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Redox signaling in cardiovascular health and disease

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Abstract

Spatiotemporal regulation of the activity of a vast array of intracellular proteins and signaling pathways by reactive oxygen species (ROS) governs normal cardiovascular function. However, data from experimental and animal studies strongly support that dysregulated redox signaling, resulting from hyper-activation of various cellular oxidases or mitochondrial dysfunction, is integral to the pathogenesis and progression of cardiovascular disease (CVD). In this review, we address how redox signaling modulates the protein function, the various sources of increased oxidative stress in CVD, and the labyrinth of redox-sensitive molecular mechanisms involved in the development of atherosclerosis, hypertension, cardiac hypertrophy and heart failure, and ischemia–reperfusion injury. Advances in redox biology and pharmacology for inhibiting ROS production in specific cell types and subcellular organelles combined with the development of nanotechnology-based new in vivo imaging systems and targeted drug delivery mechanisms may enable fine-tuning of redox signaling for the treatment and prevention of CVD.

Keywords

Reactive oxygen species; NADPH oxidases; Mitochondria; Endothelial dysfunction; Atherosclerosis; Free radicals

Introduction

Reactive oxygen species (ROS)[1] are formed from incomplete reduction of oxygen during normal respiration in all aerobic organisms. ROS are highly reactive and include free radicals containing one or more unpaired electrons, such as superoxide ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$), and nonradicals such as hydrogen peroxide (H_2O_2). It is estimated that between 0.2 and 2.0% of molecular oxygen consumed by the mitochondria in vitro may be converted to $O_2^{\bullet -}$ by the electron transport chain, but the amount of $O_2^{\bullet -}$ produced in vivo may be far less [1,2]. In addition to mitochondrial respiration, $O_2^{\bullet -}$ is generated by NADPH oxidases (Nox's), uncoupled nitric oxide synthase, xanthine oxidase, lipoxygenases, myeloperoxidase, and cytochrome P450 isozymes. Because ROS production is inherent to normal physiology, cells have evolved both enzymatic and nonenzymatic antioxidant defense mechanisms to scavenge ROS and to maintain redox balance. A shift in redox

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homeostasis to an imbalance between ROS generation and endogenous antioxidant mechanisms results in oxidative stress, which has been implicated in the pathogenesis of various diseases including those of the cardiovascular system.

Reactive oxygen species in the cardiovascular system

A vast array of data from cell culture studies and experimental animal models as well as human studies supports the role of oxidative stress in the development of cardiovascular diseases such as atherosclerosis, hypertension, cardiac hypertrophy, heart failure, and ischemia-reperfusion injury [3–12]. Upregulation of oxidative stress markers has been shown to predict cardiovascular diseases [13–16]. However, data from a majority of randomized clinical trials and meta-analysis studies failed to show any preventive effect of antioxidant vitamins on the pathogenesis of cardiovascular diseases or mortality [17–20]. The ineffectiveness of antioxidants highlights the complexity of redox reactions in biological systems including vascular cells and the limitations of our current approaches to modulating the redox signaling to effect positive outcomes against cardiovascular diseases.

Oxidative stress causes cellular damage by free radical-induced oxidation of lipids, proteins, and DNA Molecular oxygen, itself a radical, is sparingly reactive, as its two unpaired electrons are in different molecular orbitals and have parallel spins. One-electron reduction of oxygen produces $O_2^{\bullet -}$, which is membrane-impermeative and has a short half-life in an aqueous environment. Superoxide is rapidly dismutated to H_2O_2 by the action of superoxide dismutase (SOD) enzymes. As H_2O_2 is more stable than $O_2^{\bullet -}$ and membrane-permeative, it is important in cellular redox homeostasis and signaling. Reaction of H_2O_2 with transition metal ions such as Fe^{2+} generates $^{\bullet}OH$, a highly reactive and damaging ROS (Fenton reaction). Catalase, glutathione peroxidase, and per-oxiredoxins reduce H_2O_2 to water. Nitric oxide ($^{\bullet}NO$) is another important ROS in the cardiovascular system generated by the endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) enzymes. When both are produced in the cells, $O_2^{\bullet -}$ reacts with $^{\bullet}NO$ at a much faster rate than with SOD, generating peroxynitrite ($ONOO^-$), a potent oxidizing radical in vascular cells. Myeloperoxidase secreted by neutrophils and monocytes can amplify the oxidative potential of H_2O_2 at physiological chloride concentrations by generating hypochlorous acid (HOCl), which is a strong oxidant that causes chlorination of tyrosine and oxidation of lysine, cysteine, and methionine (discussed later) [21–24].

Redox signaling

The term “redox signaling” describes a process in which physiological levels of ROS/reactive nitrogen species (RNS) induce modifications to proteins that are discrete, site-specific, and reversible [25]. Data accumulated over the past 2 decades provide evidence that ROS modulate the activity of a vast array of intracellular proteins and signaling pathways and this redox signaling is spatially and temporally regulated to generate specific effects. Even in the case of phagocytosis, wherein the microbicidal action has hitherto been attributed to the direct action of ROS generated by the concerted action of NADPH oxidase and myeloperoxidase, it is now revealed that the destruction of the invading pathogen is achieved by stimulation of cellular signaling pathways involved in the activation of

proteases consequent to the ROS-induced increase in anionic charge and its compensation by the surge of K^+ ions [26].

The reversible modification of the sulfur-containing amino acids methionine and cysteine serves as a posttranslational mechanism for the regulation of protein functions. ROS-induced oxidation of the sulfur atom in methionine yields methionine sulfoxide, which can be reduced back to methionine by methionine sulfoxide reductase (Msr) in a thioredoxin-dependent reaction. Methionine oxidation together with tyrosine chlorination of apolipoprotein A-1 (apoA-1) caused by ROS impairs the ABCA1 (ATP-binding cassette transporter A1)-dependent cholesterol efflux activity of apoA-1, which might enhance foam cell formation and atherogenesis [27]. Methionine oxidation is completely reversed by Msr, suggesting that in vivo modulation of this enzyme might prevent the loss of ABCA1 activity of apoA-1 under oxidative stress conditions and attenuate atherosclerosis. Angiotensin II (AngII)-induced oxidation of methionine 281/282 activates CaMKII (calcium/calmodulin-dependent protein kinase II), causing myocardial apoptosis in vitro and in vivo [28]. Further supporting the regulatory role of methionine oxidation in cardiac remodeling, AngII-stimulated CaMKII oxidation, cardiomyocyte apoptosis, and cardiac dysfunction are enhanced in $MsrA^{-/-}$ mice. In addition to increased oxidative stress, $MsrA^{-/-}$ mice have a decreased life span [29].

The main mechanisms by which ROS generate specific cellular effects, however, are the posttranslational covalent modification of cysteine thiols within the active and allosteric sites of proteins, oxidation of iron-sulfur cluster-containing proteins, S-glutathi-onylation (disulfide link between protein thiol and glutathione), and S-nitrosylation/S-nitrosation ($^{\bullet}NO$ reacts with a thiol radical or nitrosonium ion reacts with protein thiolate to form protein-S-nitrosothiols) [30,31]. Redox signaling can induce acute effects, such as when the target proteins are ion channels and contractile proteins, or long-term effects, when the target is a protein kinase or a redox-sensitive transcription factor [30].

Modulation of protein function via alteration of cysteine thiols by H_2O_2 influences a wide variety of signal transduction cascades and diverse biological processes. H_2O_2, at low-micromolar concentrations, oxidizes catalytic cysteine residues in proteins first to generate sulfenic acid (SOH) and then disulfides (SS) [32,33]. SS formation can occur between two adjacent cysteines (intrapro-tein), between two proteins (interprotein), or as a mixed disulfide formed between a protein thiol and glutathione (S-glutathionyla-tion). Protein thiols and disulfides can undergo further oxidation by H_2O_2 to generate sulfinic (SO_2^-) and sulfonic (SO_3^-) acids. In addition, cysteine thiols can undergo $^{\bullet}NO$-dependent electrophilic and oxidative modification (S-nitrosylation) to generate protein-S-nitrosothiol (SNO), which with further oxidation can form SOH, SS, SO_2^-, and SO_3^- [34]. Oxidized or nitrosylated cysteine thiols in the cells are reduced back to cysteine by several enzymatic and nonenzymatic systems. For example, sulfenic acids, protein disulfides, and protein-S-nitrosothiols are reduced by thioredoxin, and thioredoxin reductase and S-glutathionylated protein cysteines by glutaredoxin [34,35]. Cysteine sulfinic acids, formed by the hyperoxidation of active-site Cys residues in typical 2-Cys peroxiredoxins, can be reduced by the enzyme sulfiredoxin. However, they might be irreversibly oxidized to cause damage to most proteins [36–38]. Sulfonic acids are an example of irreversible protein modification

and a marker of cumulative oxidative stress [39]. Theoretically, the posttranslational modifications of cysteines could be impeded by antioxidant enzymes as they can remove ROS before protein modification occurs. Peroxiredoxins, by virtue of their ubiquitous presence, abundance, and high rate constants, reduce H_2O_2 and other hydroperoxides far more efficiently than any other thiol-containing proteins, impeding cysteine modifications [40]. The cysteine sulfinic acid generated in this reaction in the case of glutathione-dependent peroxiredoxins rapidly forms disulfide with glutathione, which is then recycled to the reduced state by glutaredoxin or ascorbic acid. In thioredoxin-dependent peroxiredoxins, the sulfenic acid rapidly reacts with proximal thiols to form a homo-intermolecular disulfide, which is recycled to the reduced state by thioredoxin [41–44]. The progression from reversible S-nitrosylation to SOH, SS, SO_2^-, and irreversible SO_3^- represents a graded transition of cellular signaling from adaptation and maintenance of cellular redox state in the face of nitrosative and oxidative stress to toxicity [34].

$^{\bullet}$NO also modulates protein function by targeting cysteine thiols in peptides and proteins, and S-nitrosylation is a principal mechanism by which $^{\bullet}$NO regulates signaling cascades across a multitude of protein classes [34]. The basis for S-nitrosylation specificity is not in the primary sequence of the target proteins, as high-throughput proteomic approaches failed to identify a linear Cys-flanking motif that predicts stable trans-nitrosylation of cysteines across various protein classes [45]. The proximity of a protein Cys to NOS may be a determinant of S-nitrosylation [46,47], whereas the electrostatic environment, hydrophobicity, and contiguity and orientation of aromatic amino acid chains arising from the tertiary protein structure and protein-protein interactions also regulate S-nitrosylation and denitrosylation [34,48]. Redox modification of active-site thiols is a principal mechanism for dynamic posttranslational regulation of all major protein classes, including phosphatases, kinases, transcription factors, ion channels and transporters, cytoskeletal and structural proteins, GTPases, metabolic and antioxidant enzymes, and respiratory proteins [31,34].

Phosphatases

Protein tyrosine phosphorylation is a key regulatory mechanism in signal transduction, affecting many cellular functions. Sundaresan et al. [49] demonstrated a correlation between the magnitude and duration of an increase in H_2O_2 levels and the protein tyrosine phosphorylation in VSMCs treated with various growth factors. They hypothesized that increased protein tyrosine phosphorylation was due to the transient inactivation of PTPs. Several subsequent papers provided the evidence for redox regulation of PTPs by growth factor-induced ROS. Lee et al. [50] elucidated reversible inactivation of PTP1B in A431 cells treated with epidermal growth factor (EGF), and later PTP1B inactivation was attributed to oxidation by H_2O_2, as inhibition of its accumulation prevented protein tyrosine phosphorylation [51]. Reversible inactivation of Src-homology 2 domain-containing PTP (SHP2) in VSMCs treated with platelet-derived growth factor (PDGF) requires association with the PDGF receptor and is necessary for the receptor activation [52]. Treatment of VSMCs with antioxidants increased growth factor-induced activity of SHP2 and several other PTPs, further confirming their redox regulation [53,54]. It was demonstrated that rapid inactivation of PTP family members by low-micromolar concentrations of H_2O_2 as a result

of the oxidation of the essential catalytic cysteine residue to a Cys-SOH intermediate renders the PTPs inactive against phosphorylated substrates [29]. Unlike most cysteines in cellular proteins, which have a pK_a > 8.0 at physiological pH, the catalytic cysteine residues of PTPs have low pK_a (> 6.0) and are present in thiolate form at physiological pH, making them extremely reactive [55,56]. The thiolate nucleophile attacks the electrophilic phosphorus atom of the substrate, removing the phosphate group and forming an enzyme thiol-phosphate intermediate [57]. However, because of their strong nucleophilic character, the thiolate anions are susceptible to oxidation by H_2O_2.

The Cys-SOH intermediate formed during PTP1B oxidation is rapidly converted into a sulfenyl-amide species by covalent linking of a sulfur atom of the catalytic cysteine with the main chain nitrogen of an adjacent residue [58,59]. This results in large conformational changes in the catalytic site, which inhibit substrate binding as well as protecting it from irreversible oxidation to sulfonic acid and allowing redox regulation of the enzyme by promoting its reversible reduction by thiols. Glutathionylation of the Cys-SOH also protects PTP1B from irreversible oxidation [60]. Other PTPs are protected from irreversible oxidation by the formation of a disulfide either between two vicinal cysteines in the catalytic site, as reported for LMW-PTP [61,62], or between the catalytic cysteine and a nearby backdoor cysteine, as observed in Cdc25 [63], RPTPα [64], and PTEN [65]. In the case of the SHPs, rereduction of Cys-SOH is dependent on the formation of an intramolecular disulfide between two conserved backdoor cysteines [66]. Meng et al. [54] demonstrated that reversible oxidative inactivation of SHP2 is necessary for PDGF-induced mitogenic signaling in fibroblasts. Induction of endogenous •NO and exposure to •NO donors inhibited the activity of several cellular PTPs, including those in endothelial cells [67–69]. However, S-nitrosylation of the catalytic cysteine in PTP1B protected it from ROS-induced irreversible oxidation [70].

Protein kinases

In addition to indirect regulation resulting from the concomitant inhibition of PTPs, both receptor (RTK) and nonreceptor tyrosine kinases also undergo oxidation-dependent activation. Examples of RTKs that undergo direct oxidation include insulin, EGF and PDGF receptors, and Ret kinase [71]. Schmid et al. [72] reported that increased kinase activity and insulin responsiveness of the insulin receptor (IR) may require “redox priming” and results from a decrease in IR β-chain sulfhydryl groups due to oxidation. In fact, 3-D models of the IR showed that conversion of any of the four cysteine residues (1056, 1138, 1234, and 1245) into sulfenic acid produces conformational changes, bringing Tyr1158 into close contact with Asp1083, which renders the catalytic site at Asp1132 and Tyr1162 accessible and facilitates its autophosphorylation in the activation loop [73].

Among the nonreceptor tyrosine kinases, Src is regulated by many stimuli that generate ROS, including hypoxia/reoxygenation, stretch, integrins, growth factors, and vasoactive agonists such as AngII and thrombin [74]. Hypoxia-induced mitochondrial ROS production activates Src in VSMCs, resulting in increased hypoxia-inducible factor 1α (HIF1α) expression [75]. Antioxidant-inhibitable Src activation was observed in endothelial cells subjected to cyclic strain or H_2O_2 treatment [76]. Integrin-stimulated Src activation was

biphasic, with an early activation phase driven mainly by Tyr527 dephosphorylation mediated by PTPa and a subsequent Tyr418 autophosphorylation. The late phase involves oxidation of Cys245 and Cys487 by H_2O_2, resulting in the hyper-phosphorylation of Tyr418 and further activation of the kinase [77]. Src is also activated in a Tyr527-independent manner by nitrosylation and via generation of an intermolecular S-S bond, resulting in aggregation of adjacent Src molecules and Tyr416 autophosphorylation [78].

Serine/threonine kinases, such as protein kinase C (PKC), undergo redox regulation by direct oxidation of cysteine residues. All 12 PKC isozymes contain cysteine residues in the regulatory as well as catalytic domains and the stimulation or inhibition of the enzyme depends on which of the cysteines undergo redox modification [79,80]. Low levels of ROS oxidize cysteine residues in the regulatory region, promoting the release of zinc and forming intramolecular disulfide bonds, which causes Ca^{2+}, diacylglycerol, or other lipid-independent activation of PKC by the dissociation of autoinhibitory pseudo-substrate [79–82]. In contrast, oxidation of catalytic domain cysteines inhibits PKC activity [80]. On the other hand, high-glucose-induced activation of PKC increased ROS production and cyclooxygenase 2 expression and reduced •NO availability and altered prostanoid expression, causing endothelial dysfunction [83]. ROS-induced PKC activity also regulates VCAM-1-dependent lymphocyte transendothelial migration [84]. Cyclic AMP-dependent (PKA) and cyclic guanosine monophosphate (cGMP)-dependent (PKG) protein kinases are also redox-sensitive and undergo cyclic nucleotide-independent activation by forming an interprotein disulfide linking two subunits in cells on exposure to H_2O_2 [85,86]. PKG activation represents one mechanism by which H_2O_2 can act as a vasorelaxant in the cardiovascular system [76]. In addition, activities of protein kinases such as Akt [87] and JNK1 [88] are regulated by S-nitrosylation of cysteine residues, resulting in their inactivation. Independent of classic regulation by guanine nucleotide exchange factors and GTPase-activating proteins, oxidizing agents also regulate the activity of GTPases. Lander et al. [89] showed for the first time that S-nitrosylation of Cys118 enhanced the activity of Ras by promoting the exchange of GDP for GTP. Adachi et al. [90] demonstrated that S-glutathionylation of Cys118 regulates AngII-induced hypertrophic signaling in VSMCs. More recently, Burridge's group [91] showed that oxidation of Cys16 and Cys20 in the phosphoryl binding group activates RhoA and induces stress fiber formation in fibroblasts exposed to oxidants, suggesting that redox regulation of GTPases is a widespread signaling mechanism.

Transcription factors

Redox regulation of transcription factors such as NF-κB, nuclear factor E2-related factor-2 (Nrf2), AP-1, p53, and HIF plays an important role in vascular homeostasis and pathogenesis [31,92]. NF-κB regulates gene expression in immunity, stress responses, and inflammation, including in endothelial cells and cardiac myocytes [93]. Inhibitors of NF-κB (IκB) bind the inactive NF-κB p50–p65 heterodimer, the prototype of NF-κB family, and sequester it in the cytoplasm under basal conditions. Under oxidative stress conditions, activation of IκB via phosphorylation of Ser32 and Ser36 residues by inhibitory κB kinases (IKKs) targets IκB for ubiquitination and proteasomal degradation, allowing NF-κB to translocate to the nucleus and modulate gene expression [94,95]. Redox regulation of NF-

κB is complex, as Cys62 of the p50 subunit is oxidized in the cytoplasm, and its reduction, by thioredoxin or possibly by Ref-1, is essential for its DNA binding in the nucleus [96–98]. The IKK complex contains catalytic IKKa and IKKp sub-units and a noncatalytic IKKγ subunit. The Cys178 and Cys179 in the kinase domains of the IKKα and IKKβ, respectively, regulate enzyme activity by promoting phosphorylation of activation-loop serines and interaction with ATP [99–101]. These cysteine residues also mediate redox regulation of NF-κB activity, as direct binding of electrophilic compounds to them inhibits enzyme activity and this inhibition was reversed by reducing agents. S-glutathionylation of Cys179 in the IKKβ also regulates reversible inhibition of NF-κB by endogenous H_2O_2 [102]. S-nitrosylation of the Cys179 in IKKβ inhibits the enzyme activity and NF-κB stimulation, a mechanism by which •NO exerts its anti-inflammatory efforts [103]. In addition, S-nitrosylation of Cys62 in the p50 subunit inhibits NF-κB-dependent DNA binding, promoter activity, and gene transcription [104,105].

The consensus DNA *cis* elements to which NF-κB dimers bind are known as " κB sites" (5'-GGGRNWYYCC-3', where R is A or G, N is any nucleotide, W is A or T, and Y is C or T) and are present in the promoter/enhancer regions of many target genes that regulate a diverse array of functions, including inflammation, proliferation, angiogenesis, matrix degradation, and pro- as well as antiapoptosis [93,106,107]. In cardiomyocytes, functional NF-κB signaling pathways are essential for protection against apoptosis induced by cytokines and acute myocardial ischemia [108,109]. However, chronic NF-κB activation under pathophysiological settings such as heart failure exacerbates cardiac remodeling by stimulating proinflammatory and profibrotic genes and inducing myocytes apoptosis [110]. The endothelial NF-κB signal transduction system is primed for activation in regions of disturbed flow and its activity is increased by exposure to stimuli that enhance atherosclerosis [111]. Further support for NF-κB in atherogenesis is evident from the reports that its activation regulates cytokine-induced expression of the cellular adhesion molecules vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in endothelial cells [112,113]. NF-κB is activated by H_2O_2 in endothelial cells [114], whereas its activity is inhibited in H_2O_2-treated epithelial cells [92], which suggests that redox regulation of NF-κB and its attendant effects on cellular outcomes are determined by the duration and cellular context [31,93].

Nrf2 is another redox-sensitive transcription factor that helps maintain cellular redox homeostasis by upregulating antioxidant and phase II detoxifying enzymes under oxidative and electrophilic stress conditions [115]. The gene upregulation is achieved by the interaction of Nrf2 with electrophile and antioxidant (ARE) response elements and the upregulated genes include heme oxygenase-1 (HO-1), the catalytic subunit of glutamate-cysteine ligase, glutathione S-transferase, and NAD(P)H:quinine oxidore-ductase 1. Nrf2 activation and induction of downstream antioxidant genes confers protection against oxidative stress in cardiomyocytes and VSMCs and inhibits vascular inflammation [116,117]. Activation of Nrf2-dependent antioxidant gene expression by advanced glycation end products may protect the endothelium against chronic oxidative stress in diabetes [118]. Furthermore, atheroprotective laminar flow activates, whereas proatherogenic oscillatory flow inhibits, Nrf2 activity in human endothelial cells, underlying the importance of Nrf2-

regulated gene expression in vascular homeostasis [119,120]. Under redox conditions where there may be a limited availability of tetrahy-drobiopterin (BH_4), the eNOS cofactor, Nrf2 activation maintains endothelial homeostasis by downregulating eNOS levels via increased HO-1 activity and thus maintaining stoichiometric balance between BH_4 and eNOS [121].

Nrf2 is sequestered in the cytoplasm under basal conditions by a cysteine-rich protein, Kelch-like ECH-associated protein 1 (Keap1), which binds to the Neh2 domain of Nrf2 and targets it for ubiquitin-dependent proteasomal degradation [122,123]. Two cysteine residues in Keap1, Cys273 and Cys288, are necessary for the ubiquitination of Nrf2. Electrophiles and oxidants disrupt the Keap1–Nrf2 complex, perhaps by the oxidation of Cys273 and Cys288, leading to stabilization and enhanced nuclear localization of Nrf2 and increased transcription of ARE-containing genes [124]. Furthermore, Cys151 in Keap1 is required for inhibition of Nrf2 degradation during oxidative stress, perhaps by inducing confor-mational changes. Fourquet et al. [125] reported that intermole-cular disulfide formation between Cys152 residues by ROS and RNS results in Keap1 inactivation and Nrf2 stabilization. The same is observed with simultaneous inactivation of the thioredoxin and glutathione pathways.

AP-1 regulates gene expression in cells in response to a broad spectrum of environmental stimuli, including oxidative stress. It is a dimer consisting of members of the Jun and Fos families, which complex through a leucine zipper domain into homo (Jun/Jun) or heterodimers (Jun/Fos) [126,127]. Dimerization juxtaposes the conserved basic regions of constituent proteins, forming a bipartite DNA-binding domain. Classic regulation of the activity of AP-1, either by an increase in the transcription of the *Fos* and *Jun* genes or by phosphorylation of the Fos and Jun proteins, often occurs downstream of redox-sensitive protein kinase activation [128,129]. AP-1 activity is also regulated in a redox-sensitive manner, as a conserved cysteine residue in the DNA-binding domains of the Fos and Jun proteins is susceptible to oxidation resulting in the loss of DNA binding [130]. These data are supported by the loss of redox regulation observed when the conserved cysteine is substituted by a serine residue [131]. The c-Jun binding domain contains one cysteine residue (Cys269) in the basic region that directly binds DNA and another (Cys320) close to the leucine zipper domain [132]. A decrease in the ratio of reduced/oxidized glutathione under oxidative stress conditions induces S-glutathiolation of Cys269 and the formation of an intermolecular disulfide bridge between Cys320 residues, with the former enabling reversible redox regulation of c-Jun DNA binding. In addition, reversible *S*-nitrosoglutathione-dependent S-glutathiolation of Cys269 may regulate c-Jun DNA binding [133]. We and others have demonstrated that AP-1 regulates vasoactive agonist-induced expression of adhesion molecules such as CD44 in VSMCs in a redox-sensitive manner [134,135]. In isolated hearts, an increase in AP-1 activity correlates with the duration of ischemia and reperfusion, whereas in adapted myocardium AP-1 activity is at the basal level, which indicates that AP-1 stimulates oxidative stress-induced apopto[136].

Several lines of evidence suggest that DNA binding or tran-scriptional activity of p53 is highly prone sis to oxidative inactivation. For example, DNA binding of p53 to its cognate sequence in vitro requires reductants such as 2-mercaptoethanol or dithiothreitol in the binding buffers and is sensitive to H_2O_2 and other oxidants such as diamide [137]. In

addition, pharmacological oxidizing and reducing agents modulate gene transactivation by p53 in human cells [138]. More recently, Velu et al. [139] demonstrated that S-glutathionylation, in addition to other posttranslational modifications such as site-specific phosphorylation, ubiquitination, and 182 as the sites of sumoylation [140], governs the activity of p53 under stress conditions. Whereas most of the posttranslational modifications of p53 after genotoxic stress enhance its transcriptional competency to induce cell cycle checkpoints, S-glutathionylation is a negative and defensive regulatory mechanism under acute stress. Even though mass spectrometry identified cysteines 124, 141, and 182 as the sites of glutathionylation, cysteine 141 is the most reactive one on the surface of the p53 [139]. In addition, molecular modeling studies showed cysteines 124 and 141 at the dimer interface of p53, and glutathionylation of either residue interferes with protein dimerization and inhibits p53-DNA association. Inhibition of DNA binding and disruption of tetramerization under mild oxidizing conditions are correlated with the formation of a disulfide bond in p53 [141]. Reduction of disulfide bonds by thioredoxin and Ref-1 reactivates oxidized p53 and stimulates p53-mediated transactivation [142,143]. Interestingly, redox regulation of p53 in turn modulates cellular redox status. Sablina et al. [144] reported that low levels of p53 in unstressed or physiologically stressed cells upregulate several genes with antioxidant products, resulting in a decrease in intracellular ROS levels. In contrast, downregulation of p53 causes oxidative DNA damage and mutagenesis, which are prevented by an antioxidant supplement.

The role of endogenous p53 in atherosclerosis is controversial. p53 levels, cell proliferation, and apoptosis are predominant in human plaque areas with chronic inflammation [145]. An increase in macrophage p53 levels is associated with the enlargement of necrotic core, plaque rupture, and transient ischemic attacks in patients with carotid atherosclerosis [146]. Adenoviral overexpres-sion of p53 increased VSMC apoptosis and induced plaque rupture in preexisting atherosclerotic lesions [147]. However, Mercer et al. [148] demonstrated that endogenous p53 reduces atherosclerosis by protecting VSMCs and stromal cells from death and promoting apoptosis in macrophages. These data are supported by the observation of Boesten et al. [149] that macrophage-specific deletion of p53 enhances plaque vulnerability by increasing the lesion macrophage area and necrotic core formation.

Physiological roles of ROS

ROS regulate many physiological functions in the cardiovascular system under normal conditions. For example, •NO mediates endothelium-dependent vasomotor tone and flow responses in many vascular beds [150,151]. It is also suggested that •NO regulates endothelium-dependent microvascular and epicardial vasodilation under metabolic stimulation [152]. The functions of •NO include inhibition of platelet aggregation, disaggregation of aggregated platelets, and inhibition of platelet as well as leukocyte adhesion to the vascular endothelium [150,153,154]. Superoxide affects vascular tone by inactivating •NO [155] as well as by dismutating to H_2O_2 [30]. Several studies suggest that H_2O_2 is the endothelium-derived hyperpolarizing factor (EDHF) that regulates vasorelaxation in murine and human mesenteric arteries and flow-induced dilation in human coronary arterioles [156–159]. H_2O_2 and other hydroperoxides stimulate the activity of cycloox-ygenase, also known as prostaglandin endoperoxide H synthase, to produce the

vasodilator prostacyclin and other prostanoids [160–162]. This effect is termed the "peroxide tone" and is evident at very low concentrations of peroxides (2–20 nM). It is suggested that eNOS regulates the EDHF-like activity of H_2O_2 and that $^{\bullet}NO$ and H_2O_2 compensate for each other to cause endothelium-dependent relaxation [156]. SOD may play a critical role in endothelium-dependent relaxation by prolonging the half-life of $^{\bullet}NO$ and by converting the vasoconstrictor $O_2^{\bullet-}$ to H_2O_2. In this context, it is worth noting that prolonged SOD2 deficiency results in decreased agonist-induced aortic relaxation and impaired aortic compliance in mice [163]. Both $^{\bullet}NO$ and H_2O_2 regulate vasomotor tone by activating the enzyme soluble guanylate cyclase (sGC) [164–166]. $^{\bullet}NO$ activates sGC by directly binding to the ferrous (Fe^{2+}) core of the heme prosthetic group, effecting a conformational change [167]. The product cGMP causes vasodilation by relaxing VSMCs, in part, by lowering Ca^{2+} by decreasing its influx, increasing efflux, promoting sequestration in the endoplasmic reticulum, and attenuating mobilization [167,168].

Oxygen homeostasis at the tissue level is vital for development, growth, and survival, and hence, cells have evolved a number of mechanisms to sense and respond to low oxygen levels. In humans, glomus type I chemoreceptor cells of the carotid body, located at the bifurcation of the carotid artery, release neurotrans-mitters in response to hypoxia and increase alveolar ventilation [169]. The neuroepithelial bodies of the intrapulmonary airways regulate hypoxic pulmonary vasoconstriction, optimizing ventilation–perfusion matching [170]. In contrast, vasodilation occurs in response to hypoxia in systemic vascular beds, such as coronary and cerebral circulations, to maintain O_2 delivery [171]. In addition, physiological adaptation to hypoxia includes activation of transcription factor HIF1 and its downstream targets. HIF1 is a basic helix-loop-helix/PAS heterodimer, with an O_2-sensitive HIF1α subunit and a constitutive HIF1β (ARNT) subunit (Fig. 1). Hydroxylation of proline residues 402 and 564 by prolyl hydro-xylases in normoxia enables HIF1α to interact with the von Hippel-Lindau tumor suppressor protein, which has ubiquitin ligase activity, and undergo degradation [172–175]. Hypoxia decreases hydroxylation of HIF1α and stabilizes it by not allowing interaction with the von Hippel-Lindau tumor suppressor protein. In cardiovascular cells, HIF1 induces the expression of genes involved in angiogenesis and vascular remodeling, energy metabolism, erythropoiesis, vasomotor reactivity, and vascular tone [176].

The mechanisms by which cells detect a decrease in O_2 levels to cause activation of HIF1 are still emerging but considerable evidence supports the role of increased mitochondrial ROS, particularly at complex III, in the induction of HIF1 under hypoxia [177–179]. Evidence in support of this notion includes increased ROS levels, as determined using fluorescent probes and ESR spectroscopy, and decreased reduced glutathione and cysteine levels [180–182]. Inactivation of Rieske iron–sulfur protein in mitochondrial complex III abrogated hypoxic stabilization of HIF1 [183]. Mansfield et al. [184] demonstrated impaired hypoxic HIF1 stabilization in murine embryonic cells lacking cytochrome c and therefore mitochondrial activity, further supporting the necessity of mitochondrial ROS in this process.

Low concentrations of ROS generated during ischemic preconditioning, in which one or more short periods of ischemia are separated by short periods of reperfusion, confer cardiac

protection by reducing necrosis and the severity of arrhythmias and improving functional recovery when challenged with a longer period of ischemia [185–187]. Exogenous ROS mimic the protective effect of ischemic preconditioning [188] and the protective effect of preconditioning is decreased in the presence of antioxidants [189,190], suggesting that ROS generation is an innate physiologic adaptive process against potentially lethal ischemic injury. Ischemic preconditioning activates a number of signaling pathways, which converge on the mitochondria, resulting in activation of the mitoK_{ATP} channel and inhibition of the mitochondrial permeability transition pore [191]. More recently it was shown that ischemic preconditioning preserves mitochondrial efficiency by decreasing H^+ leak and ROS production during ischemia-reperfusion [192].

ROS play a critical role in the activation of mechanotrans-duction signaling pathways that regulate the physiology and pathophysiology of heart function. A physiologic stretch of cardi-omyocytes, as happens in diastole, instantaneously increases ROS production via activation of Nox2 in a microtubule-dependent manner, a process termed X-ROS signaling [193]. Nox2 activation occurs in sarcolemmal and transverse tubule membranes and the resultant increase in local ROS levels sensitizes the nearby ryanodine receptors (RyR2) in the sarcoplasmic reticulum by oxidation. This triggers a burst of Ca^{2+} sparks, which causes muscle contraction and normalization of X-ROS signaling [193,194]. When the Ca^{2+} concentration drops to basal level, the muscle relaxes, completing the cycle [195]. Thus, the release and recapture of Ca^{2+} by the sarcoplasmic reticulum in each contraction-relaxation cycle underlies the heartbeat and is regulated by X-ROS signaling under normal physiological conditions [194]. Exogenous H_2O_2 reversibly increases a Ca^{2+} spark rate similar to that in a physiological stretch, and myocytes lacking Nox2 expression do not show stretch-dependent increase in ROS levels, further supporting the role of ROS in cardiac contraction and relaxation [193]. However, hyperactive X-ROS signaling may cause cardiomyopathy through aberrant Ca^{2+} release from sarcoplasmic reticulum. Various redox modifications, such as S-nitrosylation, S-glutathionylation, and disulfide crosslinking, dysregulate RyR2 and cause abnormal Ca^{2+} in several disease states [196–199]. In addition to the ryanodine receptors, the release and recapture of Ca^{2+} by the sarcoplasmic reticulum is regulated by sarco/endoplasmic reticulum ATPase (SERCA) and several accessory proteins, including phospholamban and calsequestrin [199]. SERCA, which transports cytosolic Ca^{2+} into the lumen of the sarcoplasmic reticulum in an ATP-dependent manner, is also regulated by redox mechanisms via the oxidation of cysteines or nitration of tyrosines. SERCA is activated by low (physiologic) and inactivated by high pathological levels of ROS because of irreversible oxidative modifications [200–203]. Activation of SERCA by NO decreases the intracellular Ca^{2+} concentration, relaxing cardiac, skeletal, and vascular smooth muscle. S-glutathionylation of Cys674 in SERCA2b—the major SERCA iso-form in vascular smooth muscle—by $ONOO^-$ increases its activity in vascular smooth muscle during normal endothelium-mediated relaxation [200]. The irreversible oxidation of this residue in atherosclerosis impairs NO-induced vasorelaxation. Similarly, the positive inotropic effects of $HNO^{\bullet -}$ in normal and failing hearts involves increased Ca^{2+} into the sarcoplasmic reticulum and is mediated by reversible S-glutathionylation of Cys674 in SERCA2a —the major SERCA isoform in cardiac muscle [199,204,205].

Insulin sensitivity plays a vital role in cardiovascular health, and chronic oxidative stress is implicated in the development of insulin resistance, a state of diminished response to endogenous insulin [206]. Strong experimental evidence from human and animal models shows that increased mitochondrial ROS generation induces insulin resistance [206–208] and this can be rapidly reversed with mitochondrial uncouplers, electron transport chain (ETC) inhibitors, or mitochondrial superoxide dismutase (SOD2) mimetics or by overexpression of SOD2 [208]. The mechanisms by which mitochondrial ROS might contribute to insulin resistance include activation of JNK [209,210] and apoptosis signal-regulating kinase 1 [211]. In contrast to the role of increased oxidative stress in insulin resistance, recent evidence indicates that ROS also promote insulin sensitivity. Loh et al. [212] reported that mice deficient in the cytosolic and mitochondrial antioxidant enzyme glutathione peroxidase 1 (GPx-1) were protected from high-fat diet-induced insulin resistance. The protection resulted from ROS-induced increase in PI3K/Akt signaling and consequent AS160 phosphorylation and glucose uptake in the muscle, but not from altered insulin receptor and insulin receptor substrate 1 activation. Consistent with this idea, pharmacologic depletion of glutathione in C57BL/6J mice enhanced insulin sensitivity [213]. Interestingly, mice are also protected from diet-induced obesity in both these models. These data suggest that ROS have a physiological role in insulin sensitivity, and ROS levels, difference in sensitivity of tissues to ROS, and pathophysiological background are the major determinants of impaired versus enhanced insulin sensitivity [214,215].

Sources of ROS

Mitochondria

Mitochondria are the major source of ROS in mammals under physiological conditions [1,216] and increased mitochondrial ROS production underlies cardiovascular and many other diseases [4,217–224]. The mitochondrial ETC is the main source of ROS production in mitochondria [225]. Electrons from NADH and $FADH_2$ generated in the Krebs cycle are transferred through the ETC to reduce molecular oxygen to water, a process that involves four one-electron reduction reactions. Complex IV (cytochrome c oxidase), the terminal component of the ETC, retains all the partially reduced intermediates until full reduction of oxygen is achieved. However, other complexes may leak electrons, generating $O_2^{\bullet -}$ by the partial reduction of oxygen.

Complex I and III are the main sources of $O_2^{\bullet -}$ production in mitochondria [225], with the former being the predominant source in vivo [226,227]. Whereas $O_2^{\bullet -}$ from complex I is released into the matrix, complex III-derived $O_2^{\bullet -}$ is released into both the mitochondrial matrix and the intermembranous space [228,229]. Superoxide production from complex I occurs in vivo when NADH levels are high, from damage to the respiratory chain, slow respiration, or ischemia [2]. This may occur by the mitochondrial loss of cytochrome *c*, as happens in the failing human heart [230,231], and probably by the inhibition of cytochrome c oxidase by enhanced formation of $^{\bullet}NO$ [232]. ROS production also depends on the metabolic state of mitochondria, with more $O_2^{\bullet -}$ production in State 4 respiration (low oxygen consumption, electron flow, and ATP synthesis, limiting ADP level; high $NADH/NAD^+$ ratio) than in State 3 respiration (high electron flow, fast ATP synthesis,

partial depolarization, and decreased NADH/NAD^+ ratio) [233]. It has been suggested that an increase in pathophysiological ROS levels would occur at the extremes of overall intracellular and intramitochondrial redox potential, which in turn depends on redox couples involved in ROS generation (NADH/NAD^+) and ROS scavenging (NADPH/$NADP^+$) [234]. An increase in ROS generation occurs when mitochondrial redox potential is significantly reduced, as happens in hypoxia, or significantly oxidized, as may happen during heart failure. In the latter case, the increase in ROS levels results from a depletion of antioxidant capacity as a consequence of the decrease in NADPH levels. Recently, it was shown that elevated [Na^+] in cardiomyocytes of failing hearts reduced mitochondrial Ca^{2+} by accelerating Ca^{2+} efflux and decreased NADPH levels resulting in increased mitochondrial ROS formation [235]. A decrease in Ca^{2+} during increased workload attenuates the mitochondrial antioxidant capacity by decreasing the activity of Krebs cycle dehydrogenases.

In addition to the inner membrane, ROS are also produced at other sites in mitochondria [236]. For example, the $p66^{Shc}$ protein, partially localized in the mitochondrial intermembrane space, forms a molecular complex with cytochrome c and subtracts electrons, resulting in a reduction of oxygen and formation of H_2O_2 [237]. Data from experimental animal models suggest that activation of $p66^{Shc}$ plays a role in cardiovascular pathophysiology. Mice deficient in $p66^{Shc}$ were protected against AngII-induced myocardial damage [238] and diabetic cardiomyopathy [239] and early atherogenesis induced by a high-fat diet [240].

Monoamine oxidase, existing in two isoforms (MAO A and MAO B), is a mitochondrial outer-membrane-bound flavoprotein and is another important source of mitochondrial ROS that catalyzes the deamination of neurotransmitters and biogenic amines [241]. H_2O_2 generated during degradation of serotonin by MAO A induced receptor-independent apoptosis in isolated cardiac myocytes, and MAO inhibitors significantly decreased in vivo myocardial injury during ischemia–reperfusion [242]. Increased MAO A activity coupled with high intramyocardial norepinephrine levels plays an important role in the evolution of maladaptive hypertrophy into cardiac failure [243].

NADPH oxidases

Nox's, a family of enzymes with the sole function of producing ROS, are implicated in the pathophysiology of many cardiovascular diseases [244]. The phagocyte (neutrophils and macrophages) oxidase, the first characterized NADPH oxidase, is a multicompo-nent complex that catalyzes the formation of $O_2^{\bullet -}$ during phagocytosis [245]. In the resting cell, the phagocyte NADPH oxidase has a membrane-bound catalytic core of the enzyme, flavocytochrome $b_{55}8$, and the cytosolic regulatory subunits p47phox, p40phox, p67phox, and small G-protein Rac1 or Rac2. The flavocytochrome b_{558} is a heterodimer consisting of a large glycoprotein, gp91phox (Nox2), and a small protein, p22phox, and the close association of these two proteins stabilizes the flavocytochrome [246].

Upon cell stimulation, the regulatory subunits translocate to the membrane and assemble with the flavocytochrome b_{558} to cause activation of the enzyme. In the resting neutrophils, p47phox, p67phox, and p40phox exist as a complex stabilized by SH3 domain interactions

[30,247], whereas Rac is tethered to RhoGDI, a RhoGDP-dissociation inhibitor [248]. However, binding to the flavocytochrome is prevented because p47phox exists in an autoinhibited conformation in which its tandem SH3 domains are masked owing to intramolecular interaction with the C-terminal segment. During activation, multiple serine residues in the C-terminus of p47phox are phosphorylated, liberating the N-terminal SH3 domain for interaction with the proline-rich region of p22phox and translocation to the membrane [249–252]. This allows the proline-rich activation domain in p67phox to bind with an activation sequence in the C-terminus of Nox2 to initiate electron transfer, thus activating the enzyme [30,253].

The existence of similar, albeit lower ROS-generating, oxidases in nonphagocytic cells has been identified in the past decade, with the identification of Nox1, the first homolog of Nox2 [254]. Unlike the phagocyte oxidase, the nonphagocyte oxidases are active during normal metabolism and generate low levels of ROS even in the absence of extrinsic stimulation; however, their ROS generation is increased in response to agonist stimulation. In total, the Nox family comprises seven members, each with a distinct catalytic isoform: Nox's 1–5 and Duox1 and Duox2 [246,248]. The predicted structure of Nox's 1–4 consists of an N-terminal transmembrane region with six α-helical domains containing four conserved histidines, two each in the third and fifth domain spanning two asymmetrical hemes. The cytoplasmic C-terminus dehydrogenase domain contains conserved binding sites for FAD and NADPH. Nox5 is distinct from Nox's 1–4 by the presence of a calmodulin-like EF domain with four Ca^{2+}-binding sites in the long N-terminus, which enables rapid enzyme activation in response to elevated cytosolic Ca^{2+} levels [255,256]. The Duox proteins are further different from Nox5 in containing an N-terminal perox-idase-like domain that is connected to the EF domain by an additional transmembrane domain [257–259].

The expression of Nox catalytic subunits varies among different cell types of the cardiovascular system, with more than one subunit expression in the cell types [30] (Table 1). Nox1 is mainly expressed in VSMCs [10,254,260,261], although endothelial cell [10,262] and fibroblast [10] expression was also observed. Nox2 is present in endothelial cells [10,263,264,265,266], fibroblasts [267], cardiomyocytes [268,269], and VSMCs in human resistance arteries [270]. Nox4 expression is fairly abundant in VSMCs [10,271,272,273], endothelial cells [10,264], fibroblasts [10,274], and cardiomyocytes [275,276]. Nox5 is present in human VSMCs [277] and endothelial cells [278], whereas it is absent in rodents [279]. Nox3 and Duox2 expression was not reported in cardiovascular cells, but Kalinina et al. [280] observed Duox1 expression in the human aortic VSMCs.

Like Nox2, binding with p22phox is essential for the activity of Nox1 and Nox4. For Nox1, the cytosolic regulatory subunits are NoxO1 and NoxA1, the homologs of p47phox and p67phox, respectively, as well as Rac1. However, the subunit expression and NADPH oxidase composition may vary depending on the vascular beds and species. We and others have recently shown that Nox1 interacts with p47phox and NoxA1 in mouse VSMCs [11,281]. Nox4 does not require interaction with cytosolic regulatory subunits for activity and hence is constitutively active, with regulation mainly dependent on expression levels. Nox5, Duox1, and Duox2 activities are regulated by Ca^{2+} and do not require any subunit for activation [246].

Activated NADPH oxidases (Nox1 and Nox2) generate $O_2^{\bullet -}$ by transferring two electrons from NADPH in the cytosol to FAD and then to the two heme groups, with the second heme group reducing two successive molecules of molecular oxygen on the other side of the membrane [246,282]. Because the transfer of electrons across the plasma membrane generates depolarization, electroneutrality is ensured by the conduction of protons, which are generated from the NADPH hydrolysis in the cytosol, through a channel in the oxidase [283–285]. In contrast, Nox4 predominantly produces H_2O_2, which has been attributed to Cys226, Cys270, and a highly conserved His222 residue in the third extracytosolic loop [286]. The histidine could serve as a source of protons for the spontaneous dismutation of $O_2^{\bullet -}$ forming H_2O_2. ROS production from NADPH oxidases could be either extracellular or intracellular depending on the biological membranes in which the enzyme is expressed, which include plasma membrane, endosome, phagosome, caveolae, endoplasmic reticulum, mitochondria, and nucleus. Nox1, Nox2, Nox4, and Nox5 can be located either at the plasma membrane or within the cell and hence can generate extracellular or intracellular ROS [287].

Xanthine oxidase

Xanthine oxidase (XO) has been identified as a major source of $O_2^{\bullet -}$ in atherosclerosis [288,289] and congested heart failure [290,291]. XO and xanthine dehydrogenase (XDH) are interconvertible isozymes of the enzyme xanthine oxidoreductase (XOR) and catalyze the final two steps of the purine (adenosine) degradation pathway, reducing hypoxanthine and xanthine to uric acid. However, XDH preferentially reduces NAD^+, whereas XO reduces only molecular O_2, producing $O_2^{\bullet -}$ and H_2O_2. XDH is the predominant form in well-oxygenated tissue [292], which is converted to XO by reversible sulfhydryl oxidation or by irreversible proteolytic modification [293] under pathophysiological conditions such as ischemia-hypoxia [294,295]. Both forms of the enzymes act as NADH oxidases generating ROS, with the oxidation induced by XDH higher than that observed with XO [296,297], which may play an important role in cellular injury under conditions of increased NADH concentration, as happens in ischemia [298].

XOR has wide tissue distribution, but its plasma levels, low in healthy mammals, increase significantly under pathophysiological conditions [299]. Circulating XO binds to the vascular endothelial cells because of its affinity with the positively charged glycosaminoglycans on the cell surface [300,301], generating ROS and decreasing the bioavailability of $^{\bullet}NO$ to cause endothelial dysfunction and impair vasorelaxation [289]. This is supported by the data, which show an inverse relationship between endothelium-bound XO activity and endothelium-dependent vasodilation in patients with CAD [302]. Increased functional XOR levels were observed in monocytes/macrophages in drug- and coronary artery ligation-induced heart failure in rats [303]. However, this increase was not observed in hypertrophic ventricles, suggesting its potential role in the progression from cardiac hypertrophy to heart failure. Supporting this notion, a significant increase in endothelium-bound XO activity was observed in patients with chronic heart failure [304].

Nitric oxide synthases

The NOS family of enzymes generates $^{\bullet}NO$ from the conversion of L-arginine to L-citrulline. NOSs are homodimeric oxidoreduc-tases in which the heme-containing

oxygenase domain is linked via a calmodulin-binding linker peptide to a NADPH-cytochrome P450 reductase-like diflavin domain [305]. Upon activation, the FAD of the flavoprotein domain transfers electrons from NADPH to FMN, which reduces heme iron and results in O_2 activation followed by oxidation of the guanidino N atom of L-arginine, forming $^{\bullet}$NO and citrulline. BH_4, a cofactor and critical determinant of the enzyme activity, binds close to the heme active site at the interface of the two monomers, stabilizing the dimer [306,307].

Three NOS isoforms are present in the cardiovascular system, of which neuronal (nNOS) and eNOS are constitutive, with activity regulated at a posttranslational level [308]. The iNOS isoform is produced in response to proinflammatory agonists such as cytokines and is regulated mostly at the transcriptional level [309].

Under normal conditions, eNOS exerts antiatherogenic effects in the vascular wall, including inhibition of cell growth [310,311], leukocyte adhesion [153], and platelet aggregation [312]. Increased coronary atherosclerosis observed in eNOS-deficient apoE$^{-/-}$ mice on a Western-type diet [313,314] was attributed to increased inflammation and leukocyte-endothelial interaction [315]. $^{\bullet}$NO derived from eNOS regulates VSMC tone and blood pressure as evidenced by systemic hypertension in eNOS-knockout mice [316,317] and hypotension in eNOS-transgenic mice [318]. In the heart, eNOS is expressed in the endocardium and cardiomyocytes and eNOS$^{-/-}$ mice exhibit attenuated left-ventricular function and increased mortality after myocardial infarction and during chronic pressure overload [319,320]. However, when eNOS activity becomes “uncoupled,” as happens in pathophysiological conditions, increased $O_2^{\bullet-}$ generation occurs because the transfer of electrons from NADPH through the flavins to molecular oxygen continues [307] (discussed later in regard to endothelial dysfunction).

In contrast, a rapid and large increase in $^{\bullet}$NO generation by upregulation of iNOS expression and activity was linked to cardiovascular pathology. Wild-type mice with iNOS deficiency had increased myocardial contractility and decreased mortality after myocardial infarction [321] and were protected from systolic overload-induced myocardial dysfunction [322], whereas apoE$^{-/-}$ /iNOS$^{-/-}$ mice had a significantly reduced atherosclerotic lesion area [323–325]. However, the role of iNOS in cardiovascular pathology was questioned as its overexpression in the mouse myocardium had no effect on viability and left-ventricular function [326]. It is likely that the high flux of $^{\bullet}$NO from iNOS has pathological effects only under oxidative stress conditions, particularly with increased $O_2^{\bullet-}$ levels [327].

In addition to neurons [328], nNOS is expressed in skeletal muscle [329], kidney [330], endothelial cells and SMCs [331], and cardiomyocytes [332]. Recent evidence indicates that nNOS has a protective function against atherosclerosis and in the heart. Kuhlencordt et al. [333] reported increased atherosclerotic plaque formation and decreased survival in nNOS-deficient apoE$^{-/-}$ mice. The physical proximity of nNOS to XOR in sarcoplasmic reticulum of the cardiomyocytes regulates the activity of the latter and nNOS deficiency decreases myocardial excitation coupling via increased activity of XOR [334].

Lipoxygenases

Lipoxygenases (LOXs), non-heme iron-containing dioxygenases that oxidize polyunsaturated fatty acids released from the cell membrane under inflammatory conditions to hydroperoxy fattyacid derivatives, are another important source of ROS production in the vascular wall [335,336]. Humans have six ALOX genes (LOX genes are named “ALOX” by convention, for arachidonic acid lipoxygenase), whereas mice have seven functional genes [337] and the LOX enzymes are named for the numbered carbon atom of the polyunsaturated fatty acid that gets oxidized (e.g., 5-LOX). Of the LOXs, 5-LOX and 12/15-LOX (also known as leukocyte-type LOX and 15-LOX1; referred to as 12/15-LOX as they can form similar products from common substrates) are important for cardiovascular function and atherosclerosis because of their expression in the vascular wall and inflammatory cells [337]. Mice express only 12-LOX and not 15-LOX [338].

5-LOX catalyzes the transformation of free arachidonic acid to leukotriene A4, which on hydrolysis yields leukotriene B4 (LTB4), a potent chemoattractant and leukocyte activator [339]. The conjugation of leukotriene A4 with glutathione by the action of leukotriene C4 synthase yields cysteinyl leukotrienes, which are associated with vasoconstriction. 5-LOX and LTB4 are highly expressed in human atherosclerotic plaques and LTB4 is involved in SMC recruitment [340,341]—antagonism of its receptor decreases monocytic foam cells in mice [342].

12/15-LOX catalyzes the oxidation of arachidonic acid to yield 12- and 15-hydroperoxyeicosatetraenoic acids (12- and 15-HPETEs), which are rapidly reduced by cellular peroxidases to the corresponding hydroxides, 12-HETE and 15-HETE, respectively [336]. This enzyme also oxidizes α-linoleic acid, another polyunsaturated ω-3 fatty acid, generating 13-hydroperoxyoctadecadienoic acid (13-HPODE), which is reduced to 13-HODE. In addition to free fatty acids, 12/15-LOX oxidizes polyunsaturated acyl chains in phospholipids and cholesteryl esters, key lipid components of LDL [343,344]. In macrophages, LDL oxidation requires binding LDL particles to the low-density lipoprotein receptor-related protein [345] and translocating 12/15-LOX from the cytosol to the plasma membrane [346]. Another line of evidence for the role of 12/15-LOX in LDL oxidation comes from the 12/15-LOX, apoE double-knockout mice on a high-fat diet, which have less atherosclerosis, significantly lower titers of autoantibodies against oxidized LDL in plasma, and lower isoprostane levels in urine compared with apoE$^{-/-}$ mice [347]. Protection against atherosclerosis in the 12/15-LOX, apoE double-knockout was attributed in part to decreased adhesion of monocytes to endothelial cells [348,349], an initiating event in atherogenesis, resulting from decreased activation of RhoA and NF-κB [350,351]. As a corollary to this, the overexpression of human 15-LOX in the vascular endothelium of LDL receptor-deficient mice enhanced early atherosclerosis [352]. However, some reports suggest that 12/15-LOX products may also exert an antiatherogenic effect because of their anti-inflammatory and vasodilatory properties [336].

12/15-LOX is markedly upregulated in heart failure, and transgenic mice with cardiomyocyte-specific overexpression of the enzyme develop systolic dysfunction, aging-associated cardiac fibrosis, and increased macrophage infiltration and MCP-1 expression [353]. Supporting this, 12/15-LOX-deficient mice had significantly reduced cardiac MCP-1

expression, macrophage infiltration, and reduced systolic dysfunction during chronic pressure overload.

Myeloperoxidase

Myeloperoxidase (MPO) generates several oxidants that initiate lipid peroxidation and induce modification of amino acid residues in proteins, including nitration, chlorination, and carbamylation [31]. Brennan et al. [354] demonstrated a significant decrease in 3-nitrotyrosine levels in MPO-deficient mice in response to inflammation. In addition, MPO-deficient mice had a significant reduction in the levels of F_2-isoprostanes, HPETEs, and HPODEs in an acute model of inflammation, supporting a major role for MPO in in vivo lipid peroxidation [355]. Immunofluorescent staining revealed the presence of MPO in the neutrophils in intermediate and advanced atherosclerotic lesions of $LDLR^{-/-}$ mice [356]. MPO induces protein carbamylation in the presence of H_2O_2 at sites of inflammation and in atherosclerotic plaques by the oxidation of thiocyanate, an anion abundant in blood whose levels are elevated in smokers [357]. The product, cyanate, then covalently modifies lysine residues in proteins and lipoproteins, forming homocitrul-line (e-carbamyllysine). LDL-homocitrulline stimulates foam cell formation, VSMC proliferation, and endothelial apoptosis. In addition, the blood levels of protein-homocitrulline correlated with increased cardiovascular risk in case-control studies. An increase in LDL carbamylation in human atherosclerotic lesions by MPO causes cholesterol accumulation and lipid-droplet formation in macrophages through enhanced binding to the LDL receptor SR-A1. Similarly, an increase in HDL carbamylation by MPO induces cholesterol accumulation in macrophages through enhanced binding to the scavenger receptor SR-B1 [358]. More recently, Shao et al. [359] demonstrated that chlorination of Tyr192 in apoA-I of HDL in human plasma and atherosclerotic tissue generates dysfunctional HDL.

The proinflammatory and proatherogenic actions of MPO may include promotion of leukocyte recruitment at sites of inflammation by its positive surface charge [360]. MPO-deficient mice had reduced neutrophil infiltration in inflamed tissues, and an infusion of MPO into the circulation caused neutrophil adhesion even in uninflamed blood vessels, supporting the notion that neutrophil recruitment is mediated by the strong positive charge of MPO. However, it is suggested that MPO augments adhesion molecule-mediated interaction only between endothelial cells and neutrophils. In addition, a significant increase in systemic MPO levels was associated with coronary plaque erosion in patients with acute coronary syndrome [361]. Together, these data provide evidence that oxidative stress mediated by MPO could increase atherogenicity.

Substantial evidence also suggests that MPO converts nitrite, a major end product of $^{\bullet}NO$ metabolism, into RNS, most probably nitrogen dioxide ($^{\bullet}NO_2$), in a H_2O_2-dependent reaction [362–365]. The $^{\bullet}NO_2$ generated by the MPO-H_2O_2-nitrite system catalyzes the nitration of tyrosine residues and oxidation of tryptophan residues and promotes lipid peroxidation of LDL [363,366]. However, MPO-generated $^{\bullet}NO_2$ is not a major product, particularly in human leukocytes, and its significantly greater amounts in murine compared with human phagocytes might be due to higher local nitrite concentrations in the mice [367–370].

Endothelial dysfunction

Robert Furchgott [371] first demonstrated that relaxation of blood vessels by acetylcholine requires the presence of endothelial cells, whereas Rubanyi et al. [372] observed that this process is mediated by the release of a vasoactive substance, termed endothelium-derived relaxing factor (EDRF). The EDRF diffuses to the VSMCs and induces the production of cGMP by stimulating sGC [373]. cGMP activates PKG, causing a decline in cytosolic Ca^{2+}, which in turn suppresses the activity of myosin light chain (MLC) kinase, resulting in increased levels of dephosphorylated MLC and relaxation of SMCs [374]. Activation of MLC phosphatase by PKG also causes vasodilation by increasing dephosphorylated MLC levels. The EDRF, which is rapidly degraded by $O_2^{\bullet-}$, was identified as $^{\bullet}NO$ [375,376], synthesized by NOS, mostly eNOS in the vasculature [377,378].

In addition to the regulation of vasodilation, endothelium modulates inflammation, SMC growth, platelet aggregation, and coagulation, and the dysregulation of these processes is termed “endothelial dysfunction,” which is evident as impaired vasorelaxation in response to endothelium-dependent vasodilators such as acetylcholine [379]. The healthy endothelium responds to vasoactive agonists released from the aggregating platelets by the activation of eNOS and increased production of $^{\bullet}NO$ [380,381]. With endothelial injury, the aggregating platelets come into contact with VSMCs, causing contraction by releasing thromboxane A_2 and serotonin [382]. The endothelium-dependent response to aggregating platelets is highly active in the coronary and cerebral circulations.

Endothelial dysfunction is an initial event in the development of atherosclerosis and ischemic heart disease [383,384] and an independent predictor of CVD [385–388]. Schächinger et al. [389] demonstrated that coronary endothelial vasodilator dysfunction is a prognostic indicator of cardiovascular events, including cardiovascular death, unstable angina, myocardial infarction, and ischemic stroke. In a 30-day follow-up of nonemergent vascular surgery, patients in the upper tertile of brachial artery flow-mediated dilation (4 8.1%) had significantly fewer adverse cardiovascular events than patients with low flow-mediated dilation [384]. Similarly, endothelial dysfunction in the forearm has been shown to predict adverse cardiovascular events in subjects with no apparent heart disease [390] as well as in patients with peripheral arterial disease [391,392]. Increased ROS production and impaired endothelium-dependent and -independent vasodilator responses resulting from eNOS uncoupling in platelets were observed in patients with congestive cardiac failure [393].

The mechanisms underlying endothelial dysfunction are multifactorial [394,395], with oxidative stress playing a major role. The kinetics of the reaction of $O_2^{\bullet-}$ with $^{\bullet}NO$ are three times faster than the reaction rate of $O_2^{\bullet-}$ with SOD. Thus, it is likely that some $O_2^{\bullet-}$ always reacts with $^{\bullet}NO$ within the cells and extracellular space, but endogenous antioxidant defenses minimize this interaction [396]. However, in pathophysiological conditions such as in hypercholesterolemic rabbits, impaired endothelium-dependent vascular relaxation results from the interaction of $^{\bullet}NO$ with $O_2^{\bullet-}$, because polyethylene-glycolated SOD markedly improved endothelium-dependent vascular relaxation in these but not in normocholesterolemic animals [397]. Hypercholesterolemia also enhances oxidative stress

by upregulating the expression of the AT1 receptor, genetic disruption of which improved endothelial function and inhibited diet-induced atherosclerosis in apoE$^{-/-}$ mice [398]. Likewise, oxidized LDL but not native LDL inhibits endothelium-dependent relaxation in isolated vessels [399], whereas antioxidant vitamins enhance endothelium-dependent vasodilation in both the coronary and the forearm circulation in subjects with CVD [400,401]. Emphasizing the role of oxidative stress in endothelial dysfunction, deletion of various SOD isoforms impaired $^{\bullet}$NO-mediated arterial relaxation [402–404].

Another mechanism by which oxidative stress causes endothelial dysfunction is via the uncoupling of the eNOS. Deficiency of either eNOS substrate L-arginine or eNOS cofactor BH_4 induces eNOS uncoupling to produce $O_2^{\bullet-}$ and H_2O_2. Increased $O_2^{\bullet-}$ production in the aortas of spontaneously hypertensive stroke-prone rats was reduced by treatment with N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS, or removal of endothelium, indicating that the tissue and enzymatic sources of this increased $O_2^{\bullet-}$ are the endothelium and eNOS, respectively [405]. Activation of arginase I, which degrades L-arginine, and eNOS uncoupling were reported in diabetes, pulmonary hypertension, ischemia-reperfusion, atherosclerosis, and aging [406–410]. Arginase inhibition restores NOS coupling and reverses endothelial dysfunction [411].

Decreased BH_4 availability, resulting in eNOS uncoupling, and increased oxidative stress were observed in diabetes [412,413]. Intra-arterial infusion of BH_4 improved endothelium-dependent vasodilation in chronic smokers, supporting the notion that BH_4 depletion contributes to eNOS dysfunction [414]. BH_4 supplementation reversed left-ventricular hypertrophy (LVH), fibrosis, and cardiomyocyte dysfunction induced by pressure overload, highlighting the importance of cardiac myocyte eNOS uncoupling in hypertrophic heart disease [415,416]. Further support for the regulatory role of BH_4 concentrations as a critical determinant in eNOS uncoupling was evident in apoE$^{-/-}$ mice with endothelial-specific overexpression of eNOS [417]. These mice had enhanced endothelium- and eNOS-dependent $O_2^{\bullet-}$production and increased atherosclerosis compared with apoE$^{-/-}$ mice. BH_4 supplementation attenuated both endothelial ROS and atherosclerosis. Consistent with these data, endothelial-specific overexpression of GTP-cyclohydrolase 1, the rate-limiting enzyme for BH_4 synthesis, in apoE$^{-/-}$/eNOS-transgenic mice partially restored eNOS coupling [418] and decreased ROS levels and atherosclerosis [419,420]. In vivo depletion of BH_4 could also occur from oxidative modification by peroxynitrite [421]. Zou et al. [422] reported that the main mechanism of peroxynitrite-induced eNOS uncoupling is the release of Zn from a zinc-thiolate cluster, as this process is 10–100 times more sensitive than BH_4 oxidation.

Serum asymmetrical dimethylarginine (ADMA), an endogenous L-arginine analog, is inversely correlated with flow-mediated dilation in the brachial artery of subjects, independent of risk factors for atherosclerosis [423]. ADMA inhibits eNOS activity by competitive inhibition of endothelial cell arginine uptake. Furthermore, the activities of S-adenosylmethionine-dependent protein arginine *N*-methyltransferases and dimethylarginine dimethylaminohydrolase, enzymes involved in ADMA synthesis and degradation, respectively, are redox sensitive, with the former enhanced and the latter decreased under oxidative stress conditions [424,425].

$O_2^{\bullet-}$ required for eNOS uncoupling is generated by several sources. Administration of oxypurinol, an inhibitor of XO, improved impaired vasodilation in hypercholesterolemic [426] and CHF patients [427,428], implicating XO-derived $O_2^{\bullet-}$production in eNOS uncoupling. However, Dworakowski et al. [429] reported that increased activity of Nox, particularly of Nox4, contributes to increased $O_2^{\bullet-}$ production and vascular endothelial dysfunction in CHF patients. Impaired acetylcholine-induced relaxation of spontaneously hypertensive aged rat aortas was significantly improved by VAS2870, a pan-Nox inhibitor, by inhibiting the ectopically expressed Nox1 in endothelial cells [430]. Similarly, Nox1 overexpression in VSMCs impaired endothelium-dependent relaxation in response to AngII infusion, which was reversed by BH_4 supplementation [431].

Although vascular relaxation in large vessels mediated by endothelium-derived $^{\bullet}NO$ bioactivity is mainly dependent on $O_2^{\bullet-}$ concentrations, endothelium-dependent relaxation in small resistance vessels is mediated by H_2O_2 [31]. Matoba et al. [432] reported that endothelium-dependent relaxation and hyperpolar-ization of VSMCs in the small mesenteric arteries of mice in response to acetylcholine was inhibited by catalase and first proposed that H_2O_2 is the EDHF. H_2O_2-dependent vascular relaxation was reported in subjects with CVD. Miura et al. [159,433] reported that H_2O_2 has a more prominent role in flow-induced dilation of coronary arterioles in subjects with CAD compared with those without CAD. Also, under conditions of BH_4 depletion, H_2O_2 mediates endothelium-dependent relaxation in coronary arteries [434]. Likewise, in mice deficient in GTP cyclohydrolase H_2O_2 produced as a result of eNOS dysfunction mediates aortic relaxation in response to acetyl choline [435]. However, the regulatory function of H_2O_2 may vary depending on the vascular bed, species, age, and pathophysiological conditions. For example, the vascular cell-specific overexpression of catalase decreased blood pressure in mice, indicating the vasoconstrictive function of H_2O_2 [436]. Both genetic and pharmacological evidence suggests that H_2O_2 also impairs endothelium-dependent vasorelaxation because polyethylene glycol-catalase or transgenic overexpression of GPx-1 protected mice against AngII-induced endothelial dysfunction of carotid arteries [437].

Atherosclerosis

Endothelial dysfunction and activation in the presence of atherosclerosis risk factors such as hypercholesterolemia and hypertension induce the expression of the cell adhesion molecules VCAM-1, ICAM-1, E-selectin, and P-selectin [438,439]. Activated endothelium also permits increased permeability to macromole-cules such as LDL The induction of cell adhesion molecules enables the adherence of circulating monocytes and T lymphocytes to the endothelium and the subsequent transmigration into the subendothelial space. The activation of inflammatory cells is associated with the stimulation of oxidant enzymes such as NADPH oxidases and MPO, generation of ROS, and oxidation of phospholipids and protein in LDL, resulting in the accumulation of oxidized LDL (oxLDL), an important effector of atherogenesis [440,441]. ROS generated in the vascular wall cells as well as in inflammatory cells by the activation of oxidant enzymes stimulates redox signaling pathways that could affect atherogenesis at multiple levels [442].

It was shown recently that oxLDL increases $O_2^{\bullet-}$ generation in human aortic endothelial cells by phosphorylating $p66^{Shc}$ at Ser36 [443]. This effect of oxLDL is dependent on the binding of the lipoprotein to its LOX-1 receptor, followed by the sequential activation of protein kinase Cp_2 and c-Jun N-terminal kinase. Genetic deletion of $p66^{Shc}$ decreased oxidative stress, lipid peroxidation, and atherosclerosis in $apoE^{-/-}$ mice, indicating the important role of systemic oxidative stress in atherosclerosis [240,444]. Interestingly, the PKCp–JNK pathway is a critical effector of oxLDL-mediated induction of MMP2 expression and activity, and deletion of PKCp2 or JNK2 significantly decreases oxidative stress and atherosclerosis in $apoE^{-/-}$ mice [445–447]. Suppression of $p66^{Shc}$ expression inhibited oxLDL-induced p47phox expression as well as ROS production, indicating that NADPH oxidase is a major source of oxLDL-induced ROS in vascular cells [448]. Increases in $p66^{Shc}$ mRNA levels were reported in patients with high plasma LDL levels [433] and in angiographically confirmed CAD patients [449], implicating oxidative stress in atherogenesis.

In addition to oxidizing LDL, oxidative stress also affects cardiovascular health by inhibiting the cholesterol efflux function of HDL. Myeloperoxidase-induced chlorination of apoA-I, the major protein component of HDL, impairs the ability of apoA-I to promote cholesterol efflux through ABCA1, the macrophage ATP-binding cassette transporter [450,451]. The lecithin-cholesterol acyltransferase (LCAT) binding site on apoA-I is the preferred target for oxidative modification in atheroma, which diminished LCAT activity, resulting in a dysfunctional form of HDL [452–454]. This, in turn, could increase ROS production and inflammation, as Peshavariya et al. [455] showed that reconstituted HDL (apoA-I complexed with 1-palmitoyl-2-linoleoyl phosphatidylcholine in a molar ratio of 1:100) inhibits leukocyte NADPH oxidase activity, probably by disrupting the assembly of the enzyme subunits at lipid rafts. It is suggested that MPO catalyzes oxidation of HDL and converts it into a proinflammatory molecule [456].

Further supporting the proatherogenic role of MPO, Sugiyama et al. [457,458] reported the accumulation of a subset of MPO-containing macrophages in the subendothelial space at sites of coronary plaque erosion or rupture and suggested that MPO-positive macrophage-derived HOCl promotes acute coronary syndrome by stimulating endothelial cell death and tissue factor expression. Additional evidence for the role of MPO in the pathophysiology of atherosclerosis is evident in population-based studies of initially healthy men and women in whom high levels of circulating MPO were predictors of future risk of CHD [16,459,460].

Evidence accumulated over the past decade has shown increased expression of NADPH oxidase subunits and increased ROS levels in human atherosclerotic lesions, indicating the clinical relevance of redox signaling and oxidative stress in atherosclerosis. Sorescu et al. [10] reported increased Nox2 and p22phox expression along with increased $O_2^{\bullet-}$ generation in the shoulder region of atherosclerotic plaques. Azumi et al. [461,462] observed increased p22phox expression and ROS generation in atherosclerotic coronary arteries and in the coronary plaques of unstable angina patients. Simultaneous intravascular ultrasound and immunohistochemical analysis indicated that NADPH oxidase-derived ROS are involved in the coronary arterial remodeling associated with plaque vulnerability [463].

Evidence for the contribution of NADPH oxidase-derived ROS to atherosclerosis was also found in experimental mouse models that are deficient in various subunits of NADPH oxidase (Table 2). We demonstrated decreased aortic ROS levels in mice that are deficient in p47phox and decreased aortic atherosclerotic lesion area in apoE$^{-/-}$/p47phox$^{-/-}$ compared with apoE$^{-/-}$ mice [3,464]. A decrease in the atherosclerotic lesion area and attenuated neoin-timal hyperplasia in response to arterial injury in apoE$^{-/-}$ -/p47phox$^{-/-}$ compared with apoE$^{-/-}$ mice is associated with decreased expression of adhesion molecule CD44 in aortic/arterial cross sections [464]. An allogeneic, sex-mismatched bone marrow transplantation study demonstrated that the atheroprotective effect of p47phox deletion resulted from the inhibition of NADPH oxidase in the vascular wall cells as well as in bone marrow-derived monocytes/macrophages [465]. Absence of a functional NADPH oxidase in neointimal SMCs caused attenuated activation of redox-sensitive mitogenic proteins, including Janus kinase 2, and decreased neointima formation in apoE$^{-/-}$/p47phox$^{-/-}$ mice. Interestingly, Fenyo et al. [466] reported that the Janus kinase 2 inhibitor tyrphostin AG490 decreased the expression of Nox1, Nox2, and Nox4 subunits and NADPH oxidase activity and reduced the atherosclerotic lesion size in apoE$^{-/-}$ mice that were fed a high-fat diet. Our data on the effect of p47phox deficiency in atherogenesis in apoE$^{-/-}$ mice are supported by recent evidence of decreased aortic ROS production, increased •NO bioavailability, and significantly reduced atherosclerotic lesion size in apoE$^{-/-}$/ Nox2$^{y/-}$ compared with apoE$^{-/-}$ mice fed a high-fat diet [263]. Similarly, reduced neointima formation in response to arterial injury and decreased leukocyte accumulation was observed in Nox2$^{y/-}$ mice [467]. Recent data also indicate that Nox1 activation contributes to atherosclerosis. The aortic atherosclerotic lesion area and the macrophage content in the aortic sinus area were significantly decreased in apoE$^{-/-}$/Nox1$^{y/-}$ compared with apoE$^{-/-}$ mice that were fed a high-fat diet [468]. Nox1 is also involved in the response to vascular injury as attenuated wire injury-induced femoral artery neointima formation and decreased VSMC proliferation and migration were observed in Nox1$^{y/-}$ compared with wild-type mice [469]. Mechanistic studies revealed that Nox1 regulates VSMC migration by modulating the actin cytoskeleton via its effects on cofilin (a regulator of actin depolymerization), mDia1 (a RhoA adapter protein), and PAK1 (a serine/threonine kinase that promotes cytoskeletal reorganization). We demonstrated that adenovirus-mediated overexpression of the Nox activator NoxA1 increases neointimal hyperplasia in injured mouse carotid arteries and that NoxA1 expression is increased in aortas and atherosclerotic lesions of apoE$^{-/-}$ mice and in human carotid atherosclerotic lesions [11]. Furthermore, we showed that an inhibitor of Nox1 and Nox4, GKT136901, decreases ROS generation and expression of CD44 and its principal ligand, hyaluronan, in atherosclerotic lesions [134].

Endogenous antioxidant systems protect against atherogenesis, by virtue of their ability to scavenge ROS, facilitate endothelium-dependent vasorelaxation, inhibit inflammatory cell adhesion to endothelium, and alter vascular cellular responses, such as VSMC and endothelial cell apoptosis, VSMC proliferation, hypertrophy, and migration [470]. The protective role of SOD isoforms against atherogenesis has been elucidated using genetically modified mouse models. Overexpression of SOD1 and catalase or over-expression of catalase alone decreased plasma and aortic F2-isoprostane levels and retarded atherosclerosis in apoE$^{-/-}$ mice [471]. We showed that increased mitochondrial oxidant

generation, resulting from SOD2 deficiency, induced mitochondrial DNA damage and accelerated atherosclerosis in young apoE$^{-/-}$ mice [4]. Interestingly, mitochondrial DNA damage not only correlated with the prevalence of aortic atherosclerosis in humans and apoE$^{-/-}$mice, but also preceded atherogenesis in young apoE$^{-/-}$ mice. We also demonstrated that SOD1 and SOD2 deficiency results in SMC hyperplasia and hypertrophy, albeit through preferential activation of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinases in SOD1$^{+/-}$ SMCs and the Janus kinase/signal transducer and activator of transcriptase pathway in SOD2$^{+/-}$ SMCs [472]. It was shown that recombinant SOD3 decreases LDL oxidation by endothelial cells [473,474]. Supporting the role of SOD3 in atheroprotection, Wang et al. [475] reported that low levels of plasma SOD3 are independently associated with a history of myocardial infarction in patients with angiographically documented CAD.

Further highlighting the importance of antioxidant defenses in atherosclerosis, Torzewski et al. [476] reported increased aortic ROS levels, decreased $^{\bullet}$NO levels, and increased atherosclerosis in apoE$^{-/-}$ mice that were deficient in GPx-1. Congruent with this, overexpression of GPx-4 reduced aortic F2-isoprostane levels and decreased atherosclerosis and delayed lesion progression in apoE$^{-/-}$mice [477]. Human studies also indicate that GPx-1 is important in protection against atherosclerosis. For instance, GPx-1 activity is either decreased or absent in human carotid atherosclerotic lesions and its absence is associated with lesion severity [478]. In a prospective study of 636 patients with CAD, low GPx-1 activity in erythrocytes was associated with an increased risk of cardiovascular events independent of traditional risk factors or atherosclerosis [479]. In fact, a meta-analysis of 42 case-control and 3 prospective studies revealed that higher activities of circulating GPx, SOD, and catalase confer protection against CHD [480].

Paraoxonase, which exists in three isoforms (PON1, PON2, and PON3), is another antioxidant enzyme that has atheroprotective effects. PON1 is associated with HDL and was shown to block LDL as well as HDL oxidation in vitro ([481,482]. Congruent with this, PON1-knockout mice had higher levels of oxidized phospholipids compared with wild-type mice, and HDL from these mice was unable to block LDL oxidation in vitro [483]. Furthermore, PON1 deficiency increased aortic atherosclerosis in wild-type and apoE$^{-/-}$mice [483,484]. PON2 was shown to attenuate macrophage triglyceride accumulation and foam cell formation via the inhibition of redox-sensitive microsomal diacylglycerol acyltransferase 1 [485]. Devarajan et al. [486] reported that PON2-deficient apoE$^{-/-}$ mice develop enhanced mitochondrial oxidative stress and exacerbated atherosclerosis on both chow and Western diet. An increase in atheroprotection was attributed to decreased apoptosis of vascular wall cells and macrophages, resulting from the interaction of PON2 with mitochondrial coenzyme Q10. Correspondingly, PON3-transgenic mice had significantly smaller atherosclerotic lesions on both B6 and apoE$^{-/-}$ backgrounds when fed an atherogenic diet [487]. Likewise, adenoviral overexpression of PON3 not only significantly decreased the atherosclerotic lesion area but also lowered the serum lipid hydroperoxide levels and enhanced the ability of cholesterol-loaded macrophages to efflux cholesterol [488]. Highlighting the clinical relevance of PON1 against systemic oxidative stress and atherosclerosis, Bhattacharyya et al. [489] reported that patients in the highest PON1 activity quartile had a lower incidence of cardiac events compared to those in the

lowest quartile. Together, cell culture data, experimental mouse models (Table 2), and human studies support a contributory role for oxidative stress and redox-sensitive signaling in atherogenesis.

Hypertension

In 1991, Nakazono et al. [490] indicated a role for ROS in the etiology of hypertension when they observed a significant decrease in the blood pressure of spontaneously hypertensive rats, an animal model of hypertension, after the administration of heparin-bound SOD1. Increased oxidative stress is also associated with many other experimental models of hypertension, including spontaneously hypertensive stroke-prone rats and surgically induced, hormone-induced, and diet-induced hypertension [491]. In addition to the vasculature, the mechanisms of ROS-induced hypertension involve other organ systems such as the heart, kidneys, and central nervous system.

Supporting the role of NADPH oxidase in hypertension, an increase in the activity of this enzyme was observed in VSMCs and endothelial cells upon stimulation with AngII [492,493], as well as in various animal models of hypertension, such as those induced by AngII [494,495] and deoxycorticosterone acetate (DOCA-salt) [496]; in renovascular hypertension [497]; and in spontaneously hypertensive rats [498]. The causative role of various Nox homologs or Nox subunits in hypertension is elucidated using genetically altered mice. Genetic deletion of Nox1 in mice resulted in the loss of sustained blood pressure increase induced by AngII infusion [499,500], and L-NAME abolished the effect of Nox1 deletion on the pressor response to AngII. However, endothelium-dependent vascular relaxation was preserved in AngII-infused $Nox1^{y/-}$ aortas [499], suggesting that Nox1-derived ROS are important in hypertension. Supporting these data, overexpression of Nox1 in VSMCs increased medial $O_2^{\bullet -}$ production in response to AngII infusion, which resulted in eNOS uncoupling, decreased $^{\bullet}NO$ bioavailability, impaired vasorelaxation, and an increase in systolic blood pressure [431,500].

Nox2 expression is increased in several organ systems in hypertension and particularly in resistance arteries, the site of blood pressure control [270]. Pagano's group [501] reported that infusion of Nox2ds-tat, a competitive inhibitor of the interaction of Nox2 with p47phox, significantly decreased AngII-induced increase in ROS production and systolic blood pressure, supporting the role of Nox2-derived oxidative stress in hypertension. A decreased hypertensive response to AngII infusion in p47phox-deficient mice [502] and an increased response in mice with SMC-specific overexpression of p22phox [503] were also indicative of the role of Nox2 activation in hypertension, although these subunits also affect the activity of other Nox homologs. Increased Nox2 activity also contributes to renovascular hypertension by decreasing $^{\bullet}NO$ bioavailability [504]. Zimmerman et al. [505] reported that hypertensive response to chronic systemic infusion of AngII is correlated with a marked increase in $O_2^{\bullet -}$ production in the subfornical organ of the brain, a region lying outside the blood-brain barrier (BBB). Studies using adenoviral vectors expressing small interfering RNA demonstrated that both Nox2 and Nox4 enzymes are required for the full vasopressor effects of AngII in this region [506]. Nox4 in the kidney may also mediate hypertension or hypertension-induced renal injury as it is upregulated in several animal models of

hypertension [442]. BH_4 supplementation decreased vascular ROS production, increased •NO bioavailability, and attenuated DOCA-salt-induced hypertension, indicating that eNOS uncoupling could result in hypertension [496]. Recent evidence indicates a cross talk between Nox and mitochondrial sources of ROS, and administration of a mitochondria-targeted antioxidant, mitoTEMPO, attenuated AngII and DOCA-salt-induced hypertension in mice [7,507]. MitoTEMPO also decreased mitochondrial and total cellular O_2•−, reduced cellular NADPH oxidase activity, and restored bioavailability of •NO. Overexpression of SOD2 attenuated AngII-induced hypertension and vascular oxidative stress, further supporting the role of mitochondrial oxidative stress in hypertension [7]. Together, the various experimental animal models of hypertension support a causal role for oxidative stress in blood pressure regulation.

Cardiac hypertrophy and heart failure

Oxidative stress and redox signaling are important contributing factors for LVH, which occurs initially as an adaptive response to environmental stress to augment pump function and to reduce wall stress [508]. The heart undergoes pathologic cardiac hypertrophy in response to a long-term increase in workload, most often as a consequence of hypertension (pressure overload) or ischemic heart disease, resulting eventually in chronic heart failure [30]. Many of the molecular mechanisms of cardiac hypertrophy are redox sensitive and the transition from pathological hypertrophy to heart failure involves a decrease in contractility, ventricular remodeling and dilatation, myocardial fibrosis, and myocyte apoptosis.

Low-amplitude cyclic stretch induces hypertrophy in isolated cardiomyocytes via increased ROS production and activation of ERK1/2, whereas high amplitude causes apoptosis via activation of JNK, indicating how mechanical strain contributes to cardiac hypertrophy and heart failure [509]. Many stimuli of pathological cardiac hypertrophy act by phosphorylating class II histone dea-cetylases (HDACs), master negative regulators of cardiac hypertrophy [510] (Fig. 2). Phosphorylated class II HDACs dissociate from transcription factors such as MEF-2, NFAT, and CAMTA2, which then promote hypertrophy, whereas dissociated HDACs translocate from the nucleus to the cytoplasm. However, Sadoshima's group [511] has shown that thioredoxin 1-sensitive oxidative modification of class II HDACs is a potent mechanism for their translocation from the nucleus and induction of hypertrophy. Thioredoxin 1 forms a multiprotein complex with HDAC4 and DnaJb5, a heat shock protein, in cardiomyocytes. Treatment of cardiomyocytes with ROS-generating G-protein-coupled receptor agonists such as phenylephrine stimulates oxidation of cysteine residues in DnaJb5 (Cys274, Cys276) and HDAC4 (Cys667, Cys 669), forming intramolecular disulfide bonds that enable HDAC4 to translocate to cytoplasm, resulting in hypertrophy. Thioredoxin regulates the nucleocytoplasmic shuttling of HDAC4, independent of its phos-phorylation status, by reducing Cys667 and Cys669, which inhibits hypertrophy [511,512]. Further supporting the role of ROS in hypertrophy, antioxidant treatment inhibited pressure overload-induced LVH in mice [513] and prevented the transition from compensatory hypertrophy to heart failure in guinea pigs [514]. Congruent with this, overexpression of antioxidant enzymes GPx and heme oxygenase-1 protected against pathologic left-ventricular remodeling and dysfunction in mice [515,516]. Furthermore, myocardial

dysfunction and the severity of heart failure in patients are correlated with increased oxidative stress [30].

Cardiac hypertrophy induced in response to chronic infusion of a subpressor dose of AngII was inhibited in $Nox2^{y/-}$ mice [268]. Inhibition of Nox2 enzyme activity by cardiomyocyte-specific deletion of Rac1 also decreased AngII-induced myocardial oxidative stress, activation of the redox-sensitive Ask1-NF-κB signaling pathway, and cardiac hypertrophy, further supporting the role of this enzyme in LVH [517] (Fig. 2). Nox2 enzyme activity and expression are increased in pressure overload-induced cardiac hypertrophy [518–520] as well as in the myocardium of failing human hearts [5,521,522]. However, Nox2 is not essential for pressure overload-induced LVH although $Nox2^{y/-}$ mice had less cardiac interstitial fibrosis and less contractile dysfunction compared with wild-type mice when subjected to chronic pressure overload [523]. Cardiac hypertrophy, cardiomyocyte apoptosis, and interstitial fibrosis were substantially reduced in $p47phox^{-/-}$ mice after myocardial infarction, indicating that Nox2 activation is involved in cardiac remodeling after myocardial infarction [524].

Nox4 expression is enhanced in cardiomyocytes treated with hypertrophic stimulants such as AngII and phenylephrine and in response to pressure overload [525,526]. Mice with cardiac-specific deletion of Nox4 had less mitochondrial $O_2^{\bullet-}$, interstitial fibrosis, and cardiomyocyte apoptosis along with attenuated cardiac hypertrophy and better cardiac function in response to cardiac pressure overload compared with the wild type [527]. Correspondingly, cardiac-specific overexpression of Nox4 exacerbated cardiac dysfunction, fibrosis, and apoptosis in response to pressure overload, but did not cause cardiac hypertrophy. Also, Nox4 overexpression in vitro in cardiomyocytes induced apoptotic cell death but not hypertrophy [525], which suggests that the primary effect of an increase in Nox4 expression in a failing heart is cardiomyocyte apoptosis rather than hypertrophy. However, Shah's group [526] reported exaggerated contractile dysfunction, hypertrophy, and cardiac dilatation during exposure to chronic pressure overload in global Nox4-knockout mice, whereas Nox4-transgenic mice with cardiomyocyte-specific overexpression were protected. The protective effect of Nox4 overexpression was attributed to preservation of myocardial capillary density during pressure overload. This view is supported by the recent findings of Brandes and colleagues [528] that ischemia-induced angiogenesis was impaired in $Nox4^{-/-}$ mice in a femoral artery ligation model. The reason for the discrepancy in the reported Nox4 function is unclear but may be related to the total as well as cell-type-specific concentration of H_2O_2 and the differences in vasoactive effectors produced in various model systems.

It has been suggested that ROS generation by XO contributes to impaired myocardial energy metabolism in heart failure [290,291]. Evidence for this was obtained using transgenic mice containing a troponin I truncation, a model of progressive dilated cardiomyo-pathy in which chronic XO inhibition with allopurinol delayed heart failure progression by preventing myofibrillar protein oxidation and improving cardiac muscle force generation [529]. The improvement in contractile function with the suppression of XO activity was corroborated in mice with coronary artery ligation, using echocardiography and MRI [530,531]. In a rather small study of nine patients with idiopathic dilated cardiomyopathy, allopur-inol

significantly improved myocardial efficiency [291]. However, in a prospective OPT-CHF study (the efficacy and safety study of oxypurinol in patients with symptomatic heart failure), oxypur-inol, the active metabolite of allopurinol, failed to show improvement in the primary composite end point of heart failure clinical status and mortality [532].

Ischemia–reperfusion injury

The levels of ROS generation during ischemia are low, but cardiac ischemia–reperfusion injury occurs upon the restoration of blood flow to the ischemic myocardium, resulting from a large burst of ROS generation [191]. ROS generated during early reperfu-sion cause extensive damage to cardiomyocytes, resulting in the loss of cell viability. It was shown that heterozygous deficiency of GPx1 impaired myocardial recovery after ischemia–reperfusion injury [533]. Global knockdown of GPx1 in mice resulted in impaired cardiac recovery after ischemia–reperfusion injury [534]. GPx-$1^{-/-}$ mice had increased mitochondrial ROS production, increased oxidative mitochondrial DNA damage, and decreased expression of mitochondrial proteins including complex I, resulting in a decrease in NADH and ATP generation [534]. These results suggest that restoring homeostatic redox signaling and cardiac energy bioavailability is important for recovery from ischemia– reperfusion injury [535]. Thioredoxin 1 (Trx1; cytosolic isoform) and Trx2 (mitochondrial isoform) are key regulators of the cellular redox state, whose function is regulated by thioredoxin-interacting protein, an endogenous inhibitor. Yoshioka et al. [536] recently demonstrated improved left-ventricular function after ischemia–reperfusion injury in global as well as cardiomyocyte-specific thioredoxin-interacting protein-knockout mice compared with the wild type (Fig. 3). Protection against injury in these knockout mice was associated with a significant increase in Trx2 activity and decrease in myocardial ROS production. Furthermore, thioredoxin-interacting protein deficiency repressed mitochondrial respiration and enhanced anaerobic glycolysis, increasing cellular ATP levels.

HIF confers protection during and/or after ischemia via activation of downstream effector genes that increase glycolytic capacity, antioxidant defense, angiogenesis, and cell survival signaling [199] (Fig. 1). Cardiomyocyte-specific deletion of HIF1α in mice decreased contractility, vascularization, and high-energy phosphate content via altered gene expression, supporting a central role for HIF1α in coordinating energy availability and utilization in the heart [537]. As a corollary to this, cardiomyocyte-specific overexpression of HIF1α in mice attenuated infarct size and improved cardiac function 4 weeks after myocardial infarction, in association with an increase in capillary density as well as vascular endothelial growth factor and iNOS expression in the peri-infarct regions [538]. Similarly, activation of HIF1a by silencing HIF1a–prolyl 4-hydroxylase in the heart using small interfering RNA significantly increased iNOS mRNA expression and decreased infarct size and cardiac dysfunction after ischemia-reperfusion [539]. Furthermore, this protection was lost in iNOS$^{-/-}$ mice, indicating the critical role played by iNOS-dependent pathways against reperfusion injury. Highlighting the importance of $^{\bullet}$NO in protection against ischemia-reperfusion injury, Sun et al. [540] showed that ischemic preconditioning of rat hearts causes nitrosylation of proteins involved in Ca^{2+} handling and energetics (Fig. 4). For example, ischemic preconditioning causes S-nitrosylation and a decrease in the activity of the mitochondrial F1-ATPase, reducing the rate of decline in ATP levels. Further supporting

the role of S-nitrosylation in protection against ischemia, perfusion of hearts with S-nitrosoglutathione improved left-ventricular function.

ROS generation and redox signaling during the early preconditioning period may be involved in the eventual cardioprotective effect afforded by some ischemic preconditioning stimuli [189,199]. Diazoxide-induced cardioprotection against ischemia-reperfusion injury is mediated by ROS-induced activation of mitochondrial ATP-sensitive potassium channel, and antioxidants block the protection afforded by diazoxide as well as ischemic preconditioning [190,541]. The ROS generated during early preconditioning can be from mitochondria (slightly swollen with increased respiration) [542] or from Nox2 [543] and may act via stimulation of PKC.

Cerebral ischemia resulting from the occlusion of vessels in the brain tissue in the vast majority of strokes causes brain damage. Recanalization of the occluded vessels and restoration of the blood flow is the most effective treatment for the stroke. As in the heart, reperfusion results in increased ROS generation and oxidative stress, which significantly limit the benefits of the stroke therapies [544,545]. Intrinsic high metabolic activity, very high concentrations of the neurotransmitter-excitotoxin glutamate, and limited antioxidant defense mechanisms make the brain very susceptible to oxidative damage [546–548].

During reperfusion in the brain microvasculature, ROS break down the BBB, consisting of endothelial cells, pericytes, the surrounding basement membrane, and attached astrocyte endfeet, which can result in cerebral edema and/or brain hemorrhage, neurovascular injury, and neuronal death [544,549]. More recently, it was posited that the cell-cell and cell-matrix interaction between the BBB and neuronal and glial cells constituting the “neurovascular unit” integrates the brain function in response to various insults, including ischemia-reperfusion injury [548]. For example, sublethal levels of oxidative stress downregulate the production of cerebral endothelium-produced brain-derived neurotrophic factor, indicating the importance of cerebral endothelium as a critical source of homeostatic support for neurons and cell-cell interaction in coordinating protection against ischemia-reperfusion injury.

Mitochondria-derived ROS generation has hitherto been considered the major source of oxidative stress in the ischemia-reperfusion-induced BBB opening. Mitochondria are abundant in the brain [550] and dephosphorylation of oxidative phosphorylation complexes induced by ischemia causes hyperactive electron transport and hyperpolarization of mitochondrial membrane potential upon reperfusion, resulting in excess ROS production [551]. However, recent data suggest that Nox2 activity plays a dominant role in $O_2^{\bullet -}$ generation during N-methyl-D-aspartate receptor stimulation in ischemia in neurovascular cells and is the molecular mechanism underlying excitotoxicity-induced neuronal death [552,553]. Further affirming the important role of Nox2 in excitotoxicity, ROS generation and cell death were blocked in neurons lacking p47phox or with Nox inhibition [553]. Interestingly, Nox2 expression and activity observed in the brain after ischemia were markedly decreased in SOD1-transgenic mice, whereas they were significantly increased in $SOD1^{-/-}$ mice, indicating that Nox expression is redox sensitive [554].

Microglia, the resident macrophages of the brain that express Nox1 and Nox2, are activated very early in ischemia and their activation precedes macrophage infiltration [555,556]. However, Nox1 does not have any neuroprotective effect in severe cerebral ischemia [557,558]. Microglial Nox-derived $O_2^{\bullet -}$ enhanced astrocyte and cerebral endothelial cell death in a cell culture model of ischemia, whereas inhibition of microglia activation in mice markedly reduced BBB dysfunction and infarct volume in experimental stroke [559]. Additionally, activated astrocytes can produce large amounts of ROS and RNS via the stimulation of NADPH oxidase and iNOS [560,561], although this has not been shown under ischemia–reperfusion conditions. Infiltration of highly active Nox2-dependent NADPH oxidase-containing inflammatory cells such as neutrophils occurs for 3 days followed by that of blood-derived monocytes/macrophages, which peaks between 3 and 7 days after brain ischemia–reperfusion [555,562].

Nox4 protein is also expressed in neurons and cerebrovascular endothelial cells [558,563], with very high expression in basilar compared with systemic arteries [564]. Nox4 expression in mouse neurons is increased within 24 h after ischemia, peaks between days 7 and 15, and declines but remains high at day 30 [563]. Nox4 expression is also induced in the neurons and cerebrovascular endothelial cells of stroke patients [558]. Interestingly, Nox4 deletion had an overwhelmingly protective effect against cerebral infarction, BBB leakage, and neuronal apoptosis in mice subjected to both transient and permanent cerebral ischemia [558]. More recently, Hara and colleagues [565] have shown that Nox4 associates with Toll-like receptor 4 (TLR4; an essential component of innate immunity) in the brain tissue of mice and humans after ischemia–reperfusion. Deletion or pharmacological inhibition of TLR4 reduced Nox4 expression and suppressed ROS/RNS generation and neuronal apoptosis, indicating that activation of the TLR4–Nox4 signaling pathway is a potential pathophysiological mechanism in ischemic injury.

Conclusions and future perspectives

In conclusion, evidence from experimental and animal studies supports a decisive role for redox signaling in cardiovascular home-ostasis and disease. As mentioned earlier, translation of this knowledge into human therapy for CVD has not been particularly successful as demonstrated by the disappointing data from large antioxidant clinical trials [20,566]. Evolving consensus suggests that targeting the source of ROS using specific inhibitors might be more effective in treating CVD than the use of antioxidants [567]. The NADPH oxidases are perhaps the best example of this strategy, as direct inhibitors of various Nox catalytic subunits are in development [568]. The complexity of the regulation of these enzymes may also lend itself to therapeutic manipulation in a cell- and tissue-specific manner, with fewer off-target effects. Other potential means to modulate redox signaling and treat CVD include targeting uncoupled NOS and mitochondrial ROS and augmenting endogenous antiox-idant gene regulators such as Nrf2 and antioxidant systems such as thioredoxin and peroxiredoxins [569]. Because of the intricate nature of redox biology and the need to target ROS in specific organ systems, advances in the clinical management of CVD may depend on progress in other fields such as gene therapy, systems biology, and nanotechnology. A good example in this regard is the recent successful and safe completion of SERCA2 (a protein inactivated by high levels of ROS) gene therapy for

the treatment of heart failure [570]. Advances in nanotechnology may help in the detection of activated/dysfunctional endothelium by imaging the inflammatory markers [571–573]. Furthermore, nanotechnology-based drug delivery mechanisms targeted to specific cell types/organs (cardiomyo-cytes/heart) and organelles (mitochondria) may help transform the treatment of CVD and other diseases of oxidative stress.

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Abbreviations

AngII	angiotensin II
AP-1	activator protein-1
apoA-1	apolipoprotein A-1
Ask1	apoptosis signaling kinase-1
CAD	coronary artery disease
CAMTA	calmodulin-binding transcription activator
CHD	coronary heart disease
CVD	cardiovascular disease
CHF	congestive heart failure
EDHF	endothelium-derived hyperpolarizing factor
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ETC	electron transport chain
iNOS	inducible nitric oxide synthase
GPx	glutathione peroxidase
HDAC	histone deacetylase
HDL	high-density lipoprotein
HIF	hypoxia-inducible factor
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
L-NAME	N^{G}-nitro-l-arginine methyl ester
LDL	low-density lipoprotein
LOX	lipoxygenase
LVH	left-ventricular hypertrophy
MPO	myeloperoxidase

NF-κB	nuclear factor-κB
Nox	NADPH oxidase
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
Nrf2	nuclear factor E2-related factor 2
PDGF	platelet-derived growth factor
PKC	protein kinase C
PKG	protein kinase G
PON	paraoxonase
PTP	protein tyrosine phosphatase
ROS	reactive oxygen species
SOD	superoxide dismutase
SHP2	Src homology phosphatase 2
VCAM	vascular cell adhesion molecule
VSMC	vascular smooth muscle cell
XO	xanthine oxidase

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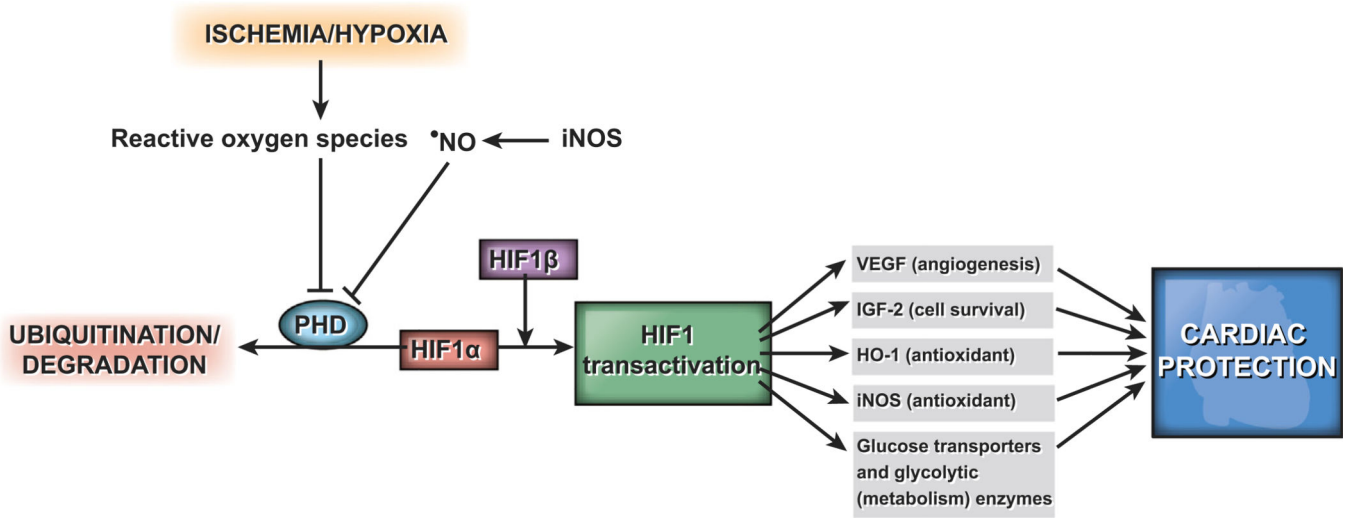

Fig. 1.

HIF transactivation confers cardiac protection during ischemia. *Abbreviations used:* HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; IGF-2, insulin-like growth factor-2; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; •NO, nitric oxide.

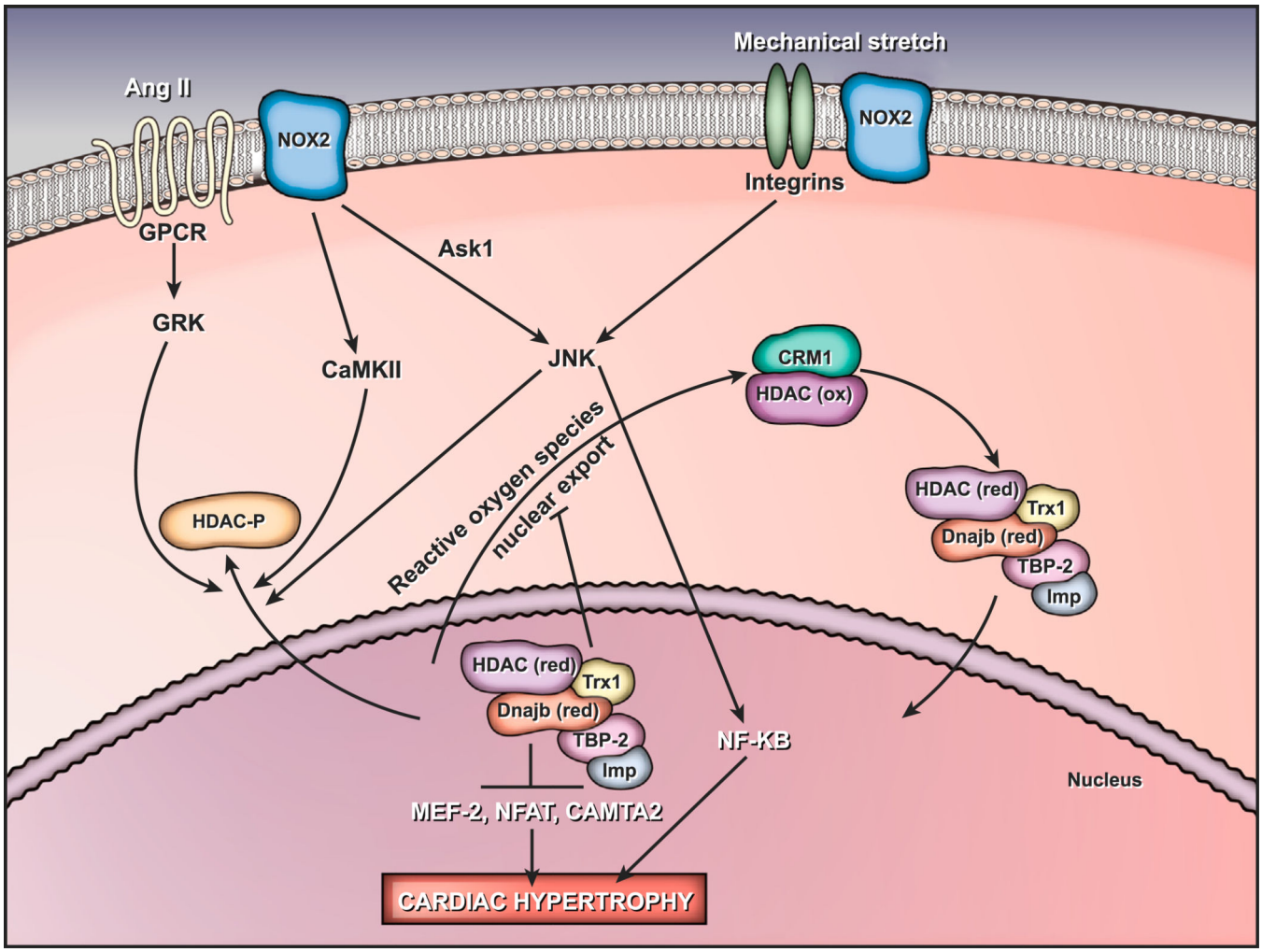

Fig. 2.

Redox signaling pathways regulate cardiac hypertrophy. Nox2 NADPH oxidase stimulation in response to G-protein-coupled receptor agonists or mechanical stretch activates the Ask1–NF-κB pathway, inducing cardiac hypertrophy. HDAC4 suppresses the activity of prohypertrophic transcription factors. Phosphorylation or oxidation of HDAC4 during oxidative stress conditions results in its export to the cytosol, leading to hypertrophy [511]. Abbreviations used: AngII, angiotensin II; Ask1, apoptosis signaling kinase 1; CaMKII, calcium/calmodulin-dependent kinase II; CAMTA2, calmodulin-binding transcription activator 2; CRM1, chromosomal region maintenance-1; Dnajb5, DnaJ homolog subfamily B member 5; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; HDAC, histone deacetylase; HDAC-P, phosphorylated HDAC; Imp, importin α; JNK, c-Jun N-terminal kinase; MEF-2, myocyte enhancer factor-2; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; ox, oxidized; red, reduced; TBP-2, thioredoxin binding protein-2; Trx1, thioredoxin 1.

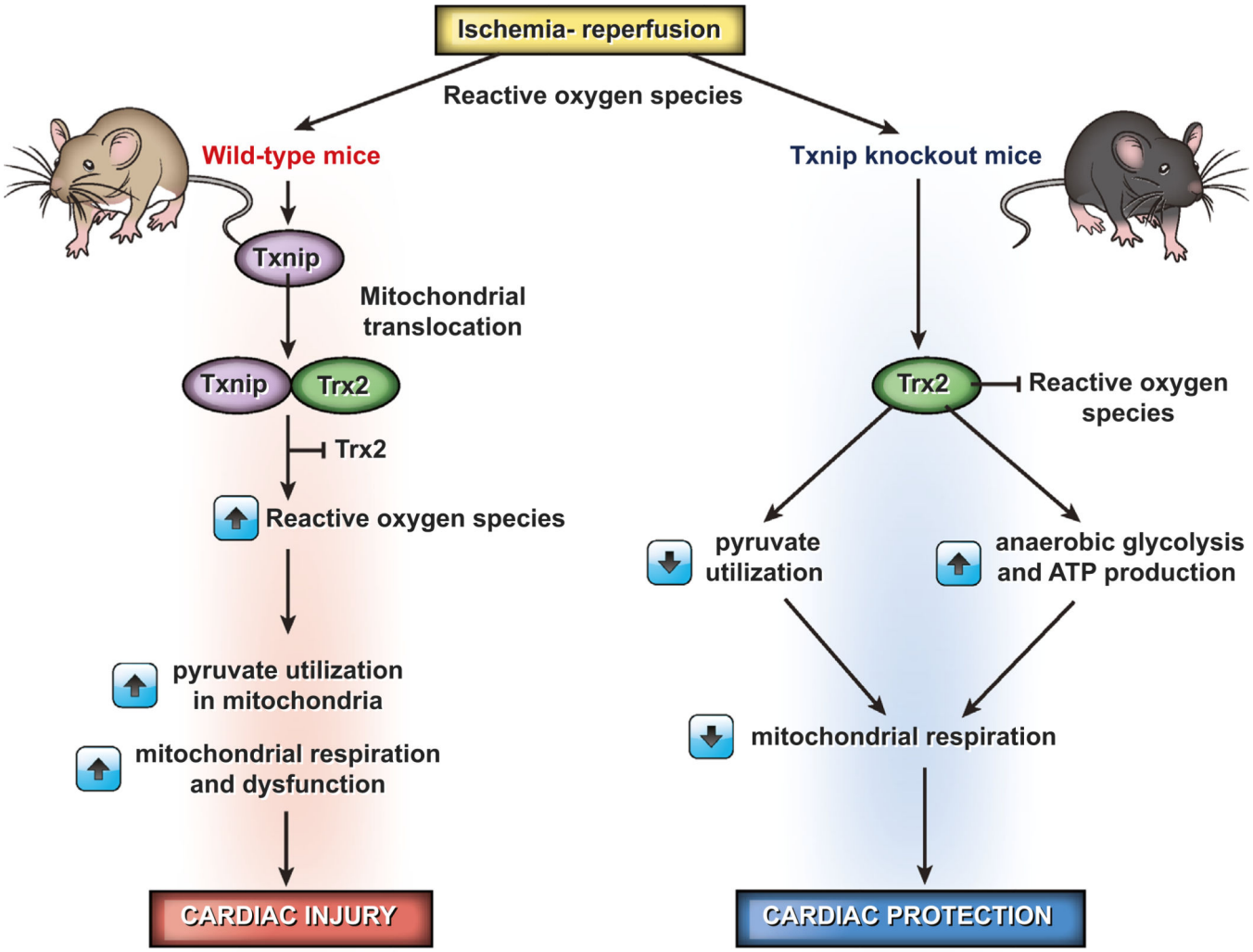

Fig. 3.

Thioredoxin-interacting protein (Txnip) regulates cardiac ischemia–reperfusion injury. In wild-type mice, Txnip translocates to mitochondria during myocardial ischemia–reperfusion and induces mitochondrial dysfunction by inhibiting thioredoxin 2 (Trx2) activity and increasing ROS levels. Txnip deficiency protects against cardiac ischemia–reperfusion injury by allowing efficient scavenging of mitochondrial ROS by Trx2 and maintaining energy homeostasis through enhanced anaerobic metabolism [536].

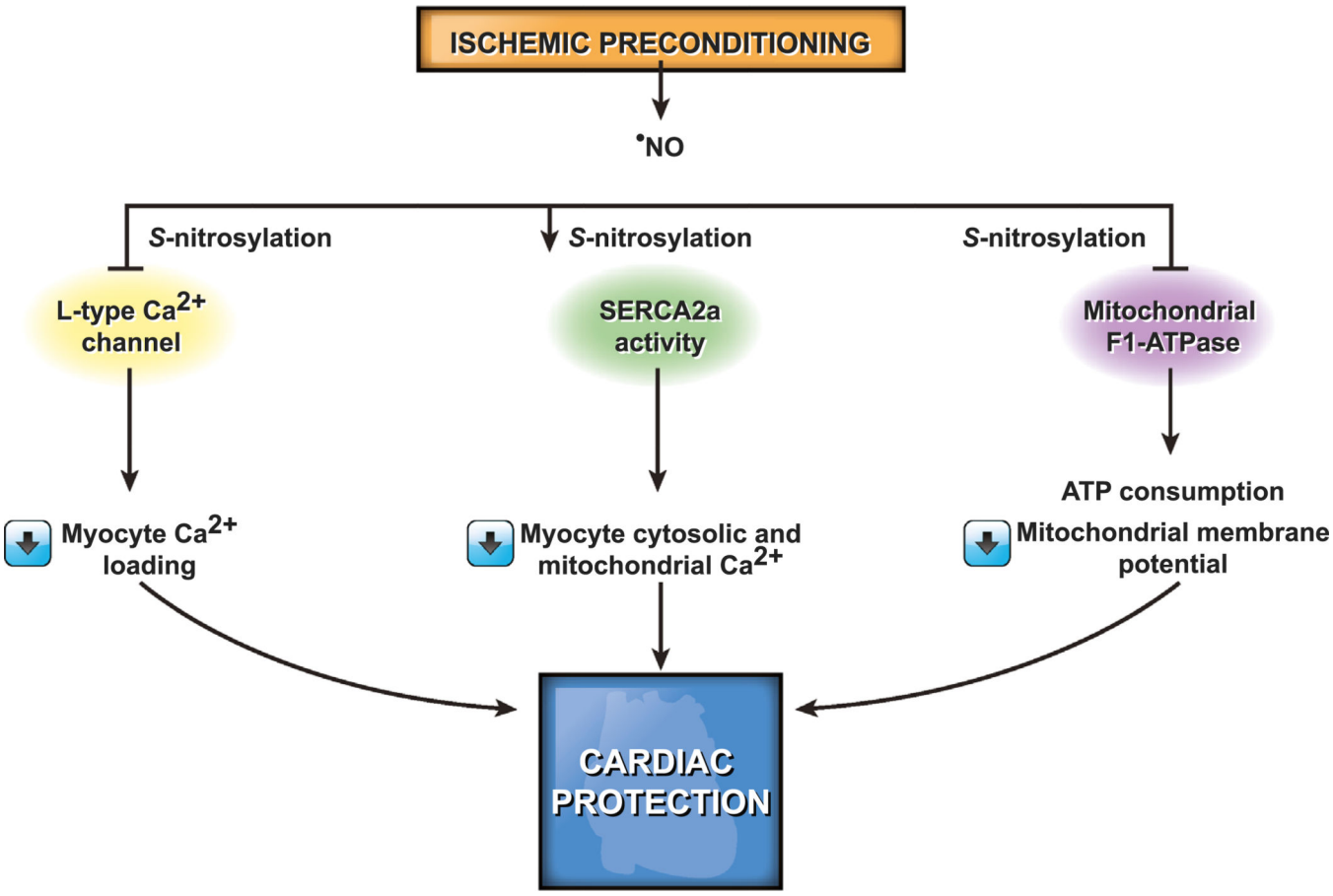

Fig. 4.

Preconditioning protects against myocardial ischemia–reperfusion injury by the S-nitrosylation of proteins regulating intracellular Ca^{2+} levels and mitochondrial energetics [adapted from 540]. Abbreviation used: SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase.

Table 1

Cardiovascular cell-specific expression of Nox isoforms.

Cell type	Nox isoform	Tissue	Ref.
VSMCs	Nox1	Mouse, rat aorta	[261,254,260]
VSMCs	Nox1	Human coronary artery	[10]
VSMCs	Nox2	Human resistance artery	[270]
VSMCs	Nox4	Mouse, rat aorta	[271,272]
VSMCs	Nox4	Human aorta	[273]
VSMCs	Nox4	Human coronary artery	[10]
VSMCs	Nox5	Human aorta	[277]
VSMCs	Duox1	Human aorta	[280]
Endothelial cells	Nox1	Human coronary artery	[10]
Endothelial cells	Nox1	Human umbilical vein	[262]
Endothelial cells	Nox2	Mouse, rat aorta	[263,264]
Endothelial cells	Nox2	Human coronary artery	[10]
Endothelial cells	Nox2	Human umbilical vein	[265,266]
Endothelial cells	Nox4	Rat aorta	[264]
Endothelial cells	Nox4	Human coronary artery	[10]
Endothelial cells	Nox4	Human heart	[10]
Fibroblasts	Nox1	Human heart	[10]
Fibroblasts	Nox2	Human coronary artery	[10]
Fibroblasts	Nox2	Human heart	[10]
Cardiomyocytes	Nox2	Mouse, rat heart	[265,269]
Cardiomyocytes	Nox4	Mouse heart	[275]

Table 2

Experimental mouse models support the role of oxidative stress in atherosclerosis.

Effector	Genetic model/pharmacologic agent	Phenotype	Ref.
$p66^{Shc}$	$ApoE^{-/-}/p66Shc^{-/-}$	Decreased oxidative stress, lipid peroxidation and atherosclerosis	[444]
PKCβ	$ApoE^{-/-}/PKC\beta^{-/-}$	Decreased MMP expression and activity; decreased atherosclerosis	[445]
JNK2	$ApoE^{-/-}/JNK2^{-/-}$	Decreased foam cell formation and atherosclerosis	[446]
p47phox (Nox1/2 NADPH oxidase activity)	$ApoE^{-/-}/p47phox^{-/-}$	Decreased vascular ROS levels and atherosclerosis	[3,464]
Nox1/2/4 expression/activity	AG490	Decreased atherosclerosis in $apoE^{-/-}$ mice	[466]
Nox2	$ApoE^{-/-}/Nox2^{y/-}$	Decreased vascular ROS levels; increased NO bioavailability and decreased atherosclerosis	[263]
Nox1	$ApoE^{-/-}/Nox1^{y/-}$	Decreased vascular ROS levels and atherosclerosis	[468]
Nox1/4	GKT136901	Decreased vascular ROS levels and atherosclerosis	[134]
Catalase; SOD1 and catalase	$ApoE^{-/-}/hCatTg^{0/+}$; $apoE^{-/-}/hSOD1Tg^{0/+}/hCatTg^{0/+}$	Decreased lipid peroxidation and atherosclerosis	[471]
SOD2	$ApoE^{-/-}/SOD2^{+/-}$	Increased mitochondrial ROS levels and mitochondrial DNA damage; increasedatherosclerosis	[4]
GPx	$ApoE^{-/-}/GPx^{-/-}$	Increased vascular ROS levels and atherosclerosis	[476]
eNOS	$ApoE^{-/-}/eNOS^{-/-}$	Accelerated atherosclerosis, aortic aneurysm, and ischemic heart disease	[314]
PON1	$PON1^{-/-}$; $apoE^{-/-}/PON1^{-/-}$	Increased atherosclerosis	[483,484]
PON2	PON2-deficient $apoE^{-/-}$	Increased mitochondrial oxidative stress	[486]
PON3	$hPON3Tg^{0/+}$; $apoE^{-/-}/hPON3Tg^{0/+}$	Decreased atherosclerosis	[487]