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Forum Review

Novel Role of Protein Disulfide Isomerase in the Regulation of NADPH Oxidase Activity: Pathophysiological Implications in Vascular Diseases

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ABSTRACT

Vascular cell NADPH oxidase complexes are key sources of signaling reactive oxygen species (ROS) and contribute to disease pathophysiology. However, mechanisms that fine-tune oxidase-mediated ROS generation are incompletely understood. Besides known regulatory subunits, upstream mediators and scaffold platforms reportedly control and localize ROS generation. Some evidence suggest that thiol redox processes may coordinate oxidase regulation. We hypothesized that thiol oxidoreductases are involved in this process. We focused on protein disulfide isomerase (PDI), a ubiquitous dithiol disulfide oxidoreductase chaperone from the endoplasmic reticulum, given PDI's unique versatile role as oxidase/isomerase. PDI is also involved in protein traffic and can translocate to the cell surface, where it participates in cell adhesion and nitric oxide internalization. We recently provided evidence that PDI exerts functionally relevant regulation of NADPH oxidase activity in vascular smooth muscle and endothelial cells, in a thiol redox-dependent manner. Loss-of-function experiments indicate that PDI supports angiotensin II-mediated ROS generation and Akt phosphorylation. In addition, PDI displays confocal co-localization and co-immunoprecipitates with oxidase subunits, indicating close association. The mechanisms of such interaction are yet obscure, but may involve subunit assembling stabilization, assistance with traffic, and subunit disposal. These data may clarify an integrative view of oxidase activation in disease conditions, including stress responses. *Antioxid. Redox Signal.* 10, 1101–1113.

INTRODUCTION

REDOX PATHWAYS ADD AN IMPORTANT DIMENSION to cell signaling networks regulating physiological and pathological events, particularly cardiovascular disorders such as hypertension (16, 99). However, mechanisms providing fine-tuning of redox equilibrium are complex and yet incompletely understood. Indeed, it has been shown that redox signaling can occur in the absence of overall changes in redox status of the major intracellular reductants glutathione or thioredoxin (37), and thus can be separated from oxidative stress, which is a disruption of redox signaling requiring some type of cellular adaptation (49). Accordingly, most intra- and extracellular redox buffer pairs are not in equilibrium, suggesting their independent regulation (49), and further indicating that oxidative stress is more complex than a simple overall imbalance between oxidants and antioxidants in favor of the former. In turn, oxidative stress activates vicious signaling circuits in several disease conditions, not only as a result of direct free radical-mediated damage to biomolecules, but—perhaps mostly—from disordered activation and/or expression of subcellular signaling targets due to excessive, uncompensated, or decompartmentalized

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reactive oxygen species (ROS) generation (49). A simultaneous requisite and corollary of this notion is the fact that cellular ROS generation is mainly a nonaccidental controlled enzymatic process. Thus, understanding how such enzymatic sources of ROS are regulated at the structural and cellular physiological levels is likely important to provide a more elaborated picture of the redox pathophysiology of vascular diseases. Among several enzymatic systems, vascular isoforms of the phagocyte NADPH oxidase complex appear to be the most relevant source of basal as well as agonist-induced signaling ROS (11, 39, 63). In this review, we discuss some novel mechanisms underlying NADPH oxidase regulation, with emphasis in the role of the endoplasmic reticulum (ER) redox chaperone protein disulfide isomerase.

The oxidase complex possesses a modular multisubunit structure, with catalytic flavin-binding Nox subunits and a number of regulatory subunits. Expression of each of them varies according to the specific vascular cell type, so that the term "Vascular NADPH Oxidase" actually stands for unequal proportions of several types of enzyme complexes belonging to each cell type (Table 1). Nox isoforms have been ascribed an important role in several pathophysiological conditions such as hypertension, atherosclerosis, restenosis after injury, and diabetic vasculopathy (reviewed in refs. 11, 16, 39, 63). Molecular structural aspects of NADPH oxidase and the known differences among Nox isoforms regarding their expression profile, type of ROS produced, and subcellular localization are reviewed in detail in excellent references (11, 39, 63).

On the other hand, comparatively less is known about mechanisms underlying the regulation of NADPH oxidase complex activity and expression. Certainly, first-order regulatory steps are the identity and intrinsic characteristics of agonist-specific Nox isoforms occurring in each vascular cell type, as well as expression, subcellular traffic and phosphorylation of known regulatory subunits (such as p22phox and p47phox or its analogs). A more refined knowledge of structural aspects of these subunits is likely to provide further insights into subcellular physiology of the oxidase, as has recently been shown for the alternatively spliced variants of the p47phox analog NOXO-1, which can account for distinct Nox complex locations within cells (103). In general, exposure to agonists is associated with increased mRNA and protein expression of such oxidase subunits, which seems to account for increased ROS generation (63). It is generally agreed that, upon activation, vascular oxidase undergoes minor, if any, further assembling of stock and newly-synthesized subunits, as the complexes seem to preassemble very early within the ER-secretory pathway (63, 64). Particularly, p22phox and Nox1/Nox4 are already assembled in resting cells (4, 11, 63). Independently of assembling, protein traffic appears to be important for oxidase activation. In fact, oxidase activation in endothelial (100) or smooth muscle cells (64) is simultaneous to shift of subunit(s) from perinuclear region to membranes. Moreover, ROS production from NADPH oxidase agonists is blocked by the Golgi disruptor agent brefeldin-A (CX Santos CX *et al*., unpublished observations). Similarly, the phagocyte oxidase undergoes traffic among subcellular vesicular compartments (54), in accordance with the recent description of p67phox-binding JFC1 protein, which appears to be involved in 3'-phosphoinositide-dependent vesicle translocation to plasma membrane (15).

It seems underappreciated that the particular multisubunit architecture of the oxidase, together with the many subcellular regulatory mechanisms discussed so far, composes a system particularly likely to display a stochastic behavior (50). Therefore, despite the desired precision of signaling and the danger of uncontrolled oxidant generation, an intrinsic degree of random variation among cells would not be unexpected. Yet, it is apparent that the attained and necessarily high degree of specificity in NADPH oxidase-dependent ROS-mediated signaling is not sufficiently explained only by selection of specific subunits (11). What is yet missing includes a better definition of mechanisms, coordinated at a cellular level, that restrict such ROS generation spatially and temporally to specific subcellular microdomains and post-translational mechanisms that account for activation of particular Nox isoforms (105). Several upstream mediators and associated proteins (reviewed in refs. 11 and 17) were described to interact with oxidase activation and might help confer targeted specificity. Furthermore, important advances in this regard were provided by a series of recent works (reviewed in ref. 105) describing scaffold-like pro-

TABLE 1. VASCULAR NAD(P)H OXIDASE

	Catalytic subunits (basal expression)				
	EC	*VSMC*	*Fib*	*Associated regulatory subunits*	*Main cellular effect*
Nox 1	+	++	0/+	p22 phox Noxo 1 Noxa 1 p47 phox (?)	Proliferation Migration
Nox 2	++	0	+++	p22 phox p47 phox p67 phox p40 phox (?)	Proliferation Migration Inflammation
Nox 4	+++	++	+	p22 phox	Differentiation Apoptosis
Nox 5	+	++	(?)	p22 phox	Ca^{2+}-mediated responses

EC, endothelial cell; Fib, fibroblast; VSMC, vascular smooth muscle cell.
The relative amount (+ to +++) represents semiquantitative estimates from the several references cited in text.
Rac 1 regulates activity of Nox 1 and Nox 2 complexes, while its role for Nox 4 and Nox 5 is unclear.

tein platforms capable of localizing NADPH oxidase-dependent ROS signals. In this context, the redox and thiol-dependent regulation of NADPH oxidase and the proposed role of protein disulfide isomerase, discussed in the following sections, add further light into the orchestration of all such regulatory mechanisms.

REDOX REGULATION OF NADPH OXIDASE

There is some evidence, albeit indirect, that nonphagocytic NADPH oxidase is regulated by redox processes. Exogenous administration of H_2O_2 triggers oxidase activity (66) and has been documented to induce expression and activity of Nox4 isoform (19, 69). After exposure of VSMC to agonists such as angiotensin II, ROS production occurs in two waves (89). The initial ROS production involves Rac activation due to the action of PKC and later to c-Src activation, EGF receptor transactivation, and PI3K activation. The second peak of ROS production is PKC-independent and depends on a more robust sustained phosphorylation of p47phox (11, 17, 89). It can be speculated that these two waves represent activation of distinct Nox isoforms (11). ROS contribute to downstream signaling pathways, including translation initiation (84), which eventually feedback positively on further oxidase-dependent ROS generation (11, 17). In fact, when the first ROS wave is abrogated by specific signaling antagonists, the second wave is also decreased (89). An analogous phenomenon occurs in an endothelial cell line after exposure to thrombin. In this case, antagonism of the initial wave of ROS generation, occurring after 15 min, was found to prevent the later increase in p22phox mRNA expression and its concomitant sustained ROS production 3 h later (24). Redox regulation of the oxidase provides a feed-forward activation mechanism able to recruit in a redox-dependent form mediators that act upstream to a later more sustained oxidase activation. In addition, it provides a redox cross-talk mechanism between the stock oxidase subunits and the later increase in expression that is known to sustain oxidase activation.

THIOL REAGENTS AND NADPH OXIDASE

It has been known for several years that the phagocyte NADPH oxidase complex is inhibited by distinct oxidizing or alkylating thiol reagents (20), an effect accompanied by decreased negativity of cytochrome b^{558} reducing potential (8). Inhibition of the oxidase with the dithiol alkylator phenylarsine oxide indicated a role for critical dithiol(s), which were assigned to two target gp91phox cysteines, accessible in resting but not activated neutrophils (23). More recently, our laboratory extended such observations to the vascular oxidase (45). We showed that incubation of cell membrane homogenates with the oxidant diamide or DTNB (5,5′ dithio-bis (2-nitrobenzoic) acid), or the alkylator PCMPS (*p*-chloro mercuryphenylsulfonic acid), but not with *N*-ethylmaleimide or glutathione disulfide, led to a strong inhibition of enzyme activity. A less evident inhibition was also found for the reductant *N*-acetylcysteine. Analogous inhibition of the apparent oxidase activity was noted with such reagents in vascular rings, in a way unrelated to the degree of cellular glutathione depletion. However nonspecific and indirect, these findings collectively suggested a critical role for redox thiols in oxidase activation, which appeared unrelated to changes in overall cellular redox status. In addition, the rapid reversibility of diamide or PCMPS inhibitory effects upon their withdrawal, or with *N*-acetylcysteine, indicated that thiolation was unlikely to explain such effects. This led to the hypothesis of a thiol oxidoreductase–mediated mechanism. In fact, as with other redox-mediated signaling pathways, there are kinetic constraints for accepting an unrestricted, direct spontaneous reaction of ROS with critical protein thiols, given the overall low rate constants (32), some of them shown in Table 2. This possibly results in rates of thiol oxidation or disulfide exchange too low to account for functionally effective signaling. Although the low pKa of critical protein thiols will make such groups much more reactive and mark them as selective targets of oxidants, effective redox regulation may require additional mediation by thiol oxidoreductases, which can accelerate thiol reaction rates by factors up to $\sim 10^4$ and thus might be required as essential players in redox signaling cascades (5, 32, 34, 74).

PROTEIN DISULFIDE ISOMERASE: PROPERTIES OF A SIGNALING THIOL OXIDOREDUCTASE

Considering the thiol oxidoreductases known to exert a role in cell signaling, the thioredoxin superfamily is particularly important and well documented (5, 18, 108, 110). The major characteristic of thioredoxin family is the catalytic dithiol motif CXXC, where X can be a wide range of amino acids. In the case of thioredoxin, the motif is CGPC. Redox properties of the thioredoxin motif, dictated in part but not solely from the amino acid composition, as well as the multiple alpha–beta folds found in the secondary structure, account for the known different properties of several members of this family. Thioredoxin itself (molecular weight 12 kDa) is known as catalytic proton donor for ribonucleotide reductase and is a known subunit of T7 DNA polymerase (18). It acts mostly as a reductase with several cellular roles, particularly apoptosis protection and NF-kappaB (and other transcription factor) binding to DNA (110).

Among the several members of the thioredoxin superfamily, PDI (molecular weight 55 kDa) and its analogs are the only

TABLE 2. RATE CONSTANTS OF ROS AND THIOL REACTIONS (IN M^{-1} s^{-1})

	$O_2^{\bullet -}$	H_2O_2	$ONOO^-$	*Reference*
Cysteine	<15*	2.9	4.5×10^3	3, 10, 81, 109
GSH	20	0.87	1.3×10^3	81, 88, 109
BSA/HSA	NR	2.26	3.8×10^3	3, 14, 80

*All rate constants were calculated at physiological pH, with exception of Cys/$O_2^{\bullet -}$, which was at pH 10.9.

NR, no reaction.

Some data were collected from http://allen.rad.nd.edu/Solnkin2/ site (last accessed 3/15/07).

ones known to catalyze isomerase reactions (*i.e.*, the ordered rearrangement of disulfide bonds through repeated cycles of reductions and oxidations) (18, 25, 30, 74, 108) (Fig. 1). Accordingly, the classic function of PDI is to promote the redox-mediated protein folding at the ER (41), which is PDI's primary location. Oxidizing equivalents for this function are provided to PDI by the ER thiol-containing oxidase Ero-1 (endoplasmic reticulum oxidase-1), which in turn is reoxidized via electron transfer to oxygen, mediated by bound FAD, generating ROS in this process (40, 101, 108). Ero-1-dependent oxidative activity is counteracted and balanced by cytosolic glutathione, suggesting that a shuttle mechanism from cytosol acts to control the ER redox environment (72). However, PDI can also act as a reductase depending on the ambient reducing potential. At the ER, given the relatively oxidizing conditions (GSH/GSSG ratios ~2–3:1), PDI acts as an isomerase/oxidase, while outside the ER, at GSH/GSSG ratios >30–100:1, PDI acts mainly as a reductase (74, 108). This significant redox versatility stems from several factors, including the low pKa of the proximal cysteine of the active N-terminal a domain. Such pKa of 4.5 renders PDI a much better oxidase than thioredoxin, which has a pKa of 7.1 and is mainly a reductase. Accordingly, the standard reducing potential of PDI is –180 mV, compared to –260 mV for thioredoxin (108). Furthermore, mutation of the active thioredoxin site sequence CGPC to the PDI sequence CGHC renders thioredoxin a 10-fold better oxidant, indicating the importance of a close positively-charged histidine residue capable of promoting thiol ionization, consequently lowering the cysteine pKa (25, 67). A similar effect is also provided by an arginine residue postulated to transiently interact with the catalytic cysteine through secondary folding (62). PDI structure shows several domain modules ordered as a-b-b′-a′-c (Fig. 2), in which a and a′ are thioredoxin domains bearing the redox-active WCGHC motif, b and b′ are thioredoxin structural folds without the redox motif, possibly related to peptide recognition and binding (25, 30, 41, 74, 98, 108), and c a putative Ca^{2+}-binding C-terminal domain, which appears to be less critical in mammalian, as opposed to yeast PDI (98). Redox-active cysteines at the a and a′ domains exist as reduced dithiols or intramolecular or mixed disulfides. Only the a and/or a′ domains are required for PDI's oxidase activity, whereas all four thioredoxin domains are required for more complex isomerizations (108). Elucidation of the crystal structure of PDI has defied several attempts and was solved for yeast PDI only recently (98), showing characteristics likely to be met also by mammalian PDI's. The enzyme has a twisted U-shaped structure, with active a domains displaying flexibility and facing each other along the arms of the U, while both are attached to a rigid substrate-binding scaffold composed by the b domains (98). The b′ domain at the bottom of the U appears to be the main substrate binding site and, together with its neighborhood, is enriched in hydrophobic residues (Fig. 2). In combination with the overall continuous hydrophobic surface of the PDI molecule, this allows binding of substrates of different sizes and disulfide bond locations. Thus, while specific domains or motifs promoting binding of other proteins to PDI have not been precisely defined, it is likely that surface hydrophobic sequences in such substrates are important (41, 98, 108). In fact, many studies have suggested that protein binding to PDI does not require the redox-active sequences, although such motifs are important to stabilize the binding (53, 73). Moreover, ATPase activity and Ca^{2+} binding may modulate PDI protein binding (42, 83, 108). In yeast, but perhaps less so in mammalian PDI, the a domain displays a lower redox potential than the a′ domain (56, 98), perhaps reflecting the predominant oxidase function of PDI in yeast, which evolved to a more elaborated isomerase function in mammals. PDI also displays a chaperone function, which is independent of the redox-active domains (98). Several (>14) PDI analogues have been described, indicating that PDI is part of a larger protein family variably displaying thiol oxidoreductase, chaperone, and isomerase properties (18, 25, 30). PDI can also display transglutaminase activity, although the importance of this property *in vivo* is unclear (27).

PDI is an abundant protein, able to reach millimolar concentrations within the liver ER (74). In addition, PDI is essential for cell survival even in yeast, so that any substantial decrease in its expression can have profound adverse effects (57, 108). In mammalian cells, PDI expression sustains cell survival, and upregulation of PDI or its novel analogue endo-PDI increases resistance to apoptosis after hypoxia in astrocytes or endothelial cells (38, 95, 96). Such essential physiological role has usually been ascribed to its important role in protein folding. However, an increasing number of functions for PDI are being described, suggesting an important role in cell signaling. PDI displays a KDEL C-terminal sequence characteristic of ER retention, which has been suggested to be required for ER retention of associated proteins (90). The most important examples in this regard are prolyl-4 hydroxylase (the rate-limiting enzyme for collagen biosynthesis) and microsomal triglyceryde transfer protein, which are well known to heterodimerize with PDI (74, 108). Such heterodimerization has additionally the functions of preventing protein aggregation (48) and providing

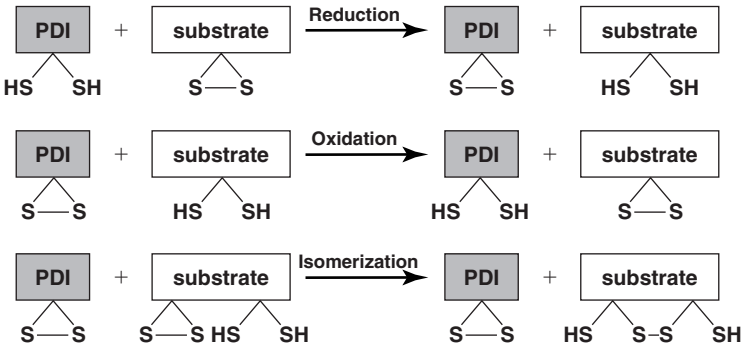

FIG. 1. Thiol redox reactions driven by PDI on peptide/protein substrates containing disulfides, defined as sufficiently close thiol groups either at primary or secondary structure. PDI can display a reductase activity at locations outside the ER in which local redox state is more reducing. In the ER, and potentially in other less reductive microenvironments, PDI tends to act as a thiol oxidase and/or isomerase, reactions that are essential for proper protein folding. However, excessive ER oxidation can cause protein misfolding and aggregation and may inhibit ER-assisted protein degradation (92).

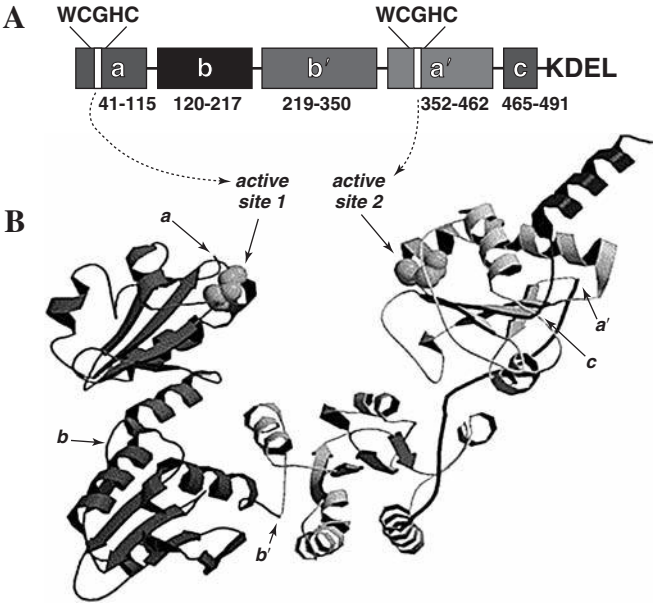

FIG. 2. Schematic representation of PDI structure. (**A**) Domain organization of mammalian PDI (adapted from ref. 74) showing the five PDI domains a, b, b′, a′, and c. The redox-active thioredoxin motifs (WCGHC) are located at domains a and a′. The b′ domain is the main peptide binding site and the c domain contains the carboxy-terminal KDEL endoplasmic reticulum retention signal. (**B**) Diagram (adapted from refs. 41 and 74) depicting the crystal structure of yeast PDI (98) showing the U-shaped form of the molecule with the active a and a′ domains at the arms of the U, and b and b′ domains at the bottom; *arrows* indicate the beginning of each domain. The active site thiols are represented as space-filling model.

oligomer assembly stability (55). PDI can also trap other proteins as aggregates [*e.g.*, the oxidase Ero-1 within the ER (77)] or other chaperones in the ER (70) or at the cytosolic side of Sec61 translocon (93). A recently described interesting function of PDI is the redox-mediated stabilization of receptive sites at the MHC-1 peptide-binding groove, allowing functionally relevant selection of optimal peptides during antigen presentation (79).

In addition, it has been well documented that PDI displays active intracellular traffic (97) and is found at the surface of diverse prokaryotic and eukaryotic cells. The mechanisms whereby PDI bypasses the KDEL receptor despite maintaining the KDEL C-terminal sequence are unclear (97, 108). It is also unclear whether PDI leaves the ER through the translocon Sec61 pore (93) and/or via secretory vesicles. In addition, in macrophages and potentially other cells, the ER may itself constitute a part of the endosome–phagosome network, thus carrying PDI, as well as other ER proteins, to such remote locations (21a). PDI seems to be loosely attached via electrostatic charges at the hepatocyte cell membrane and, after its significant rate of secretion, may eventually suffer some reuptake (97). Dithiols constitute ~25% of cell surface thiols (111). Labeling of such cell-surface dithiols indicates about a dozen proteins, including PDI, suggesting that its redox-active site(s) can be well exposed at the cell surface (22, 47). In such location, PDI appears to favor dithiol reduction, consistent with its role as reductase (47). In addition, PDI is also a major catalyst of transnitrosation reactions mediating nitric oxide internalization from extracellular *S*-nitrosothiols (82, 111). Functions for extrareticular PDI have been increasingly explored and may include roles such as cell surface recognition in cell-to-cell contact (83), shedding of L-selectin (9), chemokine receptor-dependent HIV cell internalization (7), and integrin-dependent platelet aggregation/adhesion (12). The latter effect is supported by a number of evidences. Platelet adhesion is inhibited by PDI antagonists, including the specific neutralizing monoclonal antibody RL90 (28, 29, 58, 59). In fact, enzymatic-mediated thiol exchange stabilizes platelet aggregation mediated by integrin $\alpha_2\beta_1$ (60). In addition, integrin β subunits were shown by FRET to physically interact with PDI (12). Furthermore, integrins (*e.g.*, $\alpha_{2b}\beta_3$) carry a CXXC thioredoxin motif (106), and at least some integrins (75), as well as fibronectin (61), exhibit an endogenous thiol isomerase activity, although its function is yet uncertain. PDI involvement with adhesion molecules may be a more general phenomenon, considering its analogous associations with thrombospondin (43) and cognin (78). An analogous effect of cell-surface PDI in the coagulation system was recently described. PDI disables, in an NO-dependent way, tissue factor-induced coagulation by disrupting its critical disulfide bond and activating its factor VII-related signaling branch via protease-activated receptor 2 (PAR2) (1). In addition to its roles at the cell surface, PDI has also important roles in protein traffic and secretion (97) [*e.g.*, binding and export of proteins such as thyroglobulin (21)].

An important situation in which, among several other features, PDI may be overexpressed and undergoes membrane traffic, is the *unfolded protein response (UPR)*, a complex multiple signaling cascade activated by the accumulation of unfolded or incorrectly folded proteins in the ER, potentially induced by altered ER Ca^{2+} transport, reductive stress, disturbed protein glycosylation, or simply an excess of secretory cell activity (86, 91). Accumulation of defective proteins in the ER lumen triggers ER stress, that, if sustained, can lead to apoptosis. In fact, the PDI promoter exhibits two UPR–sensitive sequences (87), and PDI expression in this situation has been linked to the removal of incorrectly folded proteins (71, 92, 93), since PDI assists the retrotranslocation of misfolded proteins to the cytosol for proteasomal degradation (92), in contrast to its analog Erp72, which tends to inhibit this process (33). In some neurodegenerative diseases, PDI S-nitrosation has been shown to abrogate its enzymatic activity and PDI-induced retrotranslocation of defective proteins, leading to ER dysfunction and cell loss (102). On the other hand, at least in the case of familial ALS, PDI appears to counteract the formation of aggregates of mutant SOD1 (6).

ROLE OF PDI IN NADPH OXIDASE REGULATION IN VASCULAR SMOOTH MUSCLE CELLS (VSMC)

Studies from our laboratory have recently provided several data suggesting a relevant role of PDI in the regulation of NADPH oxidase activity in VSMC (46). In general, VSMC ex-

posure to NADPH oxidase agonists such as angiotensin II does not change the already high baseline PDI expression; however, significant shift of PDI location toward the membrane fraction is observed. In isolated membrane homogenates exposed to diverse types of PDI antagonism, a significant (up to ~80%) decrease in NADPH oxidase activity was uniformly observed, irrespective of the method of measuring the oxidase activity, which included EPR (46), lucigenin chemiluminescence (Fig. 3A) and HPLC analysis of dihydroethidium oxidation products (Fig. 3B) (31). Strategies for PDI antagonism included bacitracin, a pharmacological antagonist of PDI oxidoreductase activity (28, 68, 76), a neutralizing anti-PDI antibody , the competitive substrate scrambled RNase (28), or the thiol reagent DTNB, known to inhibit NADPH oxidase (45) as well as PDI (36) activity. Since inhibition of PDI isomerase activity due to bacitracin, anti-PDI antibody, or DTNB is known to involve PDI thiols (28, 36, 68, 76) and all compounds were effective when incubated with membrane fraction after its separation, these results suggest together that PDI co-fractionates with NAD(P)H oxidase and contributes to assist its activity via thiol redox mechanisms.

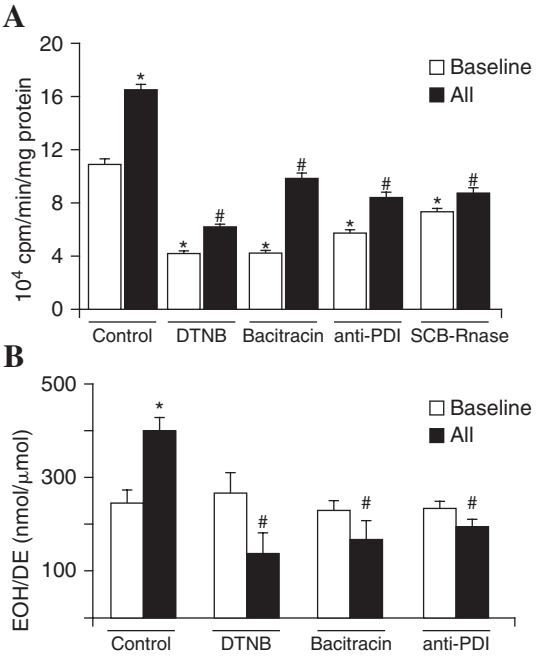

FIG. 3. Effects of diverse PDI antagonists on NADPH oxidase activity in vascular smooth muscle cell (VSMC, from rabbit aorta) membrane homogenates, obtained as described in ref. 46. VSMC were grown to 80–90% confluence in F12 medium supplemented with 10% fetal bovine serum and exposed to angiotensin II (100 n*M* for 4 h). (**A**) Effects of PDI inhibitors DTNB (500 μM), bacitracin (500 μM), neutralizing anti-PDI antibody (1:100), and scrambled (Scb)RNase (100 μg/ml), added to VSMC membrane fraction, on NADPH oxidase activity assessed with lucigenin (5 μM) chemiluminescence. (**B**) Analogous experiments in VSMC membrane homogenates using a novel NADPH oxidase assay with HPLC analysis of dihydroethidium (DHE) oxidation products (31). Results are expressed as 2-hydroxyethidium (EOH) produced/DHE consumed. ($n = 3$–6; *$p < 0.05$ *vs.* control baseline; #$p < 0.05$ *vs.* control + AII; mean ± SEM).

More specific evidence in a cellular context was obtained by examining the effects of an antisense oligonucleotide directed against PDI, which induced a decrease (average ~70%) both in baseline and angiotensin II-stimulated NADPH oxidase activity. In addition, the antisense oligonucleotide promoted a marked decrease in VSMC ROS production assessed by dihydroethidium fluorescence (46). Moreover, the antisense oligonucleotide prevented early angiotensin II-mediated phosphorylation of Akt, which is known to be mediated by NADPH oxidase-dependent ROS (104), thus indicating a functional role for PDI-assisted oxidase activation. The refolding isomerase PDI activity, assessed through the rates of scrambled RNAse renaturation (108), was increased 5-fold in the membrane homogenate during angiotensin II-induced oxidase activation. Such increase was inhibited by bacitracin or the antisense oligonucleotide against PDI. These data further support a role of thiols in functional PDI interaction with NAD(P)H oxidase.

Results of co-immunolocalization experiments with confocal microscopy provided evidence for a spatial interaction between PDI and the p22phox subunit of NADPH oxidase (46). The PDI antisense oligonucleotide transfection did not visibly change p22phox signals, but decreased PDI signals and their co-localization at baseline and after angiotensin II. In addition, co-localization was also observed with Nox 1 and Nox4, which are the main Nox isoforms expressed in VSMC. Furthermore, PDI immunoprecipitation yielded co-precipitation of NAD(P)H oxidase subunits p22phox (ref. 46 and Fig. 4), Nox1, and Nox 4. Such results were further assessed in HEK293 cells transfected with Nox1, Nox2, and Nox4-yfp coding plasmids. Precipitation of PDI revealed co-precipitation of all three Nox-yfp constructs (46). In all cases, the co-immunoprecipitation was not altered after incubation with angiotensin II, in line with the reported preassembling of p22phox and Nox1/Nox4 already in resting cells (4). In general, the association of PDI with diverse oxidase subunits indicates either direct binding to each Nox, binding to a common regulatory subunit (*e.g.*, p22phox) or to a kinase/phosphatase regulatory domain, or co-migration to a specific cellular microdomain.

In summary, results obtained so far in the VSMC indicate that PDI is associated with the NADPH oxidase complex and exerts a regulation of its activity that exhibits some degree of thiol redox sensitivity, though such redox sensitivity can be indirect. Together, the combination of versatile PDI redox properties, its known role in protein traffic and secretion, and its involvement with relevant signaling cascades open considerable perspectives regarding an integrative knowledge of mechanisms controlling NAD(P)H oxidase activation, and consequently oxidant stress, in the vascular system.

PDI AND NADPH OXIDASE INTERACTION: EVIDENCES IN OTHER CELL TYPES

We have pursued further evidence for a possible role of PDI in oxidase regulation in other cell types, and some ongoing results are already available. In endothelial cells, PDI is strongly

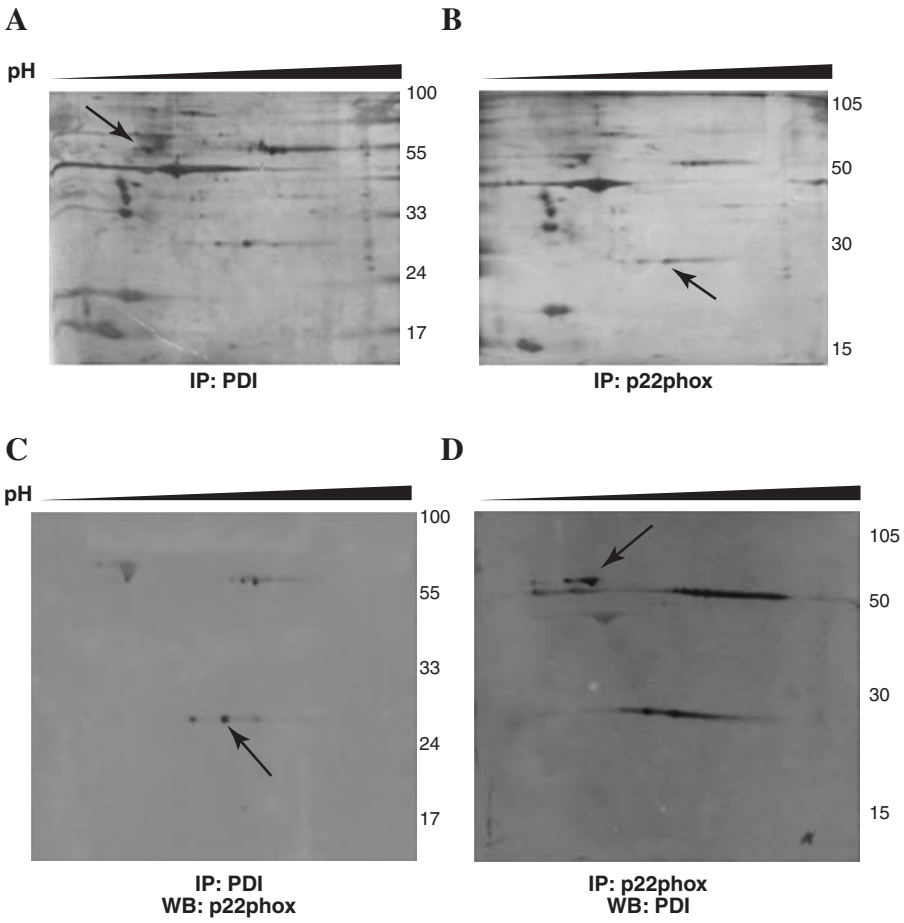

FIG. 4. Co-immunoprecipitation between protein disulfide isomerase and p22phox subunit in vascular smooth muscle cells (VSMC, from rabbit aorta). VSMC were grown as in Fig. 3 and exposed to angiotensin II (100 n*M* for 4 h). Cross-linking with Protein G-sepharose beads, antibodies, and immunoprecipitation procedures were similar to ref. 46, except that the precipitate was resolved by a 2-D gel approach, in which pI separation was performed within a pH range of 3 to 10 (corresponding to *black triangles on top of each panel*), followed by standard SDS-PAGE in a 12% gel. Additional controls were run for PDI protein and IgG standards (not shown). (**A**) and (**B**) Silver-stained gels of immunoprecipitates obtained with anti-PDI and anti-p22phox antibodies. (**C**) Western blot analysis of p22phox performed in anti-PDI immunoprecipitates, depicting corresponding band. (**D**) Reverse immunoprecipitation, in which Western blot analysis of PDI was performed in anti-p22phox immunoprecipitates, again exhibiting co-precipitation.

expressed in the membrane fraction even at baseline and does not exhibit further migration early after exposure to angiotensin II, showing a decreased membrane location and increased total expression at later time points (Fig. 5). This contrasts with VSMC, in which baseline membrane levels are lower, but there is increased migration to membranes upon angiotensin II exposure (46). A functional dependence of oxidase activity on PDI (Fig. 6) is suggested by experiments similar to those reported above for VSMC membrane homogenates in Fig. 3.

Additional ongoing data are being collected in our laboratories for macrophages and human neutrophils (unpublished observations), the latter both in whole cells and in the cell-free system. Such observations indicate that in phagocytes, PDI is also closely associated with NADPH oxidase subunits and exerts a functional regulatory role in ROS generation. Specifically in the neutrophil, PDI co-immunoprecipitates with distinct oxidase subunits at the membrane, as well as p47phox and p67phox in the cytosol (Fig. 7). The phagocyte oxidase is not only important as a well-studied model, but also because macrophages and polymorphonuclear leukocytes play an important

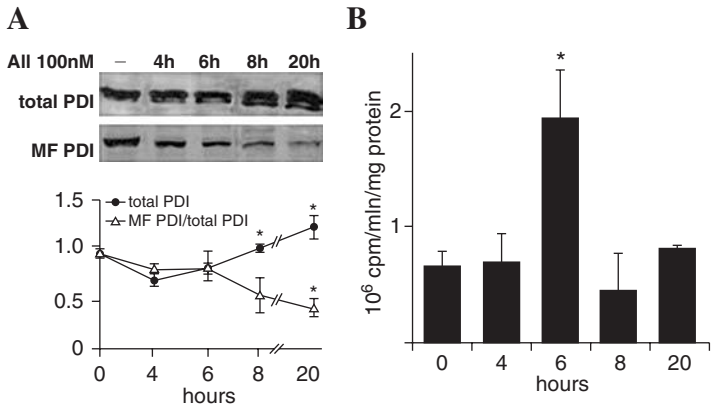

FIG. 5. (**A**) **Western blot analysis of PDI (~55 kDa) in total or membrane-enriched homogenates from rabbit aortic endothelial cells previously exposed to angiotensin II (100 n*M*, for the indicated time periods).** Cells were grown in F12 medium supplemented with 10% fetal bovine serum to 80–90% confluence. (**B**) NADPH oxidase activity at diverse time points after angiotensin II incubation, assessed with lucigenin (5 μM) technique. ($n = 3$; $^*p < 0.05$ *vs*. baseline; mean ± SEM)

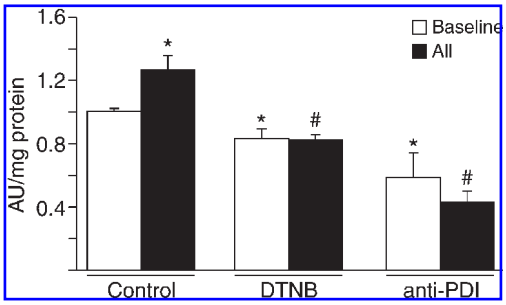

FIG. 6. Effects of diverse PDI antagonists on NADPH oxidase activity in rabbit aortic endothelial cell membrane homogenates, obtained as described in ref. 31. Endothelial cells were grown to 80–90% confluence in F12 medium supplemented with 10% fetal bovine serum and exposed to angiotensin II (100 n*M* for 6 h). NADPH oxidase activity was assessed by a novel spectrofluorometric assay for dihydroethidium oxidation products (31) in the absence or presence of PDI inhibitors DTNB (500 μM) and neutralizing anti-PDI antibody (1:100), added to membrane homogenates ($n = 3$; $*p < 0.05$ *vs*. control baseline; $\#p < 0.05$ *vs*. control + AII; mean ± SEM).

role in cardiovascular diseases, particularly atherosclerosis and hypertension (16, 99).

Altogether, such data indicate that a regulatory effect for PDI appears to constitute a more general property of the NADPH oxidase family rather than a peculiar finding of the VSMC.

MECHANISMS UNDERLYING PDI-MEDIATED NADPH OXIDASE REGULATION

The mechanisms underlying PDI-mediated regulation of NADPH oxidase remain speculative. Despite this fact, some hypotheses and inferences based on the several known properties of PDI can be made at this point.

The results obtained in vascular cell membrane homogenates incubated with exogenous NADPH render it unlikely that changes in cell redox status or NADPH availability are major factors underlying PDI effects on the oxidase. Indeed, PDI does not exhibit characteristics of a redox buffer system, due to its oxidase properties and compartmentalization (74, 108).

Figure 8 depicts two conceptual models whereby PDI may associate with NADPH oxidase subunits and potentially modulate catalytic activity of the complex. The most intuitive model (Model I) predicts that PDI would associate with the oxidase through a direct disulfide exchange via the thioredoxin motif. However, the arguments supporting this model are weak or conflictive. First, typical thioredoxin motifs are absent among known vascular cell catalytic and regulatory oxidase subunits, according to detailed database searches. Still, at least one regulatory dithiol has been postulated for Nox2 as the binding site of phenylarsine oxide (23), and four cysteines have been described in the p47phox molecule (44). In addition, as discussed above, PDI binding to other proteins, including one or more putative oxidase subunits, usually does not require the thioredoxin motifs, and occurs mainly as a hydrophobic interaction at or close to the b′ domain (53, 73, 98, 108). Even for other PDI family proteins such as Erp57, PDI binding does not involve PDI thioredoxin domain (52). The thioredoxin motifs, however, are important for subsequent stabilization of substrate binding to PDI. Such considerations implicate Model II as a more plausible, although less defined, alternative. In this model, PDI would bind to any oxidase-related subunit via its hydrophobic domain(s), and the redox-dependent part of PDI effects would involve disulfide exchange with another yet undisclosed dithiol motif, which could belong to any (co-)regulatory, structural or thioredoxin family-related protein.

Possible ways whereby PDI assists the oxidase complex likely reflect one or more of its versatile functions. First, PDI could contribute to stabilization of the binding among one or more subunits of the complex. Such a role is consistent with PDI chaperone and/or redox activities. In fact, protein complex stabilization has been increasingly recognized as one of the main functions of chaperones, in addition to their assis-

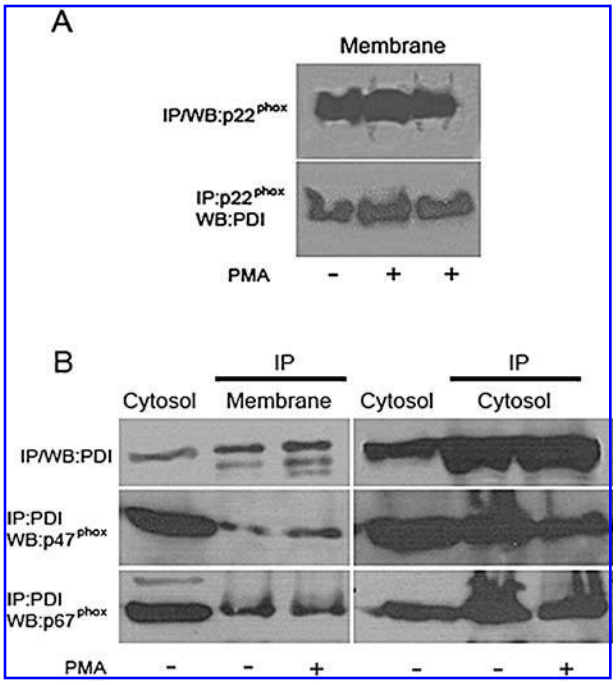

FIG. 7. Close association between PDI and human neutrophil NADPH oxidase subunits. Membrane and cytosolic fractions from neutrophils stimulated (+) or not (−) with phorbol myristate acetate (PMA, 0.1 μg/ml, 12 min) were prepared by nitrogen cavitation, followed by centrifugation through a Percoll gradient. (**A**) Neutrophil membranes were submitted to immunoprecipitation (IP) with anti-PDI antibody, followed by Western blot analysis (WB) using anti-p22phox or anti-PDI antibodies. (**B**) Neutrophil membrane or cytosolic fraction was submitted to IP with anti-PDI antibody, followed by WB using anti-p47phox or anti-p67phox antibodies, as indicated. Antibody sources (all dilutions 1:1,000) – PDI: Affinity Bioreagents; p22phox: kind gift from M. Quinn, U. Montana; p47phox and p67phox: Upstate or kind gift from B. Babior, Scripps RI, La Jolla.

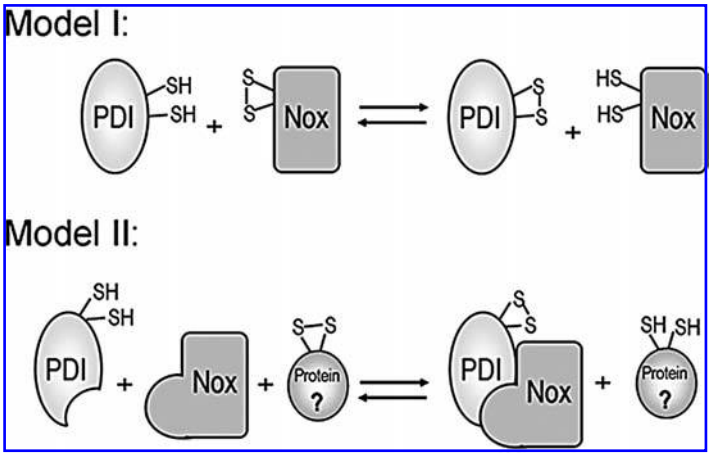

FIG. 8. Two conceptual models for PDI interaction with NADPH oxidase subunits, based on known structure and properties of PDI. In this diagram, Nox stands for one or more of the catalytic or regulatory subunits. The PDI redox configurations corresponding to active and inactive oxidase are unknown at present. In Model II, the redox status of oxidase-bound PDI thiols is only one of the possible hypothesis. For clarity, only one PDI dithiol is shown. In Model I, a possible mixed disulfide intermediate between PDI and Nox subunit is not represented.

tance in intramolecular folding (26). Second, given its known association with intracellular traffic, PDI could also contribute to deliver, stabilize binding, or remove specific Nox subunits from microdomains, thereby allowing the temporospatial control of localized ROS production. Finally, PDI might contribute to target specific oxidase subunits for proteolytic degradation, in line with its known role in endoplasmic reticulum-associated protein degradation by the proteasome (71, 91, 92).

Although redox pathways appear to be involved in PDI-mediated effects on NADPH oxidase, a more precise picture is yet undefined. The overall reducing redox ambient outside of the ER is consistent with PDI preferentially acting as a reductase at such locations. Indeed, labeling studies indicate that the presence of PDI at the cell surface is associated with reduction of most cell surface thiols, although a minority is oxidized (47). In addition, a thiol reducing effect of PDI at the platelet surface underlies its effect on integrin-mediated adhesion and aggregation (12), whereas trans-nitrosation underlies PDI-mediated disabling of tissue factor procoagulant effect (1). With the exception of Nox4, which may at least in some instances be located in the ER (69), Nox catalytic subunit locations include preferentially endosomes, caveolae, plasma membrane, or focal adhesions (105). Thus, a similar redox profile of PDI concerning its association with oxidase subunits might be anticipated. However, in this case, the exposure to ROS confined to a microvesicular environment [*e.g.*, an endosome (65)], could convert PDI into a thiol oxidase/isomerase. In fact, the low pKa of the exposed N-terminal cysteine of WCGHC motif renders PDI a preferential target of hydrogen peroxide-mediated oxidation (51) or glutathiolation (35). On the other hand, the acidic pH in the range of that found in secretory vesicles tends to increase the half-time of PDI-mediated substrate oxidation (2, 85). Thus, the redox profile of PDI accompanying its interaction with NADPH oxidase may be complex or diversified.

It is important to note that the limited data available so far do not allow establishing how specific the regulation of the oxidase by PDI might be. Given the abundant PDI expression and its multiple functions, as well as the fact that several other proteins are affected by thiol redox, including nitric oxide synthase (94) and xanthine oxidase (13), it would not be unexpected that PDI or analogous proteins exert a similar regulatory role in other enzyme systems. However, both for phagocytic and nonphagocytic oxidases, specificity is clearly not a prerequisite for relevance, considering, for example, that the subunit *rac*, well known to play an important regulatory role for the oxidase (39), also exerts many additional unrelated cellular functions. Conversely, the report that EFP1, a novel PDI-homologue of the thioredoxin superfamily, associates with Duoxs proteins of the Nox family as a component of the assembled membrane complex (107) raises the possibility that other PDI family members may also be involved with NADPH oxidase proteins. Finally, the available data do not allow to exclude that NAD(P)H oxidase might conversely exert a regulatory role on some of the multiple PDI functions. In this context, the reported association of PDI with wild-type SOD1 protein in neuronal cells (6) adds further complexity to such pathways.

TOWARDS AN INTEGRATIVE VIEW OF NADPH OXIDASE REGULATION

Although essential questions regarding PDI–NADPH oxidase interaction remain yet to be clarified, it can be anticipated that the nature of PDI effects discussed in the present review will provide novel avenues to allow a more integrative view of how NADPH oxidase is controlled in pathophysiological conditions. First, results of experiments with VSMC or endothelial membrane homogenates suggest that PDI is probably involved in mechanisms associated with a redox-mediated on-off switch of the oxidase at local membrane domains (Figs. 3 and 6). Second, the interaction with PDI can help understand mechanisms related to processing and/or traffic of Nox subunits between the ER and distal compartments of the secretory pathway. The involvement with PDI highlights the ER-secretory pathway as an important, if not the main, scenario in which NADPH oxidase is activated and exerts its signaling functions. Finally, perhaps one of the most interesting implications of the interaction with PDI is the possibility that knowledge of the intricate role of PDI in cellular physiology allows better understanding of the cell (patho)physiology of NADPH oxidase-dependent oxidative stress. Indeed, ongoing studies from our laboratory suggest that PDI–NADPH oxidase interaction may contribute to bridge UPR/endoplasmic reticulum

stress to oxidative stress, possibly in the context of an integrated adaptive stress response. This physiological connection may have implications regarding vascular response to injury (CX Santos *et al*, unpublished observations).

In summary, the novel role of PDI discussed in this review is likely to integrate with several other advances in the knowledge of NADPH oxidase control mechanisms, contributing to a more detailed model of how such enzymatic complex is regulated in vascular diseases. Clearly, this knowledge is essential to improve the accuracy and pathophysiological significance of current paradigms of redox signaling. Moreover, understanding how ROS are produced provides novel pathways for rational interventions aimed at controlling their generation in vascular diseases, an approach so far neglected in favor of interventions aimed at removing ROS after they are formed.

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ABBREVIATIONS

ALS, amyotrophic lateral sclerosis; DTNB, 5,5′ dithio-bis (2-nitrobenzoic) acid; EPR, electron paramagnetic resonance; ER, endoplasmic reticulum; Ero-1, Endoplasmic reticulum oxidase-1; FRET, fluorescence resonance energy transfer; GSH, reduced glutathione; GSSG, oxidized glutathione; PCMPS, p-chloro mercuryphenylsulfonic acid; PDI, protein disulfide isomerase; ROS, reactive oxygen species; SOD1, Cu,Zn-superoxide dismutase; UPR, unfolded protein response; VSMC, vascular smooth muscle cells.

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