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FORUM REVIEW ARTICLE

Mechanisms and Implications of Reactive Oxygen Species Generation During the Unfolded Protein Response: Roles of Endoplasmic Reticulum Oxidoreductases, Mitochondrial Electron Transport, and NADPH Oxidase

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Abstract

Cellular mechanisms governing redox homeostasis likely involve their integration with other stresses. Endoplasmic reticulum (ER) stress triggers complex adaptive or proapoptotic signaling defined as the unfolded protein response (UPR), involved in several pathophysiological processes. Since protein folding is highly redox-dependent, convergence between ER stress and oxidative stress has attracted interest. Evidence suggests that ROS production and oxidative stress are not only coincidental to ER stress, but are integral UPR components, being triggered by distinct types of ER stressors and contributing to support proapoptotic, as well as proadaptive UPR signaling. Thus, ROS generation can be upstream or downstream UPR targets and may display a UPR-specific plus a nonspecific component. Enzymatic mechanisms of ROS generation during UPR include: (a) Multiple thiol–disulfide exchanges involving ER oxidoreductases including flavooxidase Ero1 and protein disulfide isomerase (PDI); (b) Mitochondrial electron transport; (c) Nox4 NADPH oxidase complex, particularly Nox4. Understanding the roles of such mechanisms and how they interconnect with the UPR requires more investigation. Integration among such ROS sources may depend on Ca^{2+} levels, ROS themselves, and PDI, which associates with NADPH oxidase and regulates its function. Oxidative stress may frequently integrate with a background of ER stress/UPR in several diseases; here we discuss a focus in the vascular system. *Antioxid. Redox Signal.* 11, 2409–2427.

Introduction

Redox processes associated with controlled ROS generation by mitochondria or enzymes such as Nox family NADPH oxidases add an essential level of regulation to signaling pathways underlying physiological or pathological conditions (4, 7, 8, 11, 40, 42, 49, 123). Yet, cellular mechanisms associated with integration of homeostatic or disruptive (40, 42, 111) redox signaling remain insufficiently understood. In particular, it is yet unclear to what extent redox processes/oxidative stress occur within a context of cellular responses involving distinct types of stress. General stress responses are characterized by conserved interconnected signaling modules collectively referred to as the cellular stress response (46). Proteomic analyses identifying a minimal conserved core of the cellular stress response showed a key sensor and signaling role for oxidoreductases and redox-sensitive proteins (46), which integrate with expression of enzymes related to DNA damage/repair, molecular chaperones, protein degradation, fatty acid/lipid metabolism, and energy metabolism. Thus, different types of stress (*e.g.,* hypoxia, viral infection, and amino acid deprivation) converge to some common pathways promoting general responses such as redox adaptation, changes in lipid and carbohydrate metabolism, arrest in mRNA translation, inhibition of protein synthesis, and increase in protein degradation (29, 46, 82). Therefore, it is important to note that the investigation of specific pathways leading to oxidative stress in the course of other types of stress is subjected to several biases, which include (Fig. 1): (a) the occurrence of general nonspecific cell stress responses superimposed to the specific signaling targets triggered by stressors; (b) adaptive and hormetic modules of the response superimposed to the stress background (82, 86); and (c) occurrence of phenotype changes involving stress-triggered autodeterministic programs such as apoptosis, differentiation, senescence, and autophagy (74, 129). Thus, defining whether oxidative stress is a specific process during a particular type of stress is difficult, a fact that may in part explain why mechanisms of the convergence between ROS generation

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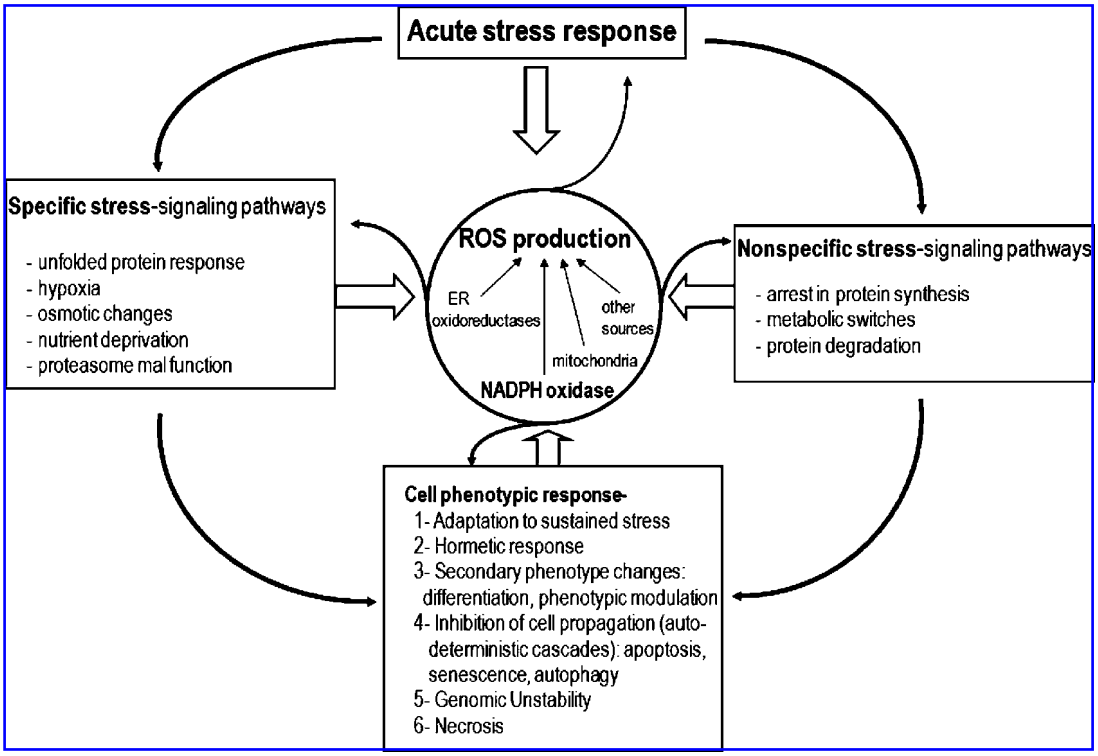

FIG. 1. Interaction between ROS generation and the cellular stress response. Scheme showing typical responses to stress. Acute stresses elicit stress-specific and general nonspecific responses. The cell may respond to acute stress in several ways. With persistence of stress, cells can adapt or even develop hormetic compensations, becoming able to tolerate a repeated stress. Secondary cell programs may ensue, such as cell differentiation (generally a result of relatively mild stresses) or phenotypic modulation [common in vascular smooth muscle cells (55)]. With persistent or unadapted stress, cells may develop strategies to limit their propagation and/or survival, namely apoptosis, senescence, or autophagy. These programs can also be adaptive to some extent. Overwhelming stresses may induce necrosis. All such processes can be influenced by and, at the same time, feed-back on ROS production. The definition of whether ROS production is "specific" to a certain type of stress is therefore subjected to several biases (see text).

and cellular stress response are not clearly understood. However, such mechanisms are relevant, considering that cellular stress response is a common feature of several pathologic processes. Moreover, portions of the cellular stress response are likely activated and integrate with signaling of physiologic cellular processes such as proliferation, metabolism, and differentiation (29, 46).

A very important modular-type stress-related signaling network, closely intersecting with cellular stress response, is the *unfolded protein response* (UPR). UPR-related signals are activated when the capacity of the ER to fold adequately and process newly synthesized proteins and/or to dispose of un/misfolded proteins is insufficiently matched to ER protein load, generating ER stress (65, 89, 90, 93). The importance of ER stress in the pathophysiology of several diseases is increasingly recognized, ranging from cancer and neurodegenerative diseases to cardiovascular diseases such as cardiac hypertrophy and atherosclerosis, as well as obesity and insulin resistance (65, 134). UPR integrates pathways aimed at providing ER and cellular adaptations to stress while improving the ER capacity to successfully process and secrete proteins and to degrade those that are terminally un/misfolded (90, 93). A number of reports have documented an association between ER stress and oxidative stress (61, 90) that, in smooth muscle cells, is at least partially dependent on the activation of Nox4 NADPH oxidase (84). While the convergence between both types of stress is now well accepted, a substantial part of the evidence for reactive oxygen species generation in the course of the UPR has been essentially indirect and circumstantial, particularly when studies are taken individually. The basic purposes of this review are: (a) to contextualize such evidences in perspective, with emphasis in recent data; (b) to discuss mechanisms whereby reactive oxygen species are enzymatically generated during ER stress, and (c) to discuss possible implications of ROS generation but upstream and downstream to UPR components.

UPR Signaling: From Activation to Apoptosis Through Adaptation

Protein folding and processing are among the major functions of the ER (99), particularly because un/misfolded proteins are extremely harmful to cells. This toxicity is due to (a) deprivation from normal protein function, (b) potentially deleterious gain-of-function, (c) tendency to form aggregates or to undergo non-native interactions, and (d) burdening of proteasomes and competition with degradation of other proteins (9). Hence, cells have developed elaborate mechanisms to prevent and/or dispatch un/misfolded proteins. Some factors are essential for ER protein processing. First, maintenance of an intra-organelle oxidative environment, discussed in the next section. Second, maintenance of high

intra-ER Ca^{2+} concentration is essential for all steps of the secretory pathway, particularly the essential function of calcium-binding chaperones such as the lectins calnexin and calreticulin (71). In addition, protein folding is connected with polysaccharide incorporation and protein traffic to Golgi and post-Golgi vesicles. These requirements are in line with some classical tools used to trigger ER stress in a variety of cell types, such as (a) dithiothreitol, a reductant; (b) thapsigargin, an ER Ca^{2+}-ATPase (SERCA) inhibitor; (c) tunicamicyn, an *N*-glycosylation inhibitor; and (d) brefeldin-A, a disruptor of Golgi transport. Accumulation of un/misfolded proteins is the canonical trigger of the UPR (64), a fact further supported by elegant recent studies (62, 69), although the molecular models of how un/misfolded proteins are sensed by the ER are only partially understood.

UPR signaling has been addressed in a number of recent excellent reviews (65, 89, 90, 93) and will not be focused in depth here. The main purposes of the UPR are to support reestablishing cell homeostasis in the course of ER stress, while amplifying the capacity of the cell to process and secrete proteins (89). Figure 2 is a scheme of the main pathways for UPR signaling, a summary of which now follows. The UPR involves proximal ER stress-sensor kinases/transcription factors located at the ER membrane, namely inositol-requiring protein (IRE1), RNA activated protein kinase-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF6), each one branching into three major arms of the UPR, which are independent but communicate extensively (90). Such redundancy may give rise to a tailored and minimally activated UPR (93). The translational arm of the UPR involves acute decreases in protein load due to arrest in translation initiation, degradation of mRNA for some ER lumen proteins, and decreased precursor translocation to the ER (89). Such responses are necessarily transient if cell function is not to be disrupted (90). Arrest in translation initiation, due to PERK-dependent phosphorylation of eukaryotic translation initiation factor 2 (eIF2α), is a converging pathway of integrated cell response to stress (29), since eIF2α is also phosphorylated by other stress-specific kinases. eIF2α is part of a multimeric eIF2 complex that initiates cap-dependent protein translation by coupling the 40S ribosomal subunit to the initiating tRNAmet, a process that involves GTP binding and is regulated by GDP exchange (121).This response is essential and common to many stresses because the processes of protein synthesis and chaperoning, as well as degradation, involve significant ATP consumption (9, 99), the avoidance of which is a uniform goal of the cellular stress response (46). On the other hand, eIF2α phosphorylation activates cap-independent translation of a few mRNAs, including UPR-related proteins such as the transcription factor ATF4 (89, 90). IRE1, the most ancestral component of the UPR, has both kinase and endoribonuclease activites, the latter accounting for the formation of an active spliced mRNA for XBP1, coding for a transcription factor. Both ATF4 and XBP1, as well as active spliced form of ATF6, promote transcription of genes for ER chaperones such as Grp78 (also known as Bip), Grp94, and calreticulin, as well as for components of ER-associated protein degradation (ERAD), protein translocation and traffic, carbohydrate and lipid synthesis, or amino acid sufficiency (89, 90, 93). In addition, ATF4, as well as pathways involving ATF6, can also activate another transcription factor, CHOP (also known as GADD153), a major contributor to ER stress-triggered apoptosis, which is executed through ER-specific initiator caspases (caspase-12 in many species and its equivalent in humans) and downstream caspases 9 and 3 (101), as well as other less well-defined pathways (90). XBP-1 also codes for genes associated with cell differentiation, as well as DNA repair pathways (1). In addition, the ER significantly expands in size during the UPR (89). The main operational markers of the UPR are summarized in Table 1.

Similar to the cellular stress response, each sensor-specific UPR arm involves both adaptive (prosurvival) and proapoptotic pathways, the latter converging to CHOP, but also involving an IRE1/JNK pathway (90, 93). While apoptosis is usually the result of sustained or overwhelming ER stress, both adaptive and proapoptotic signals appear to be activated

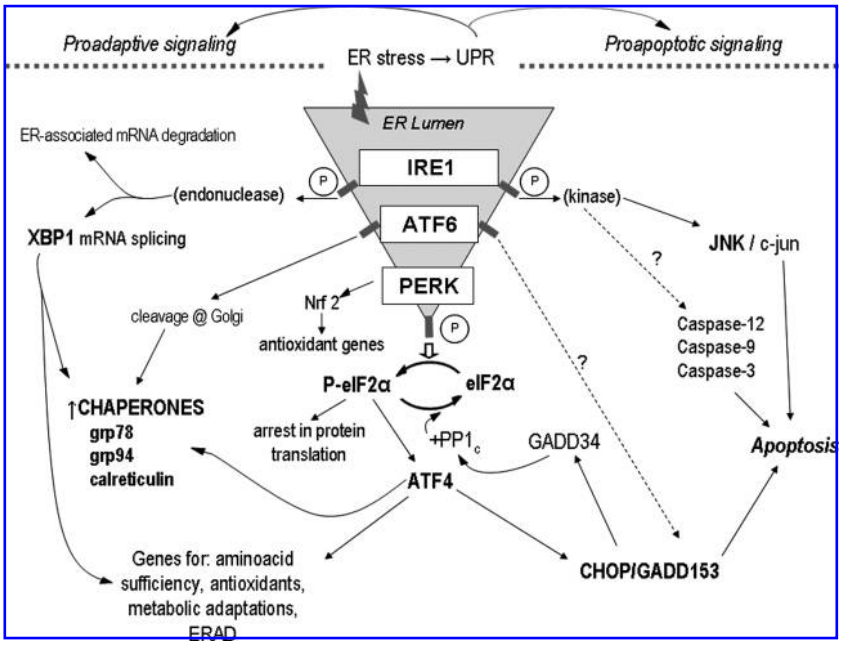

FIG. 2. Scheme depicting main pathways of UPR signaling. ER stress triggers the unfolded protein response (UPR), which is composed by proadaptive and proapoptotic signaling pathways, associated essentially to the three main arms of the UPR, dictated by ER membrane sensors IRE1, ATF6, and PERK. PERK and IRE1 are activated *via* phosphorylation mechanisms (marked as P), whereas ATF6 is cleaved in the Golgi before nuclear translocation. Proadaptive signaling tends to correct ER stress, thus inhibiting further UPR signaling to some extent. While proapoptotic signaling is usually the result of sustained or overwhelming ER stress, both proadaptive and proapoptotic signaling are co-activated during the UPR. For further details, please see text.

Table 1. Main Operational Markers of the UPR

ER stress sensors
IRE1 phosphorylation
PERK phosphorylation
ATF6 cleavage, nuclear migration
UPR pathways
eIF2α phosphorylation
XBP1 mRNA splicing
ATF4 nuclear expression
CHOP nuclear expression
Expression of KDEL chaperones: Grp78, Grp94, calreticulin, Orp150

simultaneously at all stages of UPR (90). The pathophysiological consequences of the UPR are highly model- and cell-specific and depend on factors such as protein load and differentiation status. Also, the impact of ER stress at a supracellular level depends on whether cells are terminally differentiated (*e.g.,* neuron, cardiomyocyte) or quickly renewable (*e.g.,* lymphocyte, smooth muscle cell). The factors that determine the transition from adaptation to apoptosis are not yet clear and a definite point-of-no-return—-if in fact there is one—has not been identified (90). Cessation of IRE1 and persistence of PERK signaling over time could act as an apoptosis trigger, since forced persistence of IRE1 prevents evolution to apoptosis (56). In addition, some proteins may act as regulators of apoptosis susceptibility during the UPR. For example, the normally proapoptotic (when in mitochondria) proteins Bax and Bak form a complex with IRE1 at the ER membrane, synergize with UPR signaling, and limit cell injury during ER stress (32). Proteins coding for autophagy in the course of ER expansion can be apoptosis-protective (5). Adaptation to chronic sublethal ER stress involves down-regulation of mRNA stability for proapoptotic proteins, and upregulation of adaptive ones (90). In addition, sustained induction of the integrated stress response (*via* PERK/P-eIF2α/ATF4) may protect cells *via* hormetic induction of antioxidant defenses (52, 59).

ER-associated degradation (ERAD) is part of the protein quality control system and intersects with the UPR at several levels. For example, prior to degradation by the proteasome, un/misfolded proteins are retro-translocated to the cytosol while reduced by reductase chaperones such as PDI (9) or Erdj5 (88). Also, the transcription factor XBP1 codes for many ERAD proteins (1, 67, 89). Thus, the UPR is generally associated with an increase in genes for protein degradation, although the genes/proteins responsible for UPR and ERAD superimpose only partially (67).

Protein Folding and Redox ER Homeostasis: The ER as a Potential ROS Source

Protein folding is a highly redox-dependent pathway, considering the essential role of disulfide formation in folding, processing, and assembling of membrane and secretory proteins (3). Reflecting its topologic analogy with the extracellular milieu, the ER lumen not only has high Ca^{++} concentration, but is among the most oxidizing intracellular compartments, with a GSH/GSSG ratio of 1:1 to 3:1, much lower than the cytosolic ratios of 30 to 100:1 (37, 119). The oxidizing ER ratios are close to optimal *in vitro* ratios for protein folding (which are $\sim$5:1), while more oxidizing ratios can be harmful by promoting aggregation (99, 114). This oxidizing environment is mainly sustained by the thiol oxidoreductase Ero1 and to some extent by GSSG itself (12, 114, 119), and is continuously buffered essentially by cytosolic-derived reduced glutathione, so that the ER is a net consumer of GSH (15). The oxidase Ero1, which binds to the internal face of the ER lumen (96), transfers oxidizing equivalents mainly to its substrate PDI (115, 119) and/or potentially to PDI analogs, in a way essentially independent of the redox environment (114). In mammalian cells, there are 2 Ero1 isoforms with similar catalytic mechanisms and substrate preferences, but distinct with respect to tissue distribution and transcriptional regulation, with only Ero1β being inducible by ER stress, while Ero1α is inducible by hypoxia (114, 119). The structure and properties of PDI, a dithiol–disulfide oxidoreductase and chaperone from the thioredoxin superfamily, as well as of its family members, have been previously reviewed (12, 19, 50, 122). Oxidized PDI can promote oxidation or—as its unique characteristic function—isomerization (*i.e.,* the rearrangement of disulfide bonds involving multiple sequential thiol oxidations and reductions) of disulfide bonds in client proteins. PDI can also act as a reductase, mainly in reducing environments outside the ER (122). Importantly, most substrates appear to bind to PDI through its *b′* domain hydrophobic pocket, rather than through active site thiols (19, 50, 122). The chaperone activity of PDI appears to be independent of its thiol groups (122).

The elegant catalytic mechanism of Ero1 (described for yeast Ero1p) involves direct oxidation of PDI thiols by a shuttle cysteine pair, which is reoxidized by a cysteine pair from the active site, an exchange allowed by dynamic approximation of the two sites due to flexibility of the peptide loop containing the shuttle cysteines (27, 95, 119). This mechanism, in addition to other features of Ero1 catalysis, prevents the strongly oxidizing Ero1 active thiols from introducing non-native disulfides into nonspecific substrates (95). Also, it accounts for the known capacity of Ero1 to preferentially oxidize vicinal dithiols (*e.g.,* from PDI, thioredoxin, and DTT) rather than monothiols (GSH) or multiple nonvicinal thiols (119). This mechanism also allows regulation of Ero1 activity exerted by additional regulatory-type cysteines which, when oxidized, restrict flexibility of the peptide loop containing the shuttle cysteines (96), thus providing feedback defense against excessive oxidation. The redox interaction of Ero1α with PDI occurs *via* dithiols from PDI *a′* domain, while binding requires PDI *b′* domain (119). A critical step in Ero1 catalysis is the reoxidation of its active site cysteines. This appears to occur through transfer of reducing equivalents from Ero1 reduced dithiol to molecular oxygen *via* FAD- which is tightly bound at equimolar ratio to Ero1 −, generating hydrogen peroxide according to the equation:

$$2RSH + O_2 \rightarrow RSSR + H_2O_2$$

Therefore, Ero1 is an oxygen-consuming enzyme that is able to generate ROS (27, 115, 119) *via* mechanisms that are very similar to those of sulfhydryl oxidases such as Erv2p and the QSOX family (3, 27). In the latter, the generation of hydrogen peroxide is directly linked to thiol substrate oxidation *via* structural thioredoxin domains, whereas Ero1 bears only an Erv-simile flavo-oxidase domain (95). Catalase or

superoxide dismutase do not alter disulfide formation due to Ero1, indicating that ROS are by-products, not mediators, of its catalysis (115). The potential importance and limitations of Ero1/PDI mechanism as a ROS source during the UPR is discussed below.

Close to nothing is known about ER compartmentalization of antioxidant enzymes. Remarkably, CuZnSOD levels appear to be very low or nonexistent in liver smooth and rough endoplasmic reticulum fractions, similarly to catalase (58). There is little reference to local ER roles of glutathione peroxidase(s), although some *Arabidopsis* GPx may be located in the ER (87), while glutathione reductase expression is negligible (85). Similarly, thioredoxin reductase and thioredoxin are not reported to be present in the ER, and the antioxidant role of the several ER-located PDI family members with reductase activity (12, 19) is unknown. Microsomal glutathione transferase 1 (with peroxidase activity) is important for oxidant protection and xenobiotic metabolism, but its role in extrahepatic protection against oxidants is less clear (98). The secreted heparin-binding enzymes peroxiredoxin IV, which efficiently reacts with hydrogen peroxide (123), and extracellular SOD (79), may be rapidly processed within the ER, but it is unclear whether they exert antioxidant effect within the organelle lumen. In fact, peroxiredoxin IV retained in the ER forms homodecamers with no obvious antioxidant role (109), although peroxiredoxin IV KO mice do exhibit increased oxidant-mediated sperm cell death (38). Thus, it is doubtful whether the ER is endowed with efficient enzymatic antioxidant protection, at least in basal conditions. It is possible that, similarly to the ER-analogous extracellular milieu, small molecules such as tocopherols (113), ascorbate (57), and dietary phenolic antioxidants (41), which traffic through the ER, may be relatively important, in addition to above mentioned glutathione from cytosol or cysteine (29, 42). In any case, it appears that even professional secretory pancreatic islet cells from nondiabetic individuals (known to have normal antioxidant defenses) are able to resist hyperglycemic ER stressor stimuli (63), indirectly suggesting an ability to deal with protein folding-associated oxidants.

It is important to consider that a state of ER stress is usually characterized through the increased expression of UPR markers, such as KDEL chaperones (Table 1). However, this is essentially a utilitarian definition which translates into the contradictory dilemma of defining a stress through a downstream adaptive response, conveying little about whether homeostasis has been achieved. Recently, a fluorescent indicator of ER protein oxidation was developed in yeast (69), indicating that the UPR is both necessary and sufficient to buffer the underoxidation of proteins that occurs during disturbed ER protein processing. This so-called ER oxidative folding stress is an important common pathway of challenges to ER homeostasis that cannot be inferred solely from downstream indicators of the UPR (Table 1). For example, cells deficient in specific components of UPR signaling, oxidative ER protein folding, or ER-mediated protein degradation exhibited distinct downstream indicators of UPR activation, but the redox state of ER protein oxidation indicator was normal at baseline, although it became altered after varied stress maneuvers (69). Thus, multiple ER functional pathways converge to redox protein folding, bringing about a solid basis to correlate redox processes and ROS generation with ER stress.

Convergence Between Oxidative Stress and ER Stress: Coincidence or Cooperation?

The connection between redox processes and the UPR, analyzed within the framework of specific and nonspecific stress responses, as well as primary, adaptive, and secondary events (Fig. 1), suggests a potentially multiform picture. However, there are common denominators including: a) induction of adaptive antioxidant pathways; b) production of ROS during the terminal stages of the UPR; and c) early production of ROS during the UPR.

Induction of antioxidant pathways is an important part of the adaptive response that characterizes the UPR. Pathways involving PERK-dependent activity stimulate several antioxidant defenses *via* the activating transcription factor (ATF4) (29, 52, 89, 90) and/or *via* Nrf2/Keap (13, 14). Such genes include, but are not restricted to, those involved in glutathione biosynthesis, as well as glutathione transferase, heme oxygenase-1, manganese superoxide dismutase, uncoupling mitochondrial protein 2 (*Ucp2*), amino acid/cysteine transporters and ubiquitin/proteasome system components. Induction of antioxidant defenses during the UPR indirectly suggests that oxidants can mediate noxious effects during UPR. On the other hand, while the increase in glutathione-related genes may buffer and delay decreases in cellular glutathione during ER stress in some cell types (45), intracellular glutathione levels and GSH/GSSG ratios are often decreased early during the UPR. This is the case of vascular smooth muscle cells incubated with tunicamycin (5 μg/mL, 4 h), which show $\sim$70% decrease in GSH/GSSG ratio, despite staying viable for additional 48 h (unpublished observations from our laboratory, $n = 4$). Decrease in glutathione levels during the UPR may reflect consumption due to reduction of non-natural disulfide bonds in proteins (see below), oxidation mediated by free radical or 2-electron oxidants (40), glutathiolation, increases in intracellular Ca^{2+} (88), cell leakage, or all of these. In addition, the reported decrease in mRNA level and protein processing of extracellular SOD after homocysteine incubation in vascular smooth muscle cells (79) may provide deficit of this important vascular cell-specific antioxidant.

A number of reports have addressed the generation of oxidants during the UPR and their possible implication. Here, we review some studies that investigated this issue in a more direct, paradigmatic, or mechanistic way, while this summary (Table 2) is not exhaustive. This analysis and other reviews (*e.g.*, ref. 61) indicate that several such studies have addressed a connection between oxidant generation and the terminal proapoptotic stages of the UPR. In fact, since apoptosis is usually the outcome of severe and/or sustained unadapted ER stress, it is conceivable that the level and/or time course of ROS generation might act as a switch triggering apoptosis in individual cells or a cluster of neighbor cells. Clearly, pharmacological or molecular interventions that decrease oxidant production are associated with decrease in cell death due to the UPR (29, 30, 59, 62, 75, 84, 91, 107, 127, 135). Prior studies provided some information on how ROS contribute to proapoptotic UPR stages. Consumption of reduced glutathione by protein oxidation or glutathiolation during UPR (61), as well as CHOP-mediated Ero1 induction and consequent increase in ER protein load (64) enhance ER oxidation, further compromising cells to apoptosis (68) and possibly enhancing formation of insoluble protein aggregates (64). Indeed, GSH

Table 2. A Summary of Some Studies Addressing the Convergence Between ER Stress and Oxidative Stress

Cell type	*Main biological effect*	*Stimulus*	*UPR target activated and/or expressed*	*Measured oxidant ↑*	*Antioxidant ↓ UPR targets*	*Observations*	*Ref.*
PERK −/− or ATF4 −/− fibroblasts and *C. elegans* (PEK1 −/−)	Cell death	Tunicamycin	Several genes	DCFd	+	Ero-1 silencing ↓ oxidant production in *C. elegans*	29
Yeast	Cell number and death	Misfolded carboxy-peptidase Y expression	IRE1 and others	DH-123	+	GSH suppresses ROS despite persistent ER stress	30
CHO cells	Apoptosis and other	Misfolded factor VIII expression	XBP1, ATF4, GADD34, Ero1, CHOP	DCF, HODE carbonyls,	+	Antioxidant genes ↑ in CHOP−/− mice expres. FVIII	62
VSMC	Apoptosis	7-Ketocholesterol	Ca^{++} oscillations; Grp78; Grp94, CHOP, IRE1; JNK	DCFd		Silencing of Nox-4 ↓ UPR and apoptosis	60
VSMC		7-Ketocholesterol		DCFd	+		75
L929 fibrosarcoma cells	Cell death	TNFα	eIF2α; XBP1; ATF6; PERK; UPR-responsive promoters; ER expansion	DCFd	+	Preemptive Tunicamycin-induced UPR ↓ TNFα-mediated ROS and cell death	127
		H_2O_2 (1 m*M*) Arsenite (10 μ*M*)	eIF2α, but not PERK or XBP-1	DHE			
Renal tubular cells	Apoptosis	Cadmium	Grp78; Grp94; ATF6; CHOP; ORP150		+	Forced Grp78 expression ↓ UPR but not ox. stress	131
		Menadione (1–15 μ*M*)	Grp78; Grp94				
		H_2O_2 (50–200μ*M*)	No effect				
		SIN-1	Grp78; CHOP				
MEF	↓ Protein synthesis	H_2O_2 (5–100 μ*M*)	eIF2α		+		59
HEK293		H_2O_2 (20 μ*M*)	GADD34; Grp78, CHOP		+		
MEF, HEK-293	Cell viability	Hypoxia (0.5% O_2)	PERK; eIF2α; ATF4	Protein carbonyls		Cytochrome-c KO ↓ UPR; ISR silencing ↓ viability	
Bronchial epithelial cells	Apoptosis	Cigarette smoke extract	Grp78; CHOP	DCFd	+	Antioxidants and dominant-negative CHOP ↑ survival	107
		Menadione	CHOP, not Grp78			SOD-mimetic but not catalase ↓ CHOP	
Endothelial cells	Apoptosis	Oxidized LDL 7-Ketocholesterol 4-Hydroxy nonenal	IRE-1; PERK; ATF6; CHOP; KDEL chaperones; JNK		+	Forced ORP150 expression is protective	91
PC12 cells	Cell death	Induction of mutant alpha-synuclein	Grp78; eIF2α; CHOP; caspase12	DCFd		Cell death is mitochondrial-dependent	101
H9C2 myoblasts	Cell death	Tunicamycin	Grp78; Grp94; caspase12; ATF6; Cyclophilin B	DCFd	Cyclophilin B siRNA ↑ UPR death, while overexpression ↓ ROS		43

replenishment prevents death in yeast even with continued presence of misfolded proteins in ER (30). Also, prior evidence implicates JNK and ASK1 as mediators of ROS-dependent apoptosis triggered *via* IRE1 (84, 89). Importantly, hypoxia, known to promote the UPR through pathways not completely elucidated, induces endogenous ROS-dependent PERK/eIF2α/ATF4 integrated stress response, which accounts for cell survival (59).

The studies reported in Table 2 provide also an overview of ROS generation, assessed through direct or indirect tolls, at all stages of the UPR, including earlier stages. Some conclusions derived from this analysis include the following:

1. Exogenous oxidants (such as peroxides, ROS generators, metals, lipid oxidation products, or cigarette smoke) can trigger several aspects of the UPR, which vary according to stimulus and cell type, as depicted in Table 2. Although some of these products [*e.g.,* 7-ketocholesterol in vascular smooth muscle cells (84)] can trigger the full UPR signaling spectrum, exogenous ROS have provided conflicting and less clear information. Hydrogen peroxide promoted only eIF phosphorylation in L929 tumor cell line (127) (Table 2). Meanwhile, in renal tubular cells, hydrogen peroxide promoted no UPR signaling, whereas the redox-cycling compound menadione promoted chaperone and CHOP expression (135) (Table 2). In most cases, there was no clear concentration-dependent effect. In our laboratory, vascular smooth cells incubated with 10–1,000 μM hydrogen peroxide (during 14 h) showed only minor dose-independent increases in the expression of chaperones Grp78 and Grp94 and eIF2α phosphorylation (while CHOP expression was unaltered), an effect observed only in the absence of serum. Interestingly, incubation with serum alone consistently induced moderate increase in basal chaperone expression, which was further unaffected by hydrogen peroxide (unpublished observations). Because experiments with exogenous ROS are limited by induction of nonspecific mass changes in signaling targets (23), as well as by kinetic and diffusional constraints (40, 123), such results, while providing general indication that oxidants support the UPR, must be regarded with care. Nevertheless, a general conclusion allowed from all those experiments is that exposure to ROS alone is generally not sufficient to trigger the UPR. This may at least partially hold true for endogenous oxidants, since the knock-down of NADPH cytochrome b5 reductase, which is redox-protective for pancreatic islet cells, promotes severe diabetes in a way independent of effects on endoplasmic reticulum stress (48).
2. The above considerations indicate a coincidence between UPR induction and oxidant production, with several examples suggesting ER stress downstream of oxidant production. On the other hand, there are fundamental observations indicating that classical ER stressors (29) or the introduction of misfolded proteins into the ER (30, 62) can trigger downstream oxidant generation. Specifically, the introduction of misfolded protein into the ER in yeast deficient in ERAD promoted UPR-dependent ROS generation which was normalized during UPR-silencing mutations or GSH replenishment despite continued ER stress (30). In addition, a recent elegant study showed that the *in vivo* transfection of misfolding-prone coagulation factor VIII in mice triggered several indicators of the UPR in the liver and enhanced apoptosis. All such changes were prevented by genetic deletion of CHOP or the exogenous antioxidant BHA (62).
3. The majority of reported measurements of oxidant production during the UPR make use of indirect markers of oxidant generation (*e.g.,* indexes of lipid or protein oxidation), total DHE fluorescence or fluorescence of DCF derivatives. All these indexes have limitations for the accurate detection of ROS (21, 123), the importance of which is discussed below. Total DHE fluorescence reflects the summation of products derived not only from superoxide but also from nonspecific oxidants including peroxides, peroxidase activity, and heme (21). DCF, often believed to measure hydrogen peroxide, does not in fact react directly with this species. Rather, this reaction requires catalysts such as peroxidases or metal complexes and is thus strongly influenced by their levels. Moreover, DCF is superoxide-insensitive and reacts with several nonspecific oxidants [*e.g.,* peroxynitrite, thiyl radical (123)]. Particularly, DCF can react with cytochrome-c leaking from mitochondria during early apoptosis (92). Also, reaction of DCF-derived radical intermediates with oxygen can generate reactive species that artifactually amplify the fluorescence signal and render them false positively inhibitable by scavengers such as catalase (92). Thus, while all these data, taken together, provide compelling indication that oxidants are generated during the UPR, analysis of individual results does not allow one to discriminate or to quantify any specific ROS and requires careful critical analysis.
4. Several signaling UPR markers other than those related to apoptosis signaling are mitigated by antioxidant interventions in the course of exposure not only to oxidants but also to stimuli such as hypoxia, cytokines (*e.g.,* TNFα) and forced expression of mutated misfolded proteins (Table 2). This suggests that oxidant production may mediate several aspects of the adaptive UPR signaling, in addition to terminal cell death. We investigated these aspects by means of specific loss-of-function experiments. (Supplemental Methods; see www.liebertonline.com/ars) We first assessed whether disrupting the UPR by interfering with eIF2α phosphorylation would affect UPR-induced ROS (Fig. 3A and C). Phosphorylation of eIF2α *via* PERK, an early target of UPR as well as many other stresses (121), promotes translational arrest in protein synthesis and enhances transcription of some genes. Among these, the transcription factor GADD34 is a regulatory subunit of eIF2α-directed protein phosphatase-1 (PP1c) complex promoting eIF2α dephosphorylation, thus providing negative UPR feedback (64, 80). VSMC were transiently transfected with plasmids coding for full-length GADD34 or a truncated version in which the PP1c-binding domain was deleted (64). Corresponding changes in eIF2α phosphorylation after tunicamycin incubation (Fig. 3A) showed: (a) normal eIF2α phosphorylation peaking at 2 h and sustained until 8 h of tunicamycin stimulus; (b) marked decrease in phosphorylation after full-length GADD34 transfection; and (c) more intense and sustained eIF2α phosphorylation after truncated

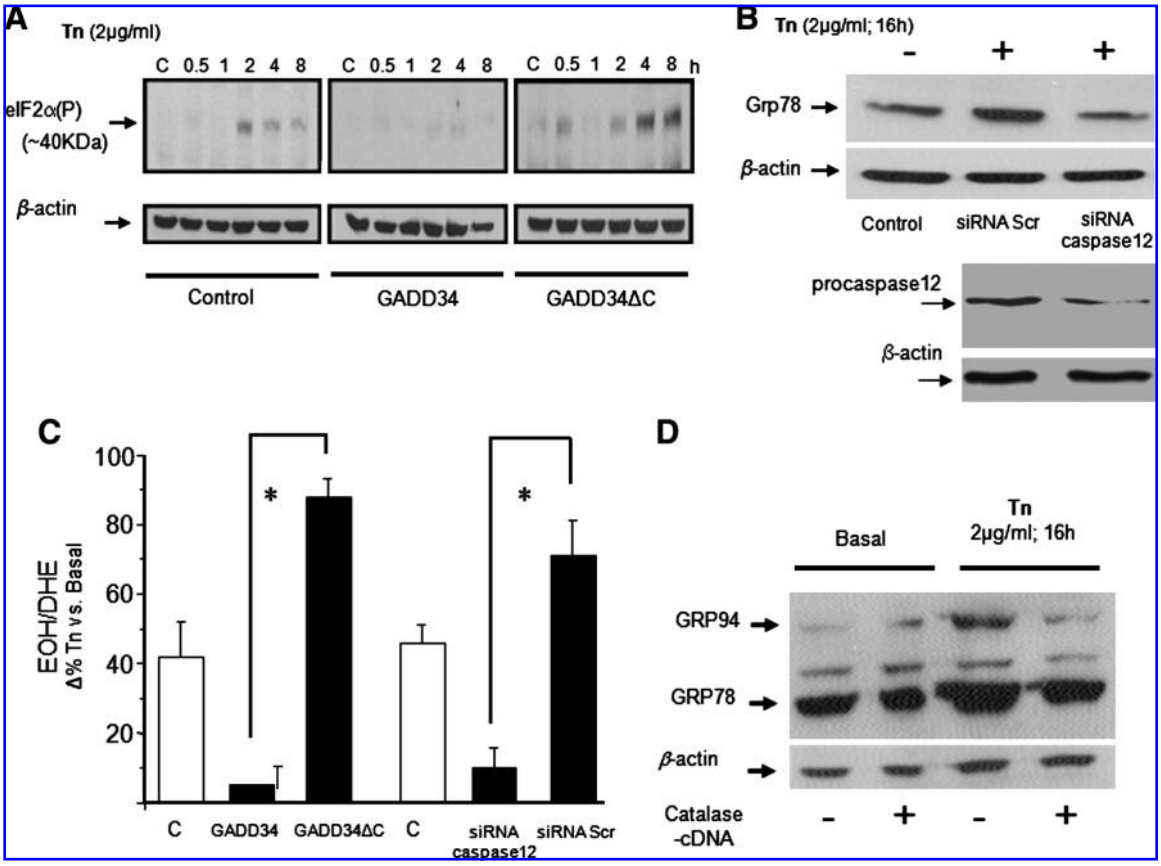

FIG. 3. Interdependence between the UPR and ROS generation. (**A**) Representative immunoblot depicting phosphorylation of eIF2α in control vascular smooth muscle cells (transfected with empty vector) or in cells overexpressing full-length GADD34 or GADD34 truncated in its C-terminal protein phosphatase 1-activating domain (GADD34ΔC), acting as a dominant negative control (64, 80). GADD34 and GADD34ΔC expressed in pcDNA3 vector were kindly provided by Dr. David Ron (New York University, New York). Rabbit aortic vascular smooth muscle cells (3×10^5 cells) grown for 24 h in 6-well dishes were transiently transfected with 5 μg cDNA using lipofectamine 2000 (10 μL, Invitrogen) according to manufacturer's instructions (51). VSMC were used for the designed experiments 24 h after transfection. Cells were exposed to 2 μg/ml tunicamycin for the indicated time periods. Prior experiments (not shown) showed that such tunicamycin concentration was associated with >95% cell viability within the time frame of such experiments. (**B**) Western analysis of Grp78 (with specific antibody) in cells transfected with caspase-12 (GCACAUUCCUGGUCUUUAUTT) or scrambled (scr) siRNA or in control cells exposed to Tunicamycin (2 μg/ml for 8 h). Efficiency of caspase-12 siRNA was confirmed by decrease in procaspase12 levels, shown, together with control scrambled siRNA, in the insert below. (**C**) Effect of GADD34 or GADD34ΔC overexpression or caspase-12 siRNA or its scrambled control in superoxide formation (analyzed through 2-hydroxyethidium = EOH) (21) after incubation with Tunicamycin (2μg/ml, 4 h). Values are mean ± SE of 3 experiments; *$p < 0.05$. (**D**) Representative western analysis depicting the response of KDEL-containing proteins to Tunicamycin (2 μg/ml, 16 h) in the presence or absence of catalase overexpression (confirmed through a ~70% increase in activity) or in Lipofectamine-exposed controls. The catalase plasmid was kindly provided by Ralf Brandes (U. of Frankfurt, Germany) Representative of 3 experiments. Control experiments showed that plasmid transfection per se did not cause expression of UPR markers *vs.* control VSMC (not shown).

GADD34 transfection. Analysis of ROS and specifically superoxide production was performed through HPLC analysis of dihydroethidium (DHE) oxidation products (21). Results showed significant prevention of ROS generation after UPR interruption with full-length GADD34, whereas increased eIF2α phosphorylation with truncated GADD34 promoted a twofold increase in ROS generation (Fig. 3C).

Apoptosis during ER stress is executed through caspase-12 and can be inhibited through its silencing (101, 112). We asked whether such a distal UPR event could positively feedback on UPR signaling, since silencing of CHOP in models of diabetes or coagulation factor VIII misfolding was recently reported not only to increase cell viability but also to decrease oxidant generation and Ero1 induction and to enhance antioxidant enzyme expression (62, 102). Using an siRNA against caspase-12, we showed that both ROS generation and, interestingly, Grp78 expression, were decreased after tunicamycin (Fig. 3B and C), suggesting that ROS generated at later UPR stages may feedback on UPR itself. Further experiments were performed in order to investigate whether intracellular ROS support UPR signaling after tunicamycin. VSMC were transiently transfected with catalase plasmids, with efficiency of transfection confirmed by ~70% increase in enzymatic activity, assessed in total cell homogenates 24 h following

transfection (data not shown). Catalase overexpression induced consistent decrease in tunicamycin-induced expression of Grp78 and Grp94 after 16 h of incubation (Fig. 3D), together with ~20% decrease in cell loss after 48 h (not shown).

Thus, our data add to previous observations (29, 30, 62, 69) indicating a mutual interdependence between the UPR and ROS generation or, in other words, that ROS play a role both downstream and upstream of UPR targets. Furthermore, these data add to several reports (Table 2) that suggest a role for ROS in synergizing with early UPR and sustaining an important adaptive arm of UPR signaling. While it may seem paradoxical that ROS simultaneously induce adaptive and apoptotic signals both in our study and in previous reports (59, 84), this is not unexpected, as both types of signals are normally co-induced at all UPR stages (90). How ROS synergize with earlier stages of the UPR is essentially unknown. Particularly interesting is the possibility that redox processes serve as a signal for misfolded proteins, as suggested by recent studies showing that client protein underoxidation is an important feature of misfolding (69) and that oxidant generation acts as an early misfolding signal in the ER (*i.e.*, a ER stress signal), able to trigger the UPR (62). Additional relevant possibilities in this context include whether ROS themselves induce some degree of misfolding/ER stress, transactivate some UPR components, or promote other indirect signaling effects. Somewhat paradoxically, ATF6 undergoes specific thiol reduction and monomerization during ER stress as a requirement for its Golgi-dependent proteolytic activation (76). Grp78 is known to bind to unfolded proteins in the ER lumen in a way that normally prevents their induced activation of ER membrane sensors such as PERK, ATF6, or IRE1 (89). Grp78 also forms large complexes with several chaperones, particularly PDI, both at ER lumen (70) and cytosolic translocon interface (103). Formation of such complexes confers a potential redox regulation to early UPR pathways, considering that Grp78 itself seems not particularly redox-active, with only one conserved cysteine (as a XCVX sequence near N-terminus). This may contrast with Grp94, which seems to be more sensitive to redox regulation (unpublished observations from our laboratory). Our data showing that GADD34 transfection decreases ROS generation after tunicamycin are at variance with prior results showing that CHOP-dependent GADD34 expression promotes counteradaptation due to eIF2α dephosphorylation and ensuing protein overloading into an already stressed ER, resulting in increased Ero1 activity and enhanced ER oxidation (64). Such differences may be due to the peculiar cell type and distinct basal ER protein loading conditions, in addition to an earlier and/or milder stage of UPR in our case. Indeed, PERK activity has been associated with cell death during the UPR (56), while sustained eIF2α phosphorylation was reported to promote antioxidant defenses during prolonged stress in neurons (52), thus stressing dichotomous roles for this pathway.

Altogether, the observations discussed in this section contextualize the notion that ROS are generated early and act as an intrinsic component of UPR, exerting both adaptive and proapoptotic effects.

ER Oxidoreductases as ROS Sources During the UPR

In this and in the next sections, we will discuss some possible mechanisms of enzyme-mediated ROS generation during the UPR. ER-dependent ROS generation during stress is connected in a yet poorly defined way to activity and upregulation of Ero1, particularly Ero1β (61), which, as discussed previously, can promote electron transfer to molecular oxygen, generating hydrogen peroxide. Clearly, client proteins are underoxidized in the stressed ER (69), while Ero1 is more active (64), thus supporting the need for feeding of oxidizing equivalents from Ero1 to PDI. This is additionally favored in terminal phases of the UPR by the action of CHOP, which further induces Ero1α and directly activates GADD34 and protein load (64). The role of Ero1 in oxidant generation is supported by decrease in tunicamycin-induced DCF fluorescence in *C. elegans* by Ero1 siRNA (Table 2 and ref. 29). Whether and how this process is sustained is not precisely known. Because GSH (and perhaps other thiol reductants) can efficiently reduce non-native disulfide bonds even during normal ER conditions (15), it has been proposed that the PDI/Ero1/ROS redox cycles operate at increased levels through repeated futile attempts to fold reduced, improperly folded, substrates, a proposal consistent with studies in yeast expressing a mutated misfolded protein (30). Thus, there is a net transfer of reducing equivalents through multiple thiol–disulfide exchanges from reduced glutathione to oxygen (15). A further source of ER reducing power consumption is ERAD, which requires protein reduction prior to their retrotranslocation and degradation (99, 117).

Despite the biochemical basis and relevant data for the proposal of such mechanism as an enzymatic source of ROS during the UPR, there are yet several critiques and/or unclear points that should be considered:

1. The introduction of constitutively active Ero1 mutated in its regulatory cysteines does not lead to detectable ROS increase, indicating that Ero1 activity alone is not sufficient to account for oxidant accumulation, at least in unstressed cells (96). Also, Ero1 structure lacks the clear channel for oxygen observed in Erv/QSOX (3).
2. The *in vivo* stoichiometry and quantitative modeling of these reactions are unclear: *in vitro* experiments suggest an equimolar H_2O_2 generation (27, 119), while substoichiometric ratios were reported for yeast Ero1p (114).
3. The rate constants of such sequential thiol–disulfide exchange reactions are unknown, and at least some of them may be slow and potentially rate-limiting. For example, the reduction of the bacterial PDI analog DsbA by GSH has a second order rate constant of $182\,M^{-1}s^{-1}$ (36).
4. Some aspects of Ero1p action may be supported by electron acceptor(s) other than oxygen (114).
5. The activity of at least Ero1p, although not of the mammalian Ero1α (119), is strongly sensitive to ER concentrations of free FAD, the sufficiency of which during the UPR is unknown. Indeed, exogenous FAD has been reported to oxidize Ero1 and PDI in liver microsome preparations in a way consistent with direct feed-forward of oxidizing equivalents for protein folding (83).
6. It is unclear whether the same futile cycles of attempted refolding would occur to a significant extent with spontaneously unfolded nonmutated substrates. In addition, underoxidative changes of a redox protein

folding ER indicator, similar to those observed with ER stressors, were still observed to some extent in yeast overexpressing cysteine-less forms of misfolded carboxypeptidase Y, indicating that such repeated folding cycles may not be the sole explanation for ER redox dysfunction during the UPR (69).

7. Some reports suggest that the thiol–disulfide ratios of at least some proteins in the ER are shifted toward a more reduced state in livers of diabetic mice, known to display chronic ER stress and enhanced ROS generation, with PDI in the reduced form and Ero1 in an oxidized form (78). While this may be an intermediate state of adaptation involving the regulatory cysteines of Ero1 [which would thus be inactivated (96)], it is not straightforward to reconcile this picture with the above proposed ER mechanisms as a single pathway for ROS generation.
8. It is unclear how the protective effect of thiol antioxidants against ROS accumulation (30, 95) and UPR are explained by mechanisms involving the mentioned feeding of Ero1/ROS redox by glutathione. The proposed explanation for this effect has been often ascribed to scavenging of ROS by glutathione. However, the rate constants of direct ROS scavenging by thiols such as GSH is exceedingly low (*e.g.*, 20, 87 and 1,300 $M^{-1}.s^{-1}$ for superoxide, hydrogen peroxide and peroxynitrite, respectively, see ref. 50 for sources. Even lower values are reported in ref. 123). Therefore, a scavenging effect of even high concentrations of glutathione against ROS is questionable.

Together, these considerations indicate some additional questions and potential drawbacks in an otherwise powerful and quantitatively important pathway for ROS generation. More investigation is needed in order to assess whether additional or parallel pathways are involved. Considering that in unstressed cells ROS generation through this mechanism could be equivalent to the amount of protein disulfides introduced into newly-synthesized secretory proteins, ROS output might be quite significant at least in protein factories such plasma cells (9) or in synthetic smooth muscle cells from atheromas (16, 75).

Mitochondrial ROS and the UPR

Evidence for a role of mitochondria in ROS generation during ER stress comes from experiments in which interference with mitochondrial respiration prevented UPR-induced ROS accumulation, as well as associated growth arrest or death. This was shown for tunicamycin-exposed Perk$-/-$ cells (23) or Nrf2 $-/-$ fibroblasts (14), as well as yeast expressing a mutant misfolded protein (30). A recent study also showed that hypoxic ROS-triggered UPR was abrogated in cytochrome-c null cells (59). The pathways connecting mitochondria and ER during the UPR comprise three categories: (a) direct physical contacts (106); (b) indirect mechanisms related to dysfunction of Ca^{++} regulation (61, 106), as well as effects of ROS, ATP depletion, and even Nox4 NADPH oxidase isoform, which is upregulated even by mild mitochondrial dysfunction (124); (c) more specific pathways such as ER stress-inducible Lon protease, which protects mitochondria by interfering with cytochrome oxidase complex assembly/degradation (34), or the bcl-2 family protein Nix, which promotes mitochondrial-mediated cardiomyocyte death *via* decreased sarcoplasmic calcium (18). The latter may be only one additional example of the important integrative role exerted by the bcl-2 family proteins, as already mentioned for Bax and Bak (32). Moreover, there is mounting evidence that mitochondria sustain a compartmental UPR, coupling mitochondrial-specific chaperone expression to perturbations in organelle protein processing (131). Although mitochondrial ROS production can induce such compartmental UPR, its induction per se does not seem to enhance mitochondrial oxidant generation (131).

ER Stress and NADPH Oxidase

While ER protein oxidation and mitochondria have been addressed in the recent past as ROS sources during ER stress, there is much less information concerning NADPH oxidase(s). Importantly, however, Nox4 NADPH oxidase isoform mRNA and protein expression has been shown to be induced in human vascular smooth muscle cells during ER stress promoted by the oxygenated lipid product 7-ketocholesterol, while Nox1 and Nox5 mRNA were unaltered (84). Similar findings were obtained in our laboratory after incubation of rabbit aortic vascular smooth muscle cells with tunicamycin (0.25–5.0 μg/ml) for 16 h, or at different times after exposure to 2 μg/ml concentration (Fig. 4A and B). While Nox1 mRNA expression showed negligible changes, Nox4 mRNA exhibited marked concentration- and time-dependent increase after tunicamycin, detectable at $\sim$4 h and reaching a 10-fold increase after 16 h of stimulus. Additional experiments with 24-h tunicamycin incubation (2 μg/ml) showed increase in Nox4mRNA up to 60-fold, (unpublished observations), indicating sustained Nox4 increase throughout late UPR stages. Incubation for 16 h with 7-ketocholesterol (84) promoted increased oxidant production, which was abolished by PEG-SOD + PEG-catalase, by the flavoprotein inhibitor diphenylene iodonium or by an siRNA against Nox4. In our vascular smooth muscle cells incubated with tunicamycin (2 μg/ml), we detected increased superoxide production (by HPLC analysis of DHE oxidation products) at 4 and 16 h of incubation. At both time points, superoxide was significantly inhibited and even decreased after an siRNA against Nox4 (Fig. 4C). These results indicate that at least a significant portion of ROS production under ER stress condition is attributable to Nox4 expression in vascular smooth muscle cells.

The functional consequences of Nox4 expression have been analyzed by Pedruzzi *et al.* (84). Remarkably, Nox 4 siRNA prevented the late onset of cell death induced by 7-ketocholesterol. Moreover, Nox4 silencing was able to inhibit or prevent several markers of the UPR, including not only the proapoptotic CHOP and Bax proteins, but also Grp78. Importantly, Nox4 siRNA was able to abolish the Ca^{2+} oscillations induced by 7-ketocholesterol 3 min after incubation (observed for 25 min), indicating a potentially very early role of Nox4 during ER stress/UPR signaling. Mechanisms of Nox4 induction during the UPR appear to involve the IRE1/JNK pathway, since Nox4 induction, as well as UPR markers, were inhibited by an siRNA against IRE1 or JNK phosphorylation inhibitors (84).

It is presently unclear whether this strong functionally relevant upregulation of Nox4 identified in vascular smooth muscle cells extends to other cell types, considering that cells

vary widely in their constitutive and inducible Nox isoform expression (3). The observed Nox4 proadaptive and proapoptotic effects (84) conform to those of ROS discussed above. This further suggests that UPR signaling does not behave as a linear crescendo system, but as a more complex network of adaptive and apoptotic pathways. The role of Nox4 in UPR is in line with some specific characteristics of this isoform. Particularly, there is increasing evidence that Nox4 has a relevant localization and compartmental action in the ER (10, 31, 84, 94). This, however, may not be its exclusive location, since other studies addressing endogenous Nox4 have consistently identified a location at the cytoskeleton or focal adhesions (33), in addition to an yet controversial nuclear staining (47, 84). It is possible that Nox4, while being primarily located within the ER, may exhibit significant intracellular traffic. This variability could also depend to some extent on technical issues, alternative spliced isoforms (26), or other unknown factors. While Nox4 requires the transmembrane topology characteristic of the Nox family (49), it is unknown whether the ROS-generating domain is at the ER lumen (the most logical arrangement) or at the cytosol. In the latter case, the oxidative ER lumen environment would not preclude NADPH-mediated O_2 reduction, since the ER NADPH and glutathione pools are independently redox-regulated due to negligible expression of NADPH-driven glutathione reductase (85). Whether ER-located Nox4 might contribute to protein folding is unclear, for an oxidative environment can indeed decrease the energetic cost of folding and facilitate achieving ideal protein conformation, but excessive oxidation leads to protein aggregation (99). In addition, because Nox4

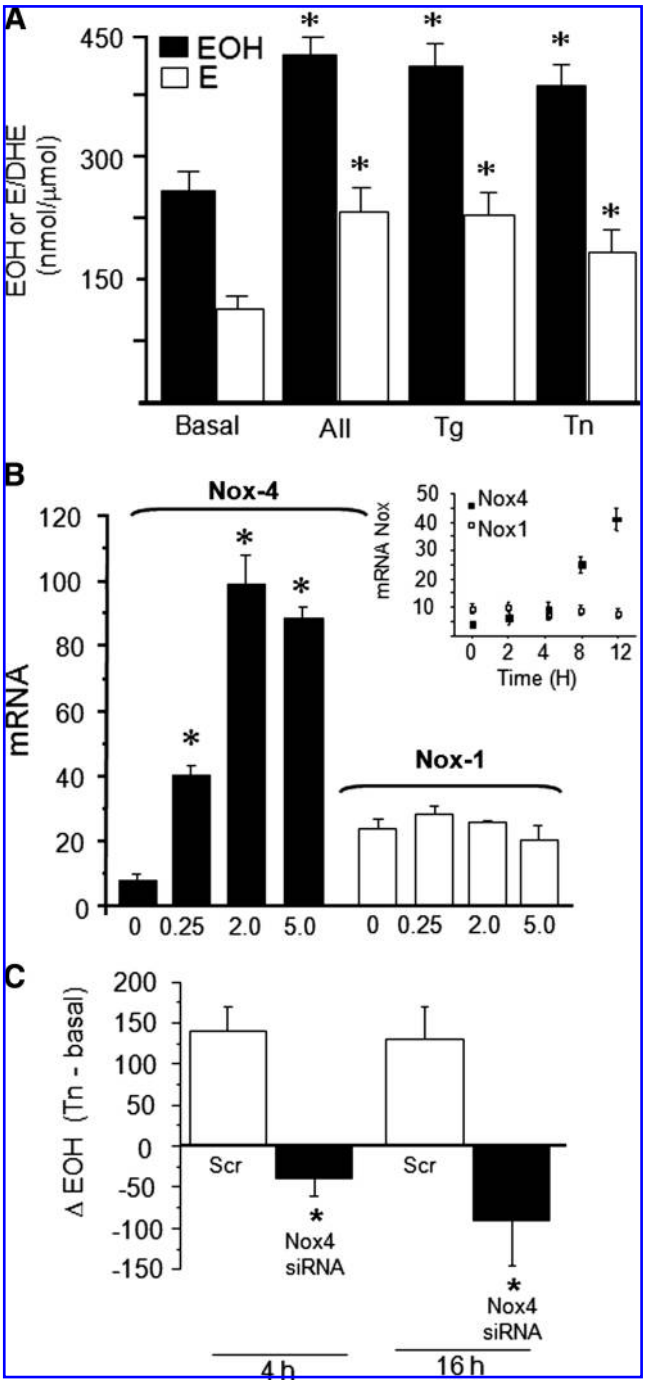

FIG. 4. Nox 4 activation and ROS production during the UPR. (**A**) Assessment of membrane fraction NADPH oxidase activity after vascular smooth muscle cell incubation with Angiotensin II (AII, NADPH oxidase agonist which does not induce the UPR in this protocol; 100 nM), Thapsigargin (Tg, 1 μM), or Tunicamycin (Tn, 5 μg/ml) for 4 h. Membrane fraction homogenates were prepared after sequential centrifugations as described, incubated with DHE (50 μM) (21, 124) and exposed to NADPH 300 μM. After acetonitrile extraction, the supernatant was run on HPLC as described in detail (21, 51). Results depict 2-hydroxyethidium (EOH, which reflects mainly superoxide) or ethidium (E, which reflects less specific oxidants) products of DHE oxidation (in nmol product formation/μmol DHE consumed). (**B**) Real-time PCR analysis of Nox1 and Nox4 mRNA expression incubated (at 80% confluence) with the indicated concentrations of Tunicamycin (in μg/ml) for 16 h. Primer sequences and PCR protocol were described in ref. 124. Data are expressed as the ratio of Nox expression/actin mRNA expression in the same sample. The inset shows results of similar experiments, but cells were exposed with Tunicamycin (2 μg/ml) at different times. Data are mean ± SE of 4 independent experiments. *$p < 0.05$ (Anova). (**C**) Effects of Nox4 siRNA(CUGU UCCUGGCCUGACAGGTT) or scrambled control siRNA (CGTACTCCTAACAGCGCTCTT) on the response of superoxide production to Tunicamycin exposure in VSMC (2 μg/ml, 4 or 16 h). The plasmid duplex (160 μmol) was transfected using Lipofectamine as described in Fig. 3 legend and cells were used 24h after transfection. We were concerned that repeated transfections over a prolonged time period, necessary to achieve substantial decrease in basal Nox4 levels (92), might interfere with subsequent cellular adaptations involved in UPR signaling, in addition to Nox1 levels. Thus, we used short-term transfection in which Nox4 mRNA levels were unaltered at baseline and decreased by ~30% after Tunicamycin (2 μg/ml, 16 h), with no changes in Nox1 mRNA levels (data not shown). This strategy is further justified by reports that Nox4 activity is closely reflected by its mRNA expression (94). Under these conditions, baseline production of ROS was, as expected, unaltered by Nox4 siRNA, but significantly abrogated or decreased both 4 and 16h after Tunicamycin incubation vs. VSMC transfected with scrambled control siRNA. Results are shown as the absolute change in formed EOH product (in nmol/μmol DHE consumed). Values are mean ± SE of 3–4 experiments. *$p < 0.05$ *vs.* Basal or scrambled siRNA.

might be glycosylated (26), it can be speculated that Nox4 traffic might help induce such an oxidative environment in post-ER compartments, thus helping to sustain some protein folding capacity outside of stressed ER, as proposed previously for mitochondrial oxidants (128). Another aspect of Nox4 is its close functional interaction with PTP1B in the ER, by promoting its oxidant-mediated inactivation (10). This phosphatase was reported to potentiate IRE1-dependent signaling during UPR and could thus be an important target of Nox4 during ER stress (28).

Which ROS Are Produced During the UPR?

The term "reactive oxygen species" (ROS) is usually employed to convey accuracy with respect to a general designation of chemical species arising from oxygen reduction and their related precursors and/or reactive reaction products. However, it should be considered that ROS are a very heterogeneous group of intermediates that differ widely with respect to reactivity, cellular location, partition, solubility, and diffusibility (discussed in refs. 40, 123). Particularly, some free radical ROS (such as superoxide) are one-electron oxidants, while several others (such as hydrogen peroxide, peroxynitrite) are two-electron oxidants. These properties turn the physiological consequences of each specific ROS significantly distinct, with important consequences regarding effects of antioxidant interventions. This emphasizes the importance of pursuing accurate identification of the precise intermediates being generated (123). Our data from Figs. 3 and 4 document (to our knowledge for the first time) the cellular production of superoxide during the UPR, using up-to-date specific and accurate HPLC methods (21, 51). On the other hand, detection of superoxide during the UPR poses intriguing questions, considering that kinetic data from sulfhydryl flavo-oxidases analogous to Ero1 argue against the production of superoxide as a relevant intermediate formed during catalysis (100). Furthermore, Nox4 is believed to generate mainly hydrogen peroxide, rather than superoxide, when its expression is induced in cells (31, 94) and possibly also endogenously (17). While an explanation for such discrepancies is unclear at present, it should be pointed out that induced Nox4 is also able to reduce nitrobluetetrazolium, a property consistent with superoxide generation (94). In addition, it can be speculated that post-translational modifications or alternative splicing (26) affecting the N-terminal portion of endogenous Nox4 might promote its superoxide generation, in line with recent elegant experiments using Nox1-Nox4 chimeras (31). In addition, evidence for NAD(P)H-triggered superoxide generation in microsomal fractions from arterial smooth muscle cells (132) or skeletal myocytes (125) has been reported. It is conceivable, therefore, that Nox4 is also able to yield superoxide under specific conditions.

All such considerations, as well as the several unknown questions still open with respect to ROS generation during the UPR, further raise the logical possibility that other mechanisms of ROS production may be operative during the UPR, although there are no data in this regard at present. Cytochrome p450 isoenzymes would appear as logical candidates, among others, due to their location and capacity to generate physiologically relevant amounts of superoxide (22). In mouse embryonic fibroblasts, thapsigargin but not tunicamycin-induced ER stress and oxidant production can be abolished by iNOS inhibitors, thus raising a possible involvement of this enzyme as an oxidant source (35).

Integration of ER, Mitochondria and NADPH Oxidase as ROS Sources During the UPR: Possible Role for Protein Disulfide Isomerase (PDI)?

Production of ROS in the ER, mitochondria, or *via* Noxes does not represent only distinct locations, but may also account for differences in the type of ROS, as well as in their amount and distribution. In this context, a conceptual but nonetheless relevant question is whether such ROS production during the UPR represents "redox signaling," as opposed to an "oxidative stress." Distinction between these two situations has been thoroughly addressed in recent reviews (40, 42, 123). While a clear state of oxidative stress occurs during established UPR, redox signaling targets might display some specificity during earlier UPR stages. Because such targets are likely multiple and configure mass physiological programs, we have come to describe this phenomenon as "redox macrosignaling." This would contrast with a restricted localized "redox microsignaling" often associated with compartmental NADPH oxidase activation (111). This likely determines distinct physiological signaling consequences and stimulus-response correlations not only related to UPR but also to other indirect cell targets. In fact, ROS production during the UPR is likely to have components specific to this type of stress (in this case, the ER oxidoreductases and possibly Nox4) and components that belong to nonspecific cell stress responses (mitochondria and eventually Nox4). The first component, contrarily to the second, is likely to some extent to be preferentially related to "microsignaling" and "macrosignaling" than oxidative stress. ER-dependent ROS production, however, also has the quantitative potential to account for significant oxidative stress (9, 114, 115). Both components are also likely to suffer the influence of adaptive and secondary phenomena (Fig. 1).

Despite all those characteristics, there is a need for the integration of redox pathways in order to achieve signaling coherence on a cell basis during the UPR (Fig. 5). Not much is known about integration of distinct ROS enzymatic sources in cells except that it probably should involve multiple sensor and effector pathways (42, 123). It is unknown but at the same time unlikely that one discrete component of the UPR can account for such a complex integrative role by itself. The cross-talk between ER and mitochondria proceeds through a number of pathways, discussed above. In addition, we recently showed that even minor mitochondrial dysfunction promotes increase in baseline Nox4 mRNA levels, together with decreased Nox1 response to angiotensin II (124), thus indicating a cross-talk between mitochondria and Nox(es).

With respect to cross-talk between ER oxidoreductases and NADPH oxidase, we have provided significant evidence for a role of PDI. Our data showed that PDI acts as a regulatory protein of NADPH oxidase complex activation in VSMC (39) or macrophages (unpublished observations and Ref. 50). In both cell types, PDI loss-of-function experiments with pharmacological antagonists, neutralizing antibodies, antisense oligonucleotides, and, more recently, with a short-interference RNA (20), provided consistent evidence for a PDI-dependent functional modulation of NADPH oxidase, particularly of its induced activation by angiotensin II. Moreover, we showed

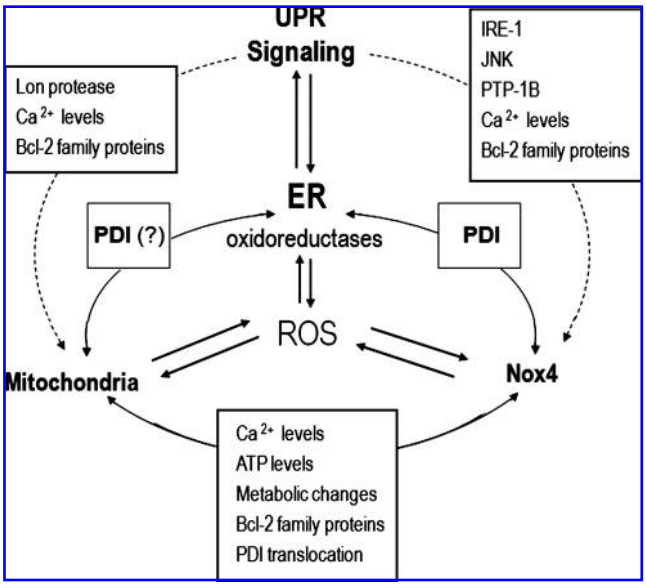

FIG. 5. Main enzymatic sources of ROS during the UPR and their integrative cross-talk. See text for details.

that PDI co-localizes and/or co-immunoprecipitates at least with subunits p22phox, Nox1, Nox2, and Nox4, indicating a close association with the oxidase complex. The primary PDI location is the ER lumen, where it assists redox protein folding and thiol isomerization and may thus be involved in ER-mediated mechanisms of ROS generation discussed above. Nevertheless, upon NADPH oxidase activation, PDI suffers translocation/partition to membrane compartment, where it seems to assist the NADPH oxidase complex (39, 50), in line with the known PDI traffic to membranes in other cell types (50, 110). Moreover, we showed recently that a 2.3-fold overexpression of PDI promotes spontaneous agonist-independent NADPH oxidase activation and Nox1 mRNA expression in vascular smooth muscle cells in a way independent of PDI thiols, thus suggestive of a chaperone-type effect (20). Furthermore, we found that overexpresssed PDI significantly sustains Nox4 mRNA levels in response to angiotensin II and/or S-nitrosoglutathione on a PDI thiol-dependent basis (20). It remains to be demonstrated whether PDI exerts a similarly important role with respect to Nox4 expression and function during the UPR, although our preliminary data are suggestive (unpublished observations from our laboratory). PDI is not usually considered to be strongly UPR- inducible (60), while it is upregulated and exerts a generally protective role in several stressful circumstances such as hypoxia (104), ischemia–reperfusion (108), development (97), or protein aggregate formation (116). Interestingly, PDI also has ascorbate reductase activity involving ER thiols, in a way that dehydroascorbate has been proposed as an oxidant for protein folding (77). The integrative role of PDI in ROS generation may also extend to mitochondrial dysfunction, which also promotes PDI translocation/partition to cell membranes in the context of interference with NADPH oxidase complex (124). Although PDI family members reportedly associate with mitochondria (44) and Grp78 translocates from ER to mitochondria during ER stress (105), their function concerning mitochondrial ROS generation is unknown. Remarkably, an elaborate thiol–disulfide redox relay carries proteins across mitochondrial intermembrane space and might be affected by PDI and ROS (6). Furthermore, PDI (50, 122) shares with mitochondria (128) the ability to modulate redox status of cell surface dithiol proteins. PDI could thus exert an important role in the integration between ER stress and redox processes, connecting in a functional and/or physical way the three major ROS sources discussed in this review: ER, mitochondria, and NADPH oxidase.

Another integrative element in this cross-talk of the three main ROS sources is ROS themselves. It is well-known that exposure to ROS (*e.g.*, from NADPH oxidase and potentially from the ER) can trigger ROS generation from mitochondria *via* mechanisms including changes in membrane permeability, ion channel function, and possibly mitochondrial DNA mutations (124). In addition, it is now accepted that ROS can trigger NADPH oxidase expression and activation (53), particularly Nox4, which is quite sensitive to hydrogen peroxide (66). Furthermore, ROS, which closely modulate the UPR, as discussed above, will very likely result in further modulation of ROS generation from the ER. These considerations strengthen the possibility of a redox-mediated cross-talk affecting ROS generation by several sources during the UPR. This proposal is in line with the regulatory role of PDI described above and potentially with roles of other redox-sensitive thiol proteins (42), as well as glutathione and cysteine. Other potential integrative elements in ROS generation are metabolic changes, bcl-2 family proteins (discussed above), and Ca^{2+} level regulation. The latter is clearly important in the cross-talk between the ER and mitochondria (61, 106), while its role in the activation of Noxes other than Nox5 and Duox1/2, particularly of Nox4, is uncertain and has been reviewed previously (25). Nox4, however, appears able to trigger early Ca^{2+} oscillations during the UPR (84).

In summary, the integration of ROS production during the UPR likely involves multiple pathways that may be coordinated by ROS themselves, PDI, Ca^{2+} levels, bcl-2 family proteins, metabolic changes, and other specific signaling proteins, as illustrated by the scheme in Fig. 5.

ER Stress and ROS Generation in Vascular Diseases

The consequences of ER stress in several diseases have been addressed in a number of reviews (93, 133, 134). Thus, in cancer, diabetes. and neurodegeneration, among other diseases, the occurrence of oxidative stress may be tightly connected to integration with other types of stress, particularly ER stress. Moreover, chemical chaperones (120) or UPR gene targeting (62) may alleviate oxidative stress in these conditions. Here, we focus in vascular diseases, since their pathogenesis uniformly involves processes of response to stress (55). In addition, there is a growing body of evidence implicating Nox4 in redox cardiovascular pathophysiology, in contexts including stem cell differentiation (73) and response to hypoxia (72). Whether such Nox4 involvement occurs in a context of UPR and ER stress remains to be tested, although the involvement of PDI (108) and some UPR pathways in hypoxia is known (59). ER stress is involved in the pathogenesis of important vascular diseases, bringing about the significance of its convergence with oxidative stress, also known to contribute to such pathology. Particularly, ER stress markers are induced at several stages of the development of

atherosclerosis in experimental models (135). Moreover, UPR markers are particularly expressed in specimens from complicated atherosclerotic plaques (75), thus suggesting a correlation between ER stress and acute coronary syndromes. Such connection is in line with the known correlation between ER stress and inflammation (133). Cytokine expression in endothelial cells by oxidized phospholipids is dependent on UPR signals involving ATF4 and XBP1 (24). In addition, several pathways involving IRE1 signaling can lead to NF-κB and AP-1 activation (133). In the liver, ATF6-analog transcription factor CREBH can promote synthesis of acute phase proteins during ER stress (133). Although not much is known about how normal levels of nitric oxide affect ER homeostasis, excessive nitric oxide levels can perturb oxidative protein folding, at least in part by PDI *S*-nitrosation and inhibition of its isomerase activity (116), as well as by deregulation of Ca^{++} homeostasis, in addition to *S*-nitrosation-dependent inhibition of mitochondrial electron transport chain. Thus, excess nitric oxide induces ER stress and ROS production (126, 133). Conversely, endogenous NO protected beta-cells from proteasome inhibitor-associated ER stress *via* activation of antioxidant-responsive element Gclc-ARE4-dependent genes (45). These pathways can be relevant not only at the local plaque milieu, but also at a systemic level, particularly considering the known role of ER stress as the pathophysiological basis of insulin resistance, obesity, and diabetes (133), known cardiovascular risk factors. Remarkably, genetic deletion of CHOP was recently shown, in distinct diabetes models, to reverse glucose intolerance and to decrease beta-cell loss, in association with increased UPR response and reduced oxidative damage (133). In addition, homocysteine, another risk factor, induces ER stress *via* mechanisms including direct interference with redox protein folding and Ca^{2+} deregulation (2). In part, ER stress-related atherosclerosis pathology may be associated with the known toxicity of free cholesterol towards the normally cholesterol-poor ER membranes, which leads to Ca^{++} release, UPR activation, and CHOP-induced apoptosis, in parallel with cytokine induction (54).

Summary and Conclusions

ROS production and oxidative stress can be considered an integral component of the UPR, being triggered by classical or noncanonical ER stressors and contributing to support UPR signaling, mainly at proapoptotic, but also proadaptive levels, in addition to a possible novel role as early misfolding signal (62). Moreover, oxidants from independent sources may also trigger at least some arms of the UPR, although experiments with exogenous ROS indicate that ROS exposure *per se* seems not sufficient to trigger the full UPR. Thus, ROS generation can occur both upstream and downstream to UPR signaling targets. Glutathione consumption is an important feature of the UPR, although the mechanism can be multiple. Antioxidant gene expression can be induced during the UPR as part of the integrated stress response. In parallel, oxidant generation and production of superoxide radical can be detected at early stages of the UPR. Many connections within this stress integration remain to be understood, but data so far suggest that ROS generation is supported by at least three enzyme-dependent sources: ER oxidoreductases, mitochondria, or NADPH oxidase isoform Nox4. How these ROS sources are integrated is unclear, but Ca^{2+} levels and ROS themselves might contribute to this role, considering the known ROS-triggered ROS release both from mitochondria or NADPH oxidases such as Nox4 (7, 49, 66, 124). Particularly, PDI might have a role in the integration between ER stress and oxidative stress, at least in part through its known close interaction with NADPH oxidase complex (20, 39, 50), as well as through its role as substrate of Ero1. Collectively, as with many other stress signals, ROS generation might have a UPR-specific component (possibly *via* ER sources and Nox4) and a nonspecific stress-related component (such as mitochondria and also Nox4). Contextualizing the evidences discussed in this review, what would be the role of ROS and NADPH oxidase activation during the UPR? At this point, perhaps the best answer to this question is that, as intrinsic components of the UPR, ROS appear to be involved in the 4 "***A****s*" of this response (described in ref. 90): activation, acute response, adaptive response, and apoptosis, while Nox4 at least in the last three "***A****s*". Altogether, a more general implication of the data discussed in this article is to suggest that redox signaling, oxidative stress, and NADPH oxidase activation should be more often analyzed against the background of their integration with UPR signaling, ER stress, and in a broader sense cellular stress response—indeed a context in which they may all have evolutionarily co-developed.

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Abbreviations Used

AngII = angiotensin II
ATF = activating transcription factor
Bref-A = brefeldin-A
CHOP = CAAT/enhancer binding protein (C/EBP) homologuos protein
DCF(d) = dichlorofluorescein (derivative)
DH-123 = dihydrorhodamine 123
DHE = dihydroethidium
DTNB = 5,5′-dithiobis-(2-nitrobenzoate)
DTT = dithiothreitol
E = ethidium
eIF2α = eukaryotic translation initiation factor 2
EOH = 2-hydroxyethetidium
ER = endoplasmic reticulum
ERAD = ER-associated degradation
Ero = endoplasmatic reticulum oxidoreductase
GADD = growth arrest and DNA damage
Grp = glucose-regulated protein
GSSG/GSH = oxidized to reduced glutathione
HEK = human embryonic kidney
HPLC = high performance liquid chromatography
IRE = inositol requiring protein
ISR = integrated stress response
JNK = c-Jun N-terminal kinase
KDEL = lys-asp-glu-leu (C-terminal ER retention sequence)
NF-κB = nuclear factor κB
Nox = NADPH oxidase
ORP = oxygen-regulated protein
PDI = protein disulfide isomerase
PERK = RNA activated protein kinase (PKR)-like endoplasmic reticulum kinase
PP1c = protein phosphatase-1
PTP1B = protein tyrosine phosphatase 1B
ROS = reactive oxygen species
siRNA = small interference RNA
Tg = thapsigargin
Tn = tunicamycin
TNFα = tumor necrosis factor
UPR = unfolded protein response
VSMC = vascular smooth muscle cell
XBP = X-box binding protein

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