

ROS-induced ROS release in vascular biology: redox-redox signaling

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Submitted 15 December 2010; accepted in final form 9 June 2011

Zinkevich NS, Gutterman DD. ROS-induced ROS release in vascular biology: redox-redox signaling. *Am J Physiol Heart Circ Physiol* 301: H647–H653, 2011. First published June 17, 2011; doi:10.1152/ajpheart.01271.2010.—The involvement of reactive oxygen species (ROS) in regulating vascular function both in normal vessels and as part of an adaptive response during disease has been intensively studied. From the recognition that ROS serve as important signaling molecules has emerged multiple lines of evidence that there is a functional connectivity between intracellular sites of ROS production. This cross talk has been termed ROS-induced ROS release (RIRR) and is supported by a variety of observations showing that RIRR is a common mechanism for ROS amplification and regional ROS generation. The compartmentalization of ROS production within a cell is critical to its signaling function and is facilitated by microlocalization of specific scavengers. This review will provide descriptions and examples of important mechanisms of RIRR.

reactive oxygen species; mitochondria; endothelial dysfunction

AEROBIC ORGANISMS continuously produce reactive oxygen species (ROS), redox active molecules with unpaired electrons derived from molecular oxygen. Cellular levels of ROS are determined by the steady-state balance between rates of formation and decomposition by endogenous enzymatic antioxidant systems and by small molecules that function as antioxidants (Table 1).

Cellular Enzymatic Sources of ROS and Stimuli for ROS Production

ROS derived from the reduction of molecular oxygen include superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{\cdot}), and peroxynitrite ($ONOO^-$). $O_2^{\cdot-}$ is converted to H_2O_2 spontaneously or more than a thousand times faster by an enzymatic process involving one of three isoforms of superoxide dismutase (SOD) (15). H_2O_2 can undergo one electron reduction to the highly evanescent and reactive OH^{\cdot} in the presence of reducing metal ions or, through two-electron reduction, can be converted to water by peroxisomal catalase. Intracellular ROS originate from multiple sites, including the mitochondrial electron transport chain, cytochrome *P*-450 oxygenase, xanthine oxidase (XO), lipoxygenase, cyclooxygenase, and uncoupled nitric oxide (NO) synthase (NOS) (7). NADPH oxidase, a prominent source of ROS in vascular tissue, has several isoforms localized to different sites within the cell. NADPH oxidase that contains the NOX2 catalytic subunit can be plasmalemmal bound and produce $O_2^{\cdot-}$ extracellularly or within the cytosol (1, 12, 14). The extracellularly generated $O_2^{\cdot-}$ can reenter the cell through anion-selective chloride channel-3 channels (16) or by conversion to H_2O_2 via

extracellular SOD. The NOX4 containing oxidase is located in endosomes (28, 39), focal adhesions, and nuclei (19) and generates $O_2^{\cdot-}$ intracellularly. Other members of the NOX family include NOX1, which can be found in various subcellular localizations such as nuclei (5) and caveolae (19), NOX3 and NOX5, which both have been shown to colocalize with the plasma membrane (42, 50). Thus subcellular localization of NADPH oxidase allows for stereospecific release of $O_2^{\cdot-}$, which is spontaneously or catalytically (SOD) converted to H_2O_2 , the primary signaling ROS. As an uncharged molecule, H_2O_2 can traverse cell membranes, is rapidly inactivated by endogenous catalase and peroxiredoxins, and can reversibly alter enzyme function through oxidative modification of susceptible residues, including arginine, cysteine, histidine, and others (24, 52). These properties strongly support a signaling role for intermediate doses of H_2O_2 . Signaling dose ranges for H_2O_2 were established in human and animal models and vary from 1 μ M to 10 mM (21, 32, 55). Interestingly, in rat coronary arterioles, sensitivity to H_2O_2 is increased with aging (21).

It has recently been demonstrated that these diverse anatomic and chemical ROS-generating systems interact to facilitate an integrated redox modulation of vascular tissue. These interactions can result in enhanced elimination or amplification of the cellular ROS signal. The latter response, termed ROS-induced ROS release (RIRR) (69), is the subject of this review.

Concentration-Dependent Effects of ROS

ROS are implicated in the etiology of aging, angiogenesis, apoptosis, and a myriad of diseases including atherosclerosis, hypertension, hypercholesterolemia, obesity, cancer, diabetes mellitus, and neurodegenerative disorders [see reviews (6, 41, 53)]. Most pathology is the result of excessive ROS formation that promotes inflammation in surrounding tissues and accelerates cell death or senescence. In these cases, levels of ROS

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Table 1. Intracellular sources of ROS and cellular defenses modulating oxidative stress

Increased Activity of Cellular Enzymatic Sources of ROS	Extracellular or Noncellular Sources of Oxidative Stress	Possible Defenses from Oxidative Stress	
		Enzymatic antioxidants	Small molecules
NADPH oxidase	Cigarette smoke	Cytosolic superoxide dismutase, containing copper and zinc (Cu,ZnSOD) and Manganese (Mn ²⁺ dependent) SOD, catalase, glutathione system (glutathione, glutathione peroxidase, and glutathione reductase), peroxiredoxins, thioredoxin reductase, heme oxygenase, and biliverdin reductase	Salicylic acid; carotenoids; vitamins C, E, and A; α -tocopherol; ascorbic acid; uric acid; thioredoxins; coenzyme Q; melatonin; flavonoids; and polyphenols
Mitochondrial electron transport	Radiation (UV, gamma)		
Nitric oxide synthase	Neutrophil activation		
Xanthine oxidase	Ischemia-reperfusion		
Arachidonic acid metabolism (lipoxygenase and cyclooxygenase)	Hyperoxia		
	Electrical current		
	Elevated glucose levels, lipid peroxidation, Metal-catalyzed oxidation		
	Catecholamine autoxidation		
	Neuromelanin		

ROS, reactive oxygen species.

can reach high concentrations, often exceeding 500 μ M for H₂O₂ in sites of inflammation or injury (17). O₂^{•-} concentrations are tightly controlled by the cytosolic Cu,Zn-SOD, which can rapidly lower O₂^{•-} levels from the nanomolar to picomolar concentration range (62). Interestingly, excessively low levels of ROS can also invoke pathological changes by interrupting the physiological role of oxidants in proliferation, vasodilation, and host defense and may promote carcinogenesis (33, 60). The presence of a “physiological window” of ROS concentrations could explain some of the negative results from clinical trials where large doses of exogenously administered antioxidants failed to improve cardiovascular outcomes [see review (20)]. There appears to be a physiological range of concentrations where intermediate levels of ROS can function as critical signaling molecules and mediate cellular growth, protein phosphorylation, and cell migration (47, 59). This dose dependency should be considered when addressing the pathophysiological relevance of ROS.

Decomposition of ROS: Role of Antioxidants

The cellular response to oxidative stress involves the elimination of, protection against, and repair of damage caused by ROS. Scavenger antioxidant enzymes including SOD, peroxiredoxin, and catalase are responsible for the direct elimination of ROS, whereas systems that reconstitute antioxidants [e.g., glutathione (GSH) and GSH peroxidase (GSH-Px)] can indirectly reduce ROS. The protective role of vitamins C and E in modulating ROS levels in athletes following maximal exercise is an example of such an indirect effect. These vitamins may upregulate GSH-Px levels, thereby ameliorating exercise-induced oxidative damage (43).

Protection from oxidative damage can be achieved by a variety of mechanisms. The driving force for oxidant generation can be reduced. Production of ROS has been shown to correlate positively with changes in mitochondrial membrane potential, such that even a small increase in membrane potential results in a rapid elevation of O₂^{•-} levels (23). Therefore, mild mitochondrial depolarization can result in more efficient electron transfer and less uncoupling, thus less diversion to oxygen to form O₂^{•-} (23). However, this effect is not universal, since mild depolarization has also been shown to have the opposite effect (45). Molecules sensitive to oxidative stress can be surrounded by decoys that are preferentially oxidized, thereby protecting key cellular molecules (e.g., GSH, α -to-

copherol, bilirubin, ascorbate, urate, albumin plasmogens, and cytosolic amino acids) (15). Direct physical quenching of O₂^{•-} or other free radicals (e.g., by carotenoids) is another biologically deployed mechanism (15). Each of these processes contributes to the local and global cellular redox balance, which has implications for cell signaling, cell growth, and cell death.

Functional Effect of ROS and RIRR

Recent investigations indicate that ROS participate in regulating vascular function both in normal vessels (36) and as part of an adaptive response during disease (57, 66). The concentration and localization of oxidant-generating and -quenching enzymes indicate high stereospecificity and compartmentalization within the cell. Given the ubiquitous nature of ROS and their involvement in signaling transduction and cell injury, their accumulation must be tightly controlled both spatially and temporally by systems capable of regulating regional ROS production. RIRR is an example of such a control, by allowing ROS formed in one region to activate specific sites in another region of the cell. While not comprehensive, the sections that follow highlight several distinct mechanisms of RIRR that have been described in a variety of cellular systems. They are grouped based on whether they represent direct or complex forms of RIRR.

Direct RIRR

Mitochondria are important sources of ROS production and a common target for the damaging effect of oxidative stress. Using mitochondria from freshly isolated adult rat cardiac myocytes, Zorov et al. (69) demonstrated that inhibition of complex III with antimycin at the inward electron transfer site diverted electrons toward the inner-membrane space leading to O₂^{•-} formation (Fig. 1A). In single mitochondria ROS production (H₂O₂ detected by dichlorofluorescein fluorescence) occurred in two distinct phases: the initial or “trigger phase,” followed by a delayed amplified release of ROS, which could be attenuated with the complex I inhibitor, rotenone (70).

Another example of feed-forward RIRR occurs through ROS amplification in smooth muscle NADPH oxidase. Exogenous exposure of vascular cells to H₂O₂ stimulates NADPH oxidase, resulting in O₂^{•-} production, which is converted to H₂O₂. The produced H₂O₂ can further stimulate O₂^{•-} produc-

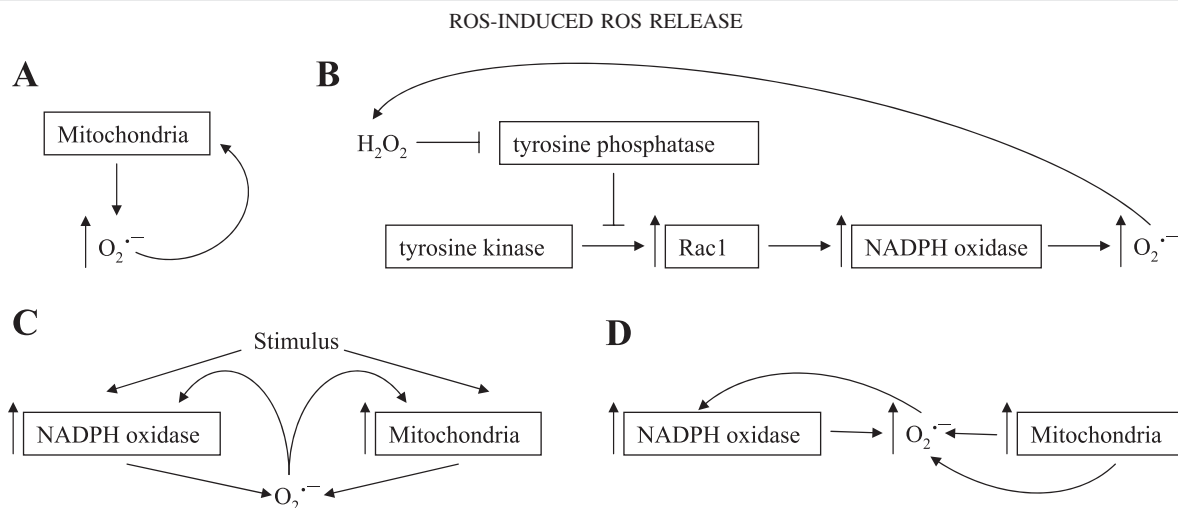


Fig. 1. A–D: sequence of events illustrating direct reactive oxygen species (ROS)-induced ROS release (RIRR). $O_2^{\cdot-}$, superoxide; H_2O_2 , hydrogen peroxide.

tion from NADPH oxidase, therefore initiating a self-promoting cycle (29). This form of RIRR signaling has been implicated in apoptosis of nonphagocytic cells (27). The mechanism responsible for this amplification of ROS might include the inhibition of tyrosine phosphatases by H_2O_2 , allowing Rac1 assembly with NADPH oxidase subunits into an active complex. These two examples describe a spatially localized but temporally sustained form of RIRR (Fig. 1B).

Redox communication between mitochondria and NADPH oxidase is one of the best-studied forms of RIRR and is involved in a variety of physiological signaling pathways.

Mitochondria initiate ROS production by NADPH oxidase. Lee et al. (26) demonstrated that complete serum withdrawal in human 293T cells rapidly elevates mitochondrial ROS production. ROS generated by mitochondria stimulated phosphoinositide 3-kinase followed by translocation of Rac1, allowing its interaction with NOX1. The dynamics of ROS generation in this model is such that initial ROS production from mitochondria is relatively transient, but the evoked activation of NOX1 is more prolonged (26). The importance of this study is twofold: 1) it demonstrates how a transient ROS signal (from mitochondria) can be converted into a more sustained ROS release (from NADPH oxidase), and 2) it also describes a pathway by which ROS generated at one subcellular site triggers ROS production in a different site through signal transduction (Fig. 1C).

NADPH oxidase triggers ROS production from mitochondria. In converse fashion, ROS generation from mitochondria can be triggered from ROS produced by NADPH oxidase. Hawkins et al. (16) demonstrated in pulmonary microvascular endothelial cells that the activation of NADPH oxidase produces extracellular $O_2^{\cdot-}$. The $O_2^{\cdot-}$ produced can then be transported intracellularly through a chloride channel-3, where it triggers rapid Ca^{2+} mobilization followed by mitochondrial $O_2^{\cdot-}$ production. These findings not only demonstrate a link between two enzymatic sources of ROS but also establish a role for $O_2^{\cdot-}$ as a signaling molecule involved in RIRR where extracellular ROS stimulate intracellular ROS production, resulting in endothelial dysfunction and cellular apoptosis (Fig. 1C) (16).

Interactions between NADPH oxidase and mitochondria represent one of the more commonly observed examples of

RIRR (8, 22, 61). Studying the cardioprotective effects of angiotensin II (ANG II), Kimura et al. identified a novel signaling pathway by which ANG-II stimulation of ANG-I receptors activates NADPH oxidase in the heart by a protein kinase C-mediated mechanism (22, 31). Although the direct contribution of ROS produced by NADPH oxidase via this mechanism does not induce preconditioning, the NADPH oxidase-derived ROS are essential for the activation of mitochondrial ATP-sensitive K^+ (K_{ATP}) channels, which elicit preconditioning through an increase in mitochondrial ROS release (22). The findings of this study reveal how ROS generated in one region of the cell (membrane) orchestrate ROS production in a separate compartment (mitochondria) to effect a physiological response (22).

As mentioned, $O_2^{\cdot-}$ from mitochondria can activate mitochondrial K_{ATP} channels to produce a substantial (over 50%) reduction in the mitochondrial membrane potential, which leads to excess ROS generation. It can also combine with NO to generate $ONOO^-$. Both $O_2^{\cdot-}$ and $ONOO^-$ oxidize electron transport chain components, uncoupling respiration and further augmenting ROS generation (8). This mitochondrial ROS production activates NADPH oxidase, creating a positive feedback loop that substantially increases ROS and reduces NO bioavailability, thereby contributing to vascular pathophysiology (8). Thus a reciprocal activation of RIRR between mitochondria and NADPH oxidase can produce a feed-forward acceleration of vascular ROS generation (Fig. 1D).

Nitrate tolerance may also occur through RIRR between mitochondria and NADPH oxidase as suggested by Wenzel et al. (61). Chronic nitrate therapy results in mitochondrial oxidative stress, perhaps by inhibiting mitochondrial SOD activity (MnSOD). Mitochondria to NADPH oxidase redox signaling may increase cytosolic $O_2^{\cdot-}$, thereby quenching NO. This concept is supported by the observation that nitrate tolerance can be attenuated either by blocking mitochondrial permeability transition pore or by treatment with rotenone, an inhibitor of mitochondrial complex I (61), and raises the therapeutic possibility of using mitochondria-targeted antioxidants as a treatment for nitrate tolerance by interrupting the RIRR tolerance mechanism.

Complex RIRR

NADPH oxidase regulates the conversion of xanthine dehydrogenase to the O₂^{•-}-generating enzyme, XO. Xanthine oxidoreductase is a critical enzyme in the metabolic pathway of purine degradation, catalyzing the oxidation of hypoxanthine to xanthine and xanthine to uric acid. It exhibits either dehydrogenase (XDH) or oxidase (XO) activities, depending on its redox state. The enzyme normally exists as XDH, which transfers electrons to NAD⁺. However, XDH can undergo redox modulation to XO, which reduces oxygen to O₂^{•-} and H₂O₂ (15). McNally et al. (37) determined that excess ROS generation in cultured endothelial cells during oscillatory shear stress is dependent on XO (37). NADPH oxidase is known to induce proteolytic cleavage of XDH to XO. Oxypurinol, a specific blocker of XO, or cells lacking NADPH oxidase activity both show reduced O₂^{•-} production in vascular areas of oscillatory shear (Fig. 2A) (37).

NADPH oxidase-derived ROS uncouples endothelial NOS, thereby increasing ROS production. Another interenzymatic form of RIRR links NADPH oxidase and NOS. Landmesser et al. (25), using a rodent model of salt-induced hypertension, showed that O₂^{•-} generated from NADPH oxidase reacts with the product of NOS, NO, forming ONOO⁻, which oxidizes the critical NOS cofactor, tetrahydrobiopterin, to dihydrobiopterin and biopterin. This induces a conformational change in the NOS enzyme subunits that uncouple the oxidase and reductase domains, allowing a diversion of electrons directly to oxygen. This process converts endothelial NOS from a NO-forming (and O₂^{•-} scavenging) enzyme to one that generates O₂^{•-}. The resulting reduction in NO bioavailability impairs endothelium-dependent vasorelaxation (Fig. 2B).

ROS-generating enzymes can overwhelm or otherwise diminish the efficacy of antioxidant defenses, leading to increased ROS production. An imbalance between ROS production and ROS decomposition constitutes an important form of RIRR. Using a well-established model of oxidative stress, global ischemia, and reperfusion, Brown et al. (4) demonstrated that oxidation of GSH to the disulfide form (GSSG) reduced the GSH-to-GSSG ratio (4). This resulted in an accumulation of ROS in the mitochondria of isolated cardiomyocytes from guinea pigs and oscillations in mitochondrial membrane potential that activated sarcolemmal K_{ATP} channels leading to arrhythmias (4). This study used diamide, a thiol-selective oxidant that promotes protein S-glutathiolation and chemically oxidizes GSH without directly producing ROS. Reduced GSH plays an important role in the detoxification of H₂O₂ via GSH-Px. Decreasing GSH-to-GSSG ratio with diamide leads to an accumulation of H₂O₂ and a collapse of the mitochondrial membrane potential, followed by increased ROS production (4). The loss of membrane potential and increase in GSSG was prevented with a ligand of the mitochondrial benzodiazepine receptor 4'-chlorodiazepam, resulting in the attenuation of associated arrhythmias (3, 4). High specificity of diamide for oxidation of internal GSH to GSSG has been confirmed by a recent study reporting similar biological responses in the diamide and H₂O₂ data sets (18). The effect of diamide can be inhibited by exogenous catalase (67). However, the pharmacological properties of diamide include cell stiffening [see review (11)], making this a complex, probably nonphysiological stimulus.

Valdez et al. (58) studied intramitochondrial pathways of ONOO⁻ disposition and found that supplementation of sub-mitochondrial particles with relatively low (1–3 μM) concentrations of ONOO⁻ elicit mitochondrial O₂^{•-} formation,

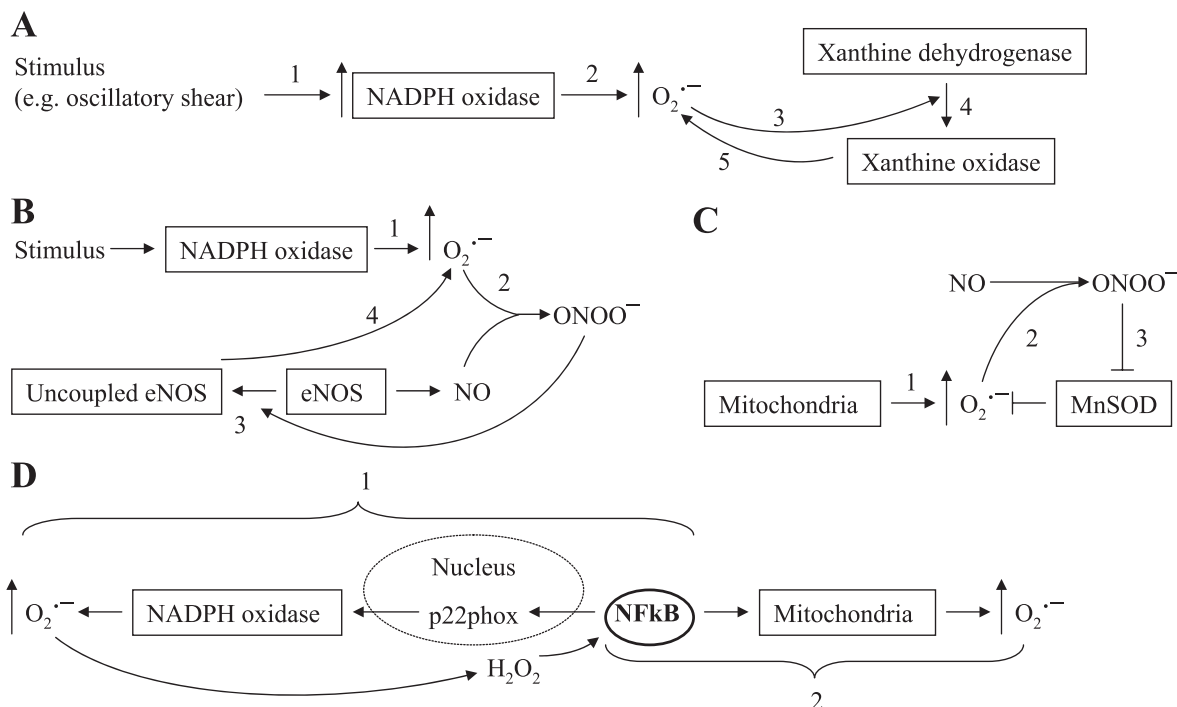


Fig. 2. A–D: sequence of events illustrating complex RIRR. ONOO⁻, peroxynitrite; NO, nitric oxide; eNOS, endothelial NO synthase.

whereas the addition of relatively high ONOO⁻ concentrations (200 μM) result in protein nitration. Previously, it has been reported that ONOO⁻ inactivates MnSOD, therefore eliminating the decomposition of O₂⁻. The excess O₂⁻ reacts with mitochondrial NO and generates more ONOO⁻, followed by an increased nitration and oxidation of mitochondrial proteins (38). The damaging effects of ONOO⁻ can be attenuated by the addition of reductants, including ascorbic acid, ubiquinol, uric acid, and GSH (Fig. 2C) (58).

ROS-induced activation of transcription factors that stimulate production of ROS-producing enzymes. ROS-induced signaling includes nuclear transcription, which can serve as a mechanism of RIRR. NF-κB signals an increase in the redox state and regulates the expression of genes involved in inflammation and oxidative stress (65, 68). In 1991, Shreck et al. (49) were the first to demonstrate that H₂O₂-treatment of Jurkat T cells results in a rapid activation of NF-κB. ROS induction of NF-κB occurs by the activation of inhibitory κB (IκB) kinases and IκB phosphorylation and degradation (10, 48). In hypertensive Dahl salt-sensitive rats, ANG-II stimulation of ROS resulted in the activation of NF-κB signaling pathway (68). Manea et al. (34) demonstrated the involvement of NF-κB in the regulation of NADPH oxidase subunit p22^{phox} in human aortic smooth muscle cells. The p22^{phox} subunit is essential for the activation of NADPH oxidase enzymes and is associated with an increased production of O₂⁻ in human coronary arteries in the presence of atherosclerosis (51). The transcriptional regulation of p22^{phox} occurs via NF-κB cis-acting elements located in the promoter of p22^{phox} gene (Fig. 2D). Therefore, NF-κB modulates the formation of NADPH oxidase subunits, leading to an increase in production of O₂⁻ and H₂O₂. Since H₂O₂ is involved in the activation of NF-κB (49), the ROS generated by NADPH oxidase will upregulate NF-κB, which in turn increases NADPH oxidase activity, creating a positive feedback mechanism for ROS production (Fig. 2D) (34).

NF-κB may also act nontranscriptionally to elicit ROS production. Recent findings of Mariappan et al. (35), using an obese mouse model of type II diabetes (*db/db*), suggest that NF-κB enters into the mitochondria of *db/db* mice, generating excess mitochondrial O₂⁻ by a nontranscriptional mechanism with decreased ATP production and complex III activity. They also found that NF-κB gene and protein expressions are elevated in *db/db* animals and demonstrated a potential therapeutic effect of NF-κB blockers in reducing mitochondrial oxidative damage and protecting against cardiac dysfunction (Fig. 2D) (35). However, we must note that because of the complexity of NF-κB signaling and lack of selective inhibitors, the NF-κB signaling pathway is still not completely understood. Studies examining the effect of ROS on NF-κB signaling in diverse cells and tissues reveal no consistent redox responses (46), suggesting that redox effects might be stimulus and cell type specific.

A recent study revealed a feed-forward interaction between TNF-α and NF-κB via the IKK-β pathway and established a link between the activation of IKK-β and oxidative stress, resulting in endothelial dysfunction in type 2 diabetes (65). The increased production of TNF-α was associated with NF-κB activation, which in turn promoted TNF-α expression and elevated O₂⁻ production by NADPH oxidase, resulting in NO

scavenging, ONOO⁻ formation, and endothelial dysfunction in diabetic mice (13).

In cultured rat aortic smooth muscle cells, H₂O₂ stimulated the expression of Ets-1, a well-known mediator of vascular inflammation and remodeling (2). Prior incubation of the cells with polyethylene glycol catalase blocked Ets-1 expression (30). Using a model of carotid artery balloon injury, Feng et al. (9) and others demonstrated that nuclear Ets-1 expression is increased in adventitial and perivascular regions in injured arteries and identified downstream targets of Ets-1, including monocyte-specific chemokine monocyte chemoattractant protein-1 and adhesion molecules (9). In the study conducted on human aortic smooth muscle cells, Ets-1 has been shown to regulate the expression of NADPH oxidase subunit p47^{phox}, therefore promoting vascular remodeling and ROS generation in response to ANG II (44). This effect was blocked by dominant-negative Ets-1 peptide, suggesting a role of Ets-1 as a critical transcriptional mediator of ROS production (44). The results of these studies allow us to propose a potential pathway in the setting of vascular inflammation where H₂O₂-stimulated Ets-1 expression results in the activation of NADPH oxidase and ROS generation.

ROS in inflammation: ROS-induced injury begets further ROS formation. Increased ROS levels are a ubiquitous consequence of chronic inflammation. The role of proinflammatory cytokines in activating NADPH oxidase is well described [see review (56)]. In microvascular endothelial cells, ROS produced by NADPH oxidase increase the expression of inducible NOS (iNOS) (63). In a murine model of abdominal aortic aneurysms (AAAs), both NADPH oxidase and iNOS activity are mechanistically linked to inflammation and aortic wall degeneration (64). In human patients with AAAs, an increased expression of NADPH oxidase and elevated O₂⁻ levels were detected not only in the regions of inflammatory cell infiltration but also within the vessel wall responsible for degradation of the extracellular matrix [stimulation of matrix metalloproteinase-2 (MMP-2)] and apoptosis of smooth muscle cell (64). The likely culprit mediating these responses in AAAs is ONOO⁻, produced from the interaction between NO (from iNOS) and O₂⁻ (from NADPH oxidase) (40, 64). The direct infusion of ONOO⁻ has similar proteolytic effects on extracellular matrix proteins (54). This sequence of events is consistent with the RIRR pathway, whereby proinflammatory cytokines activate NADPH oxidase, resulting in increased O₂⁻ production. This together with NO generates ONOO⁻, which in turn activates MMP-2, resulting in the remodeling of the extracellular matrix and the progression of AAAs.

Conclusions

Enhanced oxidative stress does not always emanate from a single cellular source but may represent a complex interplay among prooxidant enzymes, antioxidants, nuclear transcription, and disruption of normal electron transfer in key regulatory enzymes. The complexity of this system affords several potential advantages to the cell: 1) amplification of ROS generation enhances the efficiency of the process, 2) interenzymatic stimulation allows stereospecific intracellular targeting of ROS generation where small amounts of ROS from one site can activate larger amounts from a separate intracellular site, 3) one ROS can stimulate the production of another

distinct ROS within the same cell, and 4) better temporal control of ROS generation in response to diverse stimuli can be achieved. Future studies of the molecular mechanisms of RIRR should suggest treatments that allow a tighter control of ROS production, targeting of pathological sources of ROS, and preserving physiological ROS signaling.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-080704 and HL-094971.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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