (29% ± 5.69).

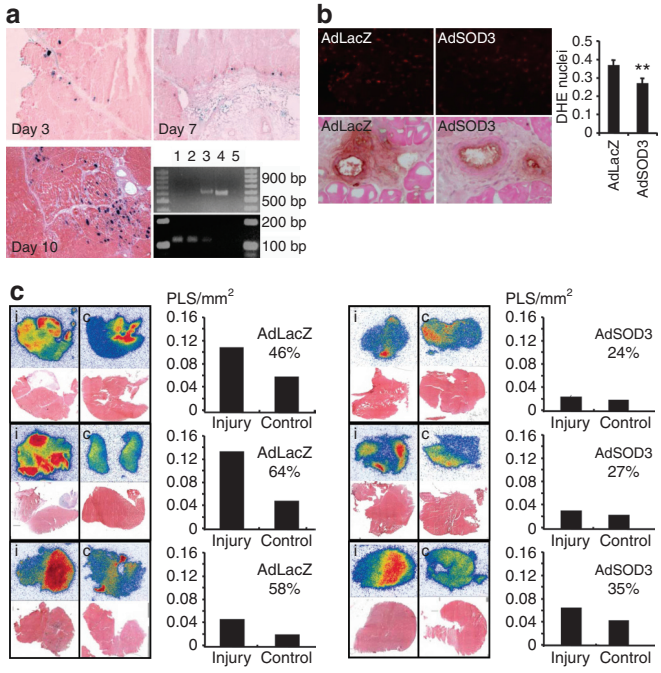

Figure 1 Detection of transgenes and their effect on glucose metabolism. (**a**) AdLacZ transgene expression at 3-day, 7-day, and 10-daytime points was analyzed with β-galactosidase staining and adenovirus superoxide dismutase 3 (AdSOD3) expression by reverse transcription (RT)-PCR analysis; 1, uninjured control muscle; 2, AdLacZ control muscle; 3, AdSOD3-transduced muscle; 4, SOD3-positive control; 5, water control. Upper-lane amplification shows specific 574 bp *sod3* RT-PCR fragment and lower lane 122 bp *β-actin* RT-PCR fragment. (**b**) Upper panels show in AdSOD3 tissues 28% decreased dihydroethidium (DHE) labeling for reactive oxygen species ($P < 0.01$). The lower panels show 3-nitrotyrosine staining for reactive nitrogen species modified proteins from 3-day tissue sections. (**c**) Positron emission tomography *in vivo* imaging. The analysis at 10-daytime point showed increased accumulation of [^{18}F]FDG in the injury leg as compared to the healthy leg from the same animal reflecting decreased metabolic performance. The percentage describes the difference in accumulation between injured tissue and normal control tissue. Each imaging figure contains digital autoradiography of injury leg (i), control leg (c), and their corresponding hematoxylin–eosin stainings.

Biological effects of *SOD3* gene transfer

To analyze the biological phenomena responsible for the SOD3-mediated injury recovery, we investigated the effects on angiogenesis and proliferation. Interestingly, the analysis of new vessel formation in tissue sections showed increased vascular development in both AdSOD3 and AdLacZ animals till 10-daytime point without significant differences (**Figure 2a**). On the other hand, when analyzing the cell proliferation in injured tissues by Ki67 staining we found a significantly ($P < 0.05$) increased cell division in AdSOD3-transduced ischemic muscles at 3-day and 7-daytime points as compared to AdLacZ controls (**Figure 2b**), even though the overall proliferation rate in skeletal muscle tissue injury was extremely low (on average 0–8 cell proliferations/histological section). These results lead us to hypothesize a proliferative effect of SOD3 as a mechanism of tissue injury healing upon *sod3* gene transfer.

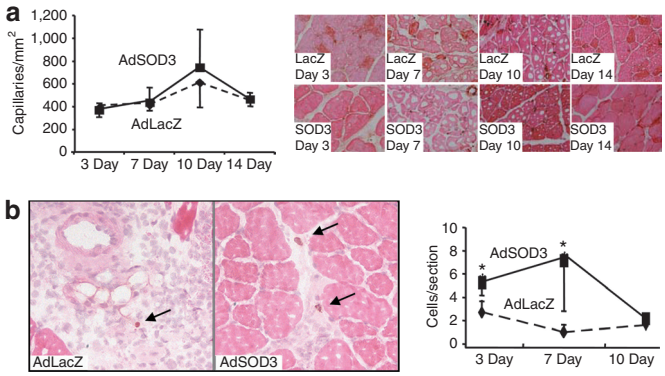

Figure 2 Determination of new vessel formation and cell proliferation. (**a**) Measurement of capillary density from ischemic tissues. Solid line represents adenovirus superoxide dismutase 3 (AdSOD3) and hatched line AdLacZ animals. The neoangiogenesis increased in both groups till 10-daytime point. (**b**) Ki68 staining showed significantly increased proliferation in AdSOD3-treated skeletal muscles at 3-day and 7-daytime points. Solid line represents AdSOD3 and hatched line AdLacZ animals. Arrows indicate Ki67+ proliferating cells.

SOD3 induces activation of the Ras-Erk and Akt signaling pathways

The analysis of the biological effects derived from *sod3* gene transfer and the finding of increased *in vivo* cell proliferation prompted us to investigate a role for SOD3 in controlling cell growth through mitogenic signaling cascades. Because it has been shown that H_2O_2 is able to activate the Ras-MAPK signaling pathway,[25] we investigated by pull-down assay the activation of Ras in response to SOD3 both *in vivo* and *in vitro*. As shown in **Figure 3a**, SOD3

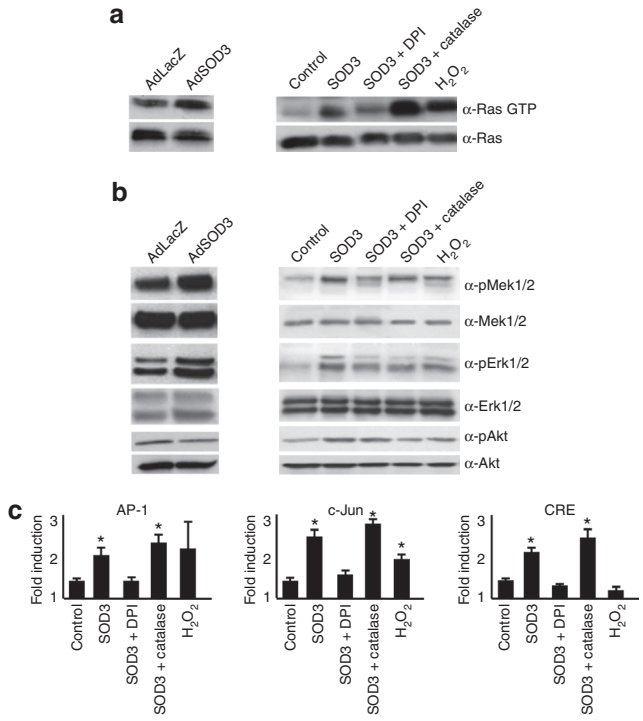

Figure 3 Superoxide dismutase 3 (SOD3)-derived signal transduction. (**a**) Pull-down assay resulted in Ras activation after *sod3* gene transfer *in vivo* (AdLacZ and AdSOD) and *in vitro* (control, SOD3, SOD3 + DPI, SOD3 + catalase, H_2O_2). (**b**) SOD3 affects the phosphorylation of growth factor signaling molecules. Analysis of Mek1/2 and Erk1/2 phosphorylation from 7-day adenovirus SOD3 (AdSOD3) tissue homogenates showed increased Mek1/2 and Erk1/2 phosphorylation as compared to AdLacZ control muscles. *In vitro* analysis SOD3 transfection increased phosphorylation of Akt and Mek-Erk signaling pathways. (**c**) Luciferase activity assay showed increased SOD3-driven activation of AP1, cJun, and CRE, which was affected by NADPH oxidase inhibitor DPI but not by catalase.

markedly increased the GTP loading on Ras 7 days after *AdSOD3* gene transfer as well as after *sod3* transfection into HEK 293T cells. Because NADPH oxidase and catalase are closely related to SOD3-driven reaction by providing the substrate and metabolizing the reaction end product, respectively, we checked their effects on SOD3-derived Ras activation. As shown in **Figure 3a**, diphenyleneiodonium sulfate (DPI) caused clear reduction in Ras GTP loading, whereas catalase, as an intracellular molecule, had no inhibitory effect on SOD3-driven Ras activation. The dismutase reaction end product, H_2O_2, strongly induced Ras supporting our data that SOD3 is able to stimulate mitogenic signal cascade (**Figure 3a**, **Supplementary Figure S1**).

GTP-bound Ras is activating a number of signaling cascades, including angiogenic and mitogenic pathways. We, therefore, investigated the status of Mek1/2-Erk1/2 and PI3K-Ak pathways in response to *sod3* gene transfer *in vivo* in tissue environment where other redox enzymes originating from different cell types are interacting with SOD3. Interestingly, *AdSOD3* gene transfer caused increased *in vivo* phosphorylation of Mek1/2 and Erk1/2 in rat hindlimb injury model at 7-daytime point, but the activation status of Akt in tissues was not affected by the virus transduction supporting our observation that angiogenesis was not increased by AdSOD3 (**Figure 3b**, **Supplementary Figure S1**).

To further characterize the signal transduction pathways and to study the interactions between different proteins *in vitro*, we chose HEK 293T cells as a model system, which show the general effect on signaling that corresponds to previously published data. In cell culture conditions SOD3 increased phosphorylation of Mek1/2, Erk1/2, and Akt showing that a single-cell–type model may be functionally different from the tissues consisting of multiple cell types with different functions (**Figure 3b**, **Supplementary Figure S1**). In line with Ras pull-down assay DPI showed modest decrease in SOD3-derived Mek1/2 and Erk1/2 activation and H_2O_2 caused increased phosphorylation of Mek-Erk and PI3K-Akt pathways.

The signal transduction cascades activated by Ras lead to changes in gene expression. Thereby, Ras signaling controls fundamental cell biological processes like proliferation, differentiation, survival, and invasion, which are mediated through the involvement of several transcription factors inside the nucleus. We analyzed whether SOD3 could stimulate the activation of different transcription factors as final readout of the Ras-Mek1/2-Erk1/2 pathway. As shown in **Figure 3c**, the co-transfections of *sod3* with *pAP1-Luc*, *pCRE-Luc*, and *pFA2-cJun* luciferase reporter genes into HEK 293T cells resulted in significantly ($P < 0.05$) increased AP1 and cJun activity, which was evident also after H_2O_2 exposure, whereas *sod3*-mediated increased CRE activation was not reproduced by H_2O_2 (**Figure 3c**). Interestingly, incubation of *sod3*-transfected cells with NADPH oxidase inhibitor DPI diminished the activation of the transcription factors, whereas catalase had no inhibitory effect on SOD3-derived activation. The activation of AP1 and CRE nuclear molecules could then explain the proliferative effect mediated by SOD3 *in vivo*.

Finally, we analyzed the expression of the CRE/AP1 target genes *cyclin D1* and *vegf-a in vivo* and *in vitro*. As shown in **Figure 4a,c**, 10 days after the injury, we found increased levels of both molecules upon AdSOD3 transduction. In AdSOD3 animals the *vegf-a* mRNA expression was increased threefold and *cyclin D1* twofold at 10-daytime point ($P < 0.01$ and $P < 0.05$, respectively) as compared to uninjured muscle preparation (**Figure 4a,c**). Interestingly, the *cyclin D1* expression in AdLacZ rats was sixfold decreased as compared to AdSOD3 tissues ($P < 0.01$) supporting the pro-proliferative effect of *sod3* gene transfer. These data are in line with our observation that the *sod3/Ras/Braf/Mek/Erk* molecules have a clear stimulatory impact on both *cyclin D1* and *vegf-a* mRNA synthesis, as shown by qRT-PCR analysis (**Figure 4b,d**).

A positive feedback loop couples Ras and SOD3

To determine the possibility for feedback stimulation of SOD3 we measured the levels and the activity of SOD3 in HEK 293T cells transiently transfected with *Ras*, *Braf*, *Mek1*, *Erk1*, and *Akt*. Interestingly, mRNA levels and protein activity of SOD3 increased upon expression of *Ras/Erk* but not of *Akt* (**Figure 4e,f**). Further on, incubation of HEK 293T cells for 6 hours with 500 µmol/l concentration of SOD3 dismutase reaction end product, H_2O_2, or 50 ng/ml NADP significantly increased ($P < 0.05$ and $P < 0.001$, respectively) *sod3* mRNA production. Finally, a Mek-specific inhibitor, U0126, and DPI showed decreased *sod3* mRNA expression ($P < 0.05$) confirming that Ras-Erk1/2 pathway is a major signal transduction cascade controlling SOD3 production, which is also controlled by

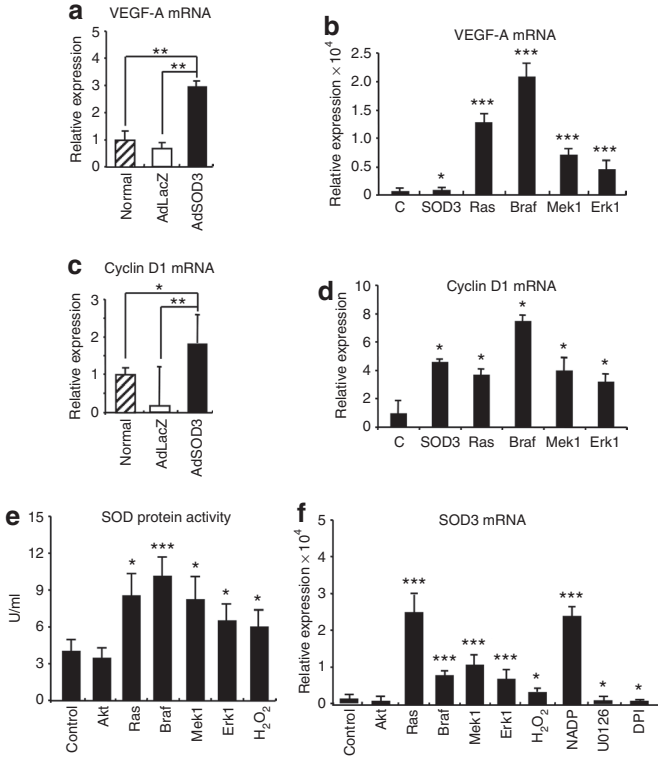

Figure 4 Gene expression analysis from 10-day tissue samples. Hatched bar represents uninjured control tissue, white bar represents AdLacZ-transduced tissue, and black bar adenovirus superoxide dismutase 3 (AdSOD3) transduced tissue. (**a**) Quantitative reverse transcription (RT)-PCR analysis for *vegf-a* expression in injured tissue. AdSOD3 increased significantly rat *vegf-a* mRNA production. (**b**) *Vegf-a* mRNA expression was significantly increased *in vitro* after transfection with *sod3* and mitogenic signaling pathway molecules *RasV12*, *Braf V600E*, *Mek1*, and *Erk1*. (**c**) Quantitative RT-PCR analysis for *cyclin D1* expression in injured tissue. AdSOD3 increased significantly *cyclin D1* expression in damaged tissue as compared to normal, uninjured control tissue. Difference was more prominent when compared to AdLacZ-treated tissue injury. (**d**) *Cyclin D1* mRNA expression after *sod3*, *RasV12*, *Braf V600E*, *Mek1*, and *Erk1* transfection *in vitro*. (**e**) SOD protein activity is activated *in vitro* by mitogenic Ras-Erk1 pathway molecules, and by H_2O_2 treatment. *Akt* transfection failed to increase the SOD3 production. (**f**) Quantitative RT-PCR analysis shows that increased SOD activity was due to increased *sod3* mRNA production.

NADPH oxidase activity. These observations together with ability of SOD3 to activate Ras-Erk and Akt led us to postulate that Ras and SOD3 activation might be coordinately regulated through a positive feedback loop most likely through H_2O_2.

DISCUSSION

Hindlimb ischemia creates an optimal *in vivo* environment to study the interaction between redox molecules and surrounding tissue in response to changes in tissue homeostasis. We have previously demonstrated SOD3-derived therapeutic effect in different injury models,[17–19] although the exact molecular mechanism responsible for the response has not been presented yet. In the current work, we investigated the mechanism of SOD3-derived healing focusing on the influence of SOD3 on intracellular signaling networks and consequent biological responses. We used adenovirus-mediated gene transfer to obtain high temporal expression and clear cellular response in the target tissue, which is further enhanced by the ability of secreted SOD3 to reversibly bind to the cell membranes,[26,27] bringing cells that are not infected by the virus vector under the influence of exogenous SOD3. Staining of AdLacZ-transducted tissue sections showed strong expression that was scattered over the injury region indicating spreading of the virus from initial injection sites expanding the injury region affected by the virus-mediated expression of the transgenes (**Figure 1a**). We have previously shown in a mouse model that relatively high virus amount, 1×10^9 pfu, is needed to significantly increase plasma SOD activity after systemic tail vein injection of AdSOD3,[17] which could be explained by tight posttranscriptional and posttranslational regulation of *sod3* affecting also transgene expression in the target tissues.[28–30] The twofold increase in the total SOD3 activity in Concanavalin A–purified muscle homogenates after *AdSOD3* gene transfer yielding a therapeutic response is in line with our previous studies showing decreased LDL oxidation *in vitro* with 10 ng/ml concentrations,[28] and decreased liver damages and attenuated neointima formation with two- to threefold increased SOD3 concentrations.[17,18]

In the current work, we observed higher *in vivo* functional muscle metabolic performance in Positron emission tomography imaging of AdSOD3-transduced tissues as compared to AdLacZ controls (**Figure 1c**), suggesting an important role for SOD3 in controlling the growth factor pathway leading to improved tissue injury healing. It is noteworthy that decreased accumulation of [^{18}F]FDG Positron emission tomography tracer demonstrated the biological functionality of the *AdSOD3* gene transfer proving that adenovirus-derived expression of SOD3 results in a therapeutic response *in vivo*. To explain the improved tissue functionality caused by *AdSOD3* gene transfer, we first focused on angiogenesis in damaged muscles. Endothelial staining analysis showed increased new vessel formation both in AdSOD3 and AdLacZ rats without significant differences (**Figure 2a**), therefore, suggesting that SOD3 is not a major angiogenic factor and that the SOD3-mediated, improved metabolic performance of injured tissue is not due to promotion of new vessel formation. We next investigated whether the *sod3* gene transfer could correspond to increased cell division. Ki67 staining for proliferating cells in injured muscle tissues showed a two- to sevenfold increase in AdSOD3-treated animals at 3-day and 7-daytime points (**Figure 2b**). Such a phenomenon could at least partially explain the observed improved performance in glucose metabolism.

To explain the mechanism causing the increased cell proliferation, we focused on the intracellular signaling activated by either AdSOD3 *in vivo* or by *sod3* transfection *in vitro*. Both AdSOD3 transduction of tissues and transient transfection of *sod3* activated Ras G-protein in the target cells, indicating a regulatory role for the enzyme in mitogenic signal transduction. Interestingly, DPI attenuated SOD3-derived Ras activation showing an upstream regulatory effect for NADPH oxidase in SOD3 signaling, suggesting that the availability of the substrate superoxide anion creates a rate-limiting step in the dismutase reaction. Hydrogen peroxide–derived Ras activation, on the other hand, suggests that SOD3 signal is mediated by the dismutase reaction end product (**Figure 3a**). Previous studies have shown that H_2O_2 is able induce the phosphorylation of a large number of cell surface molecules, such as fibroblast growth factor receptor 1,[31] epidermal growth factor

receptor,[32] platelet-derived growth factor receptor β,[33] Axl receptor tyrosine kinase,[34] and insulin receptor,[35] which all are involved in Ras G-protein activation. In addition, H_2O_2 can mediate the cellular signaling by inactivating protein tyrosine phosphatases,[36] suggesting a wide variety of cell membrane–associated molecules involved in H_2O_2 signaling. Therefore, it is likely that the effect of SOD3 on Ras-Erk activation is not mediated by a single factor but rather by a collaborative effect of tyrosine kinase receptors and protein tyrosine phosphatases.

By analyzing the Ras-Erk pathway, we detected increased phosphorylation in Mek1/2 and Erk1/2 mitogenic signal transduction pathway in AdSOD3-treated hindlimb injuries. To confirm these observations, we then investigated the same pathway *in vitro*. Transfection of *sod3* promoted the activation of Mek1/2 and Erk1/2, suggesting that SOD3 is able to modulate mitogenic growth signaling. The Akt phosphorylation was increased *in vitro* but not *in vivo*, further stressing the importance of mitogenic pathway activation after *sod3* gene transfer (**Figure 3b**).

Finally, at the nuclear level, our experiments proved that SOD3 significantly induces CRE and AP1/cJun transcription factors (**Figure 3c**) that are known to be involved in cell survival through activation of target genes such as *vegf-a* and *cyclin D1*.[37,38] In line with Ras pull-down assay, luciferase assay showed decreased CRE and AP1/cJun activation in the presence of DPI, whereas catalase had no inhibitory effect on SOD3-derived transcription factor activation, further suggesting the regulatory effect of substrate availability on SOD3 activity.

Interestingly, previous reports have shown the ability of VEGF-A to promote new myoblast generation after muscle transplantation,[39] to enhance myoblast migration, and to decrease apoptosis in hindlimb ischemia model,[40] suggesting more general proliferative effect for VEGF-A. To further analyze the role of SOD3 in tissue recovery and in increased cell proliferation in recovering muscles, we determined the effect of *AdSOD3* gene transfer on VEGF-A (**Figure 4**). The analysis of *vegf-a* expression from the damaged tissues showed that *AdSOD3* gene transfer significantly increased rat endogenous *vegf-a* mRNA expression. Accordingly, the MAPK mitogenic pathway has been shown to be involved in tissue damage recovery causing increased cell proliferation.[41] Transfection of *RasV12*, *Braf V600E*, *Mek1*, and *Erk1* into HEK 293T cells increased *vegf-a* mRNA production *in vitro*, supporting the connection between SOD3 and VEGF-A signal transduction. Even though SOD3 failed to increase Akt phosphorylation *in vivo,* increased *vegf-a* expression can be explained by SOD3-driven activation of Ras-Erk mitogenic pathway. Moreover, we also identified activation of *cyclin D1* expression *in vivo* in response to *sod3* gene transfer supporting AdSOD3-driven cell proliferation. To further characterize SOD3-related signaling, we investigated the effect of Ras-Erk pathway on SOD3. By transfecting *RasV12*, *Braf V600E*, *Mek1*, *Erk1*, and *Akt* into HEK 293T cells we observed increased SOD3 production, suggesting a possible positive feedback on SOD3-mediated Ras activation. Lastly, as a final confirmation for the regulatory role of NADPH oxidase in *sod3* gene expression, NAPD significantly increased and DPI significantly reduced endogenous *sod3* mRNA synthesis, suggesting that extracellular H_2O_2 production is linked to the availability of extracellular superoxide anion.

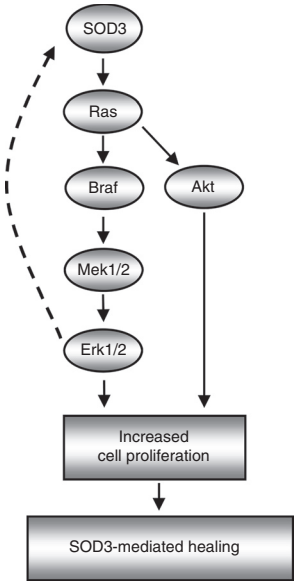

Figure 5 Suggested signaling pathway for superoxide dismutase 3 (SOD3). SOD3 transfection activates Ras GTP loading, causing increased phosphorylation of Ras-Erk and PI3K-Akt pathways. The activation of mitogenic signaling cascade could explain partly the improved healing in *SOD3* gene therapy experiments. The SOD3-driven Ras activation and Ras-Braf-Mek1-Erk1-derived increased production of SOD3 suggests positive feedback for SOD3 at the level of Ras.

In summary, we have shown that SOD3 regulates molecules of the Ras-Erk cascade and promotes nuclear signals of cellular proliferation and demonstrated how *AdSOD3* gene transfer into hindlimb ischemia affects the endogenous *vegf-a* and *cyclin D1* expression. A scheme of the suggested signaling pathway causing SOD3-mediated cellular responses is described in **Figure 5**. SOD3 activates the Ras-Braf-Mek1/2-Erk1/2 and Akt signal transduction by increasing Ras GTP loading. The activation of the cascade leads to the nuclear events through the involvement of the CRE and AP1/cJun transcription factors that, by increasing the expression of cell survival and proliferation-related target genes, induce improved healing process.

In conclusion, our findings describe a novel physiological role for SOD3 as a Ras signal transduction pathway regulatory molecule that is able to stimulate mitogenic signaling and elucidate the molecular mechanism for *SOD3* gene transfer–mediated therapeutic response in tissue damages.

MATERIALS AND METHODS

Adenovirus production. Rabbit *sod3* and bacterial *β-galactosidase* cDNAs were cloned into replication-deficient E1-partially-E3-deleted AdBglII for AdSOD3 and AdLacZ adenovirus production in 293 cells in the University of Turku Biotechnology Center as described.[28,42] The virus titer was adjusted to 1×10^{10} pfu/ml for gene transfer experiments.

Hindlimb injury. Male 4- to 5-weeks-old (86–115 g) Fisher 344 rats (n = 16) (Harlan, Horst, the Netherlands) randomized to 3-day, 7-day, 10-day, and 14-day groups were anesthetized with intraperitoneal administration of fentanyl-fluanisone (Janssen Pharmaceutica, Beerse, Belgium) and midazolame (Roche, Basel, Switzerland). Hindlimb ischemia was induced by surgical closure of the proximal femoral artery, lateral circumflex femoral artery, and distal femoral artery. The gene transfer with 0.5×10^9 pfu AdSOD3 or AdLacZ in 50 µl volume was done immediately after ligation

Table 1 PCR primers and cycling conditions

Gene	Primer	Tm
AdSOD3 for	GTT GCG TGA GCG GAA AGA TG	60
AdSOD3 rev	GTG AGC GCC TGC CAG ATC TC	
huSOD3 for	CTT CGC CTC TGC TGA AGT CT	60
huSOD3 rev	GGG TGT TTC GGT ACA AAT GG	
ratSOD3 for	GAC CTG GAG ATC TGG ATG GA	60
ratSOD3 rev	GTG GTT GGA GGT GTT CTG CT	
ratCyc D1 for	AAC GTC ACA CGG ACT ACA GG	55
ratCyc D1 rev	TGT TCC ATG GCT GGG GCT CTT	
ratVEGF-A for	CAA TGA TGA AGC CCT GGA GT	50
ratVEGF-A rev	TTT CTT GCG CTT TCG TTT TT	
huVEGF-A for	TCC GGG TTT TAT CCC TCT TC	55
huVEGF-A rev	TCT GCT GGT TTC CAA AAT CC	
ratβ-actin for	TCG TGC GTG ACA TTA AGG AG	55
ratβ-actin rev	GTC AGG CAG CTC GTA GCT CT	
huβ-actin for	TGC GTG ACA TTA AGG AGA AG	
huβ-actin rev	GCT CGT AGC TCT TCT CCA	

of the vessels. Uninjured muscle tissue was used as an additional control. Experimental Animal Committee of Turku University approved all experimental procedures according to the European Union guidelines.

Immunohistochemistry. Muscle tissues were embedded in optimal cutting temperature compound (Tissue-Tek, San Francisco, CA). Ten micrometer sections were stained with X-gal (Promega, Madison, WI), CD68 (Serotec, Oxford, UK), von Willebrand Factor (Abcam, Cambridge, UK), Ki67 (DakoCytomation, Glostrup, Denmark), 3-nitrotyrosine (Millipore, Billerica, MA), and counterstained with hematoxylin/eosin (Sigma, St Louis, MO). ROS were detected from fresh tissue sections by dihydroethidium bromide staining as described previously and ratio of dihydroethidium-positive nuclei/total nuclei was counted.

PCR. Messenger RNA was isolated from the pool of three to four animals or HEK 293T cells using Tri-reagent (Sigma) and GenElute mRNA miniprep kit (Sigma). The first strand synthesis was done using Revert-Aid M-MuLV (Fermentas, Burlington, Canada), PCR with JumpStart RedTaq ReadyMix (Sigma), and quantitative RT-PCR with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Primers and cycling conditions are shown in the **Table 1**.

Cell culture experiments. Signaling molecules and control vectors were transfected with Polyfect transfection reagent (Sigma) in to HEK 293T cells for expression studies. To determine the endogenous SOD3 expression, cells were treated with 20 µmol/l Mek inhibitor U0126 (Sigma), 500 µmol/l H_2O_2 (Sigma), 50 ng/ml NADP (Sigma), or 20 µmol/l DPI for 6 hours in serum-free media. Serum and cell samples were taken after incubation in serum-free medium for total SOD activity and quantitative RT-PCR analysis.

SOD3 protein activity assay. SOD total protein activity was determined as described.[43] Control vector transfection and serum-free media were used as controls for plasmid transfections and H_2O_2 incubations.

Concanavalin A sepharose purification. Concanavalin A sepharose (GE Healthcare, Chalfont St Giles, UK) purification was done as described.[21] Briefly, the tissues were homogenated, centrifuged, and applied to column in 500 µl volumes. The columns were washed with phosphate buffer, and SOD3 was eluted with 150 mmol/l α-methyl D-mannoside in 50 mmol/l sodium phosphate buffer.

Reporter assay. HEK 293T cells were transfected with expression vectors together with pAP1-Luc, pCRE-Luc, and pFA2-cJun luciferase reporters (Stratagene, Cedar Creek, TX). Luciferase activity was quantified with Tecan Ultra XFluor4 Fluorescence Reader (Tekan, Mannedorf, Switzerlard).

Western blotting analysis. Tissue samples or HEK 293T cells were homogenized in lysis buffer (50 mmol/l HEPES pH 7.5, 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/l EGTA, 1.5 mmol/l $MgCl_2$, 10 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 1 mmol/l Na_3VO_4, 10 µg approtinin/ml, 10 µg leupeptin/ml) (Sigma). Antibodies were rabbit anti-Braf (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Mek1/2 (Cell Signaling, Danvers, MA), pMek1/2 (Cell Signaling), 44/42 MAPK (Cell Signaling), p44/42 MAPK (Cell Signaling), Akt (Cell Signaling), and pAkt (Cell Signaling).

Ras pull-down assay. HEK 293T cells at 10 cm dishes were transfected with 5 µg of plasmids. The supernatant from lysed cells or tissue homogenates was incubated with GST-Raf1-RBD beads for 15 minutes at 4 °C and washed with lysis buffer. Active Ras G-protein-containing beads were collected, prepared for western blot, and analyzed by mouse anti-Ras (Upstate).

Analysis of* in vivo *glucose metabolism. Positron emission tomography imaging was done to 10-daytime point animals as described previously.[44] Sequential 30 µm cryo sections were exposed to imaging plate (Fuji, Tokyo, Japan) and scanned with Fuji Analyser (Fuji).

Statistical analysis. Statistical analysis was done using two-tail *t*-test for means.

SUPPLEMENTARY MATERIAL

Figure S1. Signal intensities for western blot analysis in **Figure 3a,b**.

ACKNOWLEDGMENTS

Academy of Finland, Sigrid Juselius Foundation, Paolo Foundation, and University of Turku Foundation supported this work.

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PLoS one

SOD3 Reduces Inflammatory Cell Migration by Regulating Adhesion Molecule and Cytokine Expression

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Abstract

Inflammatory cell migration characteristic of ischemic damages has a dual role providing the tissue with factors needed for tissue injury recovery simultaneously causing deleterious development depending on the quality and the quantity of infiltrated cells. Extracellular superoxide dismutase (SOD3) has been shown to have an anti-inflammatory role in ischemic injuries where it increases the recovery process by activating mitogen signal transduction and increasing cell proliferation. However, SOD3 derived effects on inflammatory cytokine and adhesion molecule expression, which would explain reduced inflammation in vascular lesions, has not been properly characterized. In the present work the effect of SOD3 on the inflammatory cell extravasation was studied *in vivo* in rat hind limb ischemia and mouse peritonitis models by identifying the migrated cells and analyzing SOD3-derived response on inflammatory cytokine and adhesion molecule expression. SOD3 overexpression significantly reduced TNFα, IL1α, IL6, MIP2, and MCP-1 cytokine and VCAM, ICAM, P-selectin, and E-selectin adhesion molecule expressions in injured tissues. Consequently the mononuclear cell, especially CD68+ monocyte and CD3+ T cell infiltration were significantly decreased whereas granulocyte migration was less affected. According to our data SOD3 has a selective anti-inflammatory role in ischemic damages preventing the migration of reactive oxygen producing monocyte/macrophages, which in excessive amounts could potentially further intensify the tissue injuries therefore suggesting potential for SOD3 in treatment of inflammatory disorders.

Citation: Laurila JP, Laatikainen LE, Castellone MD, Laukkanen MO (2009) SOD3 Reduces Inflammatory Cell Migration by Regulating Adhesion Molecule and Cytokine Expression. PLoS ONE 4(6): e5786. doi:10.1371/journal.pone.0005786

Editor: Eshel Ben-Jacob, Tel Aviv University, Israel

Received April 15, 2009; **Accepted** May 8, 2009; **Published** June 4, 2009

Funding: Academy of Finland, Sigrid Juselius Foundation, Paolo Foundation, TUBS graduation school. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The inflammatory process is initiated by endothelial cell (EC) activation comprising upregulation of chemokines and cell adhesion molecules, leukocyte activation and transmigration, and secretion of proinflammatory factors by leukocytes [1]. The inflammatory reaction is necessary for tissue recovery as it provides the correct cytokine signals and cell machinery to clear up the site for regeneration of the tissue [2]. However, uncontrolled inflammation has unfavorable effects on the course of tissue healing since the inflammatory cells are also capable of inducing tissue damage [2] and therefore many conditions involving inflammation, e.g. autoimmune diseases and tissue transplantations, are treated with immunosuppressants to reduce harmful leukocyte infiltration. Among the most potent drugs are glucocorticoids that downregulate the expression of numerous inflammatory chemokines, cytokines and adhesion molecules [3–5], which, however, are not entirely without adverse effects such as delayed myocardial tissue healing, osteoporosis, and blood vessel calcification [6–9].

The most prominent outcome in the initial phase of inflammation is the enhanced production of cytokines, such as TNF-α and IL-1, and chemokines, such as monocyte chemoattractant protein-1 (MCP-1) [10,11], which further induce expression of a number of inflammatory cytokines [4]. Many of the stimulus-specific pathways converge in the production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) as signal mediators, which in turn result in e.g. NFκB activation responsible for numerous stress-related functions [12–14]. Leukocytes are thus recruited by expression of various cell adhesion molecules, e.g. selectins, and intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1, respectively) [15,16]. They promote rolling and firm adhesion of leukocytes to endothelial wall, the necessary interactions preceding transmigration [17]. To aid leukocyte migration the vessel wall cells change their morphology by assuming cytoskeletal and cell-cell junction modifications in response to e.g. ligand binding to ICAM-1 and VCAM-1 [18–20], and when stimulated by O_2^- or TNF-α [21,22].

Previously, it has been shown that extracellular superoxide dismutase (SOD3) can attenuate tissue damage and inflammation but so far its mechanism of action has not been completely defined [23–27]. Since excess inflammation prevents the tissue injury recovery we investigated in the present study the effect of SOD3 overexpression on cell migration. We used two *in vivo* acute inflammation models to determine how SOD3 affects leukocyte extravasation, and compared the results to efficacy of the glucocorticoid immunosuppressant dexamethasone. The mouse peritonitis and rat hind limb ischemia models have been characterized previously: they induce rapid infiltration of leukocytes to the peritoneal cavity and large femoral muscles,

respectively [28–30]. We then analyzed the proportions of the infiltrated leukocyte subtypes, and determined the effects on several mediators of the inflammatory reaction.

Materials and Methods

Ischemia model

Fischer 344 rats (Harlan, Horst, Netherlands) and Balb/C mice (local colony) were maintained in specific pathogen-free conditions and had access to food and water ad libitum. All experimental procedures were approved by the Experimental Animal Committee of University of Turku.

Ischemic hind limb injury was induced to male Fischer 344 rats (5 to 6 weeks old, 86–115 g) by surgical closure of the distal femoral artery, lateral circumflex femoral artery, and the proximal femoral artery. The animals were anesthetized for the procedure by intra peritoneal administration of fentanyl fluanisone (Janssen Pharmaceutica, Beerse, Belgium) and midazolame (Roche, Basel, Switzerland). Gene transfer was done immediately after the ligation by intra muscular injection of 0.5×10^9 pfu adenovirus SOD3 (AdSOD3) or LacZ (AdLacZ) in 50 µl PBS as described [27,31,32]. Uninjured muscle tissue was used as control.

Peritonitis model

Gene transfer was done to 8–10 week old female balb/c mice with intra peritoneal injection of 0.5×10^9 pfu AdSOD3 or AdLacZ. Acute peritoneal inflammation was induced 72 hours later by i.p. injection of 1 ml PBS containing 5% proteose peptone (BD Difco, Sparks, MD, USA) and 10 ng of IL-1β (R&D Systems, Minneapolis, MN, USA). As a control treatment, animals were given 50 mg/kg of Dexamethasone (Oradexon, Organon, Oss, Holland) half an hour before proteose peptone injection. Cells from the peritoneal cavity were collected 18 hours after the induction of inflammation by washing with 10 ml of RPMI containing 5 U/ml heparin (Løvens Kemiske Fabrik, Ballerup, Denmark). Cells from peritoneal lavage were counted and cytocentrifuged at 1000 rpm for 5 minutes (Shandon cytospin 3, Shandon, Pittsburgh, PA, USA). Slides were stained with Reastain Diff-Quick (Reagena, Toivala, Finland) to analyze different leukocyte subtypes.

Immunohistochemistry

Rat muscle samples were frozen in liquid nitrogen and embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA). Ten micrometer sections were fixed in acetone and stained with rabbit anti-rat CD3 and mouse anti-rat CD68 (Serotec, Oxford, UK). The sections were counterstained with hematoxylin/eosin (Sigma, Saint Louis, MI, USA). The number of $CD3^+$ and $CD68^+$ cells were analyzed from whole sections with Zeiss Axiovert 200 M (Carl Zeiss, Oberkochen, Germany).

Reporter assay

HEK 293T cells were used for *in vitro* assay to provide a general cell model that we have previously used in our reporter, expression, and cell signalling studies allowing the comparison of the data with our previous results. HEK 293T cells were transfected with SOD3 expression vector together with pNFκB Luc reporter (Stratagene, Cedar Creek, TX, USA). Luciferase activity was quantified with Tecan Ultra XFluor4 Fluorescence Reader (Tekan, Mannedorf, Switzerlard).

Western blot analysis

HEK293 cells were homogenized in lysis buffer (50 mmol/l HEPES pH 7.5, 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/l MgCl, 10 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 1 mmol/l Na_3VO_4, 10 µg/ml approtinin, 10 µg/ml leupeptin) (Sigma, Saint Louis, MI, USA). Mouse anti-human α-IκB (Santa Cruz, Santa Cruz, CA, USA) was used to detect IκB levels from tissues.

Quantitative PCR

Total RNA was extracted from a pool of four animals using Trireagent (Sigma, Saint Louis, MI, USA). The first strand synthesis was done with Revert-Aid M-MuLV (Fermentas, Burlington, Canada), and the following quantitative PCR with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Primers and cycling conditions are shown in the Table 1.

Statistical Analysis

All results are expressed as mean±SEM. A paired t-test was used to determine differences between groups.

Results

SOD3 inhibits leukocyte migration in acute ischemia

Neutrophils, macrophages, and other inflammatory cells mediate a number of important cellular functions in injured tissue [33,34]. Phagocytotic macrophages clear cellular debris and secrete inflammatory cytokines such as MIP-2, a strong neutrophil attracting agent leading to further increase in inflammatory signaling [35]. However, excessive inflammatory reaction may also contribute to tissue damage by enhancing macrophage infiltration, which increases tissue free radical load leading to further tissue injury [36]. In addition, decreased neutrophil accumulation leads to reduced infarct size, reduced vascular permeability, and resistance in ischemia/reperfusion (I/R) injury [37,38]. Thus, it is suggested that the tissue recovery is dependent on the factors

Table 1. Primers and cycling conditions.

Gene	Primer	Tm
TNFα for	AGA TGT GGA ACT GGC AGA GG	60
TNFα rev	CCC ATT TGG GAA CTT CTC CT	
IL-1α for	TCG GGA GGA GAC GAC TCT AA	58
IL-1α rev	GAA AGC TGC GGA TGT GAA GT	
IL-6 for	CCG GAG AGG AGA CTT CAC AG	55
IL-6 rev	ACA GTG CAT CAT CGC TGT TC	
MCP-1 for	CTC ACC TGC TGC TAC TCA TTC ACT	55
MCP-1 rev	TGC TGC TGG TGA TTC TCT TGT AGT	
MIP2 for	ATC CAG AGC TTG ACG GTG AC	55
MIP2 rev	GGA CTT GCC GCT CTT CAG TA	
ICAM for	AGG TAT CCA TCC ATC CCA CA	55
ICAM rev	GCC ACA GTT CTC AAA GCA CA	
VCAM for	TGA CAT CTC CCC TGG ATC TC	55
VCAM rev	CTC CAG TTT CCT TCG CTG AC	
PSEL for	TTC CCA CAC TTC CTT CTG CT	57
PSEL rev	CAC GCT GTA GTC GGG GTA TT	
ESEL for	TTT TTG GCA CGG TAT GTG AA	57
ESEL rev	AGG TTG CTG CCA CAG AGA GT	
β-actin for	TCG TGC GTG ACA TTA AGG AG	55
β-actin rev	GTC AGG CAG CTC GTA GCT CT	

doi:10.1371/journal.pone.0005786.t001

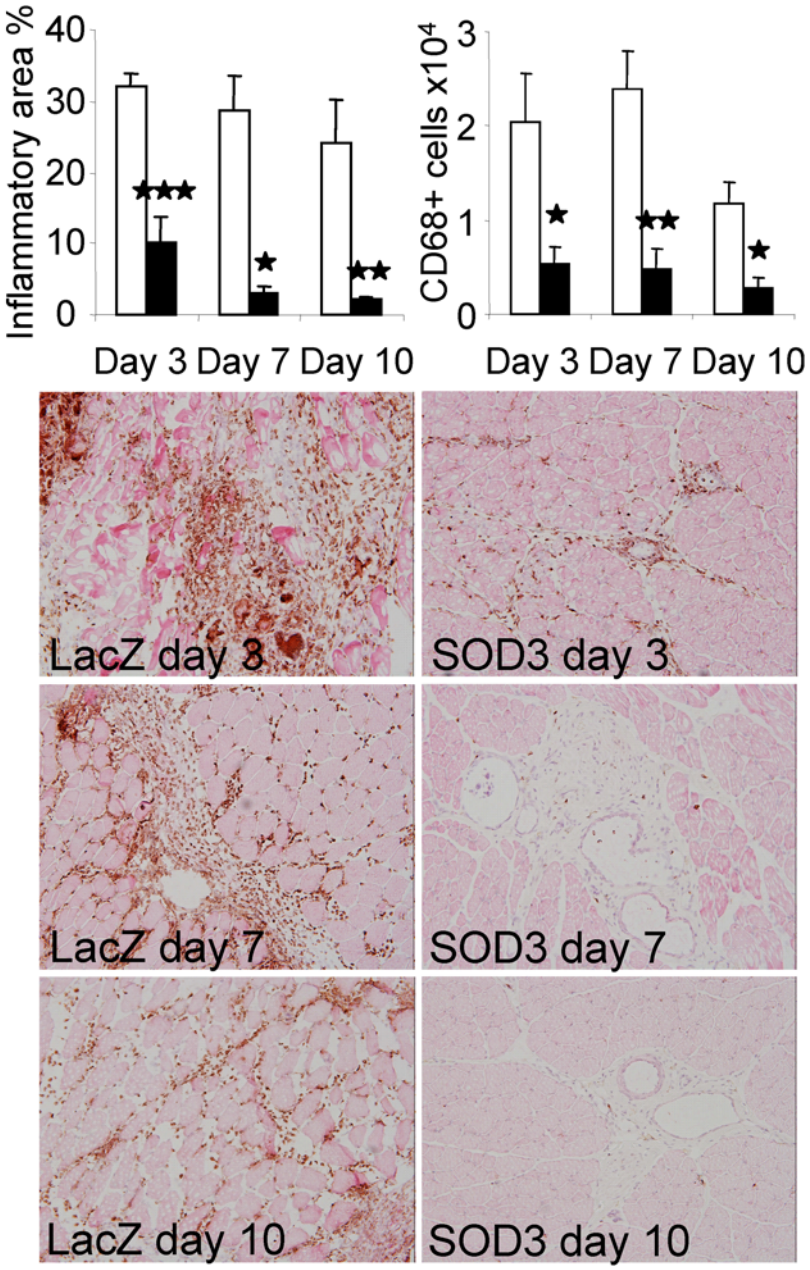

Figure 1. Reduced macrophage infiltration in to ischemic muscle. Open bars represent LacZ animals and black bars represent SOD3 animals. CD68 staining showed significantly reduced inflammatory area and macrophage infiltration in SOD3 animals at all time points studied. Histological stainings show CD68+ macrophages around the femoral artery in ischemic muscles (20× magnification).
doi:10.1371/journal.pone.0005786.g001

regulating the inflammatory cell migration into the injuries. In the present work we studied leukocyte migration in acute ischemia and peritonitis models and analyzed the contribution of SOD3 on inflammatory cytokine and adhesion molecule expression.

We determined the effect of SOD3 overexpression on the degree of inflammation by analyzing the size of inflamed tissue and the number of infiltrated macrophages and T cells in acute ischemic injury model. In a mouse model of femoral artery ligation, macrophage infiltration into ischemic muscle reaches peak values 3 days after the injury [39]. Histological analysis of the rat hind limb ischemia showed 3-fold reduction in the inflamed tissue area as determined by the presence of $CD68^+$ macrophages ($p<0.001$) in SOD3 vs. LacZ control animals 3 days after vessel ligation (Figure 1). The reduction became even more prominent in later time points reaching 12-fold difference 10 days after vessel ligation. Additionally, the number of infiltrated $CD68^+$ macrophages was 3–5 fold higher in LacZ control animals as compared to SOD3 animals ($p<0.05$). Maximal macrophage accumulation to the injured tissue was seen at 7-day time point in LacZ animals indicating that the inflammatory reaction was still developing in control animals at the initial phase of the follow-up period while the inflammation was decreasing in SOD3 animals. Throughout the experiment the number of macrophages remained higher in control animals than initially observed in SOD3 animals, which by the 10-day time point showed values close to the background levels further underlining the beneficial effect of SOD3.

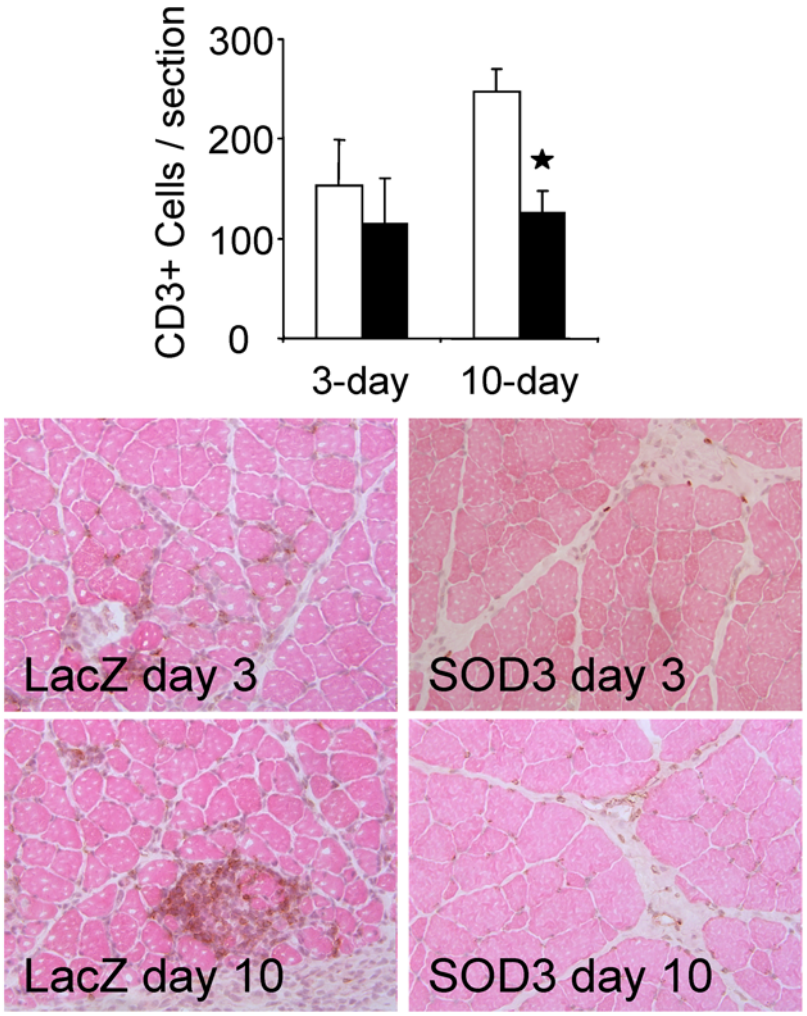

Figure 2. Inhibition of T-cell migration. Open bars represent LacZ animals and black bars represent SOD3 animals. Infiltration of CD3+ lymphocytes was inhibited in SOD3 animals 10 days after injury remaining at the level seen at earlier 3-day time point (20× magnification).
doi:10.1371/journal.pone.0005786.g002

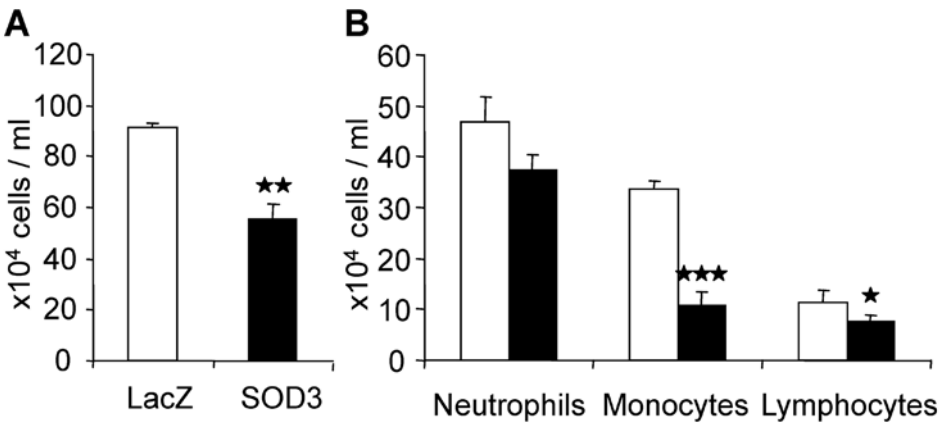

Figure 3. Anti-inflammatory effect of SOD3 affects predominantly macrophages in peritonitis model. Open bars represent LacZ animals and black bars represent SOD3 animals. (a) SOD3-mediated reduction in total leukocyte number in peritoneal lavage 18 hours after induction of inflammation. SOD3 treated animals had 55.6×10^4 (±4.1) cells/milliliters of lavage as compared to 91.6×10^4 (±6.7) found from LacZ control animals. (b) Analysis of different leukocyte subtypes showed strongest effect in macrophages although lymphocyte migration was also reduced. Monocyte accumulation in LacZ vs. SOD3 treatment was reduced from 33.5×10^4 (±1.8) to 10.9×10^4 (±2.5) cells/ml whereas lymphocytes were reduced from 11.4×10^4 (±2.2) to 7.6×10^4 (±1.2) cells/ml, and neutrophils from 47.0×10^4 (±4.7) to 37.5×10^4 (±2.9) cells/ml.
doi:10.1371/journal.pone.0005786.g003

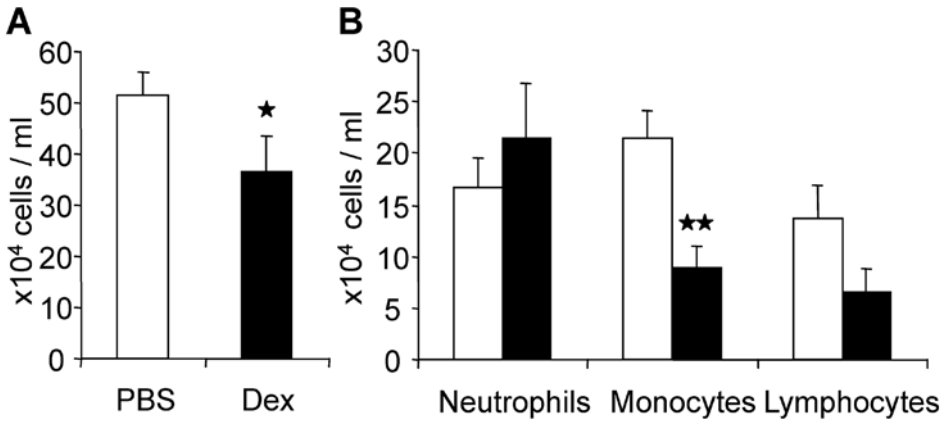

Figure 4. Anti-inflammatory effect of dexamethasone. Open bars represent PBS treated animals and black bars represent dexamethasone animals (dosage 50 mg/kg). (a) Leukocyte infiltration was reduced by dexamethasone treatment from 51.5×10^4 (±4.7) to 36.5×10^4 (±7.1) cells/milliliters of lavage. (b) Dexamethasone treatment had no effect on neutrophil migration, 16.6×10^4 (±2.9) and 21.5×10^4 (±5.2) cells/ml were found in PBS control group and dexamethasone treated animals, respectively. In contrast, accumulation of monocytes and lymphocytes were reduced from 21.4×10^4 (±2.7) to 8.8×10^4 (±2.2), and from 13.7×10^4 (±3.2) to 6.5×10^4 (±2.4), respectively.
doi:10.1371/journal.pone.0005786.g004

As compared to neutrophils and macrophages, the role of $CD3^+$ T-cells in recovery of ischemic tissue has remained uncertain. Despite relatively low level of infiltration, studies suggest an early role for T-cells in attracting neutrophils and macrophages to site of myocardial or peripheral ischemia/reperfusion injury [40,41]. The histological analysis at 3-day time point showed 114 ± 23 and 153 ± 46 ($p=$ ns.) $CD3^+$ T cells per ischemic tissue section in SOD3 and LacZ animals, respectively (Figure 2). At 10-day time point number of T cells had increased in LacZ animals by 60% to 247 ± 31 whereas in SOD3 animals T cell accumulation remained at similar level as compared to 3 day time point (125 ± 30, $p<0.05$). The analysis of leukocyte accumulation in the rat hind limb ischemia model shows selective inhibition of inflammatory cell migration and suggests SOD3 to have more prominent effect on macrophage infiltration as compared to lymphocytes.

SOD3-mediated inhibition of leukocyte accumulation in peritonitis model

To confirm the findings and to further analyze the SOD3-derived selective inhibition of cell migration we examined leukocyte migration in a mouse peritonitis model, which provides an efficient way to analyze leukocyte traffic in an acute inflammatory response. To induce peritoneal inflammation we used 5% solution of proteose peptone supplemented with IL-1β and counted the numbers of different leukocyte subtypes from the peritoneal lavage 18 hours after induction of inflammation. The analysis of SOD3 overexpression derived inhibition of cell migration showed 20% ($p=$ ns), 67% ($p<0.001$), and 33% ($p<0.05$) reduction in migrating neutrophils, monocytes/macrophages, and lymphocytes, respectively (Figure 3B). Moreover, the total number of infiltrated leukocytes was decreased by 30% in SOD3 animals as compared to LacZ controls (Figure 3A) ($p<0.01$), which is mostly caused by the effect of attenuated macrophage migration. The data effectively confirmed our findings in rat hind limb ischemia showing vastly stronger inhibition of macrophage infiltration as compared to other leukocyte subtypes.

Dexamethasone, a corticosteroid that reduces swelling and inflammation is a potent anti-inflammatory drug used to treat many bacteria-free inflammatory conditions, including rheumatoid arthritis and anaphylactic shock. Glucocorticoids exert their anti-inflammatory effect e.g. through repression of NF-κB mediated cytokine expression, which takes place after cytoplasmic glucocorticoid receptor translocates into the nucleus [5,42]. To compare SOD3 to clinically approved medication we gave an intra-peritoneal injection of dexamethasone (Oradexon) to animals 30 minutes before induction of peritoneal inflammation. Leukocyte traffic to the inflamed peritoneum was reduced by 20% ($p<0.05$) after treatment with 50 mg/kg dexamethasone (Figure 4A). Monocyte/macrophage migration was reduced by 60% ($p<0.01$), and that of lymphocytes by marked 50% indicating tendency, while no significant difference was seen in neutrophil accumulation in this setting (Figure 4B). PBS mock treated animals exhibited lower neutrophil and monocyte accumulation as compared to animals subjected to LacZ gene transfer, which may be result of the adenovirus vector used in the study. Intriguingly, SOD3 treatment reduced peritoneal monocyte and lymphocyte numbers to similar level as dexamethasone treatment although neutrophil numbers remained higher than what was observed in either PBS or dexamethasone treated animals.

SOD3 inhibits NF-κB activation and suppresses the inflammatory cytokine and adhesion molecule expression

Proinflammatory stimuli activate vascular endothelium leading to up-regulation of cell adhesion molecules and chemokines, NF-κB has been shown to be both necessary and sufficient for endothelial up-regulation of ICAM, VCAM, and MCP-1 [43]. Furthermore, ectopic expression of IκBα effectively abrogates expression of VCAM, IL-1, and IL-6 [44]. *In vitro* luciferase assay revealed 50% ($p<0.01$) decrease in NF-κB activity due to SOD3 transfection, which could at least partially be explained by increased IκBα expression (Figure 5A) suggesting that SOD3 promotes cytoplasmic localization of NF-κB rendering it incapable of binding DNA. NF-κB plays a central part in responses to inflammatory signaling by regulating the expression of cytokines suggesting that reduced NF-κB activity could lead to reduction in expression of inflammatory cytokines and chemokines. Therefore, we quantified cytokine and chemokine expression level *in vivo* from rat muscle three days after vessel ligation and SOD3 gene transfer. Quantitative RT-PCR showed significantly reduced expression of TNFα, IL1α, IL6, MIP2, and MCP1 (Figure 5B) in SOD3 animals suggesting reduced expression of several important inflammatory mediators. Specifically, MCP1 is an important macrophage

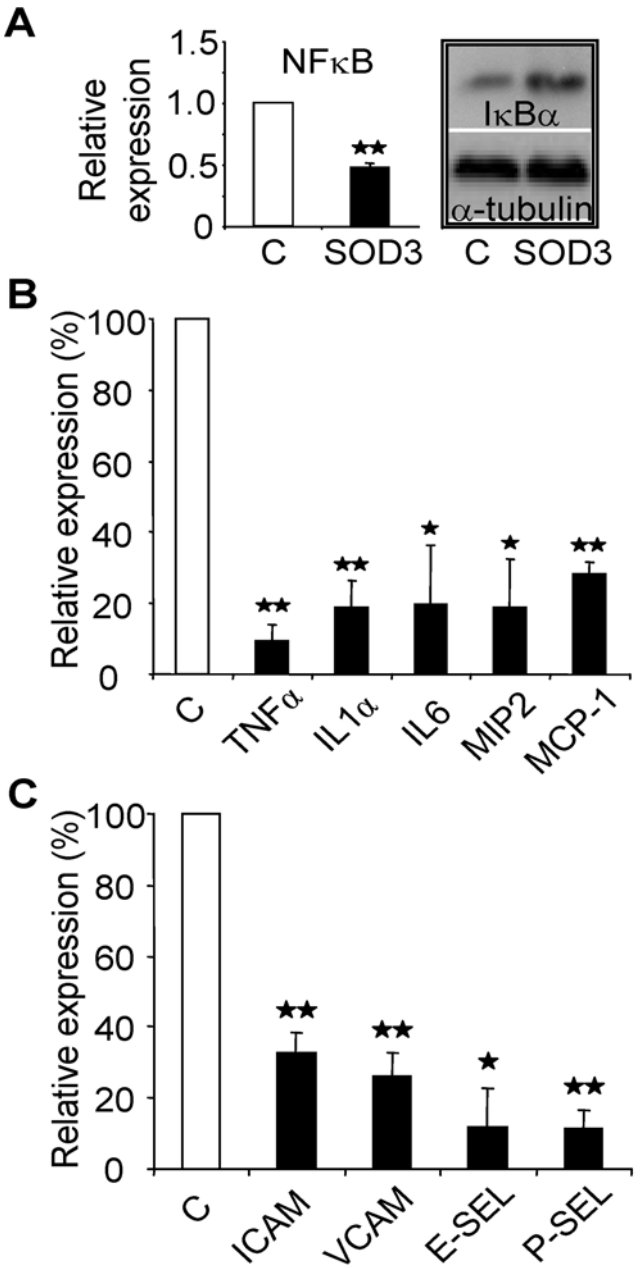

Figure 5. SOD3-mediated reduction in activity of inflammatory mediators. Open bars represent LacZ tissue and black bars represent SOD3 animals. (a) Luciferase assay shows 50% reduction in NF-κB activity *in vitro*, and western blot analysis shows increased IκBα expression. (b) Quantitative RT-PCR analysis for cytokines and chemokines. SOD3 overexpression derived reduced expression of TNFα, IL-1α, IL-6, MCP-1, and MIP2 in injured tissue. (b) Analysis of ICAM, VCAM, P-selectin, and E-selectin expression. Expression of inflammatory adhesion molecules was significantly reduced in SOD3 muscle.
doi:10.1371/journal.pone.0005786.g005

attractant [45,46], possibly explaining markedly reduced macrophage accumulation. Furthermore, since TNFα, IL1α, and IL6 are important regulators of endothelial adhesion molecule expression we analyzed expression of ICAM, VCAM, E-selectin, and P-selectin from the tissue (Figure 5C). We found significant reduction in adhesion molecule expression, which further confirms the reduction in overall inflammation in the muscle of SOD3 recipient rats as compared to LacZ control animals.

Discussion

Tissue damage launches rapid recruitment of inflammatory leukocytes into injured tissue due to activation of endothelial cells. Inflammatory reaction promotes tissue healing by eliminating pathogens, clearing cellular debris, and promoting cell proliferation. However, excessive inflammatory reaction promotes injury e.g. through neutrophil-derived superoxide production [47]. In fact, reactive oxygen species function as inflammatory mediators by activating expression of cytokines such as TNFα, IL-1, and IL-6 [48] and therefore ROS may contribute to tissue injury by not only directly damaging the tissue but also by enhancing further leukocyte accumulation.

In the current work we showed that SOD3 is an important mediator of reduced CD68+ macrophage migration into the inflammatory area. Macrophages accumulate in high numbers to ischemic muscle forming the primary leukocyte population three days after injury [39]. CD68 staining showed significantly reduced inflammatory area and macrophage migration in SOD3 treated ischemic tissue as compared to LacZ control animals (Figure 1). The SOD3-mediated reduction in macrophage accumulation was evident in all of the studied time points. T-cells accumulate to ischemic muscle in vastly lower numbers as compared to macrophages. However, their presence is required for efficient neutrophil traffic, and they attract macrophages by secreting IL-16 [40]. In our studies, SOD3 did not prevent initial low level T-cell migration, but efficiently inhibited further increase at 10-day time point (Figure 2). Late effect on T-cell migration suggests an indirect mechanism for SOD3 mediated inhibition in T-cell traffic, which might be result of general decrease in inflammation. Inflammatory cytokines secreted by infiltrating macrophages attract other leukocytes to injured tissue [49]. Thereafter, inhibition of macrophage infiltration could lead to overall reduction in inflammatory reaction.

Since the SOD3-derived reduction in inflammation showed selective inhibition of macrophage migration, we were prompted to confirm the finding and to better characterize the cell specific effect. The mouse peritonitis model supported the SOD3-derived reduction in the number of infiltrating leukocytes (Figure 3A), which was predominantly due to reduced macrophage numbers (Figure 3B). These results confirm the anti-inflammatory property of SOD3 and show a stronger inhibition of monocyte migration as compared to other analyzed leukocyte subtypes. The data suggest that reduced superoxide tissue concentrations caused by SOD3 overexpression may explain the anti-inflammatory effect of the enzyme. It has been previously shown that superoxide treatment of rat cerebral endothelial cells increases monocyte adhesion and migration, which, however, was not replicated by H_2O_2 treatment but was instead abrogated by superoxide scavengers suggesting superoxide as an inflammatory mediator [21]. We have shown in our previous works that SOD3 overexpression *in vivo* efficiently decreases the production of superoxide in cardiovascular injuries including our hind limb ischemia model [25–27].

Anti-inflammatory medications currently available for clinical use include glucorticoid drugs such as dexamethasone. Dexamethasone binds the glucocorticoid receptor, which subsequently translocates to the nucleus and represses inflammatory gene expression by inhibiting e.g. NF-κB activity [4,5,42]. To compare the efficacy of SOD3 mediated anti-inflammatory effect to existing medication we determined the effect of Dexamethasone in mouse peritonitis. As a dose of 50 mg/kg, dexamethasone reduced leukocyte traffic in comparable levels to SOD3 gene transfer (Figure 4A). Dexamethasone-mediated effect was equally effective in monocyte/macrophage and lymphocyte lineages whereas no significant effect was seen in neutrophils (Figure 4B). Neutrophil

accumulation has been shown to be at its highest as early as 4 hours after induction of inflammation in zymosan induced peritonitis [50]. Therefore, lack of effect on neutrophil migration could be due to late time point analyzed. The data suggests that SOD3 overexpression and dexamethasone administration have similar anti-inflammatory effect in acute inflammation and therefore suggesting SOD3 as a potential candidate molecule for clinical treatments.

NF-κB plays a crucial role in mediating inflammation due to its role in activating expression of pro-inflammatory genes such as cytokines TNFα and IL1α, and adhesion molecules ICAM-1 and VCAM-1 [43,44]. Since NF-κB is a redox sensitive transcription factor being activated by oxidative stress, [12,13] we analyzed the effect of SOD3 on NF-κB activity *in vitro* and showed significantly decreased activity. (Figure 5A). The data is in line with previous work in cardiovascular and liver transplantation models showing that increased NAPDH oxidase-derived superoxide production correlates with increased NF-κB activity, which is attenuated by SOD3 overexpression [51–54].

Since cytokines TNFα, IL1α, IL6, MIP2, and MCP1 are known to contain NF-κB binding sites in their gene promoters and are thus up-regulated by NF-κB activation [55–61], we analyzed their expression levels in rat muscle by quantitative PCR. All of the analyzed cytokines and chemokines were significantly down-regulated in SOD3 animals as compared to LacZ control animals (Figure 5B). TNFα, IL1α, and IL6 promote inflammatory cell migration by up-regulating E-selectin, P-selectin, ICAM, and VCAM. Furthermore, macrophage recruitment has been shown to be strongly dependent on MCP-1 secretion [46], while MIP2 is a strong attractant for neutrophils [35]. MCP-1 deficiency does not reduce the number of resident macrophages in peritoneal cavity, but prevents macrophage migration in response to acute thioglycollate induced peritonitis [46]. Lu et al. showed 3-fold reduction in macrophage migration in 2,4-dinitro-1-fluorobenzene induced skin hypersensitivity model while neutrophil numbers remained unchanged. Thereafter, marked down-regulation of MCP-1 seen in ischemic muscle could explain the observed strong macrophage inhibition. Finally, due to reduced inflammatory cytokine expression, we conducted further expression analyses and found reduced expression VCAM, ICAM, E-selectin, and P-selectin (Figure 5C). Reduced expression of common adhesion molecules highlights the anti-migratory role of SOD3. It has been shown that macrophage transmigration is strongly dependent on $\alpha_4\beta_1$ integrin - ICAM-1 interaction. Pre-treatment of recipient mice before intra venous macrophage injection with monoclonal antibodies for ICAM-1 reduced macrophage migration to atherosclerotic plaques by 65% [62]. In addition, rolling and attachment of P388D1 mouse monocyte cell line was inhibited by P-selectin and VCAM antibodies in an *ex vivo* isolated perfused carotid artery model [63] demonstrating the importance of these adhesion molecules on macrophage transmigration.

In conclusion, our novel observation shows that SOD3 gene transfer into hind limb ischemia or peritonitis results in significantly reduced leukocyte migration due to decreased cytokine and adhesion molecule expression. Further on, the data suggest more pronounced anti-inflammatory effect on macrophages as compared to other leukocyte subtypes in the models used in the current work. The observed anti-inflammatory effect in SOD3 treated mice was comparable or even higher than that of Dexamethasone, which recently has been shown to have cardiovascular side effects [64,65]. Our previous *in vivo* SOD3 overexpression models have suggested non-toxicity and beneficial effect on tissue protection and injury recovery [24–26,31] suggesting that SOD3 overexpression by exogenous administration or through increased endogenous production in injured tissues could provide a promising medication against excess inflammatory cell migration.

Author Contributions

Conceived and designed the experiments: JL MDC MOL. Performed the experiments: JL LEL MDC MOL. Analyzed the data: JL LEL MDC MOL. Contributed reagents/materials/analysis tools: MOL. Wrote the paper: JL LEL MDC MOL.

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Extracellular superoxide dismutase is a thyroid differentiation marker downregulated in cancer

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Running title: SOD3 expression in thyroid

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Abstract

Reactive oxygen species, specifically hydrogen peroxide (H_2O_2) have a significant role in hormone production in thyroid tissue. Even though recent studies have demonstrated that Dual oxidase is responsible for H_2O_2 synthesis the role of extracellular superoxide dismutase (SOD3), which is a major H_2O_2 producing enzyme in extracellular fluids, has not been investigated in thyroid environment. In the current work we determined the expression of SOD3 in vitro and in vivo thyroid models signal transduction pathways involved in SOD3 activation, and the main physiological role of SOD3 in normal thyroid. According to our data SOD3 is highly expressed in normal thyroid, which becomes even more prominent in benign rat goiter model. However, we observed differentiation dependent decrease in *sod3* synthesis during thyroid cancer development. The cellular mechanism studies showed that TSH signal transduction routes leading to hormone production and cell proliferation both increased *sod3* mRNA synthesis even though the functional analysis in normal thyroid models indicated that the main role of SOD3 in thyroid is to regulate cell proliferation. Based on the correlation to the degree of thyroid cancer and *sod3* expression we suggest that SOD3 could be used as differentiation marker for thyroid carcinogenesis.

Introduction

The G protein-coupled receptors (GPCR), such as thyroid stimulating hormone receptor (TSH-R), mediate the cellular responses of various extracellular stimuli via two principle signal transduction pathways: cAMP pathway, which mainly mediates mitogen response, and phophatidylinositol pathway that is involved in calcium signaling in the cells (1). In thyroid TSH-R cAMP pathway is responsible for the thyroid cell proliferation and differentiation where as phophatidylinositol cascade controls thyroid hormone T_3 and T_4 synthesis response to thyroid stimulating hormone (TSH). The TSH stimulation is crucially important for normal thyroid function and therefore deregulation caused by e.g. TSH-R activating mutations or external factors affecting TSH signaling essentially disrupt the cellular homeostasis in thyroid (2). The low iodide uptake leads to reduced thyroid hormone T3 and T4 production (hypothyroidism), which is compensated by increased TSH synthesis and subsequent activation of downstream cAMP mitogenic signaling leading to cell proliferation and benign thyroid enlargement known as goiter (3). Similarly toxic adenoma patients that have a constitutively activated TSH signaling due to mutations in TSH receptor with consequent permanent upregulation of cAMP pathway develop autonomous hyperfunctioning thyroid adenomas and non-autoimmune toxic thyroid hyperplasia (4).

Malignant transformations of thyroid gland vary considerably in aggressiveness ranging from relatively common benign adenomas to differentiated papillary (PTC) and follicular (FTC) carcinomas, poorly differentiated (PDC) and rapidly growing undifferentiated carcinomas (ATC) (5). Currently known thyroid cancer

differentiation markers include sodium iodide symporter (NIS), thyroglobulin (Tg), thyroid peroxidase (TPO) and thyrotropin receptor (TSH-R), which however are not in diagnostic use due to inability of an individual gene to mark different transformation degrees (6). A larger panel of markers would offer a more reliable clinical diagnostic tool and therefore discovery of new differentiation makers to reinforce the currently known genes, would improve the diagnosis of thyroid cancer.

TSH signaling has been shown to lead to increased reactive oxygen species production, especially to the synthesis of hydrogen peroxide (H_2O_2). Previously it has been shown that H_2O_2 contributes to tyrosine kinase activation, mitogen signaling, and consequent cell proliferation *in vitro* and *in vivo* models (7-9) by inactivating protein tyrosine phosphatases (10) or by phosphorylating tyrosine kinase receptors (11-15). We have recently demonstrated the ability of extracellular superoxide dismutase (SOD3), which is one of the main H_2O_2 producing enzymes in the extracellular fluids (16, 17), to promote cell proliferation *in vivo* by activating Ras-Erk mitogen signaling, transcription factor upregulation and growth factor expression suggesting growth stimulatory role in tissues (18). Therefore, in the present work we studied the effect of TSH stimulation on SOD3 expression and the contribution of SOD3 to thyroid proliferative disorders. We identified a novel TSH-R mediated signal transduction pathway activating *sod3* synthesis and demonstrated decreased *sod3* expression correlating with the degree of thyroid malignancy. Therefore, SOD3 could function as a differentiation marker in the cancer development.

Materials and methods

Cell culture experiments. Rat PC Cl3 cells were grown in Ham's F-12 medium, Coon's modification (Sigma, St. Louis, MO) supplemented with 5% calf serum (Life Technologies, Inc., Paisley, PA) and 10 nM thyrotropic hormone, 10 nM hydrocortisone, 100 nM insulin, 5 µg/ml transferrin, 5 nM somatostatin, and 20 µg/ml glycyl-histidyl-lysine. HEK 293T cells were grown in DMEM 10% fetal bone serum (Sigma). Transfections were done with Polyfect transfection reagent (Sigma) or with Fugene6 (Roche Applied Science, Indianapolis, IN). Human *sod3* cDNA, kindly provided by professor Stefan L. Marklund from the University of Umeå, Sweden, was cloned to pcDNA3 vector (Life Technologies, Carlsbad, CA) PC Cl3 cells were incubated in the presence of thyroid stimulating hormone (TSH) (Sigma), forskolin (Sigma), N(2-((ρ-Bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide (H89) (Calbiochem, San Diego, CA), or thapsigargin (Research Biochemicals International, Natick, MA).

Animals. Male 4 to 5 weeks old Sprague-Dawley rats were given 0.25 % Propylthiouracil (PTU) (Sigma) *ad libitum* in drinking water for 2 weeks, sacrificed, and tissues were collected for expression analysis. Experimental procedures were done according to the European Union guidelines.

PCR reactions. Messenger RNA was isolated from the pool of 4 animals or cells using Tri-reagent (Sigma). The first strand synthesis was done with QuantiTect Reverse Transcription (Qiagen, Hilden, Germany) and quantitative PCR with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Primers were human

SOD3-for CTTCGCCTCTGCTGAAGTCT, human SOD3-rev GGGTGTTTCGGTACA AATGG, rat SOD3-for GACCTGGAGATCTGGATGGA, rat SOD3-rev GTGGTT GGAGGTGTTCTGCT, human βactin-for TGCGTGACATTAAGGAGAAG, human βactin-rev GCTCGTAGCTCTTCTCCA, rat βactin-for TCGTGCGTGACTTAAGGAG, rat βactin-rev GTCAGGCAGCTCGTAGCTCT. Reactions were done in 60° C annealing temperature.

RNA interference. Short interfering *sod3* or *gapdh* OnTargetplus SMART pool oligos (Dharmacon, Lafayette, CO) were transfected into PC Cl3 cells according to manufactures protocol. Shortly, 20 μmol/l of siSOD3 or siGAPDH control oligo were resuspended to total volume 100 μl transfection buffer, mixed with equal volume of Optimem (Life Technologies, Carlsbad, CA), and incubated 5 min at room temperature. To prepare the transfection solution 5 μl Dhermofect 4 reagent was added to 190 μl of Optimem and incubated 5 min at room temperature. Oligo suspension and transfection regent were combined, incubated 20 min at room temperature and added to the cells for 48 h.

BrdU analysis. Cells were serum starved for 16 hours and TSH stimulated 6 hours before addition of 10 mM bromodeoxyuridine (BrdU) (Roche) for 2 hours. Subsequently, cells were fixed in 3% paraformaldehyde (Sigma) and permeabilized with 0.2% Triton X-100 (Sigma). BrdU-positive cells were revealed with FITC–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Cell nuclei were identified by Hoechst (Sigma) staining.

Calcium uptake assay. Intracellular Ca^{2+} concentrations were measured in PC Cl3 cells. Briefly, cells were detached from tissue culture plates with PBS containing 0.5 mmol/l EDTA, washed once with HEPES-buffered medium (HBM) (137 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $CaCl_2$, 0.44 mmol/l KH_2PO_4, 4.2 mmol/l $NaHCO_3$, 10 mmol/l glucose, 20 mmol/l HEPES, and 1.2 mmol/l $MgCl_2$, pH 7.4) and then loaded with 2 μmol/l fura-2 acetoxymethyl ester for 20 min at 37 °C. After loading, the cells were diluted with HBM without $CaCl_2$ to a final concentration of 0.3 mmol/l $CaCl_2$ and stored at room temperature until use. For fluorescence recordings an appropriate volume of the cell suspension was spun down, washed once in HBM, resuspended in HBM and placed in a thermostatted (37 °C) cuvette with magnetic stirring in a Hitachi F-2000 fluorescence spectrophotometer. The fluorescence was monitored at 340 nm (excitation) and 505 nm (emission). Experiments were calibrated with 60 μg digitonin/ml (F_{max}) and 10 mmol/l EGTA (F_{min}). The intracellular $[Ca^{2+}]$ was calculated from the fluorescence (F) using the equation $[Ca^{2+}] = (F-F_{min})/(F_{max}-F) \times 224$ nmol/l (Kd for fura-2), in which the extracellular fura-2 fluorescence was subtracted from F values.

Western blot. Cells were homogenized in lysis buffer (50mmol/L HEPES pH 7.5, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1mmol/L EGTA, 1.5 mmol/L $MgCl_2$, 10 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 1mmol/L Na_3VO_4, 10 μg approtinin/ml, 10 μg leupeptin/ml) (Sigma). Antibodies were human anti-SOD3 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-α tubulin (Sigma).

Immunohistochemistry. Thyroid tissues were embedded in optimal cutting temperature compound, OCT (Tissue-Tek, Torrance, CA). Ten µm sections were stained with hematoxylin/eosin (Sigma) according to the standard protocols.

Chemiluminescence assay for H_2O_2 detection. The H_2O_2 release was quantified 48 h after cell transfection by the homovanillic acid-based fluorimetric assay (19). Cos-7 cells were incubated in Krebs-Ringer HEPES (KRH) medium pH 7.4 containing 0.1 µg/mL horseradish peroxidase type II, 440 µmol/l homovanillic acid and 1 µmol/l ionomycin during 2 h 30 min at 37°C. Fluorescence intensity of oxidized homovanillic acid was measured at 425 nm after excitation at 315 nm. Results are reported as ng H_2O_2/well representing the quantity of H_2O_2 accumulated during 2 h 30 min in each well (6-well plates).

Chemiluminescence assay for $O_2^{\cdot-}$ detection. Extracellular $O_2^{\cdot-}$ release was detected by chemiluminescence using the superoxide dismutase–inhibitable Diogenes reagent (National Diagnostics, Atlanta, GA) on cells resuspended in HBSS + 10 mmol/l glucose. One µmol/l ionomycin was added to the cells to stimulate the Duox activity. Measurements were taken with 22 seconds intervals for 10 minutes in 96-well white plates ($1.5x10^5$ cells/250 µl well) at 37 °C using a Microplate Luminometer. Chemiluminescence curves were analysed and peak values of the curves were presented in Relative Light Units (RLU)/sec.

Statistical analysis. Statistical analysis was done using two-tail t-test for means.

Results

Sod3 is abundantly expressed in rat thyroid tissue

SOD3 is an extracellular H_2O_2 producing enzyme expressed in various tissues (16, 20) protecting cell surface structures against deleterious effects of superoxide. The local viral administration of *sod3* causing 2-fold increased total SOD activity in tissues or intravenous infusion of the native C-form of the enzyme with high affinity to cell surface structures results in significant attenuation of tissue damages and markedly increased tissue recovery (21-23) suggesting that even minor changes in tissue SOD3 concentration have a significant impact on the redox balance.

Comparison of mRNA expression from rat tissues (Figure 1a) demonstrated thyroid as one of the major expression sites for *sod3* showing 2.5-fold higher mRNA expression level in thyroid as compared to heart corresponding to previously reported 3-fold and 5-fold differences in heart vs. thyroid protein level in rabbit and human, respectively (16). The expression in the rat thyroid approached the levels detected in the aorta, which is one of the main expression sites for SOD3 (23), indicating participation to ROS balance and signal transduction in thyroid tissue.

Rat *in vivo* PTU model was used to investigate the expression status of SOD3 in benign thyroid enlargement caused by increased TSH secretion and compared the data to human clinical samples. PTU treatment is known to cause decreased T_3 production with subsequent increase in TSH signaling, thyroid enlargement, and activated mitogen signal transduction (24-26). PTU treated thyroids had on average 4-fold

increase in wet-weight as compared to normal untreated rat thyroids suggesting a typical goiter formation in rats (Figure 1b), which was further supported by hematoxylin-eosin staining demonstrating enlarged cellular structure and the lack of organized vacuolar network (Figure 1c,d). The expression analysis of *sod3* mRNA production showed 10-fold increase ($p<0.01$) caused by PTU-derived TSH signal transduction (Figure 1e) thereby confirming the *in vitro* data indicating the participation of SOD3-derived H_2O_2 in thyroid proliferative dysfunction. We next focused on the analysis of human goitrogenous tissues to compare the compatibility of the *in vivo* laboratory models to clinical patient samples. The qRT-PCR expression analysis from human endemic goiter and toxic adenoma patient tissues failed to demonstrate significant differences between patient groups (Figure 1f). The data therefore suggest that rat PTU model does not completely explain the regulation of *in vivo sod3* expression, which can be affected e.g. by paracrine effect of infiltrating inflammatory cells in benign thyroid enlargement.

SOD3 is downregulated in thyroid cancer models and patients

To investigate the expression of SOD3 in malignant thyroid proliferative disorders we first determined the *sod3* mRNA levels from rat thyroid tumor cell lines. The expression analysis of rat PC Cl3 derived PC PTC papillary thyroid cancer and PC E1A anaplastic thyroid cancer cell lines suggested differentiation dependent expression status (Figure 2a). Papillary thyroid cancer cell model PC PTC showed 3-fold decreased *sod3* mRNA synthesis where as more undifferentiated aggressive anaplastic PC E1A cells lacked the expression almost completely as compared to control PC Cl3 cells indicating differential regulation for SOD3 expression in benign and malignant thyroid enlargement.

Furthermore, the qRT-PCR and DNA array expression analysis from thyroid cancer patients showed significantly decreased *sod3* synthesis correlating with the differentiation degree of the tumor samples confirming the relevance of results obtained from rat thyroid cancer *in vitro* model to clinical patient samples (Figure 2 c). The *in vitro* and *in vivo* data therefore suggest that SOD3 should be considered as a differentiation marker in malignant transformation process.

TSH signal transduction increases *sod3* mRNA expression

Since SOD3 enzyme activity in human thyroid has been shown to be approximately 5-fold higher than in cardiac muscle and 3-fold above the average activity of all human tissues (16) we therefore investigated the signal transduction routes affecting *sod3* production in a versatile rat PC Cl3 cell line. The *sod3* mRNA synthesis was significantly induced by TSH (3-fold increase, $p<0.01$) stimulation suggesting signal transduction either via cAMP-protein kinase A (PKA) proliferation and differentiation pathway or via PLC-Ca^{2+} hormone production pathway (Figure 3a). The contribution of cAMP pathway in *sod3* mRNA production was studied by transient transfection of pCEF-*gs* and pCEF-*pka* into PC Cl3 cells, which resulted in 5.5-fold ($p<0.01$) and 6-fold increase ($p<0.001$) in *sod3* mRNA production, respectively. To confirm the transfection data we stimulated the TSH receptor signaling with forskolin incubation that yielded 6-fold increase ($p<0.05$) in mRNA production supporting the involvement of TSH-cAMP pathway in production of SOD3. To further demonstrate that cAMP-PKA is upstream of *sod3* we inhibited the PKA function with H89 PKA inhibitor in TSH stimulated PC Cl3 cells. Interestingly, even though TSH-H89 incubation significantly decreased ($p<0.05$) *sod3* production, it was not able to

completely diminish the messenger RNA synthesis suggesting other signaling routes for SOD3 stimulation (Figure 3a). In thyroid cells TSH-derived signaling is mediated also via G_q-PLC-Ca^{2+} signal transduction pathway, which leads to thyroid hormone production (27). To study the contribution of hormone synthesis pathway on *sod3* mRNA transcription PC Cl3 cells were transfected with pCEF-g_q or incubated in the presence of thapsigargin, which increases calcium influx, and analyzed the *sod3* expression. Transfection of pCEF-g_q increased relative *sod3* mRNA production 3-fold ($p<0.01$) and treatment with Thapsigargin 3-fold ($p<0.05$) suggesting that both G_s and G_q signal transduction pathways are involved in SOD3 activation (Figure 3b).

SOD3 has more prominent role in proliferation than in hormone production

We then aimed to investigate the physiological consequences of TSH stimulated SOD3 production. In addition to antioxidative characteristics we have recently shown that extraneously administered SOD3 is able to stimulate Ras GTP loading *in vitro* and *in vivo* leading to activation of mitogen signaling pathway and consequent growth factor expression and cell proliferation indicating that SOD3-derived H_2O_2 has growth regulatory properties (18). This is further supported by numerous reports showing that physiological concentrations of H_2O_2 can activate cell membrane receptors and downstream signaling leading to cell proliferation *in vitro* and *in vivo* (10-15).

The effect of SOD3 on cell proliferation was studied by RNA interference transfecting siSOD3 oligo into TSH stimulated PC Cl3 cells. The qRT-PCR analysis showed 95% interference effect ($p<0.001$) in *sod3* mRNA production at 48-hour time point (Figure

4a), which simultaneously corresponded to 15% ($p<0.05$) decrease in TSH-derived cell proliferation suggesting mitogenic effect for SOD3-derived H_2O_2 (Figure 4b). The data is in line with our previous results showing that SOD3 is able to stimulated Erk1/2 mitogen pathway leading to increased cyclin D1 and VEGF-A expression and consequent cell proliferation *in vivo* (18).

Since the *sod3* expression was increased also by PLC-Ca^{2+} signal transduction pathway we determined the amount of SOD3-derived H_2O_2 in Cos-7 heterologous system to predict the participation of SOD3 in hormone synthesis. The data showed significant decrease (**p<?**) in extracellular superoxide concentration indicating functionality of SOD3 in our cell model (Figure 4c). However, according to the fluorimetric assay the H_2O_2 production was 28-fold (**p<??)** lower by SOD3 than by DuoxA2 suggesting dual oxidases as the main source of H_2O_2 in thyroid hormone production (Figure 4d). Even though SOD3-derived H_2O_2 could be utilized in hormone production our data suggest that this activity is covered by dual oxidases in thyroid tissue. Therefore, TSH-cAMP-PKA signal cascade and consequent cell proliferation and differentiation could be considered to be more prominent roles for SOD3 in thyroid than the role in normal thyroid hormone production.

Discussion

Thyroid tissue physiology requires continuous high concentration of H_2O_2 for thyroid hormone T_3 and T_4 synthesis, which is according to the recent publications produced mainly by dual oxidase (28-31). Another H_2O_2 producing enzyme, SOD3, belongs to superoxide dismutase isoenzyme family that is responsible for balancing the reduction-oxidation reactions in different cellular compartments. Even though the accumulating data suggest that *sod3* expression is tightly regulated at several levels both pre-transcriptionally as well as post-translationally (18, 20, 32-37) the regulation of the expression and the physiological role of the enzyme is not completely understood. In the present study we analyzed the signal transduction activation and the effect of SOD3 in thyroid models and further compared the results obtained from *in vitro* and *in vivo* models to patient samples data.

The expression analysis of rat tissues suggested thyroid as a major expression site for *sod3*. However, in rat thyroid the expression level compared to other rat tissues was not as high as reported in human thyroid samples (16), which might influence the comparison of the results between species (Figure 1). According to our previous studies relatively low concentration of SOD3 *in vitro* or *in vivo* is able to induce a physiological response in cells and in the surrounding extracellular environment (18, 20, 23, 38) indicating that even minor differences in SOD3 concentration can have an impact on cellular signaling and consequent biological effects.

We studied the *sod3* expression in benign thyroid enlargement models to determine the expression of the enzyme in the proliferative environment. Even though previous clinical

analysis have demonstrated decreased total SOD activity in the plasma of Graves' disease patients (39-41) a recent paper suggested increased *sod3* production in thyroid caused by long-term iodine deprivation in murine models (42). Since both the synthesis of autoantibodies against thyroid hormones in Graves' disease and the diet iodine deprivation lead to increased TSH signaling and consequent hyperthyroidism we investigated the SOD3 expression in experimental *in vivo* goiter model and from hyperthyroid patient samples (Figure 1). Increased *sod3* expression in rat goiter model was in line with previous murine models (42) suggesting the participation of TSH stimulated increased SOD3 production in thyroid cells (Figure 4c). However, analysis of patient samples did not support the data from the murine model (Figure 1f). The data suggests that the expression of SOD3 in thyroid benign enlargement depends on multiple factors rather than on individual action of increased TSH production. The results further imply that murine goiter models do not completely mimic clinical patient status, which should be considered in data analysis.

The analysis of *sod3* in thyroid cancer *in vitro* and *in vivo* showed decreased expression of the mRNA suggesting that SOD3 is a differentiation marker in malignant transformation (Figure 2). Remarkably, the expression analysis pointed to distinct differentiation dependent mRNA synthesis demonstrating almost complete lack of *sod3* mRNA production in anaplastic thyroid cancer, the most aggressive form of thyroid tumors. Interestingly, a recent paper suggested that thyroid patient survival corresponded to the number of Ras oncogene activation mutations; more aggressive anaplastic thyroid cancer harbored significantly more mutations in all three Ras genes (43). Activated Ras oncogene is commonly known to cause decreased expression of several genes. Even though the exact mechanism for Ras oncogene mediated gene silencing is not clearly

understood, it has been suggested to take place by epigenetic silencing mechanism (44). We have published previously the presence of highly methylated GpC island at the *sod3* gene and showed decreased methylation in cardiovascular proliferative disease (45). Therefore, Ras-mediated epigenetic gene silencing could potentially explain the differentiation dependent decrease in *sod3* expression.

Since SOD3 related signal transduction in thyroid is not characterized previously we focused on effect of TSH signaling, the main external factor regulating thyroid function, on *sod3* mRNA synthesis. The effect of TSH is mediated by thyroid stimulating hormone receptor, a G protein-coupled receptor that activates G_s and G_q heterotrimeric G-proteins (46). TSH receptor stimulation, which principle intracellular signaling is channeled through cAMP and, to a lesser extent, by phophatidylinositol pathway, determines the main thyroid physiological functions. Activation of G_s leads to the stimulation of cAMP, a positive modulator of thyroid cell proliferation and the expression of differentiation markers such as sodium iodide symporter, thyroperoxidase, and thyroglobulin (1, 47). The G_q protein stimulation in thyroid initiates the cascade needed for Duox production and consequent increased in thyroid H_2O_2 synthesis, which is centrally important in thyroid hormone T3 and T4 production. Our present data describes a novel G protein-coupled receptor mediated activation of SOD3 through cAMP and by phophatidylinositol pathway suggesting larger role in thyroid physiology (Figure 3). Since GPCR mediated signal transduction plays a significant role also in other tissues the current data could potentially further explain our previous observations of SOD3 mediated effects in injury models (23, 48).

Lastly, we investigated the functional effect of SOD3 in thyroid environment by *sod3* RNAi and by overexpression of *sod3* in heterologous Cos7 model to study the SOD3-derived proliferation and level of H_2O_2 production, respectively. The current results showing that *sod3* RNAi knockdown is able to reduce the TSH stimulated PC Cl3 cell proliferation *in vitro* is in line with our previous data confirming the mitogen effect for SOD3-produced H_2O_2 (Figure 4). The involvement of G-protein coupled receptors, such as TSH receptor, on cell proliferation can be mediated through several routes; TSH mediated increased cAMP has been shown to activate Ras GTP loading in rat WRT cells and to increase Erk1/2 phosphorylation in rat FRTL-5 cells causing subsequent cell proliferation (49-51). It is noteworthy, that SOD3 has not been reported to have growth factor features and therefore the effect on cell proliferation likely sustain on H_2O_2 production and subsequence activation of Erk1/2 signaling (18). The present data further indicates that radical production in thyroid is not solely affecting hormone production but has also an impact on cell proliferation.

H_2O_2 production levels were studied by *sod3* overexpression in Cos-7 heterologous system to determine if SOD3 could potentially contribute to thyroid hormone production. (Figure 4). Even though transfected *sod3* reduced extracellular superoxide concentration the SOD3 derived H_2O_2 was covered by Duox produced H_2O_2 suggesting less pronounced role for SOD3-derived H_2O_2 in thyroid hormone production.

As conclusions, we have shown a novel TSH stimulation dependent GPCR-mediated signal transduction pathway activating *sod3* production in thyroid PC Cl3 cell line. We have further demonstrated differentiation dependent *sod3* expression both *in vitro* and *in*

vivo in patient samples suggesting that SOD3 could be considered as a differentiation marker in thyroid cancer diagnosis.

Figure text

Figure 1. SOD3 expression in thyroid. A. Quantitative PCR analysis for *sod3* mRNA expression from rat tissues indicated 2.5-fold increased ($p<0.05$) expression in thyroid as compared to cardiac tissue. However, the expression level thyroid was 30% lower than in aorta, which showed 4-fold higher *sod3* mRNA production ($p<0.05$) as compared to the heart. B. Thyroid weight measurement showed approximately 4-fold increase in wet weight of the PTU treated tissue indicating goitrogenous development. PTU was giving *ad libitum* in drinking water, which caused variation seen in weight development. C-D. Hematoxylin-eosin staining for PTU-treated rats showed hyperplasia and altered thyroid structure caused by the treatment. E. The *sod3* mRNA expression was significantly ($p<0.01$) increased by PTU. F. Goiter patient sample *sod3* mRNA expression level analysis insignificant differences between control, endemic goiter, and toxic adenoma patients.

Figure 2. SOD3 is a differentiation marker. A. The *sod3* mRNA analysis from rat normal and transformed PC Cl3 cells indicated decreased mRNA production correlating to the differentiation level. The *sod3* synthesis decreased 60% ($p<0.01$) in PC PTC papillary thyroid cancer cell model and was almost completely diminished ($p<0.001$) in PC E1A anaplastic thyroid cancer cell model. The *in vitro* cell model data was in line with DNA array (B) and qRT-PCR (C) patient sample analysis.

Figure 3. TSH activation increased *sod3* mRNA production in PC Cl3 cells. A. TSH-R cAMP pathway increased *sod3* production. The induction was analyzed from cells treated with TSH-R stimulants, with PKA inhibitor H89, and from cells transiently transfected

with *pka* and *tsh-r* $g\alpha_s$ subunit. B. The effect of TSH-R phophatidylinositol cascade on *sod3* synthesis was studied by *tsh-r* $g\alpha_q$ subunit transient transfection and by calcium uptake analysis using thapsigargin that blocks the calcium pumps in endoplasmic reticulum increasing the cytosolic calcium concentration. Both treatments, transfection and thapsigargin administration, increased the mRNA synthesis significantly ($p<0.01$ and $p<0.05$, respectively). C. The intracellular calcium levels went promptly up after 100 nmol/l thapsigargin administration and stayed above the baseline values over the follow-up period.

Figure 4. Functional role of SOD3 in thyroid models A. Quantitative PCR showed significant ($p<0.001$) *sod3* downregulation caused by siSOD3 intefering oligos. B. BrdU analysis for proliferating nuclei suggested significantly ($p<0.05$) decreased cell proliferation caused by siSOD3 in TSH stimulated PC Cl3 cells. C-D. Cos7 heterologous model for radical production.

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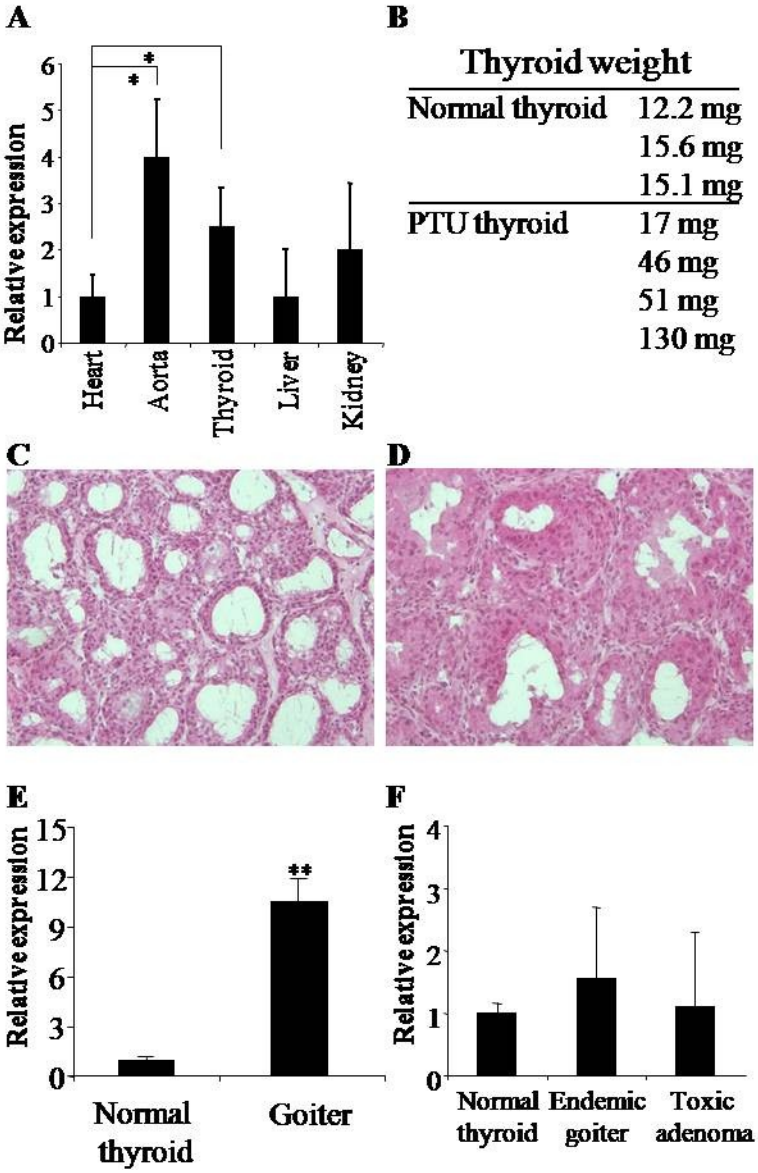
A
Relative expression
6
5
4
3
2
1
0
*
*
Heart
Aorta
Thyroid
Liver
Kidney
B
Thyroid weight
Normal thyroid 12.2 mg
15.6 mg
15.1 mg
PTU thyroid 17 mg
46 mg
51 mg
130 mg
C
D
E
Relative expression
15
12
9
6
3
0
**
Normal thyroid
Goiter
F
Relative expression
4
3
2
1
0
Normal thyroid
Endemic goiter
Toxic adenoma

Figure 1.

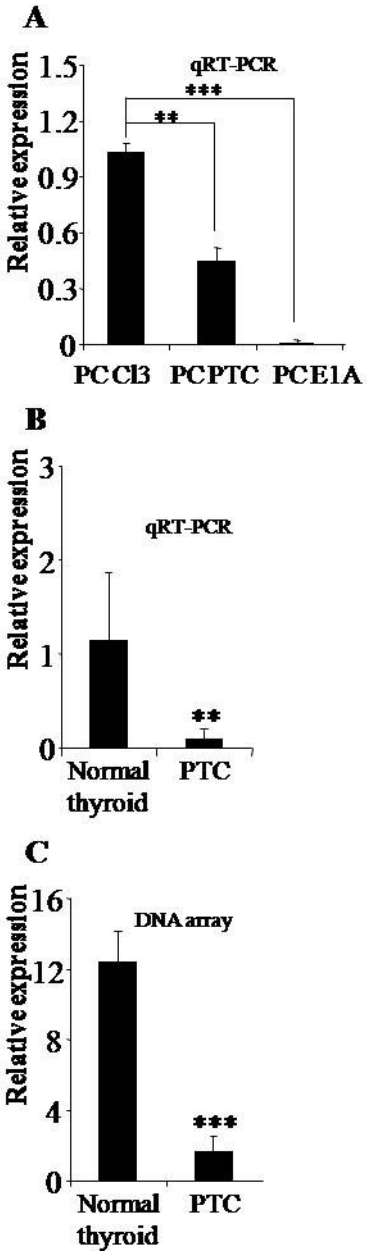
A
qRT-PCR
Relative expression
1.5
1.2
0.9
0.6
0.3
0
***
**
PCCl3
PCPTC
PCE1A
B
qRT-PCR
Relative expression
3
2
1
0
**
Normal thyroid
PTC
C
DNA array
Relative expression
16
12
8
4
0
***
Normal thyroid
PTC

Figure 2.

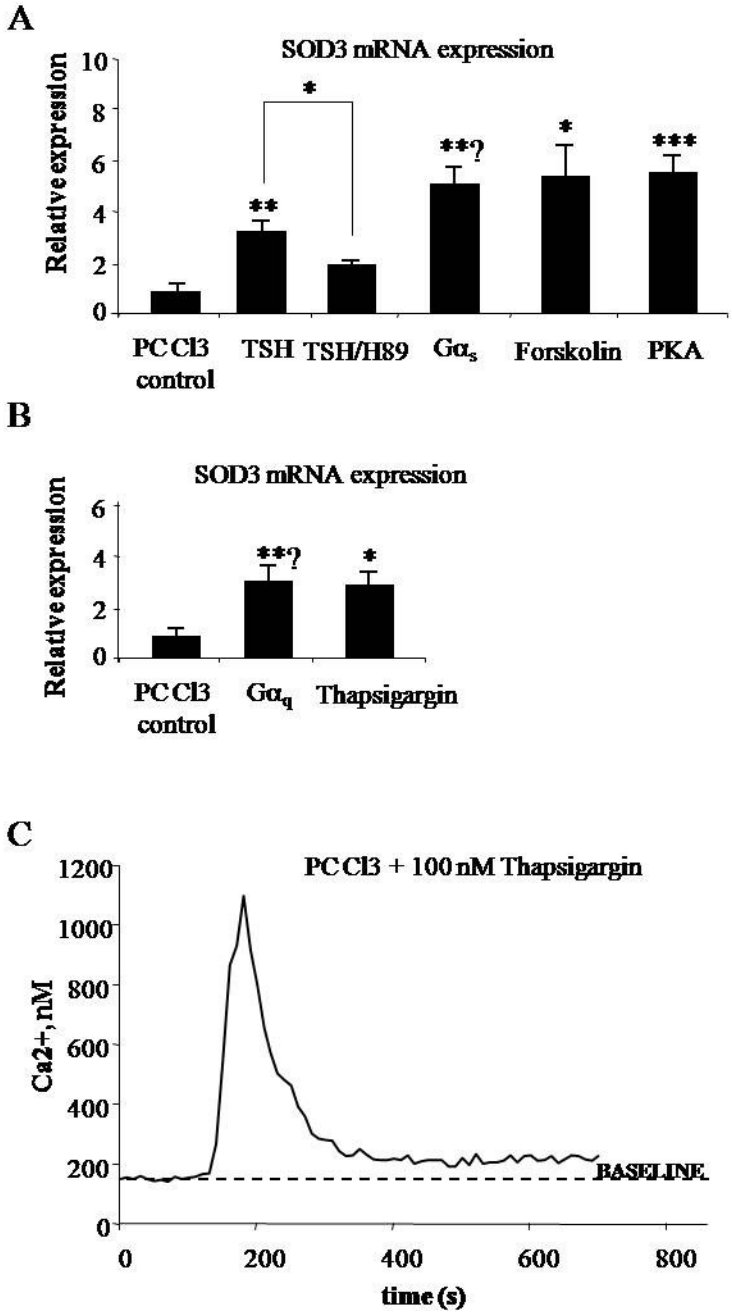
A
SOD3 mRNA expression
Relative expression
10
8
6
4
2
0
*
**
**?
*
***
PCCl3 control
TSH
TSH/H89
Gαs
Forskolin
PKA
B
SOD3 mRNA expression
Relative expression
6
4
2
0
**?
*
PCCl3 control
Gαq
Thapsigargin
C
PCCl3 + 100 nM Thapsigargin
Ca2+, nM
1200
1000
800
600
400
200
0
BASELINE
0
200
400
600
800
time (s)

Figure 3.

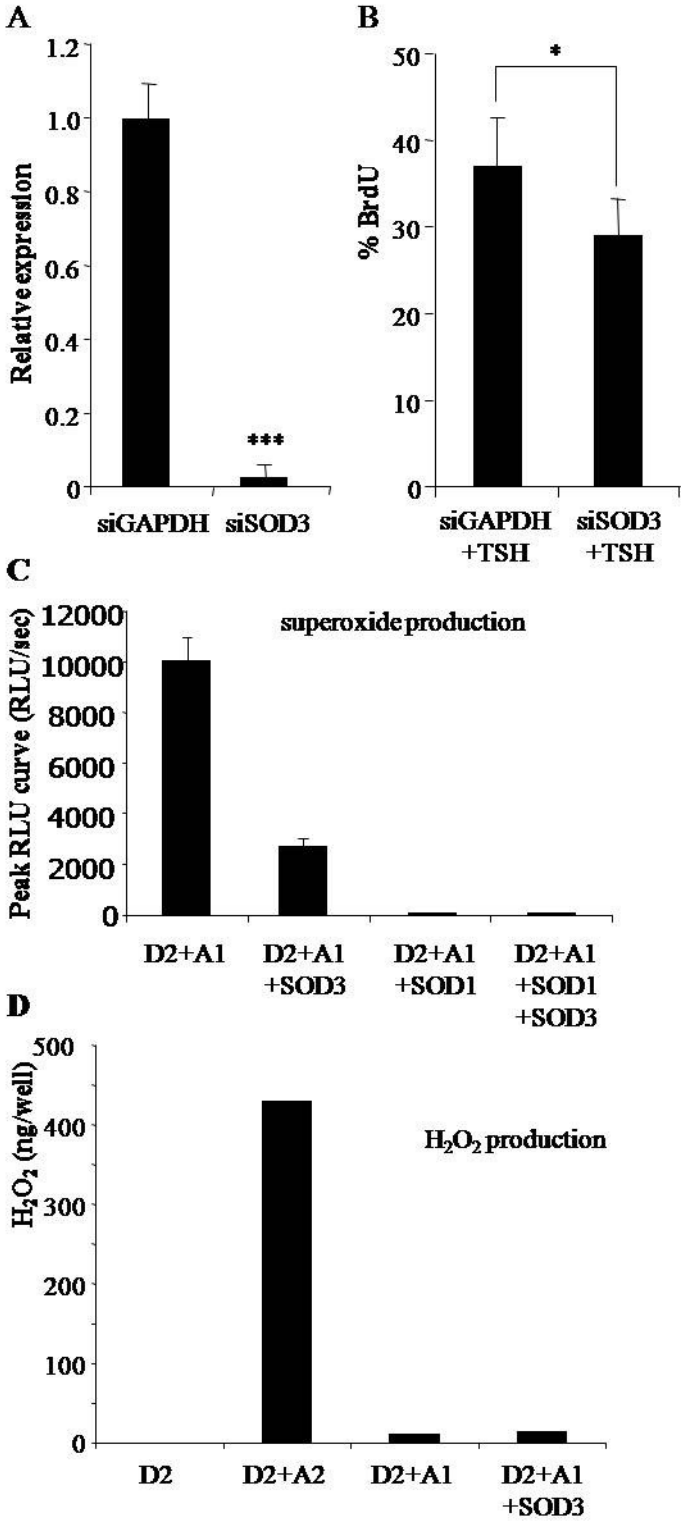
A
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0
***
siGAPDH
siSOD3
B
% BrdU
50
40
30
20
10
0
*
siGAPDH +TSH
siSOD3 +TSH
C
superoxide production
Peak RLU curve (RLU/sec)
12000
10000
8000
6000
4000
2000
0
D2+A1
D2+A1 +SOD3
D2+A1 +SOD1
D2+A1 +SOD1 +SOD3
D
H_2O_2 (ng/well)
500
400
300
200
100
0
H_2O_2 production
D2
D2+A2
D2+A1
D2+A1 +SOD3

Figure 4.