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PTPs: REDOX CONTROLLED REGULATORS OF CELL SIGNALING AND TRANSFORMATION

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ABSTRACT

Protein tyrosine phosphatases (PTPs) are important redox regulated enzymes that control the tyrosine phosphorylation status in the cell. These studies aimed to increase the understanding on how PTPs are controlled by reversible oxidation in different pathological settings such as hypoxia and restenosis and to which extent oxidized PTPs contribute to certain diseases.

Low levels of oxygen - hypoxia - can occur in tumors due to poor vascularization as well as in myocardial infarction and is associated with increased levels of reactive oxygen species (ROS). In our study we found that hypoxia followed by reoxygenation caused reversible oxidation of PTPs. This created an increased amount of inactivated PTPs and was followed by prolonged PDGF receptor activation. The hypoxia induced increase in phosphorylation lead to elevated ERK signaling as well as increased formation of cytoskeletal re-arrangements which could be inhibited by addition of the antioxidant *N*-acetylcysteine. Decreased phosphatase activity was also seen when analyzed in heart tissues from an *ex-vivo* model of rat heart exposed to ischemia-reperfusion.

Atherosclerosis is a disease characterized by artery wall thickening due to a buildup of fatty materials. A common treatment is to open up the vessel with a balloon catheter and thereby increase the blood flow. A problem with this treatment is that restenosis occur after some time. Restenosis is associated with an elevated ROS production and increase in PDGFβ-receptor signaling. Using a rabbit restenosis model we could show that restenosis could be attenuated by administration of antioxidants. Tissue analysis from vessels in combination with cell culture experiments showed that the beneficial effect of antioxidant treatment was prevention of PTP inactivation.

Peroxidized lipids have been found in increased amounts in several diseases with inflammatory components, like atherosclerosis and diabetes. The amounts of peroxidized lipids are tightly regulated by the glutathione peroxidase 4 (GPx4). In an inducible knock out model of GPx4 in cells we found that the rapid accumulation of peroxidized lipids caused PTP oxidation and lead to increased cell signaling. This was the first time peroxidized lipids were shown to oxidize and inactivate PTPs.

PTPs are often regarded as inhibitors of cell signaling and therefore as potential tumor suppressors. SHP-2 is however, an exception and is a *bona fida* oncogene in which phosphatase-activating mutations have been associated with different forms of leukemia and to a smaller proportion with solid tumors. We found that PDGF-BB dependent growth of subcutaneous tumors was compromised when SHP-2 levels were repressed by shRNA. Cell culture experiments indicated that compromised Src activity and reduced ERK activation underlie the inability of these cells to form tumors.

LIST OF PUBLICATIONS

I. **Åsa Sandin**, Boyka Markova, Frank Böhmer and Arne Östman
Evaluation of the role of the protein tyrosine phosphatase SHP-2 in PDGF-BB autocrine transformation
Submitted

II. **Åsa Sandin***, Markus Dagnell*, Adrian Gonon, John Pernow, Verena Stangl, Pontus Aspenström, Kai Kappert and Arne Östman
Hypoxia Followed by Re-Oxygenation Induces Oxidation of Tyrosine Phosphatases
Cellular Signalling, 2011, 23: 820-826

III. Marcus Conrad, **Åsa Sandin**, Heidi Förster, Alexander Seiler, Jeroen Frijhoff, Markus Dagnell, Georg Bornkamm, Olof Rådmark, Rob Hooft van Huijsduijnen, Pontus Aspenström, Frank Böhmer and Arne Östman
12/15-lipoxygenase-derived lipid peroxides control receptor tyrosine kinase signaling through oxidation of protein tyrosine phosphatases
Proc Natl Acad Sci, 2010, 107: 15774-15779

IV. Kai Kappert, Jan Sparwel, **Åsa Sandin**, Alexander Seiler, Udo Siebolts, Olli Leppänen, Stephan Rosenkranz and Arne Östman
Antioxidants relieve phosphatase inhibition and reduce PDGF signaling in cultured VSMCs and in restenosis
Arteriosclerosis, Thrombosis, and Vascular Biology. 2006, 26: 2644-2651

V. **Åsa Sandin**, Manuela Schneider, Jeroen Frijhoff, Heidi Förster, Alexander Mannes, Olof Rådmark, Arne Östman and Marcus Conrad
Novel links between arachidonic acid metabolism, PTP oxidation and growth factor signaling
Manuscript

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CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic Acid
AML	Acute Myeloid Leukemia
Csk	C-terminal Src kinase
COX	Cyclooxygenase
DEP-1	Density Enhanced Phosphatase-1
DUOX	Dual Oxidase
ecSOD	Extracellular SOD
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
ERK	Extracellular Regulated Kinase
FGF	Fibroblast Growth Factor
GF	Growth Factor
GPx	Glutathione Peroxidase
Grx	Glutaredoxin
GSH	Glutathione
HER-2	Human Epidermal Growth Factor Receptor 2
HETE	Hydroxyeicosatetraenoic Acid
HpETE	Hydroperoxyeicosatetraenoic Acid
IAA	Iodoacetic Acid
JMML	Juvenile Myelomonocytic Leukemia
LOH	Loss of Heterozygosity
LOX	Lipoxygenase
MAPK	Mitogen Activated Protein Kinase
NAC	*N*-acetylcysteine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOX	NADPH Oxidase
NRPTP	Non Receptor PTP
PDGF	Platelet Derived Growth Factor
PI3K	Phosphatidyl-Inositol-3-Kinase
Prx	Peroxiredoxin
PTEN	Phosphatase and Tensin Homolog
PTP	Protein Tyrosine Phosphatase
PUFA	Polyunsaturated Fatty Acids
ROS	Reactive Oxygen Species
RPTP	Receptor PTP
RTK	Receptor Tyrosine Kinase
SFK	Src Family Kinases
SH2	Src Homology 2
SHP-1/2	SH2-containing Phosphatase 1/2
SOD	Superoxide Dismutase
TK	Tyrosine Kinase
Trx	Thioredoxin
TrxR	Thioredoxin Reductase
VSMCs	Vascular Smooth Muscle cells

1 INTRODUCTION

1.1 TYROSINE PHOSPHORYLATION AS A CONTROL MECHANISM FOR CELL SIGNALING PROCESSES

Phosphorylation of proteins is a regulatory mechanism that plays an important role in cellular processes such as proliferation, migration, differentiation, fertilization, apoptosis and metabolism. Phosphorylation is the covalent binding of the energy rich phosphate group (PO_4) to a protein. Three amino acids that can be phosphorylated are serine, threonine and tyrosine. Serine phosphorylation is most abundant, whereas tyrosine phosphorylation represents only a small fraction of the total protein phosphorylation.

The first tyrosine kinase (TK) was discovered over 30 years ago by Tony Hunter and colleagues (Eckhart et al 1979) and since then TKs have been shown to participate in many signaling processes (Lemmon and Schlessinger 2010). Receptor tyrosine kinases (RTKs) are trans-phosphorylated at different sites when ligand stimulated. These phosphorylated sites recruit other proteins which in turn, in some cases, also become phosphorylated and thereby promote the downstream signal transduction of RTKs. The phosphorylation status is strictly regulated to prevent uncontrolled signaling, which otherwise can give rise to diseases such as cancer (Brognard and Hunter 2011).

Approximately one decade after the first TK was found, Edmond Fisher and colleagues purified the first protein tyrosine phosphatase (PTP) (Tonks et al 1988). PTPs have since then been shown to regulate many signaling processes (den Hertog et al 2008, Ostman et al 2006, Tonks 2006). As the name suggests, the PTPs dephosphorylate tyrosine-phosphorylated proteins. Individual PTPs display specificity for certain substrates, and in some cases also to specific phospho-tyrosine sites, and thereby contribute to a precise fine-tuning of the receptor signal (Ostman and Bohmer 2001). PTPs thus act as important regulators of RTK signaling.

1.2 THE PTP FAMILY

The PTP family consists of 107 PTPs encoded by the human genome. Two major subgroups are the dual specificity phosphatases that dephosphorylate serine, threonine and tyrosine, and the phosphotyrosine-specific PTPs. The first PTP to be purified and cloned was PTP-1B (Charbonneau et al 1989, Guan et al 1990, Tonks et al 1988). Since then, 37 additional classical phosphatases have been found. The classical PTPs can be further divided into receptor PTPs (RPTPs) and non receptor PTPs (NRPTPs) which consist of 17 and 21 members, respectively (Fig 1.) (Alonso et al 2004).

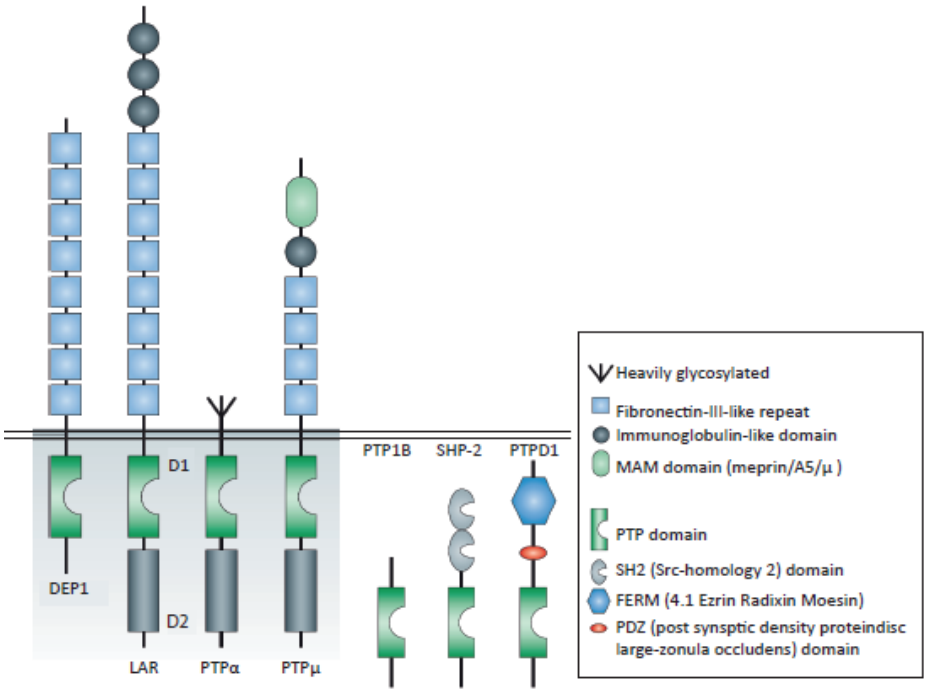

Figure 1. ***Schematic representation of some of the classical PTPs***

The left part of the illustration shows representative RPTPs. RPTPs contain a membrane spanning region and an extracellular domain which often contains domains involved in cell adhesion, such as immunoglobulin-like, fibronectin-III-like repeat, or MAM domains. The RPTPs also contain one or two intracellular PTP domains. The right part displays some NRPTPs which consist of one single PTP domain and often domains involved in protein-protein interactions such as SH2, FERM and PDZ domains (Figure adopted from (Ostman et al 2006)).

All the classical, phosphotyrosine specific PTPs, contain a 240–250 amino acid PTP domain with an active site sequence. In addition to the catalytic domain they contain many different regulatory domains or motifs which make the PTPs a diverse group of proteins. The RPTPs contain a transmembrane α-helix which directs them to the plasma membrane and an extracellular part that allows the transfer of signals through the membrane. The NRPTPs contain many protein-protein interacting domains, e.g. the Src homology 2 (SH2) domain of SHP-1 and SHP-2, that directs them to their target proteins as well as regulating their enzymatic activity. Some of the NRPTPs also contain sequences targeting them to the sub-cellular locations, e.g. the endoplasmatic reticulum-targeting (ER) sequence of PTP1B (Alonso et al 2004).

1.2.1 Regulation of PTPs

Reversible oxidation of the active site cysteine causes an inactivation of the catalytic activity of PTPs, and is a major regulatory mechanism which is described in more detail below (section 1.4) (Salmeen and Barford 2005).

Dimerization

Dimerization of RPTPs can lead to inactivation of the enzymatic activity as has been described for RPTPα (Bilwes et al 1996). Dimerization-induced inhibition of PTPRO activity was also demonstrated when a chimeric fusion protein was analyzed (Hower et al 2009). In contrast, activating dimerization of DEP-1 has been seen. Using an antibody against DEP-1, Takahashi et al could detect an increased DEP-1 activity, which also reduced intracellular signaling and blocked proliferation. When the antibody was used in mice, corneal angiogenesis was decreased. The effects were not seen when a monovalent form of the antibody was used (Takahashi et al 2006).

Ligand induced regulation

Ligand induced regulation of RPTPs can occur. One example is pleiotrophin that causes a reduction of the RPTP β/ζ catalytic activity (Meng et al 2000). RPTPs are important for development in neuronal synapses. In a Drosophila model, syndecan (a heparan sulfate proteoglycan) has been shown to bind to PTP-LAR and increase signaling (Fox and Zinn 2005). In another Drosophila model of synapse development, a different heparan sulfate proteoglycan, dallylike, had opposite effects on LAR signaling (Johnson et al 2006).

Phosphorylation

Phosphorylation is a common modification of PTPs leading to different effects on the catalytic activity of PTPs.

PTP1B has been shown to be inactivated by insulin in an Akt dependent manner, due to serine-phosphorylated residues in the catalytic site (Ravichandran et al 2001). Insulin stimulation *in vivo* could also inactivate PTP1B, involving tyrosine phosphorylation (Tao et al 2001). However, increased activity of PTP1B has also been found due to EGF-induced tyrosine phosphorylation, which created binding to Grb-2 (Liu and Chernoff 1997) .

RPTPα was found to be serine phosphorylated (Tracy et al 1995) which increased the catalytic activity (den Hertog et al 1995). In contrast, another study showed that RPTPα serine dephosphorylation by PP2A increased the ability of RPTPα to dephosphorylate Src on its inhibitory phosphorylation site (Vacaru and den Hertog 2010). Also tyrosine phosphorylation has been found on RPTPα. The tyrosine

phosphorylation was concluded to be essential for the binding and dephosphorylation of Src (Zheng et al 2000).

RPTPs have also been shown to dephosporylate each other. Both CD45 and RPTPα regulate Src family kinases (SFKs), but the expression pattern of the two RPTPs is different. CD45 is the major PTP expressed in T-cells whereas RPTPα is more widely expressed but can also be found in T-cells. When analyzing T-cells without CD45, RPTPα phosphorylation was increased, indicating that RPTPs can cross-talk and regulate each other (Maksumova et al 2007).
Platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) receptor stimulation has been found to phosphorylate SHP-2 on tyrosine residues (Feng et al 1993, Vogel et al 1993). The tyrosine phosphorylation on SHP-2 was found to be required for the PDGF- and FGF-induced ERK activation. Nevertheless, mutation of the tyrosine residues on SHP-2 could not completely decrease the ERK activation (Araki et al 2003). Biochemical studies explained that tyrosine phosphorylation of SHP-1 and SHP-2 increases the catalytic activity, possibly through changes in intramolecular inhibition (Lu et al 2001, Zhang et al 2003). SHP-2 can also be serine phosphorylated in a manner not affecting catalytic activity (Strack et al 2002).

Expression

PTPs are differentially expressed both in different cell types and tissues. A recent study in immune cells showed that different immune cell lineages had their own PTP expression signature, probably affecting specific signaling pathways important for each cell lineage (Arimura and Yagi 2010). Increased expression of DEP-1, PTP-LAR, PTPμ, RPTPκ and PTPβ was seen in cells cultured at high densities (Campan et al 1996, Fuchs et al 1996, Gaits et al 1995, Longo et al 1993, Ostman et al 1994). PTP expression levels have also been shown to change during the pathological process of restenosis (Kappert et al 2007).

Another mechanism that alters the PTP expression profile is the alternate usage of different promoters. This has been shown to give rise to tissue-specific forms of SHP-1 (Banville et al 1995) and to different isoforms of RPTPε (Elson and Leder 1995).

Proteolysis

The extracellular parts of some RPTPs are cleaved, sometimes causing translocation of the intracellular PTP domain to distinct cellular locations (Aicher et al 1997, Jiang et al 1993, Streuli et al 1992). The cleavage can be a consequence of high cell density and has been described for PTPmu (Campan et al 1996). Cleavage due to high cell density has also been shown for RPTPκ. The resulting intracellular PTP-containing domain can translocate to the nucleus and dephosphorylate β-catenin (Anders et al 2006). Calpain

cleavage of PTP1B redirects the catalytical domain of PTP1B from the ER to the cytosol and increases its catalytic activity (Frangioni et al 1993).

Subcellular localization

The localization of PTPs in the cell is important, since that will determine access to their substrates. PTPs contain different localization signals, as described in section 1.2. This directs e.g. RPTPs to the cell membrane and PTPs with ER targeting sequences to the ER and a compartment specific regulation of tyrosine phosphorylation is thereby achieved.

RPTPs are located in the membrane and have been found in complexes with TKs thereby regulate their signaling. Two examples are RPTP LAR in complex with TrkB (Yang et al 2006a) or together with c-Met (Machide et al 2006).

Some PTPs contain sequences that direct them to the ER. PTP1B is one example and most of its activity is found in the peri-nuclear area (Frangioni et al 1992). The participation of PTP1B in dephosphorylation of RTKs might therefore be due to the internalization of RTKs in endosomes (Yudushkin et al 2007). TC-PTP on the other hand is subjected to alternative splicing that gives rise to two different isoforms containing different carboxy terminals, TC45 and TC48, localized to the nucleus and the ER, respectively (Lorenzen et al 1995).

SH2 domains recognize and bind to phosphorylated tyrosine residue in a sequence-specific way. Binding of SH2-domain proteins is important for initiating the intracellular signaling of RTKs (Manning et al 2002). The cytoplasmatic SHP-1 and SHP-2, are recruited to cell surface proteins via their SH2 domain (Neel et al 2003). After ligand stimulation, SHP-2 has been shown to translocate to the activated PDGFβ receptors (Meng et al 2002). In addition to the SH2 domain, SHP-1 has a C-terminal motif that binds acidic phospholipids. This recruits SHP-1 to the cell membrane (Frank et al 1999) and has been shown to regulate T-cell receptor signaling in T-lymphocytes (Sankarshanan et al 2007). SHP-1 has further been shown to have a nuclear localization signal and translocates to the nucleus after epidermal growth factor (EGF) stimulation (He et al 2005). SHP-2 was first believed to be a cytosolic PTP but has also been found associated to STAT5 in the nucleus (Chughtai et al 2002).

This spatial dissection of PTPs might allow proteins to stay phosphorylated and participate in signaling at a certain compartment.

1.3 *IN VIVO* FUNCTION OF PTPs

Animal models have been generated to explore the *in vivo* function of PTPs. Nearly all members in the "classical" PTP family have been deleted in animal models by various techniques and some of them were found to be essential for life and therefore embryonic lethal when depleted (Hendriks et al 2008). SHP-2, PTP-PEST, VE-PTP and PTP-MEG2 null mice have been found to die during embryogenesis (Dominguez et al 2007, Sirois et al 2006, Wang et al 2005, Yang et al 2006b).

Generation of TC-PTP knock-out mice has shown that this knock out is not embryonical lethal. However, TC-PTP null mice died soon after birth. Analyses showed that TC-PTP is important for hematopoiesis and immune function (Heinonen et al 2004, You-Ten et al 1997).

Two mouse strains with different SHP-1 mutations have been described, the motheaten (me) and the viable motheaten (mev). Both mutations were found to be at the same gene but on different positions, creating a protein with differential deficiency (Green and Shultz 1975, Shultz et al 1993, Tsui et al 1993). The mev mouse with functionally deficient SHP-1 protein is viable and display enhanced insulin sensitivity compared to wild type (Dubois et al 2006).

One study explained that DEP-1 null mice were viable (Trapasso et al 2006). However, another DEP-1 model, with a replaced intracellular domain, was shown to be embryonic lethal (Takahashi et al 2003). This might depend on the remaining extracellular domain that could participate in signaling and prevent vascular development.

1.4 PTP OXIDATION

Already in 1974, Czech et al showed that a component of the glucose transport system becomes sulfhydryl oxidized upon binding of insulin to cell surface receptors (Czech et al 1974). In 1988 Koshio at el showed an increase in insulin receptor phosphorylation after exogenous addition of H_2O_2 (Koshio et al 1988). Two years later it was shown that the increased phosphorylation was due to inactivated phosphatases (Heffetz et al 1990). Since then, many studies have demonstrated inhibitory oxidation of the active site of different PTPs (Boivin and Tonks 2010, den Hertog et al 2008, Meng et al 2002, Salmeen et al 2003).

1.4.1 Maintenance of cellular redox balance

The levels of reactive oxygen species (ROS) are balanced by the actions of synthesis of reactive oxygen species and antioxidant capacity to scavenge the ROS.

Reduction of ROS can occur directly through H_2O_2 degrading enzymes (e.g. catalase, glutathione peroxidases or peroxiredoxins). Superoxide can be removed by superoxide dismutase (SOD) which exists in three different isoforms, the cytoplasmic SOD1, mitochondrial SOD2 and extracellular SOD (ecSOD). These SODs convert superoxide to H_2O_2 which can be converted to water by catalase or peroxidase (Chen et al 2009b).

Cells have two major systems to maintain the cellular thiol-disulfide redox status, they are the thioredoxin (Trx)/thioredoxin reductase (TrxR) system and the glutathione (GSH)/glutaredoxin (Grx) system (Lillig and Holmgren 2007, Lillig et al 2008).

In addition, removal of ROS can be performed by treatment with cell-permeable antioxidants such as *N*-acetylcysteine (NAC). The ROS mentioned below mostly consists of H_2O_2, superoxide and the hydroxyl radical.

1.4.1.1 ROS scavenging and reducing enzymes

Low molecular weight antioxidants

Glutathione and ascorbate are found at high concentration in cells where they are active against H_2O_2 and superoxide, respectively. Glutathione is a tri-peptide that consists of glutamate, cysteine and glycine, and is important both for protection against ROS and for maintaining ascorbate in the reduced state (Chen et al 2009b).

Peroxiredoxins (Prxs)

Prxs are believed to be the main converters of H_2O_2 to water. The Prxs exist as homodimers that gain intermolecular disulfide bonds in the H_2O_2 scavenging process and can be reduced by Trx. Oxidized Trx is then reduced by TrxR using NADPH as the electron donor (Chen et al 2009b).

Both Prxs and PTPs contain a thiolate anion with a low pKa which makes them reactive with H_2O_2 at neutral pH. A kinetic model system displayed that H_2O_2 was much more likely to react with PrxII than with PTP1B, taken in to consideration protein concentration and rate constants for both proteins (Winterbourn 2008). Nevertheless, it is clear that PTPs are inactivated by ROS produced by different stimulis. Explanations have been proposed to involve inactivation of Prx either through hyper-oxidation or phosphorylation of Prx, as discussed below. This might create sub-cellular compartments with transient accumulation of H_2O_2 leading to inactivated PTPs.

Reversibly oxidized Prx forms intermolecular disulfides that are reduced by Trx. This is a slow process and sometimes Prx is further oxidized to the hyper-oxidized sulfinic acid form. The sulfinic form of Prx can be reduced by sulfiredoxin (Woo et al 2003).

Woo et al discovered that PrxI and PrxII are regulated by two different mechanisms. They found that PrxI was tyrosine phosphorylated after PDGF and EGF stimulation and that the phosphorylation decreased PrxI activity (Woo et al 2010). The phosphorylation of PrxI was further increased when NOX1 was over-expressed. EGF stimulation was shown to induce co-localization of NOX1 and phosphorylated PrxI in the cell membrane. To study the role of PrxI phosphorylation *in vivo* wound healing was investigated and found to be associated with increased PrxI phosphorylation, especially in the edge of the wounds where RTK signaling was also very active. The PrxI phosphorylation declined at the same rate as wound healing. PrxII, on the other hand, was not tyrosine phosphorylated but found to be inactivated by hyper-oxidation. This growth-factor-induced PrxI inactivation might be an important way to facilitate RTK signaling during certain circumstances.

Glutathione peroxidase 4 (GPx4)

GPx4 belongs to a family of 8 members. Embryos from GPx4 knockout mice die in early embryogenesis. GPx4 can reduce small hydroperoxides (e.g. H_2O_2), fatty acid hydroperoxides, cholesterol and complex membrane lipid hydroperoxides using glutathione as the electron donor (Conrad 2009). Depletion of GPx4 in cells has been shown to induce apoptosis due to an increased 12/15-lipoxygenase activity and concomitant generation of lipid hydroperoxides (Seiler et al 2008).

1.4.1.2 ROS production

The two main sources of ROS in the cell are the mitochondria and NADPH oxidases.

Mitochondria and ROS production

Cellular respiration is associated with ROS production (Rigoulet et al 2011). This is believed to predominantly occur in the "Q cycle" of complex III and involves superoxide production both at the inner and outer surface of the inner membrane. Another part of the respiratory chain that contributes to ROS production is complex I.

The TCA cycle represents another mitochondrial source of ROS. Dehydrogenases such as alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase are implied as ROS generators. Furthermore, a series of studies have demonstrated that mitochondrial metabolism of lipids, including palmitoyl-carnitine, leads to ROS production independent of the respiratory chain (Rigoulet et al 2011).

Regulation of mitochondrial ROS production remains poorly characterized. However, “uncoupling” is recognized as one state associated with reduced ROS generation.

As discussed in study II of this thesis, hypoxia and hypoxia/reoxygenation are associated with increased mitochondrial ROS production. In this context it can be noted that a recent study, using a redox-sensitive protein sensor (RoGFP), targeting different cellular localizations, showed that hypoxia caused increased ROS production in the intermembrane space of the mitochondria (Waypa et al 2010).

NADPH oxidase (NOX)

NOX is a membrane protein and catalyzes the transfer of electrons from nicotinamide adenine dinucleotide phosphate (NADPH) on the inside of the cell across the membrane. When electrons come in contact with oxygen on the outside superoxide or H_2O_2 are formed.

Mammalian cells consist of five different NOX family members (NOX1, NOX2, NOX3, NOX4, or NOX5). Some of them require different subunits (p22phox, p47phox, p67phox and the small guanosine triphosphatase Rac1) to be activated although NOX5 exists as a monomer (Fig 2). Some subunits are common for some of the members, while others are isoform-specific. Therefore different NOX enzymes display difference in sub-cellular localization, tissue distribution and cellular functions (Chen et al 2009b).

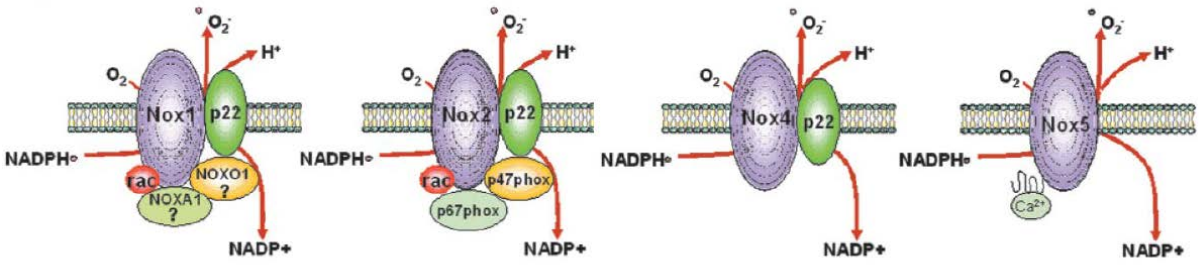

Figure 2. ***Schematic picture of representative NADPH oxidase members in endothelial cells*** *(figure adopted from (Frey et al 2009)).*

Other sources of ROS

There are also other sources of ROS in the cell. Glutamate, serum deprivation or UV irradiation are examples of ROS inducers. Metabolism of drugs or xenobiotics has also been shown to produce ROS. Furthermore, anticancer drugs, like doxorubicin, react with cytochrome P450 which creates a radical that reacts with oxygen and generates superoxide (Winterbourn 2008). As discussed in studies III and V of this thesis, peroxidized lipids can also act as oxidizing agents. These are formed in the eicosanoid synthesis pathway which ultimately converts arachidonic acid (AA) to eicosanoids. A key enzyme in this pathway is 12/15-LOX which oxygenates arachidonic AA or other

poly unsaturated fatty acids (PUFAs). The main product from 12/15-LOX derived AA oxidation is hydroperoxyeicosatetraenoic acid (15-HpETE) (Kuhn and O'Donnell 2006).

1.4.2 ROS as signal transducers

For a long time ROS were believed to only have cytotoxic effects in e.g. phagocytotic cells and in neurodegenerative diseases. However, accumulating evidence indicate that ROS can also participate in cell signaling. In order for a particular ROS to function as a signaling molecule, it should not be too reactive, since the half-life will be too short to react with a target. An example is the hydroxyl radical ($OH^{\cdot}$) which has a very short half-life. Superoxide and H_2O_2 on the other hand, have more favorable chemical profiles to function as signaling molecules.

Superoxide ($O_2^{\cdot}$-) cannot diffuse through membranes because of its negative charge and therefore needs to be transported through anion channels. Superoxide dismutase converts superoxide to the two electron oxidant H_2O_2 that can easily diffuse through membranes. H_2O_2 can then react with protein thiols to produce different sulfur oxidation states, such as disulfides (S-S), sulfenic (-SOH), sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid products. The first two are regarded as reversible post-transcriptional modifications that are important for cell-signaling (Chen et al 2009b).

The first example of ROS as a signaling molecule after RTK stimulation came from Sundaresan et al. They found that treatment of cells with antioxidants such as catalase or NAC inhibited receptor phosphorylation and downstream signaling (Sundaresan et al 1995). Later the same group found that the small GTP-binding protein Rac1 created a significant increase in intracellular ROS. They also found that growth factors such as PDGF and EGF and cytokines like tumor necrosis factor alpha or interleukin could induce ROS formation in NIH3T3 cells (Sundaresan et al 1996). Subsequently, PI3K has been shown to be required for PDGF mediated ROS formation (Bae et al 2000).

1.4.3 Biochemistry of PTP oxidation

The PTP active site (VHCSAGxGR[T/S]G) contains a conserved cysteine residue with a low p*K*a value. Therefore, at physiologic pH, the catalytical cysteine is deprotonated which makes it a good nucleophile that actively participates in the dephosphorylation process. This also makes the cysteine easily react with ROS. Oxidation of the catalytic cysteine destroys the nucleophilic effect and the phosphatase activity is lost. The oxidation of the cysteine thiolate anion to sulfenic acid is a reversible process. Further exposure to ROS can convert the sulfenic acid to sulfinic- or sulfonic- acid forms that represent irreversible modifications (Fig 3.) (den Hertog et al 2005).

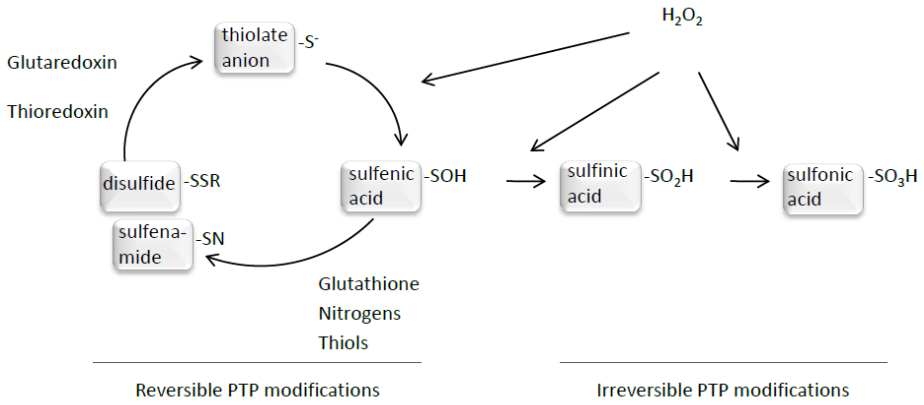

Figure 3. ***Protein tyrosine phosphatase regulation by reversible oxidation***

There appear to be mechanisms to protect PTPs from irreversible oxidation. In PTP1B and RPTPα a reversible sulfenylamide bond has been described between the oxidized cysteine and an adjacent serine residue (Salmeen et al 2003, van Montfort et al 2003, Yang et al 2007). Additional variants of oxidized PTPs have also been described, including catalytically inactive forms of SHP-2 containing a thiol-disulfide-bridge between two cysteines, but not including the active site cysteine (Chen et al 2009a).

The normally occurring oxidized PTPs are believed to be reversibly oxidized and susceptible to reduction by e.g. glutathione or thioredoxin. Some early studies suggest that PTPs differ with regard to "reduction-susceptibility" and also that they display differential sensitivity to different reducing agents. A recent study showed that SHP-2 but not SHP-1 could be reactivated by thioredoxin (Chen et al 2009a), whereas another study showed that PTP1B could be re-reduced by thioredoxin but not by glutaredoxin (Lee et al 1998).

Biochemical studies also suggest that PTPs differ in susceptibility to oxidation. Such examples include demonstrations of different oxidation sensitivity of the two PTP domains of RPTPα, as well as between SHP-1 and SHP-2 (Chen et al 2009a, Persson et al 2004, Weibrecht et al 2007). Furthermore, dual specificity protein phosphatases and lipid phosphatases differ with regard to oxidation susceptibility (Ross et al 2007). Finally, analyses of transfected PTPs and recombinant GST-PTP fusion proteins also uncovered big variations in oxidation susceptibility (Groen et al 2005, Persson et al 2004).

1.4.4 Control of GF signaling by ROS regulators

Lee et al showed that EGF stimulation of cells caused inactivation of PTP1B. The inactivation was transient, greatest 10 minutes after addition of EGF and returned to base line after 40 minutes (Lee et al 1998). Insulin can also cause a reversible oxidative inhibition of PTPs, including TC-PTP and PTP1B (Meng et al 2004). Transient

inhibition has also been seen in SHP-2 after PDGF stimulation, but only in the pool of SHP-2 that was recruited to the PDGF receptor (Meng et al 2002).

Also non-RTKs have been shown to induce inhibitory PTP oxidation. In anti-IgG stimulated B-lymphocytes an increased ROS production that inhibited SHP-1 has been observed (Singh et al 2005). Stimulation of antigen receptor of T cells caused transient oxidation of SHP-2. The oxidation of SHP-2 could be prevented by expression of PrxII or addition of antioxidants. By usage of the PrxII over-expressing cells they observed that prevention of SHP-2 oxidation led to increased adhesion (Kwon et al 2005).

A pattern is thus emerging suggesting that stimulation of cells with growth factors induces production of ROS, which in turn oxidize and inactivate different PTPs that can no longer dephosphorylate the RTKs. However, cells have a very efficient machinery to maintain cellular redox balance. It is therefore puzzling how RTKs can mediate ROS induced inactivation on PTPs. Mechanisms, also discussed above in section 1.4.1 and 1.4.2, that have been proposed to account for growth-factor-induced elevation of ROS are local inactivation of Prx and increased NOX activity (Fig 4).

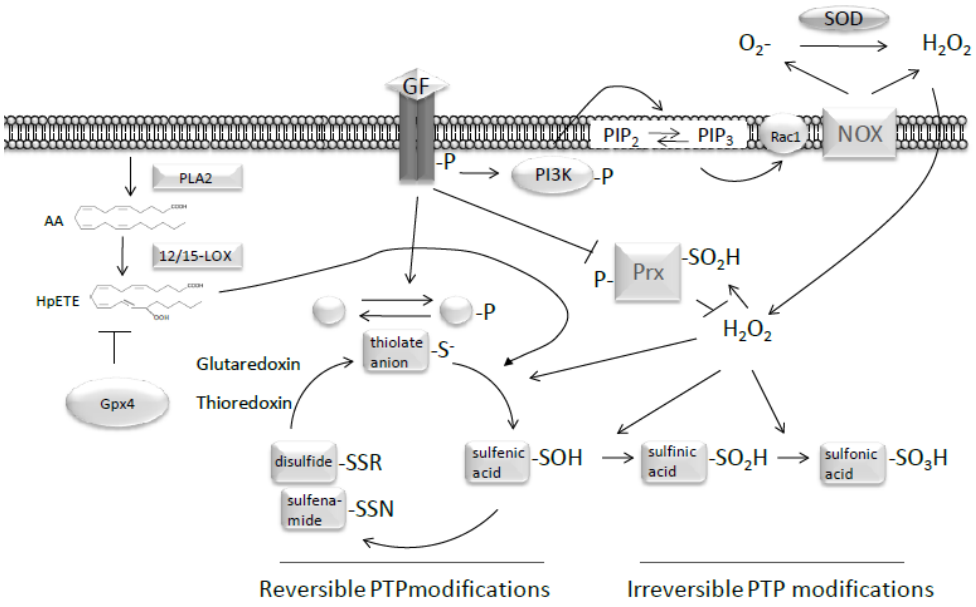

Figure 4. ***Mechanisms for growth-factor induced elevation of ROS levels***

Growth factor stimulation can activate PI3K which promotes Rac1 activation of NOX (Bae et al 2000). NOX can produce H_2O_2 or superoxide that is converted to H_2O_2 by SOD. Prx that normally scavenge H_2O_2 can be inactivated by oxidation upon elevated levels of ROS (Woo et al 2003). An alternative pathway for PrxI is the inactivating tyrosine phosphorylation (Woo et al 2010). An additional pathway, for PTP inactivation is production of peroxidized lipids from AA (data from study III and V).

The discussions above have emphasized growth-factor-induced PTP oxidation as a critical component of RTK signaling. A related, but distinct phenomena, is that growth factor responses are determined by the particular redox state of the target cell. This concept has received experimental support through a series of studies which have showed alterations in growth factor signaling in cells where expression levels of various redox enzymes have been perturbed.

PTP1B activity has been shown to be inhibited by insulin stimulation. The inactivation of PTP1B and the elevated insulin signaling cascade could be reverted by addition of catalase (Mahadev et al 2001). Juarez et al showed that cells treated with a SOD1 inhibitor decreased the amount of produced H_2O_2. This protected PTPs from oxidation and minimized the effects of growth factor stimulation (Juarez et al 2008).

Cells over-expressing glutaredoxin (Grx) displayed a decreased response to PDGF stimulation both with regard to PDGF receptor phosphorylation and proliferation. Depletion of Grx induced the opposite effect. It was also shown that LMW-PTP acted as a PDGF receptor targeting phosphatase and that LMW-PTP was protected from oxidation in cells with Grx over-expression. *In vitro* studies revealed that LMW-PTP formed reversible oligomers after H_2O_2 stimulation, and that this could be prevented by the presence of Grx. However, Grx needed the glutathione regenerating system to inhibit the oxidation of LMW-PTP (Kanda et al 2006).

Manipulations of NOX members have also been shown to affect growth factor signaling. Mahadev at al showed that NOX4 is important for insulin receptor signaling due to its ROS production that in turn inhibited PTP1B (Mahadev et al 2004). Another study showed that PTP1B was inhibited by ROS from NOX4, but only when both proteins were located in the ER. This was important for EGF signaling, since the EGF receptors that remained phosphorylated due to PTP1B inactivation, were recycled to the cell membrane instead of being degraded (Chen et al 2008). In a lens epithelial cell model Wang et al showed that $p22^{phox}$ (subunit of NOX) is involved in ROS production affecting LMW-PTP oxidation and PDGF receptor phosphorylation (Wang and Lou 2009).

Dual oxidase 1 (DUOX1) expression was found to be increased in epidermal keratinocytes after interleukin stimulation. The increased levels of DUOX1 protein was accompanied by increased H_2O_2 levels. The increased ROS production caused oxidation of PTP1B and increased phosphorylation of STAT6. DUOX knockdown or usage of a NOX inhibitor decreased the interleukin induced oxidation of PTP1B (Hirakawa et al 2011). Interleukin stimulation was also shown to, via NOX5, produce ROS which caused an increase in STAT 6 activation. PTP1B was the main PTP

responsible for the increased STAT6 signaling and was found to be oxidized from the interleukin stimulation (Sharma et al 2008).

Focal complexes are formed prior to cell migration and are enriched in tyrosine phosphorylated proteins. The NOX subunit, $p47^{phox}$, was also found to be enriched in these structures, and found to oxidize PTP-PEST (Wu et al 2005).

1.4.5 Tissue based evidence for PTP oxidation

A set of recent studies have also implicated ROS-induced PTP oxidation in *in vivo* models.

One example is PrxII that was shown to be recruited to activated PDGF receptors where it attenuates the PTP oxidation and decreases PDGF receptor signaling *in vitro*. PrxII knockout mice also displayed increased thickening of the vessel wall compared to wild type mice in a model of restenosis, which is consistent with a negative role for PrxII in PDGF signaling through inhibition of PTP oxidation (Choi et al 2005).

Mice lacking GPx1 showed increased ROS and increased PTEN oxidation. The consequence was elevated intracellular signaling which created increased insulin sensitivity compared to wild type. Treatment with the antioxidant NAC attenuated the enhanced signaling and insulin sensitivity in the GPx1 knockout mice (Loh et al 2009).
Oshikawa et al used a hind limb ischemia model to display increased angiogenesis when ecSOD was expressed. Cell culture studies revealed that the ecSOD increased oxidation of PTP1B and DEP-1 in caveolae lipid rafts which in turn increased the VEGF receptor signaling (Oshikawa et al 2010).

As discussed below, study II and study IV also presents evidence for *in vivo* oxidation of PTPs in the context of tissue ischemia and in restenotic vessels.

1.4.6 Methods for detecting oxidized PTPs

Most methods that analyze PTP oxidation in cell lysates or tissues share two features. Firstly, they require that experiments are done under low or oxygen-free conditions, since PTPs are highly sensitive to oxidation induced by atmospheric oxygen. Experimental procedures therefore need to be performed under very low oxygen conditions using reagents that contain as little oxygen as possible. Secondly, most assays involve the differential reaction of a chemical with reduced and oxidized PTPs that will allow these two pools to be distinguished from each other. Most often this involves the reaction of the reduced active-site cysteine with alkylating agents, such as iodoacetic acid (IAA), which do not react with the oxidized cysteine.

Some studies analyzing PTP oxidation has relied on ***in vitro* dephopshorylation assay**, using pTyr-peptides as substrate. Assays, performed on total cell lysates or on immunoprecipitated PTPs, are done in the absence or presence of exogenously added reducing agents. The difference in activity observed under these conditions is assumed to reflect the fraction of oxidized PTPs. An intrinsic problem with this approach is the difficulty in controlling the experimentally induced PTP oxidation occurring during the analytical steps.

The **OxPTP assay** is an antibody-based method for detection of oxidized PTPs (Persson et al 2004, Persson et al 2005). It uses an antibody recognizing a stable derivative of the oxidized PTP active site. In this assay all reduced PTPs are alkylated with iodoacetic acid during cell lysis. The PTP of interest is subsequently immunoprecipitated and subjected to pervanadate treatment which converts the oxidized active site cysteine to a sulfonic acid (SO_3). This derivative is finally detected by immunoblotting using the designated oxPTP antibody, originally raised against a PTP active site peptide with the cysteine in its sulfonic acid form (Fig 5.). The assay has been used to detect oxidation of endogenous PTPs, when antibodies of sufficient quality were available, and epitope-tagged recombinant and transfected PTPs.

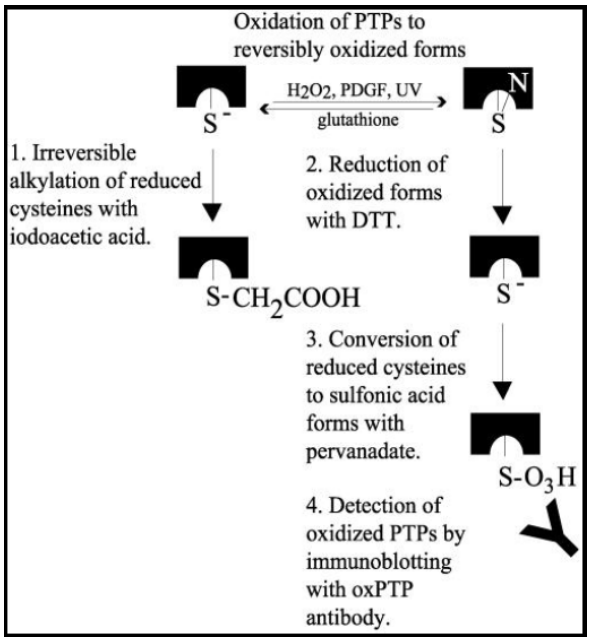

Figure 5. ***oxPTP antibody based method for detection of oxidized PTPs*** *(adopted from (Persson et al 2004))*

The **In gel assay for PTP oxidation** also uses initial alkylation of reduced PTPs as a strategy for counter-selection of the reduced variants. The reversibly oxidized PTPs are detected based on their ability, following renaturation, to dephosphorylate [γ-^{32}P]-labeled Glu_4Tyr peptides integrated in an SDS-polyacrylamide gel (Meng et al 2002). This method has the disadvantage that oxidation of RPTPs are not properly detected since these large, membrane proteins do not properly refold in the gel. The assay has

been used to monitor oxidation of multiple PTPs, by analyses of total cell lysates, or of individual PTPs using immuno-precipitation or immune-depletion approaches (Markova et al 2005).

The **cysteinyl-labeling assay** uses two alkylating agents that are used to differentially label reduced and oxidized PTP active sites. In brief, reduced cysteines in PTPs are blocked with an alkylating agent, whereas the oxidized PTPs - after exposure to reducing agents - are labeled with a biotin-coupled sulfhydryl-reactive probe and thereafter recovered by a streptavidin-pull-down procedure, SDS-PAGE and immunoblotting (Boivin et al 2008).

In **oxoMRM** (multiple reaction monitoring) non-oxidized cysteines are differentiated from reduced cysteines through labeling with two different forms of *N*-ethylmalemide (NEM) (Held et al 2010). First, non-oxidized cysteines are labeled with un-tagged NEM. This is followed by a reduction step and a second labeling step using isotope tagged-NEM. After immuno-precipitation and trypsin cleavage the peptides are separated with high-performance liquid chromatography and detected with mass spectrometry. Due to its design, the method will also identify irreversibly oxidized cysteines. In its present format the assay still depends on availability of specific PTP antibodies. Furthermore, early applications have indicated that different PTPs will display variations in susceptibility to trypsin cleavage which introduces another confounding factor.

When discussing PTP oxidation it should be mentioned that this term commonly remains vaguely used. Many PTP oxidation assays that are used do not describe the actual oxidation of the PTP but rather the inactivation of the PTP activity or inability to bind to alkylating agents. A decreased activity or less binding to alkylating agents could also depend on other inactivating factors such as nitrosylation. If evidence for reversible PTP oxidation is warranted methods involving antibody based detection of oxidized PTPs or identification with mass spectrometry is to prefer. Next best is activity based assays, especially if used with and without reducing agents. Analyses of phosphorylation or activation state of different signaling pathways downstream of the oxidized PTP are also a good complement. Depletion of different ROS producing or ROS scavenging enzymes can increase or decrease PTP oxidation, respectively, and can be a valuable tool to achieve information about biological significance of PTP oxidation. Finally, depletion of specific PTPs might also help to identify PTPs involved in different settings.

1.5 TYROSINE PHOSPHATASES AND CANCER

Cancer is the general name for many diseases with the common feature of deregulated cell signaling. PTPs are normally occurring as negative regulators of cell

signaling and are therefore predicted to be involved in tumor progression by loss of function. However, increasing evidence predicts that PTPs can also have oncogenic functions (Julien et al 2011).

1.5.1 PTPs as tumor suppressors

Different approaches have been used to study the role of PTPs in cancer. Wang et al performed a systematic sequencing of colorectal cancers to search for mutations in PTPs. Screening of 18 colorectal cancers for mutations in all PTPs revealed that six of the PTPs had somatically derived mutations. These six PTP genes were analyzed in an additional 157 colorectal cancers where 77 different mutations were found, meaning that 26% of the tumors had one or more mutation(s). When the same genes were examined in other tumor types mutations were found in some cancer types, whereas others were unaffected. The predicted outcome from the 15 mutations was loss of PTP expression or loss of PTP activity. The mutations in the most frequently mutated PTP, the *PTPRT* (coding for PTPρ), were further analyzed and were also found to have decreased PTP activity (Wang et al 2004).

Hyper-methylation of DNA can cause gene silencing and has been found in regulatory regions upstream of *PTPRG*, *PTPRD*, *PTPRO*, and *PTPN13* genes in lymphoma, leukemia, glioblastoma and different carcinomas. This caused a loss in gene expression compared to normal tissue (Motiwala et al 2004, Motiwala et al 2007, van Doorn et al 2005, Wang and Dai 2007, Veeriah et al 2009, Yeh et al 2006). *PTPN6* also displayed hyper-methylation and a subsequent loss of SHP-1 expression in lymphomas and leukemias (Koyama et al 2003, Oka et al 2002, Reddy et al 2005).

PTPRT null mice were shown to have hyper-phosphorylated paxillin which ultimately led to increased Ras signaling. The *PTPRT* null mice were also shown to be more sensitive to develop carcinogen induced colon tumors than wild-type mice. (Zhao et al 2010).

DEP-1 has also been shown to have tumor suppressor functions in several studies. The first evidence was that different cancer cells decreased their growth rate after re-expression of the DEP-1 protein. Other studies found loss of heterozygosity (LOH) in breast, colon and thyroid cancers (Ostman et al 2006). *PTPRJ* (encoding DEP-1) was found in the cancer susceptibility locus (Scc1) in a mice strain with increased colon cancer susceptibility. Further analyses in colon, lung and breast cancer samples revealed that *PTPRJ* had LOH in 49-78% of the cases (Ruivenkamp et al 2002). LOH of *PTPRJ* has also been found in meningiomas. *In vitro* studies revealed that the mobility of cells with loss of DEP-1 was increased. Tumors from DEP-1 depleted cells grew rapidly when transplanted in to mice and also displayed an increased invasion (Petermann et al 2010).

In addition, TC-PTP has been proved to be a tumor suppressor in T cell acute lymphoblastic leukemia due to deletions in the *PTPN2* gene. The *PTPN2* deletion was found in combination with the TLX1 transcription factor oncogene important for the activation of the mitotic checkpoint (Kleppe et al 2010).

A recent study used a pool of shRNAs against all PTPs. They screened transformed cells and found *PTPN12* as the top candidate. Quantitative proteomics revealed that 69 proteins displayed increased phosphorylation with loss of *PTPN12*. The 69 phosphorylated proteins were found to cluster in EGFR/HER2 pathways. Screening for mutations in breast cancer revealed that triple negative breast cancer had mutations in 4.8 % of the cases and that *PTPN12* was lost in ~60% of the cases. In comparison, the groups with HER2 amplification or PR positive breast cancers displayed no *PTPN12* mutations or loss of *PTPN12* protein. This indicated that *PTPN12* is a tumor suppressor that is lost or inactivated in triple negative breast cancers (Sun et al 2011).

1.5.2 PTPs as oncogenes

Some PTPs have been found to promote, instead of inhibiting cell signaling. It is therefore not surprising that some PTPs are also oncogenic. This is best described in the case of SHP-2. The role of PTP1B in cancer appears more complicated and PTP1B has been described both as tumor suppressor and tumor promoter (Lessard et al 2010).

Two independent mouse models using activated ErbB2 (HER2) as promoter for mammary carcinogenesis showed that PTP1B null mice had a delayed onset of tumor formation (Bentires-Alj and Neel 2007, Julien et al 2007). The study performed by Bentires-Alj and Neel explained the positive role for PTP1B as an activator of the ERK pathway, however, only in the pre-malignant mammary tissue. Furthermore, if polyoma middle T were used to induce mammary tumors, PTP1B status did not affect tumor formation, indicating that PTP1B act only on specific signaling pathways (Bentires-Alj and Neel 2007). Julien et al found that PTP1B null tumors had decreased RAS-MAPK and Akt signaling. The positive role of PTP1B in the ErbB2 induced mammary tumor formation was also confirmed by the use of a small molecule inhibitor of PTP1B (Julien et al 2007).

SHP-2 is a NRPTP that contains two SH2 domains, a PTP domain and a C-terminal tail with two tyrosine phosphorylation sites. It has been shown that SHP-2 requires both the PTP domain and the SH2 domains in order to carry out biological actions (Neel et al 2003). The crystal structure of SHP-2 revealed that the basal state of SHP-2 is a closed and inactive conformation in which the amino terminal SH2 domain is interacting with the PTP domain. Upon binding of the SH2 domain to pTyr, this closed structure is released and the catalytic activity is increased (Fig 6.) (Hof et al 1998).

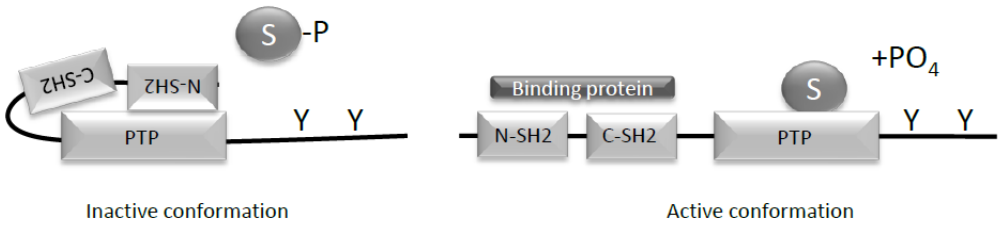

Figure 6. ***Structure of SHP-2***

When SHP-2 binds to a phosphorylated substrates via the SH2 domains it opens up the structure and can dephsoporylate tyrosine phosphorylated substrates (S).

SHP-2 has been shown to have a positive role in RTK signaling and to be required for cell transformation driven by a number of TKs, such as Bcr-Abl, EGFR, FGFR and Ret (Agazie et al 2003, Chen et al 2007, D'Alessio et al 2003, Zhan et al 2009). In a PDGF-dependent glioma model, transformation was reverted if the binding sites for SHP-2 and PI3K were deleted. Cell culture experiments confirmed that a SHP-2 inhibitor or SHP-2 shRNA could prevent transformation of the glioma cells (Liu et al 2011).

Speculations on how SHP-2 mechanistically mediates the positive role in cell signaling and tumorigenesis have generated many suggestions, most of them involving activation of the Ras-MAPK pathway. One study revealed that SHP-2 mediates direct dephosphorylation of the binding site for Ras-Gap on the PDGF receptor and thereby induces Ras signaling (Klinghoffer and Kazlauskas 1995). Another study proposed a mechanism where SHP-2 dephosphorylate the binding site for Ras-Gap on Gab1 and thereby induce activation of Ras (Montagner et al 2005). Others suggested that SHP-2 impairs C-terminal Src kinase (Csk)-mediated inhibition of Src, thereby also giving rise to Ras activation (Ren et al 2004, Zhang et al 2004). Finally, SHP-2 has been shown to dephosphorylate and thereby inactivate sprouty, an inhibitor of RTK signaling (Hanafusa et al 2004).

Sequencing of *PTPN11* (encoding SHP-2) revealed activating mutations in SHP-2 in one third of the cases of sporadic juvenile myelomonocytic leukaemia (JMML) and also, at lower frequencies, in myelodysplastic syndrome and acute myeloid leukemia (AML) (Loh et al 2004, Tartaglia et al 2003). Analyses of solid tumors displayed only a few cases with *PTPN11* mutations (Bentires-Alj et al 2004). The disease-associated mutations were found to be clustered in the SH2 domains or in the catalytic site of SHP-2. Most of the mutations created an open protein conformation with a higher catalytic activity than wild type SHP-2 (Fig 6). Some of the mutations displayed a comparable activity to wild type in their basal state, but upon substrate binding increased their activity much more compared to wild type (Keilhack et al 2005, Tartaglia et al 2003).

To gain further insight into the SHP-2 variants found in leukemia, primary bone marrow was transduced with SHP-2 constructs. Transformation occurred after addition of the leukemia-variants of SHP-2, but only after a significant period of time. However, addition of cytokines caused hyper-activation of ERK, AKT and STAT5, suggesting that mutated SHP-2 works together with other mutations (Mohi et al 2005).

In breast cancer, mutations in SHP-2 are uncommon. On the other hand, Gab2 which requires SHP-2 for transformation, is often amplified (Bentires-Alj et al 2006). Another cancer setting in which SHP-2 has been implied is Helicobacter Pylori-induced gastric cancer. During Helicobacter Pylori infection, the CagA protein is delivered to the gastric epithelial cells where it becomes tyrosine phosphorylated by Abl and Src kinases. The phosphorylated CagA protein can then bind to SHP-2 leading to the open and active conformation which in turn transforms the epithelial cells. Support for this model has also been provided using SHP-2 siRNA (Hatakeyama 2009).

2 PRESENT INVESTIGATION

2.1 AIMS OF THE THESIS

- To investigate the requirement for SHP-2 in PDGF-BB dependent autocrine transformation
- To characterize PTP oxidation in different pathological settings that involve increased ROS production, such as hypoxia and restenosis
- To explore the role of oxidized lipids in PTP oxidation and cell signaling
-

2.2 KEY FINDINGS

2.2.1 Paper I

Evaluation of the role of the protein tyrosine phosphatase SHP-2 in PDGF-BB autocrine transformation

Since SHP-2 has been reported to act as a positive regulator of cell signaling downstream of different RTKs, we aimed to analyze the role of SHP-2 in PDGF dependent transformation. Tissue culture based transformation assays and xenograft experiments demonstrated that SHP-2 is required for PDGF-BB induced transformation of NIH3T3 fibroblasts. Downregulation of SHP-2 attenuated both Src activity and ERK phosphorylation. In addition, we found that SHP-2 repression impairs acidification of cell growth media.

2.2.2 Paper II

Hypoxia followed by re-oxygenation induces oxidation of tyrosine phosphatases

Heart tissue ischemia, induced in the Langendorff model of *ex vivo* heart circulation, was found to induce oxidative inactivation of PTPs. Inactivation was detected both in the total pool of PTPs, and in immunoprecipitated SHP-2.

In vitro experiments confirmed that hypoxia followed by reoxygenation caused PTP oxidation of receptor like PTPs and cytosolic PTPs. The elevated PTP oxidation was associated with a decreased PDGF receptor dephosphorylation and increased PDGF-induced formation of lamellipodia structures. The latter was possible to attenuate with antioxidants.

2.2.3 Paper III and V

12/15-lipoxygenase-derived lipid peroxides control receptor tyrosine kinase signaling through oxidation of protein tyrosine phosphatases and **Novel links between arachidonic acid metabolism, PTP oxidation and growth factor signaling**

GPx4 is an efficient scavenger of H_2O_2 and phospholipids in the cell. The aim was to explore the consequence of GPx4 depletion, and the concomitant increase in peroxidized lipids, on PTP oxidation.

GPx4 knock out cells displayed an increased level of peroxidized lipids and caused elevated PTP oxidation and inactivation, thus uncovering a novel mechanism for PTP oxidation. *In vitro* assays demonstrated that the peroxidized lipids were more potent than H_2O_2 with regard to the ability to oxidize PTPs. The PTP inactivation in the GPx4-/- cells occurred together with an increased ligand-independent PDGF receptor phosphorylation, reversible by addition of vitamin E, and PDGF receptor inhibitor-sensitive lamellipodia formation.

Addition of exogenous HpETE and AA to cells induced ligand independent PDGF receptor phosphorylation and AA-induced PTP oxidation was also detected. 12/15-LOX null cells displayed reduced response to PDGF-BB with regard to phosphorylation and proliferation. These findings, obtained independent from the use of GPx4-/- cells, support the general notion of a crosstalk between AA metabolism and growth factor signaling.

2.2.4 Paper IV

Antioxidants relieve phosphatase inhibition and reduce PDGF signaling in cultured VSMCs and in restenosis

Vascular disease involves activation of vascular smooth muscle cells (VSMCs). We aimed to investigate if inhibitory oxidation of PTPs is part of the increased activation of VSMC seen in restenosis. Neointima formation after vessel injury was found to be decreased in animals that received either of two antioxidants, *N*-acetylcysteine or tempol. Analysis of the injured vessels revealed that antioxidant treatment increased PTP activity and decreased PDGF receptor phosphorylation, compared to control treated animals. The protective effect of antioxidants on VSMCs could be confirmed *in vitro* using H_2O_2 as oxidizing agent. Interestingly, only *N*-acetylcysteine had a protective effect on PTP activity. In contrast, tempol, which had a protective effect against vessel injury *in vivo* had no effect on the PTP activity in cells. *In vitro* studies using VSMCs also showed that addition of antioxidant decreased PDGF induced migration and proliferation.

2.3 FUTURE PERSPECTIVES

We have demonstrated that SHP-2 is required for PDGF-BB dependent transformation of mouse fibroblasts. These studies should be extended to other PDGFR-dependent cancer models, e.g. primary cultures of glioblastoma cells or models of PDGF-dependent GIST tumors. Further characterization of the pathways regulated by SHP-2 in PDGF-dependent transformation is also warranted. SHP-2 displayed an interesting connection to acidification of the cell growth media. It would be interesting to further analyze if this phenotype is linked to the transformation-supportive roles of SHP-2.

The results from the hypoxia study showed that PTPs display oxidative inactivation after ischemia and hypoxia that were followed by reoxygenation. The finding that ERK phosphorylation and lamellipodia formation could be attenuated by the usage of antioxidant make it of interest to see if antioxidant mediated re-activation of PTPs could be a useful strategy in pathological settings that involve an increased ROS production. In addition, it would be most interesting to see if hypoxia-mediated PTP inactivation also occurs in hypoxic cancers and if this also leads to enhanced RTK signaling.

The finding that knock-out of GPx4 created massive PTP oxidation in combination with the fact that 12/15-LOX knock-out cells did not respond to PDGF-BB indicates that a crosstalk occurs between the two pathways. One hypothesis that would be interesting to investigate is the possibility that GF stimulation possibly activates the 12/15-LOX enzyme, or blocks the GPx4 activity, to provide a feed forward loop for RTK activation. It would also be interesting to further investigate the PTP oxidation status in 12/15-LOX cells. Concerning disease relevance it appears motivated to investigate if conditions associated with increased levels of peroxidized lipids also display increased PTP oxidation and enhanced RTK signaling.

The restenosis study describes that ROS formation and PTP oxidation participate in the pathological processes of increased vessel thickening after injury. Future studies on the mechanisms for ROS production would be of high interest. It would also be interesting to obtain a more detailed description on which PTPs that are most important for PDGF-dependent and -independent processes involved in vessel remodeling after injury.

Several methods have been used to detect PTP oxidation in this study. However, a need still remains for better methods to analyze oxidized PTPs in tissue sections. A potential method would be the proximity ligation assay with a format combining one binder against the PTP and one targeting the oxidized cysteine. Such an assay would make it possible to localize oxidized PTPs. Together with information about localization of ROS producers and ROS scavengers this should give better information about the role(s) for PTP oxidation in physiological and pathological settings.

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4 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kroppens signaler till dess celler är ett viktigt instrument för att styra om cellen ska dela sig, förflytta sig eller dö. Till cellen kommer signaler i form av proteiner, som kallas tillväxtfaktorer. Dessa binder till receptorer på cellens utsida. Aktivering av receptorer sker genom att fosforgrupper sätts på receptorn. Dessa fosfatgrupper kan också avlägsnas från receptorerna med hjälp av en klass proteiner som kallas tyrosinfosfataser (PTPs).

Nettosignaleringen från cellyte-receptorerna bestäms alltså både av aktiviteten hos PTPs och mängden tillväxtfaktorer som når cellen. Ett genomgående tema i denna avhandling är analyser av hur PTPs kontrolleras och i vilken utsträckning störd PTP-aktivitet bidrar till sjukdom.

Flera sjukdomstillstånd, inklusive cancer och hjärtinfarkt, kännetecknas av syrebrist (hypoxi) i vävnaden. Vi har studerat hur hypoxi påverkar PTPs. Vi fann att hypoxi orsakade minskad aktivitet hos PTPs, vilket medförde att cellsignalering från cellyte-receptorer ökade. Detta kunde påvisas både i hjärtvävnad och i celler odlade utanför kroppen. Dessa effekter kunde blockeras genom tillsatser av antioxidanter.

Arteroskleros är ett tillstånd av förträngningar i kärl. Normalt behandlas arteroskleros med ”ballongsprängningar” som öppnar upp kärlen och därigenom förbättrar blodcirkulationen. Ett vanligt problem efter detta ingrepp är att kärlen växer igen och att cirkulationen då åter blir nedsatt. Denna process kallas restenos och orsakas av överaktivitet hos en viss typ av cellyte-receptorer - PDGF receptorer - som stimulerar celldelning hos kärlväggsceller. Med hjälp av en restenos-djurmodell visades att restenos kunde minskas genom behandling med antioxidanter. En serie analyser antydde att dessa goda effekter av antioxidanter orsakades av en aktivering av PTPs, som i sin tur motverkade den sjukdomsframkallande PDGF receptor signaleringen.

Peroxiderade lipider är en sorts molekyler som tidigare kopplats till vissa sjukdomar. I en studie användes en celltyp som producerar extra höga nivåer av peroxiderade lipider eftersom den saknar ett enzym – GPx4 – som normalt bryter ner dessa skadliga molekyler. Utan GPx4 ökade dramatiskt mängden inaktiverade PTPs och detta ledde till ökad cellsignalering. Kopplingen mellan peroxiderade lipider och PTPs hade tidigare inte visats och öppnar för en mängd studier för fortsatta undersökningar av kopplingar mellan peroxiderade lipider, PTP inaktivering och sjukdomsuppkomst.

De flesta PTPs betraktas som ”bromsproteiner” för celldelning eftersom de stänger av cellyte-receptorer. Ett undantag från denna regel är SHP-2 som tidigare kopplats till olika tumörtyper. I en tumörmodell, som härmar tumörer med överaktiv PDGF

receptorsignalering kunde vi visa att cellernas tumöregenskaper blockerades om man förhindrade produktionen av SHP-2. Resultaten antyder att SHP-2-hämmare kan vara av nytta i vissa cancer-typer, t.ex. hjärntumörer, som är beroende av PDGF-receptorsignalering.

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