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Review

Two axes in platelet-derived growth factor signaling: tyrosine phosphorylation and reactive oxygen species

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Abstract. The tyrosine phosphorylation cascade is a hallmark of platelet-derived growth factor (PDGF)-induced signal transduction. The amplitude and propagation of the tyrosine phosphorylation signal relies on the balance between tyrosine kinase and tyrosine phosphatase. The tyrosine kinase is latent in the absence of stimulation, whereas the tyrosine phosphatase is highly and constitutively active. Therefore, the kinase activation should be accompanied by temporal and spatial inactivation of tyrosine phosphatase to achieve the robust amplification of tyrosine phosphorylation. For the past decade, reactive oxygen species have been receiving a great deal of attention with regard to their ability to shut down tyrosine phosphatase activities in a reversible manner. In this article, the crosstalk between tyrosine phosphorylation and reactive oxygen species in PDGF signaling is discussed.

Keywords. PDGF, tyrosine phosphorylation, reactive oxygen species, protein tyrosine phosphatase, peroxiredoxin, NADPH oxidase.

Introduction

Platelet-derived growth factor (PDGF) is a peptide growth factor that signals the proliferation of target cells [1, 2]. PDGF isoforms consist of different combinations of two polypeptide chains (A- and B-chain), e.g. PDGF-AA, -AB and -BB. PDGF receptor (PDGFR) is a representative member of the receptor tyrosine kinase (RTK) family. The kinase domain of PDGFR is responsible for phosphorylation of its own tyrosine residues, as well as substrate signaling molecules associated with the receptor cytoplasmic tail. There are two isoforms of PDGF receptors, α- and β-receptor, in the plasma membrane, and the receptors have different affinities for the PDGF isoforms, e.g. β-receptor interacts only with PDGF-B chain. Therefore, upon PDGF engagement, various dimeric receptor complexes are formed. Both receptor isoforms are involved in induction of mitogenic responses, whereas only β-receptor triggers stimulation of chemotaxis. In the case of β-receptor, 12 out of a total of 16 tyrosine residues in the cytoplasmic tail of the receptor are known to be phosphorylated by PDGF engagement, one in the kinase domain (Tyr 857, based on human β-receptor sequence) and 11 in the non-kinase domain [3]. One intriguing feature is that each phosphorylated tyrosine residue in the non-kinase domain is a specific docking site for a defined SH2-containing counterpart molecule. There is no other signaling process involved besides tyrosine phosphorylation at the level of the receptor in this pathway. Therefore, the phosphorylation/dephosphorylation of each tyrosine site on the receptor is a key step for turning the corresponding downstream signaling pathways on or off (Fig. 1). The receptor phosphorylation is catalyzed only by its own tyrosine kinase activity,

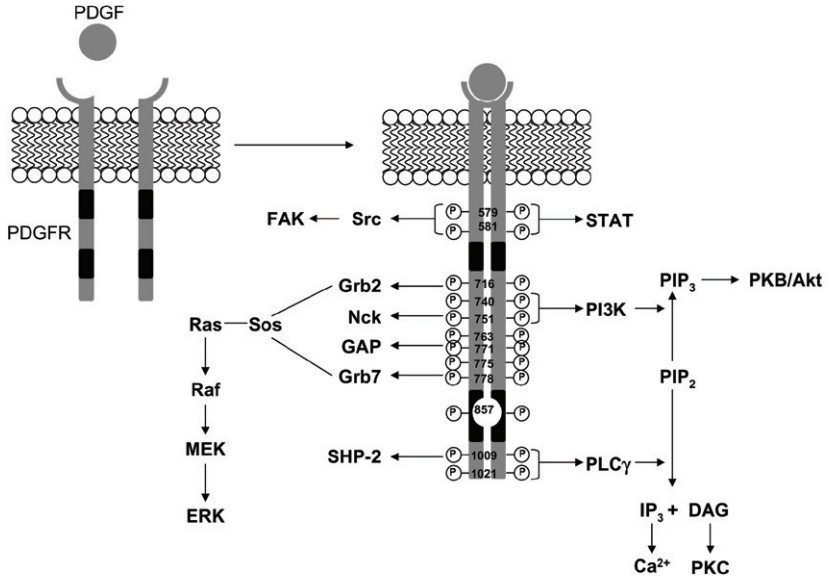

Figure 1. PDGF-induced signaling pathways. Upon PDGF binding, the PDGF receptors are dimerized and autophosphorylated on tyrosine residues. Each phosphorylated tyrosine residue induces activation of the designated downstream signaling pathways. FAK, focal adhesion kinase; GAP, GTPase-activating protein; SHP-2, SH2-containing phosphatase; STAT, signal transducer and activator of transcription; PIP_2, phosphatidyl inositol-3,4-bisphosphate; PIP_3, phosphatidyl inositol-3,4,5-triphosphate; PLCγ, phospholipase C-γ; PI3K, phosphatidyl inositol-3-kinase; IP_3, inositol-3,4,5-triphosphate; DAG, diacylglycerol. Dark *boxes* represent a kinase domain on PDGFR.

whereas the receptor dephosphorylation is relatively complex because a number of protein tyrosine phosphatases (PTPs) participate [4]. In addition, the basal PTP activity overwhelming the activity of the activated kinase has to be overcome in order to initiate tyrosine phosphorylation upon receptor stimulation. The involvement of reactive oxygen species (ROS) in platelet derived growth factor (PDGF) signaling was observed for the first time in 1995, when Sundarasan et al. reported that PDGF induces the intracellular production of ROS in vascular smooth muscle cells [5]. A cell-permeable ROS-sensing dye, named 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probes), which is non-fluorescent in the reduced state and subsequently becomes fluorescent upon oxidation by ROS [6], was used in the experiment. This dye is used quite frequently, although it is non-specific to the type of ROS and is qualitative. The authors, however, proved using bovine catalase that the ROS produced by PDGF was H_2O_2. The most striking discovery in their study was that the introduction of catalase into the cells diminished PDGF-induced tyrosine phosphorylation. This study brought forth a new paradigm that ROS could function as a signal messenger in receptor-mediated signal transduction. Similarly, Bae et al. demonstrated that epidermal growth factor (EGF) receptor, another RTK similar to PDGF receptor, also induced intracellular production of ROS in an RTK-dependent manner [7]. Since these pioneering works, a large number of studies have reported the intracellular production of ROS by a variety of extracellular stimuli and proposed signaling roles of ROS [8, 9]. Now, several questions have been raised and still remain to be answered: how ROS are produced and regulated in a receptor-dependent manner, whether a number of PDGFR-mediated signaling events are influenced equally or differentially by ROS, and what the true *in vivo* target molecules of ROS are.

Cellular systems producing intracellular ROS

ROS, including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), can be generated sequentially by reducing the oxygen molecule with unpaired electrons. Mammalian cells have diverse cellular sources of unpaired electrons (Fig. 2). Mitochondria are the biggest cellular reservoir of these electrons because they operate the respiratory electron transport chain to produce ATP energy. The electrons can leak from the mitochondrial electron transport chain and react with dissolved oxygen molecules to produce superoxide anion. The site of the leakage is likely to be ubiquinone, which is a small lipid-soluble electron carrier that delivers the electrons from complex I (NADH:ubiquinone oxidoreductase) or complex II (succinate dehydrogenase or glycerol-phosphate dehydrogenase) to complex III (cytochrome bc_1). Mitochondrial-dependent generation of ROS is also absolutely dependent on the metabolic rate, so that this source of ROS is likely to be an end result, rather than a second messenger, in proliferative signaling. In fact, mitochondrial-derived ROS have been proposed to play a role in negative feedback inhibition of cell growth [10]. Recently, a family of superoxide-producing enzymes acting in non-phagocytic cells, enzymes which are homologous

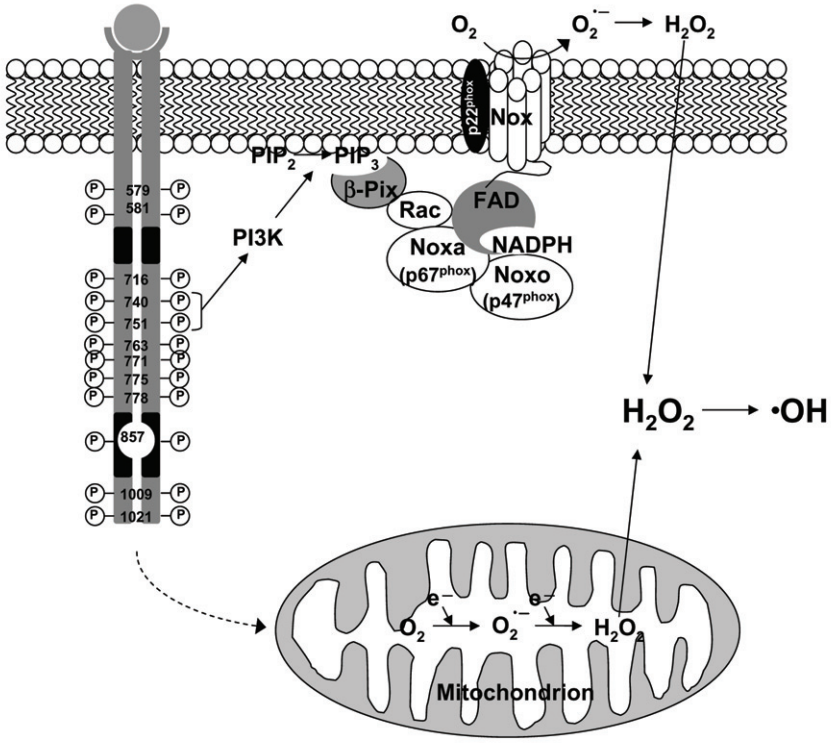

Figure 2. PDGF-induced ROS production. The phosphorylation of PDGFR on Tyr740/751 leads to activation of Nox enzymes via sequential activation of the PI3K/β-Pix/Rac pathway. As known in Phox system, Nox enzymes produce superoxide anions in the cell surface, which may enter into the cell after being converted to hydrogen peroxide. Although nothing is known about whether electron leakage in mitochondria is linked to PDGF signaling, if it occurrs, mitochondria can release ROS into the cytosol. Noxa, Nox activator; Noxo, Nox organizer.

to the gp91phox subunit of phagocytic NADPH oxidase (Phox), has been discovered. These enzymes, including the gp91phox (also called phagocytic Nox or Nox-2), are classified as a new family of NADPH oxidase/Dual oxidase enzymes (NOX/DUOX) [11]. NOX/DUOX family consists of seven members, Nox-1 thru Nox-5, Duox-1 and Duox-2. All of these enzymes are typical flavoproteins that contain six helical transmembrane domains, which coordinate two hemes, and a carboxyl-terminal domain, which has binding sites for flavine adenine dinucleotide (FAD) and NADPH. Nox-5 has an additional EF-hand domain for calcium binding, and Duox-1/2 has an extracellular peroxidase domain as well as an EF-hand domain. The NOX/DUOX family is recognized as the sole cellular system that produces ROS in a regulated manner. The well-studied biochemical functions of the prototype isoform Nox-2 have provided insight as to how the other Nox/Duox enzymes work. Newly discovered Nox isoforms share several constituents for activation with the Nox-2 system, in that they form a flavocytochrome b_{558} via constitutive interaction with another integral membrane component, p22phox, to produce ROS [12–15]; the small-GTPase Rac1 is also required [15–17]. The homologues of two cytosolic factors, p47phox and p67phox, which are essential for Nox-2 activation, have been discovered and named as Nox organizer (NOXO) and Nox activator (NOXA), respectively [18–20]. Therefore, recent studies collectively suggest that regulation of the superoxide-producing activity of Nox/Duox enzymes is quite similar to those known in the Nox-2 system.

PDGF-mediated ROS production

In general, the second messenger molecules are basal in resting cells but transiently increase in stimulated cells, which is a necessary step to allow them a signaling role via their action on the desired target molecules. Such a transient increase can be achieved through seesaw-like balance between a production system and an elimination system. For example, in the case of cyclic AMP (cAMP), the regulated balance of adenylate cyclase and cyclic nucleotide phosphodiesterase determines the transient increase of cAMP upon hormone stimulation. Likewise, the transient burst of intracellular ROS in a receptor-dependent manner is also determined by temporal and spatial regulation of a ROS-producing enzyme and a cellular antioxidant enzyme. Since PDGF was known to induce intracellular production of ROS in smooth muscle cells [5], there have been some studies dealing with signal communication between PDGFR and ROS (Fig. 2). First, Bae et al. demonstrated that

phosphatidyl inositol 3-kinase (PI3K) has an essential role in PDGF-induced ROS production [21]. Taking advantage of the fact that each phospho-tyrosine residue on PDGFR is engaged with specific signaling pathways, a series of PDGFR Tyr-to-Phe mutants were examined for their ability to facilitate PDGF-induced ROS production. Second, several reports have implicated Nox enzymes as a possible ROS-producing system responding to PDGF stimulation. Nox-1 (also known as Mox1) was initially discovered as a mitogenic oxidase that produces ROS and that is induced by PDGF [22]. A guanine nucleotide exchanger factor (GEF) for Rac, β-Pix, has been shown to be associated constitutively with Nox-1 and required for PDGF-dependent ROS production via Rac1 activation [23]. It seems that Nox-1 is required for PDGF-induced ROS production and proliferation in hepatic stellate cells [24]. Re-introduction of Nox-4 gene recovers the PDGF-induced tyrosine phosphorylation that is otherwise impaired in skin fibroblasts isolated from leprechaunism patients [25].

However, it has not been shown whether the knock-down of Nox/Duox expression can diminish PDGF-induced tyrosine phosphorylation. Moreover, it remains to be answered why the PDGFR mutant lacking the tyrosine residues for PI3K binding, Tyr740/Tyr751, is fully autophosphorylated upon PDGF stimulation [21, 26]. This suggests that tyrosine phosphorylation could occur even in the absence of ROS or there are distinct pools of ROS. In fact, the localized role of Nox-produced ROS for cell migration was recently discussed [27]. As mentioned above, the activation of Nox enzyme by PDGF is likely to depend on the sequential activation of PI3K/GEF(β-Pix)/Rac1/NOXO1 (or p47phox). In this activation pathway, the product of PI3K, phosphatidylinositol-3,4,5-triphosphate (PIP_3), is enriched in the new leading edge of the migrating cells. Rac1, p47phox and Nox-2 are all found in focal complexes in lamellipodia and membrane ruffles. Although there are currently no techniques for monitoring the local production of ROS, this situation draws speculation that Nox-derived ROS can be accumulated at the leading edge of the cell and thus may regulate the cytoskeletal rearrangement. This point of view restricts the role of Nox-derived ROS to tyrosine phosphorylation in focal complexes. Consequently, extensive studies of the mechanism by which ROS are produced are needed for better understanding of a second messenger role for ROS in PDGF-induced tyrosine phosphorylation. Recently, a systematic study via manipulating the cellular peroxidase (Prx) systems proposed a new model for the function of intracellular ROS in PDGF signaling [28]. Intriguingly, a 2-cys Prx isoform called Prx II has turned out to be the sole endogenous regulator of PDGF-induced tyrosine phosphorylation, although the level of intracellular ROS measured using DCF fluorescence is altered by all three mammalian peroxidase systems discussed below. Even though this phenomenon should be investigated further in a diverse cellular system, this study does leave a question about the source of ROS visualized by DCF fluorescence.

Antioxidant enzyme systems regulating intracellular ROS

Mammalian cells do not have any enzyme system responsible for removing hydroxyl radicals. Therefore, once formed, hydroxyl radicals attack cellular macromolecules, including proteins, nucleic acids and lipids. The cytosolic compartment of the mammalian cell is in the reduced state and equipped with high antioxidant power supported by glutathione and the peroxidases. This is thought to provide the protection of the cellular substances against unexpected oxidative damage by blocking the formation of hydroxyl radicals. In principle, ROS playing a signaling role should also be controlled by cellular antioxidant systems (Fig. 3). Superoxide anion can be converted to H_2O_2 by the reaction of superoxide dismutase (SOD). There are two SOD isoforms, Cu/Zn-SOD in cytosol and Mn-SOD in mitochondria. H_2O_2 is then converted to water by the three types of peroxidases. Catalase is a H_2O_2 dismutase that contains a heme group and is exclusively present in the peroxisome [29, 30]. Glutathione peroxidase (GPx) catalyzes the reduction of the hydroperoxides by utilizing the electrons transferred from NADPH via glutathione reductase and glutathione (GSH) [31]. Of the five isoforms, GPx-1 and GPx-4 are cellular enzymes utilizing H_2O_2 and phospholipid hydroperoxide, respectively, as substrates. Peroxiredoxins constitute the most recently discovered peroxidase family [32]. The peroxiredoxin family is classified into three subfamilies, called typical 2-cys Prx, atypical 2-cys Prx and 1-cys Prx, based on the number and position of the conserved cysteine residues in the primary structure that are involved in the peroxidase activity. 2-cys Prx reduces hydroperoxides to water by utilizing electrons transferred from NADPH via thioredoxin and thioredoxin reductase [33]. Consequently, there are two major peroxidase systems in cytosol, each of which is supported by glutathione or thioredoxin. Glutathione- and thioredoxin-dependent peroxidases have distinctive enzymological features. First, GPx has a selenocysteine residue in the active site that is known to be naturally more reactive ($pK_a \cong 5.2$ for selenol group) than the cysteine residue ($pK_a \cong 8.2$ for

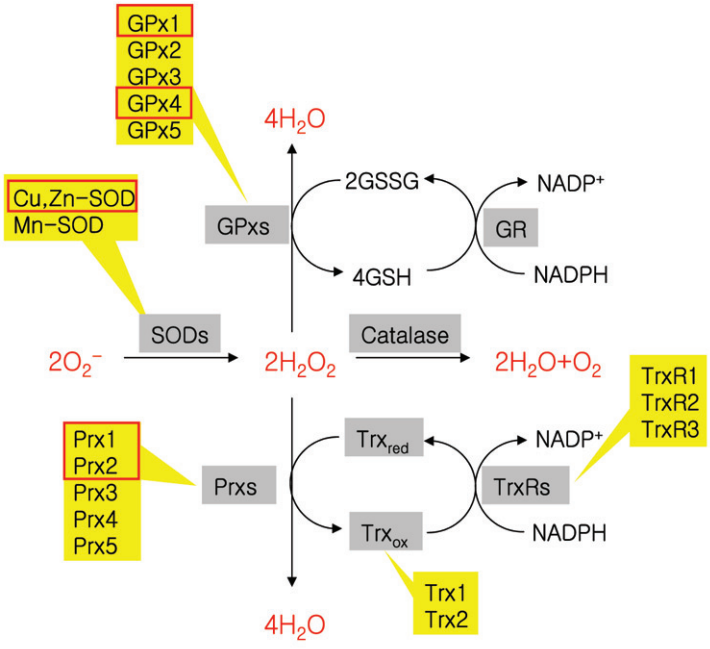

Figure 3. Mammalian antioxidant enzymes. GPx, glutathione peroxidase; SOD, superoxide diamutase; GSH, reduced glutathione; GSSG, oxidized glutathione; Prx, peroxiredoxin; Trx, thioredoxin; TrxR, thioredoxin reductase. Red *boxes* represent enzymes present in cytosol.

sulfhydryl group), which is the conserved amino acid residue in the active site of 2-cys Prx. This leads us to assume that GPx is a more efficient enzyme than Prx in terms of the kinetics of the H_2O_2 reduction reaction. In fact, in the reaction with H_2O_2, the catalytic efficiency of bovine GPx-1 is between 10^7 ~ 10^8 [34], whereas that of yeast 2-cys Prx (also named as cTPx) is ~10^4 [35]. Although the catalytic efficiency of the mammalian 2-cys Prxs has not been examined, it is thought to be similar to that of the yeast enzyme. Second, GPx has a relatively high Michaelis-Menton constant (K_m) for H_2O_2 (approximately millimolar range), although it relies on GSH concentration, whereas yeast 2-cys Prx has a micromolar K_m value for H_2O_2. In particular, the K_m value of mammalian 2-cys Prx for H_2O_2 is even below micromolar [33]. Consistent with the intriguing feature of 2-cys Prx as a sluggish enzyme with a high affinity for the substrate H_2O_2, it is readily inactivated by overoxidation of the active site cysteine residue from –SH to $–SO_2$ in H_2O_2-treated cells as well as during the *in vitro* reaction, e.g. inactivated by H_2O_2 below 1 μM at the rate of 0.072% per turnover [36, 37]. In fact, the cytosolic 2-cys Prxs are likely to be designed for efficiently removing H_2O_2 produced intracellularly with growth factor and cytokine [28, 38, 39]. Taken together, these two peroxidase systems are thought to play roles in different pools of cellular H_2O_2; for example, GPx is adequate for removing stress-induced H_2O_2, whereas Prx is adequate for receptor-induced H_2O_2.

ROS targets in PDGF signaling

A common feature in the cellular target proteins of ROS is the presence of a reactive cysteine residue in primary structure; the pK_a value of the side chain (–SH group) of this residue is low. At cellular pH, the side chain of the reactive cysteine is deprotonated to thiolate ion ($–S^-$) and vulnerable to oxidation by ROS. The oxidation products of the sulfur atom include the sulfenic acid (–SOH), sulfinic acid ($–SO_2H$) and sulfonic acid ($–SO_3H$). Related to tyrosine phosphorylation signaling, the best-known ROS target molecule is the protein tyrosine phosphatase (PTP), in which the catalytic site is the reactive cysteine residue [40–42]. PTPs found in the literature related to PDGF signaling include PTP1B, phosphatase and tensin homologue (PTEN), low-molecular weight PTP (LMW-PTP), SHP-2 (also called Syp or PTP1D), density-enhanced phosphatase-1 (DEP-1, also called CD148 or rPTP-η) and T-cell protein tyrosine phosphatase (TCPTP). PTP1B is known to be inactivated through the reversible oxidation of the catalytic cysteine residue by ROS produced in response to epidermal growth factor EGF [43, 44]. The cysteine sulfenic acid of PTP1B forms the sulfenyl-amide intermediate by reacting with the main chain nitrogen atom of an adjacent residue; this intermediate is resistant to further oxidation of the catalytic cysteine by ROS, but reversible, as it can be reduced with cellular reducing thiols [45, 46]. Another lipid and protein phosphatase, PTEN, is also reversibly oxi-

dized by ROS induced by PDGF, such that the oxidized catalytic cysteine forms a disulfide bond with an N-terminal cysteine [47, 48]. The reversible oxidation of LMW-PTP is similar to that of the PTEN, in that formation of a disulfide bond contributes to the reversibility of the oxidized catalytic cysteine. PDGF treatment induces the formation of a disulfide bond between the two cysteine residues in N-terminal catalytic pocket of the LMW-PTP, which is subsequently reduced by GSH [49]. Meng et al. have developed a modified in-gel PTP activity assay to screen for oxidized PTPs, in which the PTPs containing the reduced catalytic cysteine are irreversibly inactivated by alkylation with iodoacetic acid. The activities of the remaining oxidized PTPs are measured after reduction with the reductant [50]. Using this method, they have demonstrated that SHP-2 is reversibly oxidized by PDGF stimulation. This oxidation is PDGF-specific, at least in Rat-1 fibroblast cells. The PDGF-induced oxidation of the catalytic cysteine in SHP-2 has also been demonstrated using a peptide antibody that recognizes the conserved PTP signature motif containing the cysteine sulfinic acid, $VHC(SO_2)SXG$ [51].

It seems that studying ROS-mediated regulation of PTP activity is not a bottleneck in this field of research because molecular techniques, like the aforementioned in-gel assay, are available. Rather, a more important scientific hurdle is to define the physiological significance of the redox regulation of PTPs in a category of tyrosine phosphorylation signal transduction. Related to this matter, there have been numerous efforts to re-evaluate the ability of PTPs to dephosphorylate the PDGFR with respect to site specificity.

Until a decade ago, the site selectivity of PTP had been studied *in vitro* through phosphopeptide spanning. For example, *in vitro* experiments have demonstrated that SHP-2 has a site preference for p-Tyr771 and p-Tyr751 on PDGFR, whereas PTP1B has no selectivity for the various phosphorylation sites [52]. It has also been shown that a receptor-like PTP, DEP-1, prefers p-Tyr1009/1021 with much less preference for p-Tyr857 [53]. However, recent *in vivo* studies have been improved using site-specific phosphotyrosine antibodies [54]. LMW-PTP exhibits site selectivity for p-Tyr857 and 751, but not for pTyr716 [55]. When several phosphorylation sites on PDGFR were examined in TCPTP-null mouse embryonic fibroblasts, the levels of p-Tyr771 and p-Tyr1021 were more obviously increased than those of p-Tyr579 and p-Tyr751 [56]. Unfortunately, none of the previous *in vitro* and *in vivo* studies have shown authentic site-selectivity. There are theoretical reasons for this; one is that the PTPs may have intrinsically low site (or substrate) specificity. It seems similar to the poor sequence specificity of tyrosine kinases [57]. Another is that the site selectivity of PTPs may be determined temporally and spatially *in vivo* (Fig. 4). Our study mentioned above supports the latter hypothesis [28, 58]. An intriguing observation is the site-selective regulation of PDGFR phosphorylation by Prx II-dependent H_2O_2, which appears only at Tyr579/581 and Tyr857, as well as the reversible oxidation of a membrane-associated PTP in a Prx II-dependent manner. This proposes a possibility that ROS can inactivate the particular PTP, possibly in a localized area, that is responsible for the dephosphorylation at Tyr579/581 and Tyr857. So, if such a hypothetical membrane-associated PTP, whose oxidation is PDGF and Prx II-dependent, is uncovered, the redox regulatory roles of ROS in PDGF signal transduction will be established.

Conclusion and perspectives

It is now clear that, upon PDGF stimulation, intracellular ROS are produced and involved in the induction of tyrosine phosphorylation. Although follow-up studies are needed, the 2-cys Prx may be a prime candidate for a ROS regulator in PDGF signaling. Nonetheless, extensive studies about the ROS-producing system and PTP as direct links between the ROS and tyrosine phosphorylation should be undertaken (Fig. 4).

The urgent, but difficult, need is the development of a live cell probe capable of monitoring ROS in a real-time and quantitative manner. With fluorescent dyes, including DCF, that are semiquantitative and diffusible, one cannot interpret where the ROS came from or what produced the ROS.

The 81 genes that encode catalytically active PTPs have been clarified in the human genome [59]. PDGFR interacts with a number of PTPs, including PTP-PEST, SHP-2, PTP1B, T-cell PTP (TCPTP) and so on, in resting or stimulated cells [60]. However, the correlation of each PTP with the phosphotyrosine residues on PDGFR is largely unknown. It is generally believed that the dephosphorylation of a phosphotyrosine residue that results in turning the signal off in a linked downstream pathway must be catalyzed by a designated PTP. Given that one particular type of PTP may be involved in various cellular signaling pathways, the total reduction in the cellular levels of a certain PTP of interest using RNA interference or gene knockout is an unsuitable approach for elucidating its role in the site-selective regulation of tyrosine phosphorylation. Therefore, monitoring the temporal and spatial interaction of PTP with a phosphorylated

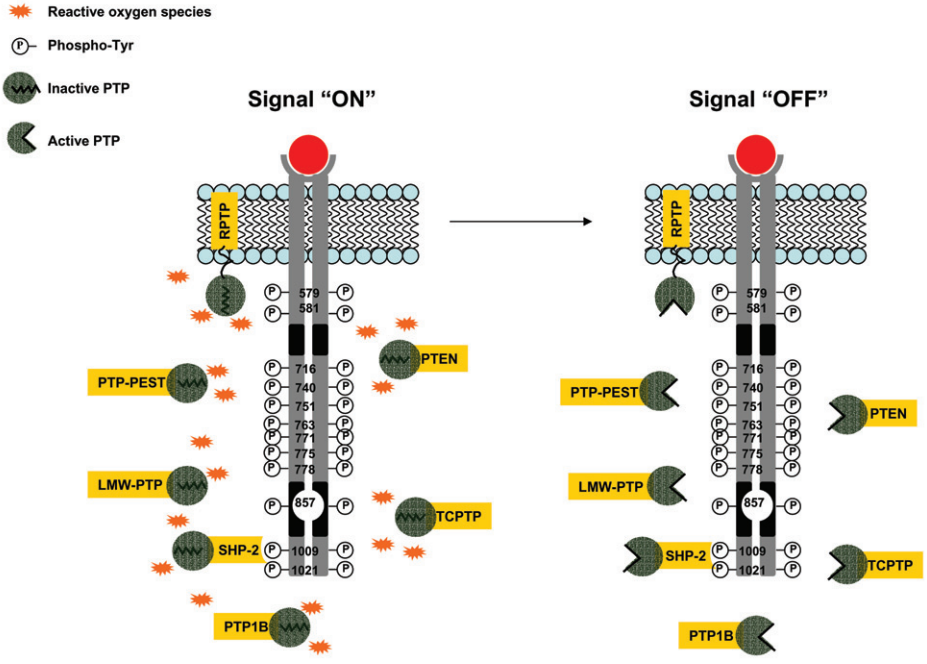

Figure 4. Cooperation of ROS and tyrosine phosphorylation via PTP in PDGF signaling. ROS produced in response to PDGF inactivate PTPs, which are responsible for dephosphorylating PDGFR, and thus assist PDGFR phosphorylation. When PDGF signaling is completed, ROS are removed by an antioxidant enzyme, possibly 2-cys Prx. Immediately, PTPs are reactivated and strip out the phosphoryl groups from PDGFR.

site (or substrate) is promising. In fact, such experimental attempts have recently been initiated [61, 62]. By putting these together, the future studies should focus on visualizing and studying the signaling triad, composed of ROS, PTP and a specific phosphotyrosine, as a whole.

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