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# Nox NADPH Oxidases and the Endoplasmic Reticulum

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## Abstract

***Significance:*** Understanding isoform- and context-specific subcellular Nox reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase compartmentalization allows relevant functional inferences. This review addresses the interplay between Nox NADPH oxidases and the endoplasmic reticulum (ER), an increasingly evident player in redox pathophysiology given its role in redox protein folding and stress responses. ***Recent Advances:*** Catalytic/regulatory transmembrane subunits are synthesized in the ER and their processing includes folding, *N*-glycosylation, heme insertion, p22phox heterodimerization, as shown for phagocyte Nox2. Dual oxidase (Duox) maturation also involves the regulation by ER-resident Duoxa2. The ER is the activation site for some isoforms, typically Nox4, but potentially other isoforms. Such location influences redox/Nox-mediated calcium signaling regulation *via* ER targets, such as sarcoendoplasmic reticulum calcium ATPase (SERCA). Growing evidence suggests that Noxes are integral signaling elements of the unfolded protein response during ER stress, with Nox4 playing a dual prosurvival/proapoptotic role in this setting, whereas Nox2 enhances proapoptotic signaling. ER chaperones such as protein disulfide isomerase (PDI) closely interact with Noxes. PDI supports growth factor-dependent Nox1 activation and mRNA expression, as well as migration in smooth muscle cells, and PDI overexpression induces acute spontaneous Nox activation. ***Critical Issues:*** Mechanisms of PDI effects include possible support of complex formation and RhoGTPase activation. In phagocytes, PDI supports phagocytosis, Nox activation, and redox-dependent interactions with p47phox. Together, the results implicate PDI as possible Nox organizer. ***Future Directions:*** We propose that convergence between Noxes and ER may have evolutive roots given ER-related functional contexts, which paved Nox evolution, namely calcium signaling and pathogen killing. Overall, the interplay between Noxes and the ER may provide relevant insights in Nox-related (patho)physiology. *Antioxid. Redox Signal.* 20, 2755–2775.

## Introduction

Working paradigms of cellular redox signaling increasingly suggest compartmentalization of transduction pathways, in which oxidative stress can be modeled as a loss of such modular architecture (51). This pattern of organization assumes that reactive oxygen species (ROS) generation is highly regulated on a basis of space, time, and type of species being produced (27, 126, 167, 178), thus implying an important role of enzymatic ROS sources in this process. Nox family reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are the prototypical source of signaling ROS in most cells [reviewed in Refs. (12, 58, 96, 97)], even though in quantitative terms, mitochondria can predictably surpass Noxes as ROS sources in most, although likely not in all, cell types (10, 25). While in mitochondria, the modular architecture is provided by the organellar structure itself, in the case of Nox NADPH oxidases, this is achieved through a regulated set of multiple protein interactions and/or post-translational modifications associated with Nox complex assembling and traffic to specific subcellular locations, which vary according to the type of Nox isoform [reviewed in Refs. (12, 58, 96, 97)]. Thus, understanding isoform- and context-specific subcellular location of Nox(es) is relevant to allow inferences on their functional pathways.

The endoplasmic reticulum (ER) has emerged as an increasingly evident player in redox physiology and pathophysiology (110, 145). This is due to at least three facts, the first of which being the ER-dependent production of ROS *via* enzymes, such as ER oxidoreductin 1 (Ero1) and its thiol

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redox partner(s) protein disulfide isomerase(s) (PDI). Such ER oxidoreductases engage in thiol-disulfide exchange reactions that culminate with the downstream insertion by oxidized PDI of disulfides into nascent proteins, coupled at the upstream end to electron transfer from reduced Ero1 to oxygen, thus generating hydrogen peroxide and oxidized Ero1—the latter step re-enabling the redox protein folding cycle (14, 68, 159). The significance of this potentially enormous source of hydrogen peroxide is not yet clear, but peroxide can be used at least in part for productive protein folding by promoting parallel oxidations of ER-resident peroxiredoxin IV (199) or glutathione peroxidase 7/8 (122), in addition to glutathione (158)—all converging toward PDI oxidation. These processes have been previously reviewed in detail (99). Second, the ER closely communicates and interacts with mitochondria *via* specific membrane tethering structures composing the ''mitochondria-associated membrane'', a subcompartment involved in calcium exchange, Ero1-mediated protein folding, and apoptosis regulation (21). Finally, the ER is closely involved in multiple aspects with Nox NADPH oxidases, in a way that extrapolates its expected role as the site of synthesis and protein processing of the transmembrane catalytic subunits. Increasing evidence points to the ER or ER-associated structures as an activation compartment for some isoforms, a pattern bearing possible ancestral connections and evolutionary relevance. Furthermore, ER chaperones, such as PDI, have been shown to display functionally relevant interactions with Nox(es) in distinct cell types (99). The aim of the present review is to discuss in more detail the mechanisms and implications of such interactions between the ER and Nox NADPH oxidases, an exercise we believe is likely to shed light on important aspects of Nox (patho)physiology.

## Synthesis and Processing of Nox NADPH Oxidases at the ER

Excellent previous reviews (8, 9, 12, 58, 96, 97) have addressed in-depth the structural and functional aspects of Nox NADPH oxidases, which will not be covered here. As transmembrane proteins, the prototypical catalytic Nox isoforms are synthesized and processed within the ER, similar to the regulatory transmembrane subunit p22phox. Contrarily, the canonical cytosolic regulatory subunits p47phox, p67phox, p40phox, and Rac1 or Rac2 are likely synthesized at the cytosol. Full maturation of the Nox complex includes several steps: glycosylation (and putatively other post-translational modifications), p22phox heterodimerization, insertion of heme and flavin adenine dinucleotide (FAD), and traffic to relevant locations within the distal secretory pathway up to membrane-containing subcompartments. Distinct functions of the ER are involved to a variable extent in each of these steps. In addition, these steps are isoform-specific: for example, p22phox incorporation is not known to occur with Nox5, and traffic distal to the ER, as well as post-translational modifications, may be restricted in some aspects for isoforms active at ER membranes, such as Nox4. In addition, as discussed below, the incorporation of cytosolic subunits seems to occur as a process concomitant to the traffic of nonphagocytic Noxes toward membrane(s) (162), as opposed to a clear activation step for the phagocytic complexes [reviewed in Refs. (8, 9)].

The most well-known NADPH oxidase complex regarding synthesis and processing is the phagocytic Nox2 (8, 9). Flavocytochrome $b_{558}$ contains two integral membrane proteins: gp91phox (a synonym for Nox2) and p22phox (127). The former is the redox center of the electron transfer, which binds heme and FAD, whereas the latter provides stability to gp91phox and fulfills the role of an adaptor protein working as an activation platform able to mediate interactions with the cytosolic regulatory subunits p67/p47 phox (35). The 91 kDa glycoprotein gp91phox weighs 50–55 kDa when fully deglycosylated (64, 127), whereas a 65 kDa precursor is most often detectable, even as the only form in cells, such as B cells (132) and PLB-985 human promyelocytic leukemia cells (190). Such gp65 precursor cosediments with ER, but not with Golgi, marker-containing fractions (188) and is susceptible to digestion with endoglycosidase H, suggesting that it contains ER-related high mannose-type carbohydrates (188, 190). Therefore, the synthesis of the larger subunit of flavocytocrhome $b_{558}$ occurs in the ER, where initial glycosylation takes place (40, 188, 190). Surprisingly, less than one third of newly synthesized gp65 pool appears to be processed to gp91phox and a significant fraction is degraded by the proteasome (40). The functional importance of ER-dependent gp91phox *N*-glycosylation is supported by the fact that patients with congenital deficiency of ER membrane-located glucose-incorporating enzymes have neutrophil dysfunction in connection with impaired NADPH oxidase activity. Such Nox dysfunction is associated with normal protein expression of all subunits but decreased $gp91^{phox}$ molecular weight, indicating a failure of glycosylation, and in fact the *N*- and *O*-glycomes of such neutrophils exhibit a failure of galactosylation (69). In addition to ER-dependent glycosylation, gp91phox presents complex carbohydrates, likely added at the Golgi, in line with the fact that, contrarily to gp65 precursor, endoglycosidase H is unable to digest gp91phox carbohydrate chains (40).

Full processing of mature stable gp91phox involves, besides glycosylation, the interrelated events of heme addition and heterodimerization with p22phox. Coexpression of gp91 phox and p22phox is important for gp91phox maturation and increases its cell surface expression without influencing gp65 expression in Chinese hamster ovary cells (197). Gp91phox binds two heme groups by itself (189). Accordingly, proteolysis study of purified neutrophil plasma membrane reveals that the portion of cytochrome b bound to heme groups is located within the lipid bilayer (135) and critical histidines at the N-terminal region are involved in the coordination of the bis-heme structure (53, 189). Importantly, these histidine residues are conserved across the Nox family. Cells expressing gp91phox mutated in critical histidines (H101L, H115L, H209C, or H222L) do not process gp65 to gp91phox nor do they achieve plasma membrane expression of flavocytochrome b, whereas their NADPH oxidase activity is completely abolished. (18). Relevant to the present discussion, in these mutated cells, not only gp65 but also p22phox were normally synthesized, but degraded with time, suggesting that sustained binding to the latter requires heme insertion and that heme insertion is a post-translational process (18). Such post-translational nature is further suggested by experiments with the heme synthesis inhibitor succinyl acetone, which abolishes superoxide generation (72), but does not affect the expressions of gp65 protein or gp91phox and

p22phox mRNA, while decreasing p22phox and mature gp91phox protein expression. This indicates a fundamental heme function in heterodimer assembly, which occurs in the ER with the gp65 precursor (188, 190). Golgi disruption or inhibition of *N*-linked glycosylation, respectively, did not impair the associations between p22phox and the precursors gp65 or gp58, although preventing gp91phox maturation (40). Induction or superexpression of heme oxygenase in murine macrophages significantly decreases superoxide production, in connection with enhanced proteasomal degradation of gp91phox and p22phox, indicating that post-translational heme incorporation is not only essential for adequate electron transfer by cytochrome $b_{558}$ but also for its stability (157). After ER synthesis and Golgi processing, human flavocytochrome $b_{558}$ is constitutively expressed on monocyte and neutrophil plasma membranes, as well as neutrophil secondary granules (54), and Nox activation at either or both locations depends on the stimulus [reviewed in Ref. (87)].

The neutrophil NADPH oxidase cell-free system requires FAD to be active and cytochrome $b_{558}$ is the FAD-binding site (140). Neutrophil membranes from X-linked CGD patients have FAD content below the normal FAD/heme 1:2 ratio (146). FAD replenishment to FAD-depleted cytochrome $b_{558}$ promotes 10-fold increase in the NADPH oxidase activity in the cell-free system, whereas FAD binding to cytochrome $b_{558}$ increases in phorbol ester-stimulated whole neutrophils. Probably, in resting cells, FAD is loosely bound to cytochrome $b_{558}$ and their interaction enhanced upon activation (66). The H338Y gp91phox mutation described in a patient (186) promotes FAD depletion as well as decreased gp91phox expression. His338 is located in the HPFT motif (His338ProPheThr), which is involved in FAD binding in a ferredoxin-$NADP^+$ reductase. FAD replenishment restored phorbol myristate acetate (PMA) stimulation of the NADPH oxidase activity in such H338Y CGD granulocytes. Retention of nascent mutant H338Y gp91phox by the ER glycoprotein-processing chaperone calnexin prevents mutated gp91phox to be detected at the cell surface, implicating FAD binding in calnexin dissociation from gp91phox. In normal cells, gp65 interaction with calnexin is detectable 1 h after its synthesis and totally abolished within 5 h, whereas gp65 from H338Y mutant cells remains bound to calnexin up to 5 h after synthesis. Surprisingly, calnexin also associates with p22phox even in the absence of gp91phox, probably *via* an unknown glycoprotein (106). Interestingly, NADPH oxidase as well as *in vitro* and *in vivo* bactericidal activities were further restored in H338Y mutants in the concomitant presence of FAD and thapsigargin, which efficiently redistribute ER-retained gp91phox to plasma membranes (77). Thapsigargin effect was apparently unrelated to increases in cytosolic calcium levels, but rather on its action on ER calcium-dependent protein quality control, allowing relief of calnexin-mediated ER retention of mutant gp91phox (33). On the other hand, FAD presence was not detected in resting or stimulated T341K, C369R, G408E, and E568K gp91phox mutants displaying $X^+$-CGD phenotype with loss in the NADPH oxidase activity. Surprisingly, the NADPH oxidase assembly is normal in T341K, although not in the other mutants, indicating that FAD binding is dispensable for the translocation of cytosolic factors. Of note, Thr-341, located at the predicted FAD-binding site, and Glu-568 are conserved in Nox1, Nox3, and Nox4 (39). Overall, these data indicate that FAD is not strictly required for gp91phox maturation and cell surface expression on the cell surface, although probably contributing in yet unclear aspects to sustain gp91phox expression. FAD binding to gp91phox likely occurs concomitantly or upon activation of NADPH oxidase and consequently not within the ER.

Lipid metabolism is an important ER-related function, and the association with lipid compartments appears important for the distribution and assembly of active Nox2 complex since mature gp91phox has been documented in lipid rafts, as opposed to gp65 precursor (170). Moreover, lipid raft disruption impairs NADPH oxidase assembly, as seen in the cell-free system, in which cholesterol is required for efficient p67phox and p47 phox translocation. Cholesterol depletion in Ra2 microglia and HL60 cell membranes changes the distribution of gp91phox and p22phox in detergent-resistant membranes from low- to high-density fraction, in association with the decrease in stimulated superoxide production (170).

Therefore, there are many levels of organization to be reached until NADPH oxidase becomes a functional enzymatic complex (48). These include (i) incorporation of redox components like heme and FAD, which are essential to proteins stability and heterodimer formation; (ii) correct localization on the membrane; and (iii) assembly of cytosolic components, which includes translocation and association of factors: p40phox, p47phox, p67phox, and Rac with flavocytochrome $b_{558}$.

Little is known about the synthesis and early processing of the other Nox isoforms within the ER (the Duoxes will be discussed in the next section). There is, however, no apparent reason why Nox1, Nox3, and in some aspects Nox4 processing and assembling should be significantly distinct from that of Nox2. Nox4 location at the ER and the lack of downstream interaction with cytosolic subunits, in addition to the lack of Nox5 heterodimerization with p22phox, are, of course, obvious differences. Since Nox4 does not require cytosolic subunits for activation, the role of heterodimerized p22phox is likely of stabilization rather than activation. Of note, the mutation of Nox4 histidine 115 abolishes its interaction with p22phox, similar to Nox1 and Nox2 (4). In addition, there is no reason why these isoforms are not glycosylated in a manner analogous to that of Nox2, although objective evidence for this is presently lacking.

### Nox NADPH Oxidase Localization and Activation Within the ER

A good deal of research in the Nox field has focused on understanding isoform localization as a conceivable window to reveal their possible functions. This approach, somewhat disappointingly, has proven so far only partially successful. No Nox isoform has a clear localization signal in its structure, and their subcellular location appears quite dynamic and stimulus-related, consistent with its preferential dependency on post-translational processes, such as protein modifications and protein–protein interactions, which remain to be better understood. In addition, most Nox isoform antibodies have questionable specificity, rendering localization studies a challenge. Few studies report controls with inhibitory peptides and Nox silencing to probe antibody specificity. In the case of the ER, the issue of location is further limited because,

Table 1. Selected Studies on Nox4 Subcellular Localization

| *Cell type* | *Model* | *Detection method* | *Subcellular localization* | *Obs.* | *Ref.* |
|---|---|---|---|---|---|
| Epithelial cells<br>HEK293, COS7 | Nox4 overexpression | Confocal IF, colocalization with $p22^{phox}$ | ER | Nox4 is constitutively activated. | 114 |
| HEK293<br>HEK293 T-Rex™ (tet-inducible Nox4) | Nox4 inducible with tetracyclin | TIRF microscopy<br>Confocal microscopy | Plasma membrane<br>ER | | 194 |
| HEK293 T-Rex (tet-inducible Nox4) | Nox4 overexpression | Light microscopy superoxide detection by NBT | Perinuclear<br>Nuclear envelope | Detection of superoxide production in inducible Nox4-overexpressing cells. | 147 |
| HUVEC | Nox4-GFP overexpression | TIRF microscopy, Hyper sensor | ER | Nox4 mediates UPR and ER signaling through local Ras activation. | 181 |
| HEK293<br>Human ASM | Nox4 overexpression | Confocal IF, colocalization with $p22^{phox}$ | Cytoskeleton<br>ER | Nox4 heterodimerizes with p22phox in the ER. | 4 |
| HAEC | Overexpression of AdNox4-V5 | Confocal IF, colocalization with GRP78<br>Electron microscopy, immunogold labeling<br>Nicodenz gradient | ER | Nox4 in the ER regulates oxidation and inactivation of PTP1B and ER resident proteins. | 28 |
| Human monocyte-derived macrophage) | oxLDL-stimulated macrophages | Immunohistochemistry, confocal IF, colocalization with $p22^{phox}$ and PDI | ER<br>Nucleus | Nox4 increases oxidized LDL-induced macrophage cytotoxicity and apoptosis. Nox4 is increased with oxLDL stimulus. | 100 |
| Adult and neonatal cardiomyocytes | Endogenous and overexpressed Nox4 | Confocal IF, colocalization with PDI | Perinuclear region, probably ER | Nox4 is a regulator of myocardial angiogenesis. | 195 |
| Rat primary VSMC | Primary culture, endogenous Nox4 | Confocal IF, colocalization of endogenous Nox4 with vinculin | Focal adhesions, stress fibers, nucleus | Colocalization of Nox4 and p22phox<br>Compartmentation of ROS production<br>Redox regulation of focal adhesion proteins | 74 |
| Rat VSMC<br>Human VSMC, HEK293 | Endogenous Nox4 | Endogenous Nox4 in confocal IF | Focal adhesions<br>Stress fibers, nucleus | Nox4 is activated by p22phox/Poldip2 recruitment | 109 |
| Primary cardiomyocytes | Nox4 Tg mice endogenous Nox4 | Confocal IF, colocalization of Nox4 with F1F0 ATP synthase (complex V) and mitotracker | Mitochondria | Nox4 promotes apoptosis and mitochondrial dysfunction when upregulated under hypertrophic stimuli.<br>Truncated Nox4 without mitochondria localization signal does not colocalize with mitochondria. | 1 |

(*continued*)

Table 1. (Continued)

| *Cell type* | *Model* | *Detection method* | *Subcellular localization* | *Obs.* | *Ref.* |
|---|---|---|---|---|---|
| Rat kidney cortex and mesangial cells | Rat model of diabetes induced by streptozocin | Mitochondria purification<br>Confocal IF | Mitochondria<br>ER | Nox4 is an important source of ROS in diabetes. | 19 |
| Cardiomyocytes | Tg mouse with cardiac-specific Nox4 overexpression × Nox4 KO | SOD-inhibitable ROS production by lucigenin chemiluminescence in purified mitochondria | Mitochondria | Nox4 is the major source of superoxide and/or hydrogen peroxide in the heart, mediating mitochondrial dysfunction during pressure overload. | 93 |
| HUVEC | Endogenous Nox4 | Immunocytochemistry<br>Electron microscopy with 3 antibodies<br>Nuclei fractionation | Nucleus | Nox4 activity is enhanced in the nuclear fraction after PMA. | 94 |
| Adult Cardiomyocytes | Nox4 KO mice and Tg mice with cardiac specific overexpression of Nox4 | Nuclei fractionation<br>WB, confocal IF | Nucleus<br>Mitochondria<br>Microsome | Nox4 is involved in cysteine oxidation and nuclear exit of HDAC4, mediating cardiac hypertrophy in response to phenylephrine and pressure overload. | 115 |
| Mice hepatocytes | shRNA for Nox4 silencing | Nuclei fractionation, WB, electron microscopy, confocal IF | Nuclear envelope—inner and outer membranes | Nox4 most significantly contributes to NADPH-dependent hepatic nuclear superoxide production. | 151 |
| THP-1 monocytes | LDL and high glucose-induced metabolic stress *in vitro* | Confocal IF, colocalization with phalloidin | F actin | Nox4-derived $H_2O_2$ remodels actin, leading to chemotactic activity of monocytes. | 166 |
| Human airway smooth muscle cells | Primary culture | Confocal IF with dye that shifts to red in the ER | ER<br>Nucleus | No colocalization with mitochondria or focal adhesions. Nox4 induced by TGF-$\beta$ mediates airway smooth muscle cell proliferation and asthmatic airway remodeling. | 153 |
| Rabbit/mouse hindlimb skeletal muscle | Primary culture | Subcellular fractionation of sarcoplasmic reticulum with sucrose gradient and WB | Microsome<br>Sarcoplasmic reticulum | SR Nox4-derived H2O2 regulates $O_2$-coupled RyR1, promoting changes in $pO_2$. | 154 |
| HUVEC<br>HMEC (microvascular) | Nox4-GFP overexpression by microinjection | Confocal IF, colocalization with calreticulin | ER | No Nox4 localization in cytoskeleton, lisosomes or mitochondria. Noxes play a role in EC cell–cell adhesion/motility | 168 |

ASM, aortic smooth muscle; ER, endoplasmic reticulum; GFP, green fluorescent protein; HAEC, human aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; IF, immunofluorescence; KO, knockout; oxLDL, oxidized low-density lipoprotein; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PDI, protein disulfide isomerase; PMA, phorbol myristate acetate; ROS, reactive oxygen species; SOD, superoxide dismutase; Tg, transgenic; TGF-$\beta$, transforming growth factor beta; TIRF, total internal reflection fluorescence; UPR, unfolded protein response; VSMC, vascular smooth muscle cells; WB, western blot.

as the place of synthesis, all isoforms have at least a transient maturation stage within the ER, whereas in many cases, they do not become fully active at the ER. Importantly, many studies addressing Nox location make use of transfection of tagged isoforms, whereas overexpression of any protein can lead to their artifactual retention in the ER as they may not be correctly folded and/or they can overload the ER folding machinery.

An emerging discussion in this context is the role of dehydrogenase domains, which are common to all Noxes. Such domains canonically mediate electron transfer from NADPH to FAD, that is, diaphorase activity. Normally, electron transfer from FAD to transmembrane heme groups is optimized by changes in protein conformation, as suggested for Nox2 (37). However, electron transfer from FAD to artificial substrates has been reported for Nox2 (62) and Nox4 (80, 121, 124). Whether *in vivo* the corruption of such dehydrogenase activity can generate ROS if these electrons are transferred to oxygen is interesting but yet speculative. However, the demonstration of cellular ROS generation for short alternatively spliced Nox4 isoforms lacking the transmembrane domain, such as Nox4D (56), which has recently been shown to display nuclear location (6), is consistent with such possibility. Similar findings have been shown for short alternatively spliced Nox2 beta isoform, which has only two transmembrane domains and lacks FAD-binding as well as all heme-binding domains (65). These facts allow some speculations. Since some of these short isoforms may lack domains responsible for traffic and membrane anchoring, they could behave as immature Noxes, being retained at the ER. An analogous mechanism could account for ROS generation by immature and/or slightly misfolded Noxes retained at the ER, even for otherwise normally membrane-located isoforms.

### *Nox4*

Nox4 is the isoform most consistently associated with the ER according to many studies, some of them detailed in Table 1. However, even in the case of Nox4, several other locations have been described: mitochondria, focal adhesions and cytoskeleton, and nucleus, the latter associated to one of its short spliced isoforms (6). In addition, recently developed monoclonal antibodies have detected Nox4 localization also at the plasma membrane by total internal reflection fluorescence (TIRF) microscopy (194) (Table 1). High glucose and palmitate induce Nox4 translocation to lipid rafts in adipocytes (63). Overall, a seemingly emerging consensus about the yet controversial topic of Nox4 location is that this isoform is primarily an ER-targeted Nox but undergoes traffic to many other compartments depending on unknown factors that likely include cell type, triggering stimuli, proliferative status, and probably culture conditions. Each of these Nox4 locations, including the ER itself, may require specific protein–protein associations or post-translational modifications since Nox4 does not depict a known ER retention signal, whereas a software prediction algorithm yielded high score for mitochondrial location (19). Studies with chimeric Nox1 and Nox4 indicate that location, as well as type of produced ROS, are determined by the N-terminal portion of each Nox, whereas constitutive activity is regulated by cytosolic tail (70).

Since Nox4 is constitutively active, evidence for Nox4 location at the ER implicates that Nox4 is active within this organelle. However, there is not a wealth of data reporting direct evidence for this fact. This is suggested by Nox4 loss- or gain-of-function experiments utilizing ER-localized sensors, such as Hyper (175, 181). Although an important advance, studies with redox sensors should yet be analyzed with caution, as their precise *in vivo* reaction mechanisms and limitations are not understood (116). Table 1 summarizes some specific aspects addressed in studies focusing on Nox4 within the ER. Since the ER lumen is topologically analogous to the extracellular milieu, it is expected that Nox4 is oriented at the ER membranes to release ROS toward the ER lumen. Interestingly, the opposite orientation cannot in principle be ruled out since the NADPH pool is preserved and reportedly thiol-independent at the ER lumen (130). Whether Nox4-derived ROS contribute to protein folding is possible but unknown.

The implications of ER location regarding Nox4 functions are not clear-cut, similar to Nox4 functions themselves. However, some specific ER-related targets have been identified, apart from Nox4 involvement with ER stress responses, discussed in the next section. One such target is sarcoendoplasmic reticulum calcium ATPase (SERCA), an ER membrane-located enzyme, which upon nitric oxide (NO)-stimulated cys674 glutathiolation increases calcium uptake into the ER, thereby impairing cytosolic calcium-dependent cell migration. Nox4 upregulation in vascular smooth muscle cells (VSMC) of prediabetic rats promotes irreversible SERCA cys674 oxidation after transforming growth factor-$\beta$ stimulation, preventing NO-mediated inhibition of cell migration. Knockdown of Nox4 prevented SERCA cys674 oxidation and neointima formation after carotid artery injury (161). In endothelial cells, Nox4-derived hydrogen peroxide synergizes with NO to promote SERCA glutathiolation and increased vascular endothelial growth factor-dependent migration. Interestingly, SERCA glutathiolation also required Nox4-induced generation of Nox2-derived superoxide (50). Another potential Nox4 target is the ryanodine receptor. In striated muscle myocytes, a $p(O_2)$-dependent generation of Nox4-derived oxidants targets ryanodine receptor cysteines to promote calcium release and muscle contraction. These data place Nox4 as a novel oxygen sensor, at least in skeletal muscle (154). Other observations in coronary artery myocytes, however, place the ryanodine receptor upstream of Nox4 ROS generation (192). Protein-tyrosine phosphatase-1B (PTP-1B), another ER-located enzyme, is oxidatively inactivated by Nox4. Through this effect, Nox4 is able to sustain epidermal growth factor (EGF) receptor signaling by negating PTP-1B-mediated inhibition of this tyrosine kinase, which occurs through its dephosphorylation at the ER (28). PTP-1B can also potentiate IRE1-dependent signaling during ER stress (59) and thus can be a potential target of Nox4 in this condition (see below).

### *Nox1*

The fact that Nox1 colocalizes with PDI in smooth muscle cells (82) has been sometimes taken to implicate an ER location for this isoform. The evidences for Nox1 location at plasma membrane, endosomes, and caveolae are compelling, however, and the ER pool of Nox1 may represent to some

extent immature enzyme—in addition to the fact that not all PDI may localize at the ER lumen (164). In gastrointestinal cancer cells, a major Golgi-located pool of Nox1 and its regulatory subunits has been described (160). Nevertheless, in lipopolysaccharide (LPS)-stimulated human colon cancer cells, diphenylene iodonium-sensitive oxidant generation displays a pattern suggestive of ER location, on the basis of colocalization between oxidant-sensitive 2′,7′-dichlorofluorescein (DCF) and ER-tracker dyes. Although both Nox1 and Nox2 are expressed in these cells, oxidant signals and increased cell adhesion were completely abrogated by Nox1 siRNA (125).

### *Rac1 and p47phox*

Although Rac1 and p47phox are cytosolic subunits, they have been associated (probably indirectly) with dysfunction of calcium regulation. A Rac1-dependent NADPH oxidase controls intracellular calcium oscillations in histamine-simulated endothelial cells, *via* possible ER sensitization to inositol 1,4,5-trisphosphate (76). Also, the oxidation of calcium/calmodulin-dependent protein kinase II (CAMKII) has been associated with cardiac sinus node dysfunction, an effect significantly inhibited upon genetic deletion of p47phox (155). These results implicate a role for distinct Noxes, in addition to Nox4, at several levels of calcium signaling regulation.

### *Nox2*

Phagocytic Nox2 is clearly functional at the plasma membrane, phagosomes, and specific granules (8). In macrophages, LPS stimulation promotes internalization of Nox2 *via* clathrin-coated pits for recycling within the endosomal secretory compartment (46). In contrast, nonphagocytic Nox2 displays locations that resemble the ER, for example, in endothelial cells (102, 168). It is unclear, however, whether the enzyme can be active at this location or whether this represents just a maturation stage of the active complex. In the cardiomyocyte, Nox2 localizes at the sarcolemma, as well at transverse T-tubules, which are deep sarcolemmal invaginations toward the cytosol juxtaposed to terminal cysternae of the smooth ER. Physiological stretch activates, *via* microtubules, Nox2 at those locations, promoting a flash of oxidants that act locally at the ryanodine receptor from junctional ER, releasing a calcium spark for contraction. This circuit integrates and tunes excitation–contraction coupling (133, 134) in cardiac as well as skeletal muscles.

### *p22phox*

Recent evidence indicates close correlation between p22phox and ROS generation detectable by an ER-specific aryl-boronate probe in a human acute myeloid leukemia cell line, for which Nox2 may contribute at least partially to the phenotype. ROS generation is inhibitable by the Nox inhibitor VAS2870 or siRNA against p22phox. The expression of p22phox is reduced, through proteasome degradation, upon tyrosine kinase inhibition, thus providing a connection between p22phox and the leukemic phenotype, further reinforced by demonstration of p22phox-related downtream signal transducer and activator of transcription 5 (STAT5) signaling (179).

### *Nox5*

The location of Nox5 seems variable and dynamic, similar to other Noxes, but there are some evidences for an ER location. Importantly, Nox5 has many distinct variants with potential location-related functions (13). Within the cell, either endogenous or overexpressed Nox5 displays an ER-resembling perinuclear distribution pattern, colocalizes with calreticulin in endothelial cells (11, 13), and has been reported at the detergent-resistant microdomains of the ER (81). When overexpressed in HEK293 cells, Nox5 seems located not only at the plasma membrane but also at the perinuclear region, generating superoxide in response to protein kinase C activation (148). Despite the evidences for Nox5 in the ER, the strategies of Nox5 to bind to the plasma membrane indicate the functional relevance of such location. One such strategy is the binding to p22phox: Nox5 coimmunoprecipitates with p22phox when both are overexpressed, but not with endogenous p22phox, whereas p22phox is not essentially required for Nox5$\beta$-dependent ROS production (13, 88). Also, Nox5 has two polybasic regions, one N-terminal and another C-terminal. Extracellular production of ROS by Nox5 is modulated by PtdIns4,5P2, which targets Nox5 location at the plasma membrane through its N-terminal polybasic region (89). The nonreceptor tyrosine kinase c-Abl comigrates with Nox5 to the plasma membrane in a calcium-mediated process (47). Finally, some studies indicate a cytosol location for Nox5 in myocardium (61), myometrium (34), and esophageal carcinoma cells (75). In the latter case, colocalization and functional interplay between the short Nox5S isoform and Rac1 has been reported.

### *Duox1 and Duox2*

While mature Duoxes clearly locate at plasma membranes, maturation and processing of these glycoproteins involve a tight connection with the ER. Oxidative folding is the rate-limiting step for Duox2 maturation and exit from the ER, which is facilitated by the specific maturation factor DuoxA2 (57). Interestingly, the partially glycosylated form of Duox2 is located at the ER and generates, in a calcium-dependent way, superoxide detectable with electron paramagnetic resonance techniques, contrarily to the mature enzyme, which releases mainly hydrogen peroxide (5). Duox2 targeting to plasma membrane is preceded by its exit from the ER, which is regulated by an N-terminal ectodomain segment including the first transmembrane domain. Such exit is completely inhibited by mutation of four specific cysteine residues (119).

Overall, the data discussed in this section indicate that Nox4 (constitutively) and possibly other isoforms (circumstantially) do exhibit ER localization, and there is emerging evidence that at least Nox4 in fact becomes active at this organelle. At the same time, this discussion indicates that, for each isoform, focusing on location as a window to define function is a limited strategy in the absence of a specific (patho)physiological context in which this is inquired. In fact, Noxes appear to have evolved to display varied subcellular locations, which are not only isoform-specific but also stimulus-specific, likely in connection with acquisition of many specialized functions.

### ER Stress and Nox Activation

Adequate protein folding and processing is likely to be one of the most fail-safe and conserved cell functions, as judged by the number and nature of known defensive strategies to assist, preserve, correct, or balance it (23, 150). Protein processing within the ER lumen includes three major general activities: folding, disulfide introduction, and *N*-glycosyl residue insertion (23, 33). Failure at any of these steps will promote an imbalance between protein load and the ER capacity to process such cargo, leading to ER stress. ER stress counteractivates homeostatic signaling known as the ''unfolded protein response (UPR),'' a complex network of signals arising from ER membrane sensors toward the nucleus and back to the ER and several other cell compartments. Details of UPR signaling have been reviewed elsewhere (139, 143, 156, 180) and are briefly summarized in Figure 1. The UPR aims (i) to prevent, at least temporarily, protein synthesis, (ii) to improve the ER folding capacity, (iii) to remodel the ER, and (iv) to adjust cell metabolism to a defensive mode. Some features of the UPR recapitulate general cell stress responses, such as the arrest in protein synthesis, some metabolic adjustments, and enhanced protein degradation, whereas other aspects are more specific, for example, increased expression of ER chaperones, such as Grp78, Grp94, and calreticulin (139). Although such responses are primarily homeostatic, they can, as with any stress response, lead to cell damage and apoptosis if ER stress is sufficiently sustained or strong (143). Experimental studies often address the acute UPR (145). In many disease states, however, there is a state of chronic protracted UPR activation, in which sustained or oscillating ER stress coexists with variable combinations of adaptive as well as apoptotic signaling. Such state, which is so far not well understood, is a hallmark of many disease states, including atherosclerosis, diabetes, obesity, and cancer (112). Oxidative stress and ROS generation is an integral component of the acute as well as some chronic states of UPR signaling, whereas ROS generation by itself can apparently trigger the UPR in some, but not every case (145). Antioxidant compounds have been repeatedly shown to protect against apoptosis, especially during the acute UPR, in many distinct circumstances (110, 145). This, however, should not be taken as a rule, and protective effects of ROS during the UPR have been reported as well (15, 181, 182). Overall, it is increasingly clear that the UPR is a complex response activated in highly specific patterns, often

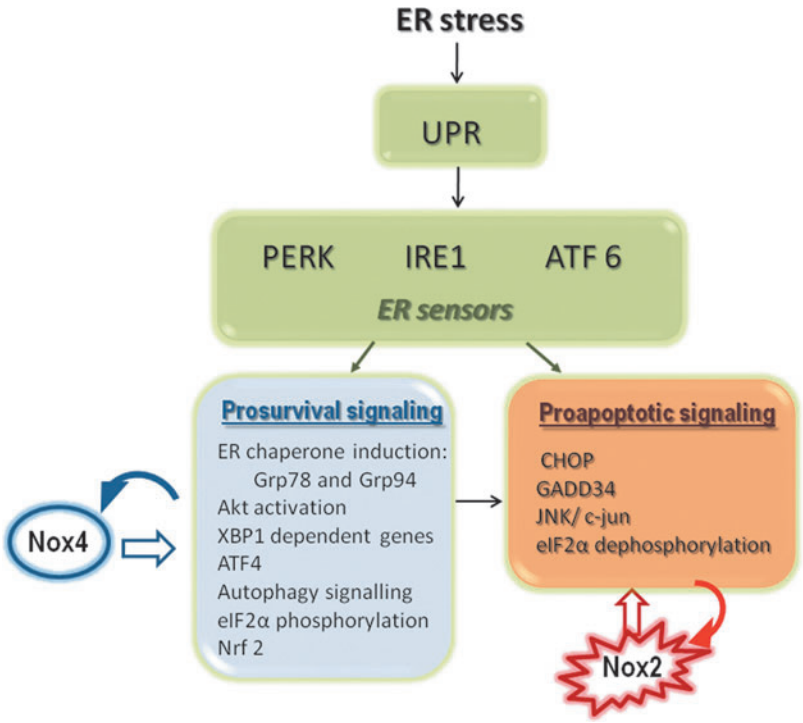


**FIG. 1. Organization of the unfolded protein response (UPR) signaling and interplay with Nox NADPH oxidases.** ER stress triggers UPR signaling from three main arms based on distinct ER transmembrane sensors: the endonuclease/kinase IRE1, the kinase PERK, and transcription factor ATF6. Each one triggers signals emerging from the cytosolic surface of ER, which integrate pathways for decreasing global protein synthesis load, improving protein folding, adjusting metabolism and amino acid sufficiency, and inducing misfolded protein degradation. Such responses can be classified as either prosurvival or proapoptotic, although both types are triggered simultaneously. Among several UPR-responsive homeostatic genes, the chaperones Grp78 (BiP), Grp94, and calreticulin are regulators, as well as operational markers, of the UPR. The most specific apoptosis pathway is the transcription factor CHOP (GADD 153). Nox4 is induced early during the UPR and is upstream to several signaling events that may induce autophagy, survival, or eventually apoptosis. Nox2 is induced through a CHOP/CAMKII pathway to mediate apoptosis. CAMKII, calcium/calmodulin-dependent protein kinase II; CHOP, CCAT/enhancer binding protein homologous protein; ER, endoplasmic reticulum; Grp, glucose-regulated protein; JNK, c-Jun N-terminal kinases; NADPH, reduced nicotinamide adenine dinucleotide phosphate. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

partially, and depends highly on the cell type and context. Clearly, however, ER stress is a major context in which transient or sustained oxidative stress arises in many disease states.

The mechanisms accounting for ROS generation during the UPR appear to be multiple [reviewed in Ref. (145)]. Prior suggestive evidence has been published with respect to ER folding-related oxidoreductase cycles and mitochondria. This is, however, not without criticism since compelling evidence that the ER is underoxidized during the UPR in yeast (117) or quite resistant to redox imbalances in mammalian cells (7a) argues against a predominant luminal ER source of oxidants during ER stress. More recently, increasing evidence suggests that Nox NADPH oxidases are important ROS sources during the UPR in distinct cell types (Fig. 1). Nox4 has been the most studied isoform in this context, with consistent evidence for increase in its gene and protein expression during ER stress induced by distinct stimuli. In VSMC, incubation with the oxidized cholesterol product 7-ketocholesterol induced 3-fold increase in Nox4 mRNA and protein expression (128), similar to the classical ER stressor tunicamycin, which increases Nox4 mRNA levels about 10-fold (145). Neither stimulus significantly affects Nox1 expression. In this scenario, ROS generation is virtually abolished by Nox4 knockdown, implicating this isoform as ''a'' and eventually ''the'' major ROS source in this circumstance. Intriguing evidence indicates that Nox4 is a proximal trigger of the UPR in endothelial cells exposed to tunicamycin or the ER stressor protein HIV-tat (181). In this case, the ER surface constitutes a platform for spatial organization of Nox4 signaling and downstream K-Ras activation, both as upstream UPR triggers (182). Nox4-dependent signals activate autophagy as a protective mechanism since autophagy inhibition promotes apoptosis. Transfection with an ER-located Hyper plasmid allowed detection of hydrogen peroxide production within the ER triggered by tunicamycin or HIV-tat, in a Nox4-dependent way, even though Nox4 could still affect hydrogen peroxide production indirectly. Moreover, the Hyper probe can be influenced by pH changes and is heavily affected by thiol-disulfide redox (116). Intriguingly, hydrogen peroxide production was not locally detected after thapsigargin or DTT-induced stress, even though shRNA for Nox4 decreased UPR signaling triggered by them (181). Thus, Nox4 can have a central role as upstream regulator of the UPR, although the mechanisms of Nox4 effects appear to be dependent on the nature of the UPR trigger. The distinct outcomes—survival *versus* apoptosis—regarding Nox4 within the UPR context is in line both with the conflicting evidences for protective *versus* damaging effects of Nox4 (1, 195), discussed above, as well as with the known dichotomy of ER stress endpoints. To what extent Nox4 itself is at the crossroads of UPR-related cell decisions is unknown.

Nox2 has also been implicated in the UPR (Fig. 1). ER stress induction with cholesterol or 7-ketocholesterol promoted apoptosis and oxidative shift in macrophages, which were abolished by Nox2 siRNA (101). Such apoptosis signaling involved a CCAT/enhancer binding protein homologous protein (CHOP)-CaMKII-c-Jun N-terminal kinases (JNK) pathway. Remarkably, the expression of CHOP (the canonical effector of UPR-related apoptosis) was dependent on double-strand RNA-dependent protein kinase (PKR) and was decreased in stressed macrophages from $Nox2^{-/-}$ mice. Mitochondrial pathway of apoptosis was also diminished in $Nox2^{-/-}$ macrophages. $Nox2^{-/-}$ mice also displayed kidney protection upon an *in vivo* challenge with tunicamycin, in connection with abrogated CHOP induction (101). Close convergence between ER stress signaling and Nox subunits also occurs in central nervous system regulation of hypertension. Loss-of-function of p22phox in the subfornical organ eliminated hypertensive responses to angiotensin II (AngII) (107) in association with decrease in UPR marker expression (187).

In addition to the above-discussed investigations, a relatively large body of studies report coincidences between Nox or Nox subunit effects and the occurrence ER stress, without showing clear evidence that such Nox plays a direct role in UPR signaling. In most such studies, loss-of-function of Nox promotes decrease in some ER stress-associated targets or another pathway promotes both Nox activation and ER stress. Such results should be interpreted bearing in mind the complex pathways connecting Nox to ER stress-dependent target responses, which can be indirect and involve collateral signaling, such as inflammatory mediators (193), metabolic adaptations, cell adhesion processes, and many others. A few examples can be listed regarding these studies: Nox2 in atherosclerosis (104); Nox2 in neurodegenerative diseases (16); p47phox in impaired cardiac contractility (196); p47phox in cytokine-induced inflammatory angiogenesis and autophagy (141); Nox4 in glycation product-induced fibroblast apoptosis (108); Rac1 in saturated fat-induced JNK activation in hepatocytes (149); Rac1 in diabetes-induced cardiac hypertrophy, collagen deposition, and contractility (103); Rac1 in high glucose-induced ER stress in culture cardiomyocytes (103); Nox activity in vascular cell calcification (105). ER stress plays a prominent role in *Toxoplasma gondii*-induced trophoblast apoptosis, whereas an increase in Nox1 expression and oxidant generation preceded UPR signaling and downstream activation of caspase 12, and UPR-related apoptotic signaling was abolished by N-acetylcysteine (183).

The UPR closely converges with the ''endoplasmic reticulum-associated degradation (ERAD)'' to promote enhanced degradation of un/misfolded proteins in the cytosol by the proteasome (22). ERAD is the executive arm of essential protein quality control mechanisms that can exert control on Nox subunit expression, although information for this is incipient. Such is the case not only of defective forms, for example, mutated Nox2 (106), but also of normal regulation of p22phox (20) or Rac1 (92), both cases seemingly involving redox-dependent steps (42, 92). The proteasome also helps sustain Nox4 mRNA expression during the UPR (3).

In summary, evidence for the involvement of Noxes in UPR signaling, as well as in protein quality control, is growing, but the mechanistical links are yet fragile. As judged from the convergence between Noxes and the ER discussed in this review, it can be speculated that more chapters of this story are to be written.

### PDI Interaction with Nox NADPH Oxidase

Another point of convergence between the ER and Nox NADPH oxidases is the interaction between Noxes and ER chaperones from the PDI family. There are more than 20 PDI isoforms, with PDI itself, also called PDIA1 or *P4HB* gene product, as the founding member. These proteins belong to

the thioredoxin superfamily and have, as hallmarks, a thioredoxin fold and in most cases dithiol groups. Most studies addressing PDI convergence with Noxes concentrate on PDIA1 (or simply PDI), which has two dithiols with the typical –CXXC– sequence. PDI displays oxidase, reductase, and—as the hallmark of this family—thiol isomerase activity (7, 68, 78, 99, 176). In addition, PDI has a chaperone effect—preventing protein aggregation and sustaining their folding—which in itself is independent on its thiol groups, although cooperative with thiol oxidoreductase activity. Such chaperone effect is mostly attributable to a hydrophobic pocket within the main protein core (67, 68). Access to this core is flanked by a mobile arm, which opens when PDI is oxidized and closes upon its reduction (172, 173). The canonical function of PDI in the ER lumen is to introduce and/or reshuffle disulfide bonds into nascent proteins destined to membrane insertion or secretion (176). PDI composes with other isoforms or related chaperones to integrate protein folding, disulfide introduction, and *N*-glycosyl residue attachment, the three main steps of protein processing in the ER. All such PDI effects provide it with the intrinsic function of being the convergent hub of ER redox homeostasis and, consequently, an important player in ER-dependent redox signaling processes (99). The role of PDI in redox signaling, as well as ER and cellular redox homeostasis, was reviewed in detail recently (99).

In addition to its canonical ER location, PDI, similar to some other ER chaperones, is also present at the cell surface and pericellular compartments (''peri/epicellular PDI'' or pecPDI), where it mediates a number of thiol-disulfide exchange events (reductase and possibly oxidase and isomerase activities) (86, 99, 164). Such redox effects mediate a number of physiological or pathophysiological processes related to thrombus formation (30, 31, 84, 137), tissue factor functional regulation (2, 137, 169), phospholipid asymetry (131), platelet adhesion, and aggregation [reviewed in Ref. (49)], ADAM activity (177), citoxicity of diphtheria toxin (111), uptake of viral particles (55, 113, 171), and galectin regulation (17), among others. The putative route through which PDI gains access to its epi/pericellular location remains elusive (71, 86). This remains a crucial question since it is possible that PDI effects on Nox regulation outside the ER take place at the physical route of PDI externalization.

Convergence between PDI and the NADPH oxidase complex was described by our group in the context of addressing mechanisms underlying thiol sensitivity of nonphagocytic NADPH oxidase (82). Evidences suggesting such an interaction have been obtained in both the context of functional interdependence and physical association, with the functional data showing the most clear results so far. In VSMC, antagonism of PDI by pharmacological compounds, as well as loss-of-function experiments with specific protein knockdown induces loss of NADPH oxidase activation and superoxide production in response to AngII (52, 82). Moreover, silencing PDI expression even partially is sufficient to prevent the enhanced expression of Nox1 due to AngII (52) or platelet-derived growth factor (129), whereas in both cases, the expression of Nox4 remains unaltered. Importantly, gain-of-function experiments in which PDI was overexpressed about 2–2.5 times depicted an enhanced spontaneous baseline activation of NADPH oxidase, reflected by superoxide and hydrogen peroxide detection, in a preemptive way, that is, further incubation with AngII did not enhance the NADPH oxidase activity. Such Nox activation was associated with enhanced expression of Nox1, but not Nox4 at baseline, whereas after AngII incubation, Nox4 expression was also enhanced (52). PDI requirement for NADPH activation was also observed in endothelial cells challenged with AngII (98), as well as PMA-activated macrophages (144) and neutrophils (38) and in glial cells (83). This indicates that the functional support of the NADPH oxidase activity by PDI occurs in distinct cell types and with nonphagocytic as well as phagocytic Noxes.

Importantly, PDI-dependent support of the Nox activity was shown to have functional implications. In response to AngII incubation in VSMC, PDI loss-of-function abrogates Akt phosphorylation (82), a response known to be associated with Nox1. Accordingly, PDI silencing with siRNA significantly impairs Nox1-dependent VSMC migration in response to PDGF (129). In macrophages, lowering PDI expression inhibits *Leishmania chagasi* phagocytosis (144).

Of note, the partial PDI knockdown in these experiments in VSMC and macrophages was unassociated with evidence of UPR activation, a result in line with other studies indicating that PDI loss-of-function does not necessarily trigger the UPR (120, 142) and that PDI is generally not considered an ER stress-responsive gene (68, 145, 176). However, in some cell types such as mouse embryonic fibroblasts (unpublished observations from our laboratory), PDI silencing associates with UPR marker expression and, indeed, PDI silencing can possibly sensitize most cells to other ER stressors. In fact, in neuronal cells (165) and oxidized low-density lipoprotein-challenged macrophages and endothelial cells (120), loss of PDI function promotes or amplifies the UPR, leading, respectively, to the formation of insoluble protein aggregates and CHOP expression/apoptosis. The extent to which PDI down-expression induces that UPR is likely dependent on cell type, secretory status, ER redox state, and concomitant expression of other PDI family members (99). PDI has a possible role to couple ER stress to Nox-dependent ROS generation (99). Evidence for this logical hypothesis is however poor, even though preliminary (unpublished observations) data from our group indicate that PDI silencing, while increasing apoptosis signaling, prevents ROS generation in tunicamycin-challenged VSMC, a situation in which Nox4 expression accounts for essentially all detectable ROS (145). Interestingly, UPR-induced increase in mRNA levels of both PDI and Nox4 are essentially abrogated by nonlethal concentrations of proteasome inhibitors, which nonetheless increase UPR-induced loss of VSMC (3).

The above results suggest a clear functional interplay between PDI and either Nox1 or Nox2, in distinct cell types, whereas the functional interplay between PDI and Nox4 is unclear at present. Evidences for physical interaction between PDI and Nox subunits further reinforce these functional data. A word of caution is important here since PDI is highly expressed in cells and displays a client-type interaction with a large number of proteins, although such interactions tend to be quite rapid and generally do not yield detectable protein complexes unless PDI is artificially mutated to trap substrates (85). Also, the large PDI hydrophobic core may interact nonspecifically with unfolded proteins (67) and even small substrates, such as estrogens, bisphenol A, insulin [reviewed in Ref. (78)], antigen peptides (32), and

somatostatin (91). With these caveats in mind, there is substantial evidence for confocal colocalization and/or coimmunoprecipitation between PDI and many Nox subunits, including Nox1, Nox2, Nox4, p22phox, p47phox, and Rac1 or GTPase regulator RhoGDI (38, 82, 99, 144). Since these associations do not imply functional relevance, they may implicate a similar share of location at the ER or downstream ER-derived compartments. Such may be the case of Nox4, which clearly associates with PDI (82, 195), but does not show expression modulation in response to PDI loss- or gain-of-function, as discussed above. Another important point is that none of these associations has shown a clear pattern of modulation in response to Nox activation. Overall, the mode of PDI association is likely to be transient and possibly multiple, more akin to that expected for a peripheral regulatory protein rather than a more stable subunit of the Nox NADPH oxidase complex.

The mechanisms whereby PDI affects Nox NADPH oxidases are yet unclear and likely to be multiple, reflecting the diverse functions of PDI and its versatile array of interactions. Within contexts in which PDI exerts the regulation of AngII-mediated Nox1 activity, overall PDI expression exhibits little or no change, whereas there is a translocation of PDI toward particulate fraction containing membrane subcompartments (82). In such homogenates obtained after AngII stimulation, incubation with the thiol isomerase inhibitor bacitracin or a neutralizing antibody against PDI induces decrease in the NADPH oxidase activity, indicating that PDI segregates into locations close to the oxidase complex milieu and exerts Nox control *via* thiol redox mechanisms (82). Given PDI role as chaperone and folding catalyst, a role for PDI in complex stabilization and/or organization is possible, but remains to be proven. In addition, nonredox mechanisms also play a role in PDI effects since overexpression of cysteine-less PDI in VSMC induces acute activation of the NADPH oxidase activity and Nox1 expression similar to the wild-type counterpart (52). This indicates that at least acutely a PDI-mediated chaperone effect, known to be thiol-independent, may contribute to NADPH oxidase complex organization. In fact, it is well known that PDI binding to most client proteins occurs through a chaperone-like mechanism involving the hydrophobic PDI groove, whereas thiol redox pathways subsequently stabilize the interaction (67, 68, 99). Of note, redox sensitivity of any PDI interaction does not implicate that binding to target necessarily occurs *via* PDI thiols, as this may simply reflect the profound redox-dependent plasticity of PDI. When its thiols are oxidized, PDI is in open configuration, exposing its hydrophobic core and enhancing its chaperone activity. As PDI thiols are reduced, for example, following disulfide introduction into a client protein, the hydrophobic PDI groove shifts to closed configuration. These configurations are in line with PDI functions. Although oxidized PDI is expected to accomodate variable client proteins, reduced PDI configuration is consistent with an impaired binding to complex proteins, while allowing exposure of aromatic residues that bind the oxidase Ero1, thus potentially allowing PDI reoxidation (99, 173).

Surprisingly, not much is known about the existence and possible function of reactive thiols within Nox subunits. Classical thioredoxin-like dithiol motifs are absent in their structure, although a dithiol specific for Nox2 has been reported in its cytosolic domain (36, 37, 43). In addition, up to four cysteines may contribute to modulate p47phox function (79). Mutations of two Nox4 E-loop cysteines switch the ROS product from hydrogen peroxide to superoxide, with no change in its expression or subcellular localization (158). The absence of a clear Nox dithiol target, plus the somewhat protean nature of PDI association with Nox subunits discussed above, do not allow at present to highlight any particular catalytic or regulatory subunit as a specific PDI target, although possible candidates have recently emerged. We detected in human neutrophils a thiol redox-dependent association between PDI and p47phox in pull-down assays, whereas in the cytosol fraction during neutrophil activation, PDI shows a reductive shift, contrarily to the oxidation shift depicted for p47phox (38). In addition, we recently identified

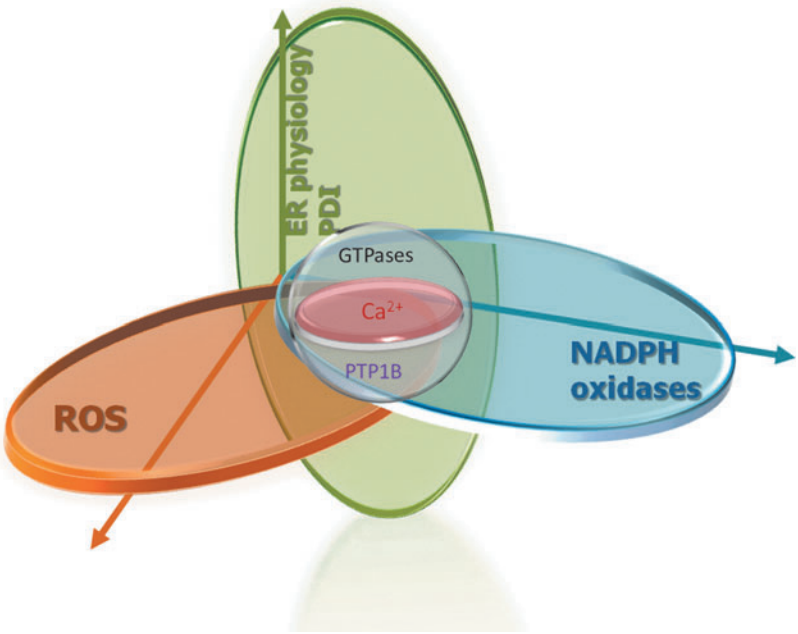


**FIG. 2. PDI and ER (patho)physiology confer a novel dimension to Nox NADPH oxidase/ROS signaling.** The mechanisms involved in these connections have been discussed throughout the text, whereas here we highlight: calcium signaling convergence with Nox(es), RhoGTPase convergence with PDI, and Nox(es) and PTP1B convergence with Nox4. PDI, protein disulfide isomerase; ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

RhoGTPases (Rac1 and RhoA) and their regulator RhoGDI as possible mechanisms of PDI-mediated support of platelet-derived growth factor-induced, Nox1-dependent, VSMC migration (129). Such interaction was proposed from physical protein–protein interaction system biology maps, which identified high bottleneck score values for GTPases and GDI in PDI interactome. In fact, impaired cell migration after PDI silencing closely correlated with decreases in Rac1 or RhoA activity and profound disruption of cytoskeletal structures, such as stress fibers, focal adhesions, and adhesion vesicles. Importantly, Rac1 and RhoA can be strongly regulated by redox mechanisms, although it is yet unclear to what extent this occurs *in vivo* (118). Overall, these emerging findings allow the proposal that PDI may act as novel redox-related organizer of the NADPH oxidase complex. Together, PDI and ER physiology confer an additional dimension to Nox/redox-dependent signaling (Fig. 2).

The interaction between PDI and Noxes is not an isolated example, and other PDIs may also be involved in distinct ways. Erp72 associates with Nox1 at the plasma membrane and is oxidized by it upon EGF exposure, leading to decreased Erp72 reductase activity (29). The thioredoxin superfamily protein EFP1 interacts with Duoxes as a component of the assembled membrane complex (174). Endothelial cell incubation with atorvastatin promotes enhanced migration of endoplasmic reticulum protein 46 (Erp46, also known as endoPDI) to lipid rafts, the same location of Nox2 (60). In addition, Noxes may interact with other chaperone families, such as heat shock protein 90 (Hsp90), which binds to the C-terminal region of Nox1–3 and 5, though not of Nox4, to enhance their stability (26).

### Evolutive Perspectives of ER-Nox Convergence: Implications for Calcium Signaling

Evolutive analysis provides a quantum leap in the quality of biological inferences. It is thus opportune to raise some evolutive perspectives about Nox convergence with the ER, even though evidences for such correlations are indirect. The major ER-related function connected to Nox NADPH oxidases is calcium handling. Calcium flashes are ancestral early injury signals that converge with Duox-type Noxes in zebrafish to trigger hydrogen peroxide generation after mechanical injury as a required step for leukocyte recruitment (123), which is dependent on a specific cysteine from the leukocyte Src-family kinase Lyn (185). The early calcium response as well as the ensuing Duox/redox-dependent activation of the epithelial Src-family kinase FynB are essential for adequate injury response since ablation of either prevents late epimorphic fin regeneration (184). Of note, in the same zebrafish model, epithelial Duox-derived hydrogen peroxide is not required for leukocyte recruitment after infection with *Pseudomonas aeruginosa* (41), thus suggesting some degree of specificity to this highly conserved stress response. In Drosophila embryos, calcium flashes are the earliest upstream response to laser injury, triggering Duox-dependent hydrogen peroxide generation (136). In filamentous fungi, mechanical injury promotes calcium signals associated with Nox1/NoxR activation and production of oxylipin metabolites (73)—with lipid metabolism being another important ER-related function. The high conservation of such Nox-dependent responses, likely to have strong evolutive pressure, is in line with evolutive analyses identifying calcium-sensitive Nox NADPH oxidases (Duoxes, Nox5) as the most ancestral Noxes in eukaryotes (90). Ancestral convergence between Nox regulation and calcium-dependent signaling is indicated by the fact that, in *Arabidopsis*, phosphorylation of a calcineurin B-like protein, physically associated with the Nox NADPH oxidase RBOHF, and its coexpression with the interacting protein CIPK26 strongly enhance ROS generation (44). The convergence between Noxes and calcium signaling in upper eukaryotes, regarding SERCA function, ryanodine receptor, muscle contraction, cell migration, oxygen sensing, and ER stress signaling, discussed throughout this article and summarized in Table 2, provides further links between Noxes and calcium signaling. Moreover, the roles of *N*-glycosylation, which is predicted or demonstrated for most Noxes, interactions between Noxes and PDI, as well as subcellular localizations of Noxes, all discussed previously, further corroborate those functional interplays.

Whether ancestral Nox NADPH oxidases from lower eukaryotes localize at the ER is yet poorly known, although emerging evidence indicates an ER location for Nox-related enzymes from yeast (138) and many fungi (163). On the other hand, a membrane location has been suggested for *Arabidopsis* (44, 191), *Aspergillus* (95), and *Caenorhabditis elegans* (45) NADPH oxidases, in association with specific functions, such as immune regulation, sexual differentiation, and cuticle strengthening, respectively. These considerations allow one to speculate that the ER has been a default subcellular localization of Noxes, whereas compartmentalization

Table 2. Nox NADPH Oxidases and Calcium Regulation: A Summary of Discussed Evidences (References)

| |
|---|
| Nox5 and Duoxes exhibit calcium-binding EF hands involved in enzyme activation and required for ROS generation (5, 8, 9, 12, 58, 81, 96, 97). |
| Calcium flashes are ancestral signals triggering activation of specific Noxes (36, 44, 73, 123, 136, 185). |
| ER calcium levels and related chaperones (ex. calnexin) control Nox processing in the ER (33). |
| ER calcium uptake by the ER *via* SERCA is redox/Nox-regulated (161). |
| Nox4 can affect calcium release *via* ryanodine receptor (154). |
| Nox2 at transverse T-tubules regulates ryanodine receptor and calcium-mediated muscle contraction (133, 134). |
| Rac1 interplays with calcium oscillations (76). |
| p47phox-related CAMKII oxidation impairs cardiac function (155). |
| Nox2 supports apoptosis during ER stress in macrophages *via* CHOP/CAMKII/JNK pathway (101). |
| Intracellular calcium levels mediate effects of Nox activation and can to some extent substitute for its effects: p47phox-related, VEGF-dependent c-Src, Akt and FAK phosphorylation in endothelial cells (100a); Nox1-dependent, thrombin-mediated VSMC migration (198). |

Akt, protein kinase B; CAMKII, calcium/calmodulin-dependent protein kinase II; CHOP, CCAT/enhancer binding protein homologous protein; Duox, dual oxidase; JNK, c-Jun N-terminal kinases; SERCA, sarcoendoplasmic reticulum calcium ATPase; VEGF, vascular endothelial growth factor.

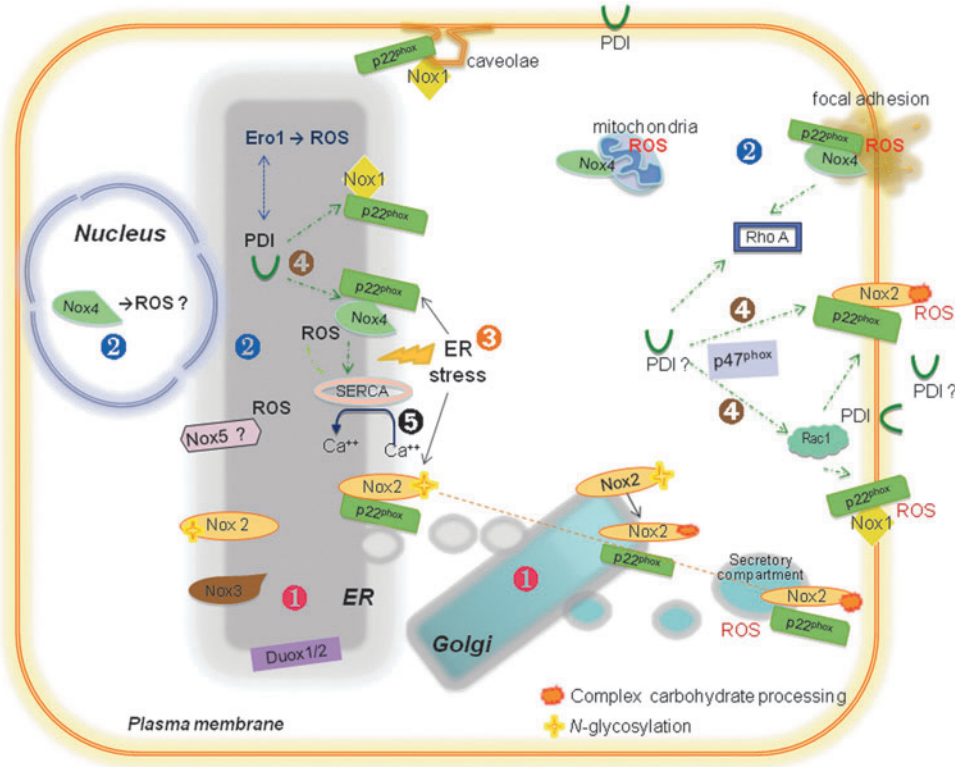


**FIG. 3. NADPH oxidase synthesis/processing, trafficking, and activation are connected to the ER.** (1) Transmembrane catalytic Nox subunits, as well as p22phox, are synthesized in the ER. Nox2 is folded, *N*-glycosylated (p65), and heterodimerizes with p22phox in the ER and then migrates to Golgi apparatus, in which processing mannosidases add complex side chain carbohydrates. Mature gp91phox moves to secretory vesicles and/or to plasma membrane. The lumen of Nox2-containing vesicles is topologically analogous to the extracellular milieu regarding superoxide production. It is likely that other isoforms share at least to some extent the same sequence of processing, except for Nox4, which often seems to become active at the ER membrane. (2) Some Noxes become active at the ER, such as Nox4, and may be in specific cases other isoforms (Nox5 and other immature Noxes?). Nox1 can translocate to caveolae and plasma membrane, whereas Nox4 can also be found at locations other than ER, such as nucleus, focal adhesions, and mitochondria. (3) ER stress leads to the UPR, which associates with oxidative stress, reportedly related to Nox4 or Nox2. (4) ER chaperones such as PDI associate with and functionally regulate Nox activation. Although PDI is canonically involved in redox protein processing at the ER, PDI interaction with Noxes likely occurs at other cell locations and even at the cell surface. Mechanisms of PDI effects may include its association/interaction with p47phox and RhoGTPases Rac1 and RhoA. (5) Noxes converge with calcium level regulation at several levels, here exemplified by the effects of Nox4 on the oxidation of sarcoendoplasmic reticulum calcium ATPase (SERCA). Please see text for further details.

to other locations was associated with acquisition of specialized functions, in some cases involving alternative splicing (6). An additional arguable context around which Noxes evolved is pathogen killing, another function in which, among other organelles, the ER has a prominent role as a site for intracellular pathogen targeting and chaperone-mediated host–pathogen interaction (152), antigen presentation (32), and particularly as a possible but much-debated source of the Nox2-containing phagosome (24). Overall, Noxes appear to have evolved around prominent ER-related functions and conversely the ER itself has played a possible role in contexts associated with Nox evolution.

## Conclusions and Perspectives

The data reviewed here place the ER at an important position of Nox physiology and pathophysiology (Figs. 2 and 3). This organelle is the site of synthesis and processing of all catalytic subunits, as well as p22phox. Several processing steps crucial for further Nox maturation, traffic, and regulation also occur at the ER, such as heme insertion, p22phox heterodimerization and, particularly, N-glycosyl residue attachment. On a second realm, some Noxes, such as Nox4—but maybe also other Nox isoforms in specific circumstances—are active at the ER. This location exposes functionally relevant ER-resident proteins to Nox-derived ROS: such is the case of calcium-related proteins such as SERCA and signaling effectors such as PTP-1B. In addition, Noxes interplay with ER stress responses and are integral components of the UPR, with documented roles for Nox4 and Nox2. In line with these events, ER chaperones such as PDI associate with and are involved in Nox regulation and can possibly further connect Noxes to ER-dependent functions (Fig. 3).

Much emphasis in previous years has been placed on Nox ''localization.'' Although this review further highlights the importance of understanding accurate Nox subcellular localization, it also raises the limitations of a monolithic approach to this question, indicating that such localization is

highly dynamic and influenced by cell type, specific stimuli, protein and lipid interactions, and the occurrence of alternative splicing isoforms. In this context, the ER emerges as an important structural and functional reference to help understand the complex regulation of Nox NADPH oxidases.

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**Abbreviations Used**

Akt = protein kinase B
AngII = angiotensin II
c-Abl = Abelson murine leukemia viral oncogene homolog
CAMKII = calcium/calmodulin-dependent protein kinase II
CHOP = CCAT/enhancer binding protein homologous protein
cys = cysteine
DCF = 2′,7′-dichlorofluorescein
Duox = dual oxidase
EGF = epidermal growth factor
ER = endoplasmic reticulum
ERAD = ER-associated degradation
Ero-1 = ER oxidoreductin 1
Erp46 = endoplasmic reticulum protein 46
FAD = flavin adenine dinucleotide
GFP = green fluorescent protein
Grp = glucose-regulated protein
GTPase = guanosine triphosphate hidrolase enzyme
HAEC = human aortic endothelial cell
Hsp90 = heat shock protein 90
HUVEC = human umbilical vein endothelial cell
IF = immunofluorescence
JNK = c-Jun N-terminal kinases
KO = knockout
LPS = lipopolysaccharide
NADPH = reduced nicotinamide adenine dinucleotide phosphate
NO = nitric oxide
PDI = protein disulfide isomerase
PMA = phorbol myristate acetate
PTP-1B = protein-tyrosine phosphatase-1B
ROS = reactive oxygen species
SERCA = sarcoendoplasmic reticulum calcium ATPase
STAT5 = signal transducer and activator of transcription 5
Tg = transgenic
TGF-$\beta$ = transforming growth factor beta
TIRF = total internal reflection fluorescence
UPR = unfolded protein response
VEGF = vascular endothelial growth factor
VSMC = vascular smooth muscle cells