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# Impact of Oxidative Stress on the Heart and Vasculature Part 2 of a 3-Part Series

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## Abstract

Vascular disease and heart failure impart an enormous burden in terms of global morbidity and mortality. Although there are many different causes of cardiac and vascular disease, most share an important pathological mechanism: oxidative stress. In the failing heart, oxidative stress occurs in the myocardium and correlates with left ventricular dysfunction. Reactive oxygen species (ROS) negatively affect myocardial calcium handling, cause arrhythmias, and contribute to cardiac remodeling by inducing hypertrophic signaling, apoptosis, and necrosis. Similarly, oxidative balance in the vasculature is tightly regulated by a wealth of pro- and anti-oxidant systems that orchestrate region-specific ROS production and removal. ROS also regulate multiple vascular cell functions, including: endothelial and smooth muscle cell growth, proliferation, and migration; angiogenesis; apoptosis; vascular tone; host defenses; and genomic stability. However, excessive levels of ROS promote vascular disease via direct and irreversible oxidative damage to macromolecules, as well as disruption of redox-dependent vascular wall signaling processes.

### Keywords

cardiac; reactive oxygen species; vascular

With the basic biology of oxidative stress and telomeres already reviewed in Part 1 of this series, here in Part 2 we turn our attention to the impact of oxidative stress on the heart and

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vasculature, and, in particular, to the pathophysiological role of oxidative stress in heart failure and vascular disease (Central Illustration).

## PATHOPHYSIOLOGICAL ROLE OF OXIDATIVE STRESS IN HEART FAILURE

### INTRODUCTION

Heart failure (HF) is characterized by activation of the sympathetic nervous and renin-angiotensin-aldosterone systems. This neuroendocrine activation is associated with oxidative stress in the myocardium and vasculature. As outlined in Part 1 of this review, oxidative stress is an imbalance between the generation and detoxification of reactive oxygen species (ROS) (1,2). In patients with HF, oxidative stress occurs in the myocardium (3,4) and plasma, and correlates with left ventricular (LV) dysfunction (5). ROS negatively affect myocardial calcium ($Ca^{2+}$) handling, cause arrhythmias, and contribute to cardiac remodeling by inducing hypertrophic signaling, apoptosis, and necrosis (for review see (6,7)). Enzymatic sources for ROS, such as the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), uncoupled nitric oxide (NO) synthase, and mitochondria are all considered relevant sources of ROS in HF, causing vascular and myocardial dysfunction (2). Importantly, mitochondria amplify ROS derived from NOXs and may thereby function as “redox hubs” in cardiac physiology and disease (8,9).

In the initial part of this review, we focus on the crucial role of ROS in HF causing vascular and myocardial dysfunction. We also address the vitamin paradox by exploring why attempts to reduce oxidative stress in patients at cardiovascular risk (e.g., with vitamin E) caused, rather than prevented, the development of HF (2).

## ENZYMATIC SUPEROXIDE SOURCES IN HF AND FUNCTIONAL CONSEQUENCES FOR THE VASCULATURE AND MYOCARDIUM

### NADPH OXIDASE

In experimental models and patients with HF, myocardial superoxide ($O_2^-$)-generating NOX activity is increased (3,4). Specific NOX isoforms exist in endothelial cells (ECs), smooth muscle cells (SMCs), adventitial cells, and cardiac myocytes (10). In the latter, physiological stretch activates the sarcolemmal and transverse (T)-tubule–localized NOX2 (X-ROS signaling), and these ROS sensitize nearby ryanodine receptors to trigger $Ca^{2+}$ release from the sarcoplasmic reticulum (11). This *mechano-chemotransduction* also involves NO synthases and calmodulin-dependent protein kinase II (12), raising the possibility that peroxynitrite ($ONOO^-$) and methionine oxidation are involved.

Canonical activation of NOX2 is through $G_q$-coupled angiotensin II (ATII) receptors. Subpressor doses of ATII induce cardiac hypertrophy that is abolished by NOX2 deletion, although NOX2 deletion does not prevent HF development in response to severe pressure overload induced by aortic constriction (6). In animal models of myocardial infarction, NOX2 inactivation reduces infarct size and ameliorates HF development, but it is unclear whether this is related to vascular NOX or phagocytic NOX located in inflammatory cells. At higher ATII doses that induce hypertension, depletion of inflammatory cells attenuates

some features of oxidative stress, such as endothelial dysfunction, vascular ROS formation, and also arterial hypertension (13).

In contrast to NOX2, NOX4 does not require any regulatory subunits, and it constitutively produces hydrogen peroxide ($H_2O_2$), rather than $O_2^-$ (6). In the heart, NOX4 is located in the endoplasmic reticulum (ER), nucleus, and possibly also in mitochondria (6). Stimuli that activate NOX4 include ischemia, hypoxia, and adrenergic stimuli, which are all present and enhanced in HF. However, the role of NOX4 in HF development is controversial, as mice lacking cardiac NOX4 have been shown to exhibit both reduced and aggravated maladaptive remodeling in pressure overload-induced HF using differing model systems (6,14). Furthermore, NOX4-deficient mice exhibit partial protection against ischemic myocardial injury (15), whereas double knockout of NOX2 and NOX4 increases susceptibility to ischemic myocardial injury, perhaps through altered ROS production and down-regulation of stress-response pathways, such as hypoxia-inducible factor (HIF)-1α (15). As both myocardial and endothelial NOX4 promote angiogenesis, their absence from cardiac myocytes or vessels could also lead to capillary rarefaction and sensitivity to ischemic injury (16,17).

## MITOCHONDRIA

In mitochondria, $O_2^-$ is generated by the electron transfer chain (ETC), but is rapidly dismutated to $H_2O_2$ by manganese-dependent superoxide dismutase (Mn-SOD). $H_2O_2$ is then eliminated by antioxidative enzymes (i.e., glutathione peroxidase and peroxiredoxin), which require NADPH for regeneration (Figure 1). In HF, a functional block of complex I provokes excessive production of $O_2^-$, which is transformed to $H_2O_2$ and hydroxyl radicals ($OH^•$; via the Fenton reaction) (18). Besides increased production, the elimination of ROS in the mitochondrial matrix is compromised by dynamic oxidation of the antioxidative capacity, resulting in increased net emission of ROS from mitochondria (Figure 1) (concept of “redox-optimized ROS balance”; for reviews see (19,20)).

In mitochondria, the Krebs cycle generates nicotinamide adenine dinucleotide (NADH), which donates electrons to the ETC to produce adenosine triphosphate (ATP). However, the Krebs cycle also produces substrates that regenerate NADPH, which, in turn, regenerates antioxidative enzymes (Figure 1) (20,21). In HF, defects in cytosolic $Ca^{2+}$ and sodium ($Na^+$) handling in cardiac myocytes (e.g., reduced sarcoplasmic reticulum $Ca^{2+}$ release, elevated $Na^+$) reduce mitochondrial $Ca^{2+}$ accumulation and thereby hinder the regeneration of NADH and NADPH, which, on the one hand, compromises ATP production, while, on the other hand, provoking the emission of ROS from mitochondria (20).

In HF, limited substrates for ATP production (i.e., NADH) and ROS elimination (i.e., NADPH) face an increased energetic demand imposed by elevated cardiac preload, afterload, and heart rate. Workload increases adenosine diphosphate (ADP) production, and ADP accelerates respiration, which consumes NADH (Figure 1). In this scenario, the mitochondrial transhydrogenase (nicotinamide nucleotide transhydrogenase [NNT]), which under physiological conditions regenerates NADPH, then paradoxically consumes NADPH towards NADH and ATP production, but at the cost of antioxidative capacity (21). The resulting increase in mitochondrial $H_2O_2$ emission induces necrosis, LV dysfunction, and

death (21). In fact, targeting the $H_2O_2$-eliminating enzyme catalase to mitochondria prevents the development of HF in response to pressure overload and/or neurohormonal activation (22,23). Taken together, these data indicate that in HF, increased production and decreased elimination of ROS in cardiac myocytes conspire to elevate net mitochondrial ROS emission, which plays a causal role in the pathobiology of HF.

## UNCOUPLED ENDOTHELIAL NITRIC OXIDE SYNTHASE IN HF

In patients with HF, concomitant endothelial dysfunction can be improved with acute administration of oral or intra-arterial vitamin C, suggesting that oxidative stress contributes to endothelial dysfunction in this condition (24); an observation that is also supported by preclinical studies. In fact, uncoupling of endothelial NO synthase (eNOS) in both the vasculature and the myocardium contribute to HF development in animal models, a condition where NO production by the synthase is converted to $O_2^-$ generation (25). Similarly, several other enzymes downstream in the NO cascade are also inhibited (e.g., soluble guanylyl cyclase or cyclic guanosine monophosphate [cGMP]-dependent kinase I), leading to an impairment of NO signaling in vascular SMCs (4).

## XANTHINE/XANTHINE OXIDASE

In chronic HF, serum uric acid levels rise with increased purine catabolism resulting from tissue hypoxia, apoptosis, and/or enhanced or up-regulated xanthine oxidoreductase activity (reviewed in (26)). Xanthine oxidase (XO) activity and expression are also increased, and its inhibition improves coronary and peripheral endothelial dysfunction, as well as contractile efficacy in patients with HF due to coronary artery disease (27). However, despite these promising effects in smaller trials (27), larger multicenter trials of XO inhibitors in patients with HF were neutral (28).

## CROSSTALK BETWEEN DIFFERENT ROS SOURCES

ROS biology likely includes self-supporting mechanisms in which ROS trigger further ROS formation from various sources through the activation of “redox switches”. For instance, oxidation of thiol residues of xanthine dehydrogenase converts this enzyme to a $O_2^-$ producing oxidase, and uncoupling of eNOS also involves redox-sensitive kinases, tetrahydrobiopterin ($BH_4$) depletion, and disruption of the enzyme’s zinc-sulfur complex (8). These mechanisms may be initiated by the “escape” of mitochondrial ROS, which may occur in a burst-like fashion as a consequence of increased membrane permeability under de-energized conditions in the failing heart. Conversely, ROS derived from NOX2 require further mitochondrial ROS release to trigger maladaptive remodeling and diastolic dysfunction in response to ATII infusion (9), a process termed *ROS-induced ROS release* (Figure 1). In these studies, ROS scavenging in the cytosol and/or peroxisomes (by N-acetyl cysteine or targeting catalase to these compartments) was ineffective, whereas reducing mitochondrial ROS (by targeting catalase to mitochondria or by the tetrapeptide SS-31, see later discussion) prevented pathology (9,23,29). These data indicate that mitochondria could resemble a final “afterburner” that integrates ROS signals from various cellular sources and accounts for detrimental ROS-induced remodeling and HF development (Figure 1) (for a more mechanistic review, see also (9,20,29)).

## THERAPEUTIC STRATEGIES IN HF

### ANTIOXIDANTS/VITAMINS

Despite a predominant role of ROS in the pathophysiology of HF, large clinical trials have failed to show any benefits of antioxidants such as vitamins E and C (1,2). A possible explanation is that these vitamins are not specifically targeted to the sites of ROS generation (e.g., mitochondria) that are most important in pathological conditions. Furthermore, vitamins react much more slowly with ROS than ROS can interact with their direct counterpart NO to induce damage, and therefore exceptionally high concentrations of vitamins would be required to override these kinetic constraints (1). However, when administered in high doses, vitamin E worsens, rather than improves vascular function (30), which may be related to the formation of the pro-oxidative vitamin E radical (31). This may also explain why long-term treatment with vitamin E does not prevent, but rather induces HF and acute left heart decompensation, as shown in the HOPE (32) and HOPE-TOO trials (33).

### TARGETING MITOCHONDRIAL REDOX AND ROS

On the basis of this experience with vitamins C and E, a more promising approach may be to target more specifically the most relevant compartments for ROS production (Figure 1). As reviewed previously (34), mitochondrial ROS can be targeted in at least 3 different ways. First, due to the negative mitochondrial membrane potential ($\Delta\Psi_m \approx -180$ mV), antioxidants can be coupled as “cargo” to lipophilic cations, which accumulate several hundred-fold in the matrix. This is discussed in greater detail in a subsequent section.

Second, some drugs, such as the tetrapeptide SS-31, which binds to cardiolipin (35), accumulate in mitochondria because they bind to mitochondria-specific structures. Cardiolipin is an important phospholipid of the inner mitochondrial membrane that controls the assembly of ETC complexes (35). Oxidation of cardiolipin in HF (36) hampers ETC assembly, and thereby provokes deviation of electrons to oxygen, which increases $O_2^-$ formation. SS-31 is not a direct antioxidant (37), but binds specifically to cardiolipin and prevents its oxidation, thereby improving electron transfer along the ETC and reducing aberrant production of $O_2^-$ (35). In various preclinical HF models, SS-31 reduced oxidative stress, preserved mitochondrial function, and prevented necrosis, maladaptive remodeling, LV dysfunction, and death (21,35). Although, in a first clinical trial involving 297 patients with acute myocardial infarction, a single application of SS-31 did not reduce infarct size, it was, however, safe (38), and is currently undergoing evaluation for longer-term treatment in patients with HF (NCT02814097, NCT02788747 and NCT02914665) (35).

A third option to target mitochondrial ROS is to interfere with cellular ion handling. Because an elevated cytosolic $Na^+$ concentration accelerates mitochondrial $Ca^{2+}$ extrusion, inhibition of the mitochondrial $Na^+/Ca^{2+}$ exchanger with CGP-37157 (Figure 1) prevents oxidation of NADH and NADPH, emission of ROS, maladaptive cardiac remodeling, and arrhythmias in a guinea pig model of systolic HF (20,39). This compound, however, is yet to be evaluated in humans.

Although the listed interventions target oxidative stress, there is also some evidence that in HF, reductive stress may be relevant to disease development and a potential target for

treatment. Down-regulation of complex I in HF may reduce the redox state of NADH/$NAD^+$, thereby limiting the availability of $NAD^+$ to the mitochondrial $NAD^+$-dependent deacetylase sirtuin 3 (SIRT3) (40). The ensuing hyperacetylation of mitochondrial proteins induces an energetic deficit and contractile dysfunction, whereas elevating $NAD^+$ synthesis via the $NAD^+$ salvage pathway by supplementing with the $NAD^+$ precursor, nicotinamide mononucleotide (NMN), prevented these changes (40). Due to its deacetylase activity, SIRT3 is therefore protective in various cardiac pathologies (40,41). Although hyperacetylation of mitochondrial proteins can occur in common forms of HF (40), it has also been identified as an important downstream consequence of mutations in the human αB-crystallin gene that are responsible for the development of desmin-related cardiomyopathy (42).

### ANGIOTENSIN-CONVERTING ENZYME INHIBITORS AND ANGIOTENSIN-1-RECEPTOR BLOCKERS

As ATII is a key upstream trigger of ROS formation from NADPH oxidase and, in turn, mitochondria (9), it is reasonable that antiotensin 1 (AT1) antagonists and angiotensin-converting enzyme (ACE) inhibitors may exert their beneficial clinical effects in part through antioxidative mechanisms. In fact, ACE inhibitors ameliorate inflammatory processes in the vessel wall (4), prevent SMC proliferation, and, in particular, prevent activation of the vascular and phagocytic NADPH oxidase (4,43). Thus, although vitamin E therapy can be considered as a secondary therapy that scavenges already formed ROS, ACE inhibitors (and also AT-1 receptor blockers) can be thought of as a primary therapy that blocks ROS production at the enzymatic source (43).

### HYDRALAZINE

Another drug with often underestimated beneficial effects on the balance between $O_2^-$ and NO (the “nitroso-redox balance”), which is impaired in HF (44), is hydralazine. Clinical treatment with nitrates (e.g., nitroglycerin or isosorbide dinitrate [ISDN]), induces tolerance to its vascular and hemodynamic effects, mediated largely by endothelial dysfunction (for review see (45)). When combined with hydralazine, however, nitrate tolerance is prevented because hydralazine inhibits nitroglycerin-induced vascular $O_2^-$ and $ONOO^-$ formation both in vitro (46) and in vivo (47) (Figure 2). Also, in failing cardiac myocytes, hydralazine improves cytosolic $Ca^{2+}$ handling, in part by scavenging $O_2^-$ formation (48). Thus, the antioxidant action of hydralazine and the prevention of tolerance development in response to ISDN (49) may explain, at least in part, why this combination improves morbidity and mortality in patients with chronic congestive HF (50).

## CONCLUSIONS REGARDING OXIDATIVE STRESS IN HF

Although at lower concentrations, ROS serve physiological (protective) functions (6), higher levels of oxidative stress triggered by neurohormones and hemodynamic alterations play a causal role in the initiation and progression of HF. In recent years, the importance of ROS compartmentalization has been progressively recognized, and instead of global, nonspecific antioxidative approaches (e.g., with vitamins), specific treatments targeted toward these compartments (in particular, mitochondria) may hold promise for ongoing and future drug

development. These treatments may add to the established benefit provided by the upstream therapy of neuroendocrine antagonism.

## OXIDATIVE STRESS IN THE VASCULATURE

### PATHOPHYSIOLOGY OF ROS IN THE VASCULATURE

Vascular dysfunction or disease refers to different pathological states affecting the venous and/or arterial system that may give rise to adverse cardiovascular events. Among these pathologies are atherosclerosis, arterial remodeling, restenosis, and thrombosis. Different risk factors predisposing to cardiovascular disease (CVD), such as hypercholesterolemia, diabetes, obesity, hypertension, and aging, lead to vascular dysfunction and disease, partially through oxidative stress, making the latter a common denominator to risk factor–induced vascular disease, as well as an attractive therapeutic and preventive target (51,52). Under physiological conditions, oxidative balance is sustained by a tightly regulated wealth of pro- and anti-oxidant systems distributed within different cellular compartments to orchestrate region-specific ROS production (Figure 3). In fact, ROS have been shown to regulate multiple cellular functions, including: EC and SMC growth, proliferation, and migration; angiogenesis; EC and SMC apoptosis; vascular tone; host defense; and genomic stability (53). Excessive levels of oxidants over antioxidants, however, cause a deviation in the redox state, defined as oxidative stress. This mediates vascular disease through direct and irreversible oxidative damage to macromolecules, as well as disruption of redox-dependent signaling processes in the vascular wall (54).

Among the signal transduction pathways and targets modulated by ROS are proximal tyrosine kinases, such as c-Src, platelet-derived growth factor receptor, epidermal growth factor receptor, the low molecular weight guanosine-5′-triphosphate (GTP)-binding protein Ras, mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinases (ERK1/2), p38, and c-Jun N-terminal kinases, as well as the serine/threonine kinase Akt/protein kinase B (55). Furthermore, ROS directly regulate several classes of genes that are crucial for vascular function (e.g., adhesion molecules, chemotactic factors, vasoactive substances, and antioxidant enzymes), whereas ROS also indirectly modulate genomic activity through transcription factors such as activator protein 1, nuclear transcription factor kappa-light-chain-enhancer of activated B cells (NF-κB), and HIF-1.

In conditions of oxidative stress, however, ROS mediate pathological vascular changes, such as endothelial dysfunction, by disrupting the vasoprotective NO signaling cascade (56). NO is a key antiatherogenic player, which inhibits platelet adhesion and aggregation, powerfully augments vasodilation through stimulation of cGMP production by SMCs, decreases leukocyte-endothelial interactions, and reduces SMC proliferation (25). It is generated from L-arginine by eNOS in the presence of its essential cofactor $BH_4$. Superoxide ($O_2^-$) rapidly scavenges NO to produce $ONOO^-$ (57), a potent oxidant in its own right. This rapid reaction between $O_2^-$ and NO, yielding $ONOO^-$, is about 3 to 4 times faster than the dismutation of $O_2^-$ by the superoxide dismutases (SODs). Therefore, depending on the rates of tissue $O_2^-$ production, this pathway can be of major importance, particularly as $ONOO^-$ is cytotoxic and causes damage to proteins, lipids, and DNA (58,59). Furthermore, in these settings, NO is not only depleted, but peroxynitrite-induced oxidation of $BH_4$ leads to a state of eNOS

uncoupling (60), where oxygen reduction takes place independently from NO production, turning eNOS into a pro-oxidant enzyme (61).

ROS also play a key role in the inflammatory component of atherosclerosis, where they mediate inflammasome formation, which, in turn, supports secretion and processing of the proinflammatory cytokines interleukin (IL)-1β) and IL-8 via caspase 1 activation (62).

## IMBALANCE IN VASCULAR ROS PRODUCTION AND SCAVENGING: ROS-PRODUCING ENZYMES

Oxidative balance is tightly regulated by several key enzymes that either produce or dispose of free radicals (Figure 3). Although many enzymes are known to produce ROS in the vascular wall, 4 enzymes in particular seem to play an especially prominent role, namely NADPH oxidase, enzymes of the mitochondrial respiratory chain, XO, and uncoupled eNOS. Further sources of vascular ROS include lipoxygenase, cyclooxygenase, and cytochrome P 450 monooxygenase.

**NADPH oxidases—**NADPH oxidases are major sources of vascular ROS and are unique in their sole commitment to ROS-production. They produce $O_2^-$ from molecular oxygen using NADPH as an electron donor, and are found in various vascular cell types, including ECs (NOX2 and 4), SMCs (NOX1 and 4), and fibroblasts (NOX2 and 4) (62,63). The multisubunit enzyme complexes rely on up to 5 regulatory subunits and, in some cases, on a small GTPase (Rac1 or 2) for correct assembly and activation. Evidence for NADPH oxidase activation in vascular disease stems from animal models of hypertension (genetic and ATII-induced), diabetes, and hypercholesterolemia.

Genetic disruption or overexpression of NOX1 in mice is associated with reduced or increased blood pressure responses to ATII, respectively (64,65). The systemic deletion of NOX2 or the p47phox subunit in apolipoprotein E-deficient ($ApoE^{-/-}$) mice was shown to decrease atherosclerotic burden (66,67) and diminish blood pressure response to ATII (67). In humans, atherosclerosis correlates with increased coronary expression of NADPH oxidase subunits (p22phox, NOX2, p47phox, and p67phox) (68).

**Xanthine oxidase—**Xanthine oxidase (XO) produces $O_2^-$ and $H_2O_2$, and is a source of $O_2^-$ in diseased human coronary arteries (68). It is expressed by ECs in response to several stimuli, including ATII (69). It is also produced in a circulating form by the liver, a mechanism of production that is promoted by hypercholesterolemia, and can adhere to ECs through interaction with glycosaminoglycans (70). The activity of both endothelial and plasma XO is elevated in experimental models of atherosclerosis and in human atherosclerotic plaques. Consistent with this, XO inhibitors improve endothelial function in aortic ring explant assays from hyperlipidemic animals (70), attenuate endothelial dysfunction in heavy smokers (71), and reduce the development of atherosclerosis in $ApoE^{-/-}$ mice (72). However, clinical data concerning XO inhibitors are controversial (56).

**Mitochondrial dysfunction and contribution to ROS—**Under physiological conditions, mitochondria represent a key source of $O_2^-$ by reducing approximately 1% of consumed oxygen by only a single electron to $O_2^-$. In this respect, the ETC enzyme

complexes NADH dehydrogenase (complex I) and ubiquinone-cytochrome b-c1 (complex III) play an integral role, whereas the amount of mitochondrial $O_2^-$ released also depends on Mn-SOD activity. In pathophysiological conditions, evidence suggests that mitochondrial dysfunction (73) and mitochondrial ROS generation could be linked to atherogenesis through promotion of other ROS sources and the release of mitochondrial apoptotic signals (74,75). In ECs, mitochondrial dysfunction due to Mn-SOD deficiency was indeed correlated to accelerated atherogenesis in *ApoE*$^{-/-}$ mice (76). Accordingly, specific targeting of antioxidants to mitochondria has raised hopes in preclinical studies, but has yet to be translated to treatment of human vascular disease.

**Dysfunctional eNOS and inducible NO synthase—**As mentioned, oxidative stress causes endothelial dysfunction through $O_2^-$ scavenging of NO to produce $ONOO^-$, followed by uncoupling of oxygen reduction from NO synthesis by eNOS, causing it to produce not NO, but $O_2^-$. This pathological process, termed eNOS uncoupling, is evident in several animal models and in humans with endothelial dysfunction. The peroxynitrite-mediated depletion of the essential cofactor $BH_4$ is considered a significant (but not the only) cause of eNOS uncoupling. In addition, the inducible form of NO synthase (iNOS) is expressed in the vasculature in pathological states such as inflammation, oxidative stress, or sepsis. iNOS produces excessive amounts of NO and mediates impaired vasoconstriction and endothelium-dependent vasodilation. Proposed mechanisms for impaired contractile responses include continuous guanylyl cyclase activation (77), disturbed vascular calcium regulation (78), and oxidative alteration of catecholamines (79). Concurrently, impaired vasodilation may be further worsened by decreased eNOS activity, resulting from competition with iNOS for $BH_4$ and NO scavenging by $O_2^-$ (80). Additionally, continuous NO exposure can impair the link between endothelial receptors and calcium-calmodulin–dependent activation of eNOS (81) and the sensitivity of SMCs to NO (82,83). In vivo, genetic deficiency of iNOS was seen to reduce atherosclerosis in *ApoE*$^{-/-}$ mice (84). In humans, iNOS expression in plaques from patients with transplant coronary artery disease correlates with nitrotyrosine residues and protein nitrosylation, both markers of $ONOO^-$ formation, which is an important mediator of atherogenesis (85).

## IMBALANCE IN VASCULAR ROS PRODUCTION AND SCAVENGING: ANTIOXIDANT ENZYMES

Counterbalancing the ROS-producing factors discussed in the previous section, the following act as the major defenses against vascular ROS.

**Superoxide dismutase—**Three SOD isoforms exist in humans. SOD1 (copper, zinc [Cu-Zn]-SOD) is a soluble enzyme of the cytoplasm and mitochondrial intermembrane space. SOD2 (Mn-SOD) exists in the mitochondrial matrix, whereas SOD3 (extracellular [EC]-SOD) is secreted into the extracellular space. All isozymes catalyze the dismutation of $O_2^-$ to $H_2O_2$ and oxygen, thus blunting superoxide-mediated NO inactivation. High levels of superoxide dismutation products, however, can cross cellular membranes to form proatherogenic molecules, thus rendering the SOD effect dose-dependent. Accordingly, SOD1-overexpressing mice develop more pronounced fatty streaks on an atherogenic diet

(86), whereas combined overexpression of SOD and catalase retards atherogenesis in *ApoE*$^{-/-}$ mice (87).

SOD2 is the frontline defense against mitochondrial $O_2^-$. The role of this enzyme is underlined by the fact that SOD2 knockout mice die within days of birth, whereas heterozygous SOD2 deficiency mediates mitochondrial dysfunction and DNA damage, as well as atherosclerosis in *ApoE*$^{-/-}$ mice (74). Vascular SOD3 is produced by macrophages and SMCs, the same cell types implicated in iNOS induction (88). Thus, SOD3 may also prevent formation of $ONOO^-$, as well as $O_2^-$. The functional role of SOD3 in atherogenesis, however, remains poorly understood (89).

**Catalase—**Catalase, a tetramer of 4 polypeptides, catalyzes the decomposition of $H_2O_2$ to oxygen and water by virtue of 4 porphyrin heme iron groups. The enzyme's overexpression blunts atherosclerosis (87) and inhibits ATII-mediated aortic wall hypertrophy (90). The differing contributions of various ROS to vascular disease are underlined by the observation that some atherosclerosis models, like *ApoE*$^{-/-}$ mice or a high fat diet, are attenuated by catalase, but not SOD1 overexpression alone (87,91).

**Paraoxonases—**Three members make up the paraoxonase (PON) family: PON1, 2, and 3. Despite their varying functions, localizations, and activities, all exhibit antiatherogenic properties, most likely through inhibition of oxidative stress and lipid peroxidation (92). PON1, mainly secreted by the liver, associates with high-density lipoprotein (HDL) in the plasma, where it protects HDL and low-density lipoprotein (LDL) against peroxidation and degrades cholesteryl esters and lipoproteins found in oxidized lipoproteins. It inhibits ROS production by myeloperoxidase (MPO), an enzyme secreted by immune cells upon activation and an accepted biomarker in acute coronary syndrome (93), thereby reducing oxidative stress, inflammation, and monocyte attraction.

PON2 is localized intracellularly in membranes of the ER and mitochondria, and exerts antioxidative effects in vascular cells (94,95). It has been proposed that PON2 interacts with coenzyme Q10 (96,97) and thus inhibits mitochondrial $O_2^-$ generation, which is of relevance both to atherosclerosis and cancer. In line with this, PON2 has antiatherogenic effects in mice (92) and possibly humans, where its expression is lost in vessels with advanced atherosclerotic disease (98). PON3 is found both in serum and cells, and appears to exert similar effects to PON2 (99). Like the other members of the family, PON3 attenuates atherogenesis in mice (100) and is lost in vascular cells from patients with atherosclerosis (101).

**Glutathione peroxidase—**Glutathione peroxidase (GPx) exists in 8 isoforms, and reduces $H_2O_2$ to water and lipid peroxides to their respective alcohols. GPx1, among the most abundant GPx enzymes, is expressed in many cell types. GPx1 deficiency in mice raises sensitivity to myocardial ischemia/reperfusion injury and atherosclerotic burden in *ApoE*$^{-/-}$ and diabetic mice (102–104). In humans, GPx1 activity in erythrocytes correlates inversely to the risk of cardiovascular events in patients with coronary artery disease (105).

GPx4 is unique in function and structure. It is dispersed among various subcellular locations, such as the ER, cytoplasm, mitochondria, and plasma membrane. Apart from $H_2O_2$, it can detoxify a broad selection of lipid and cholesterol hydroperoxides. GPx4 overexpression in *ApoE*$^{-/-}$ mice blunts atherogenesis by inhibiting lipid peroxidation and vascular cell sensitivity to oxidized lipids (106).

**Heme oxygenase—**Heme oxygenase (HO) catalyzes the first part of the disintegration of heme to carbon monoxide (CO), biliverdin, and free ferrous iron. It exists in 3 isoforms, with HO-1 representing the inducible, HO-2 the constitutive, and HO-3 the enzymatically-inactive variant. HO-1 expression is induced by oxidative stress, hypoxia, and certain cytokines. It is implicated in protection from adverse vascular remodeling and atherosclerosis (107). Mechanistically, the breakdown of pro-oxidant heme to the ROS scavenger bilirubin is thought to be of importance. Apart from radical scavenging, bilirubin also inhibits the assembly and activity of NADPH oxidase (108). Reduced heme availability can additionally interfere with the formation of NOX2, and thereby NADPH oxidase (109). Although toxic at very high levels, CO produced in small amounts by HO exhibits anti-inflammatory, antiproliferative, and vasodilatory functions, with each of these being implicated in adverse vascular remodeling (109). Different genetic models of HO deficiency and overexpression support its role in atherosclerosis (110).

**Thioredoxin—**Thioredoxin (Trx) consists of 2 systems, Trx1/thioredoxin-reductase (TrxR1) and mitochondrial Trx2/TrxR2, and is found in ECs, SMCs, and fibroblasts. It has direct and indirect antioxidant actions, and plays an accepted vasoprotective role. Recently, Trx was shown to reverse age-related hypertension and arterial stiffness by improving vascular redox and restoring eNOS function (111).

**Small nonprotein antioxidants—**Nonenzymatic, low molecular weight compounds, including metabolic products like uric acid, bilirubin, and glutathione, as well as exogenous composites, such as antioxidant vitamins (mainly C and E) and polyphenols, add to cellular antioxidant systems. Glutathione determines the intracellular redox state, whereas bilirubin and uric acid scavenge extracellular ROS. Evidence for a protective role of these small nonprotein antioxidants in vivo, however, is still limited. Vitamin C (L-ascorbate) scavenges several reactive oxygen and nitrogen species, and restores α-tocopherol from its radical state (112). Furthermore, vitamin C stabilizes $BH_4$ (113) and, in turn, eNOS. Among the 8 tocopherols and tocotrienols constituting vitamin E, α-tocopherol is the most active and its major antioxidant action is thought to be scavenging of lipid peroxides. Nevertheless, upon radical-scavenging, α-tocopherol itself is converted into a pro-oxidant radical (tocopheroxyl radical), potentially limiting its in vivo efficacy. Finally, some of the polyphenolic antioxidants in food (e.g., vegetables, cocoa) and beverages (e.g., green tea, red wine) have been demonstrated to inhibit the activity of NADPH oxidases or support the activation of antioxidant enzymes, possibly explaining positive data from epidemiological studies (114,115).

## CARDIOVASCULAR RISK FACTORS, ATHEROSCLEROSIS, AND ROS

Although dealt with at the level of the entire cardiovascular system in Part 3 of this review series, it is important to note from the vascular perspective that classical cardiovascular risk factors associate with increased oxidative stress and specifically with endothelial dysfunction. Indeed, oxidative stress is likely to be the common pathological mechanism through which different risk factors contribute to the development of vascular disease, rendering it an attractive therapeutic target (Figure 4).

Atherosclerosis, as a chronic arterial inflammatory state, is strongly linked to oxidative stress (116). Indeed, increased ROS favor proinflammatory gene expression, including vascular cell adhesion molecule-1, intercellular adhesion molecule-1, monocyte chemotactic protein 1, and E-selectin, which in turn, are transcribed by redox-sensitive transcription factors like NF-κB, activator protein-1, HIF-1β, and early growth response protein 1. These genes are crucially involved in the early phases of atherogenesis, such as endothelial-leukocyte interactions (117). The same transcription factors also play a role in the proliferative signaling associated with SMC growth and vascular remodeling (118). MAPKs, as well as receptor and nonreceptor tyrosine kinases, are known to be activated through ROS. For instance, p38 MAPK is activated by ATII-mediated $O_2^-$ production through NADPH oxidase, which, in turn, stimulates the fibroblast migration and SMC proliferation that contribute to the atherogenic process (Figure 5).

We have recently shown that oxidative stress can directly promote endothelial-to-mesenchymal transition (EndMT) (116). Although indispensable for the normal development of the cardiovascular system (119), EndMT in the adult is generally considered to be pathological and may arise in in the setting of chronic inflammatory diseases, such as atherosclerosis. We identified that high concentrations of $H_2O_2$ cause EndMT, with the extent of EndMT-derived mesenchymal cells (fibroblasts, SMCs) in atherosclerotic plaques related to an advanced plaque phenotype and human plaque instability (116). We are currently engaged in further unraveling the relevant signaling pathways, and are actively investigating possible ways to manipulate EndMT for therapeutic gain.

Oxidative stress is also a critical component of hypertension, where NADPH oxidase represents the primary source of ROS. Mechanistically, ATII, through angiotensin 1 receptor (AT1R) signaling, mediates the vascular up-regulation and activation of NADPH oxidase (120,121) promoted by rapid translocation of the small GTPase Rac 1 (Ras-related C3 botulinum toxin substrate 1) to the cellular membrane (122), or by phosphorylation and membrane translocation of the p47phox subunit (123). Mechanical stretch, as observed in hypertension, has also been directly linked to p47phox translocation and NADPH oxidase activation (124,125). The role of oxidative stress in hypertension is further supported by the blood pressure-lowering effects of genetic deletion or pharmacological inhibition of NADPH oxidase (67,69,126).

Hyperglycemia promotes cellular oxidative stress (127) with the mitochondrial respiratory chain as the primary source of $O_2^-$ (128). Mitochondrial $O_2^-$ mediates stimulation of protein kinase C and generation of advanced glycation end-products (128). Protein kinase C and advanced glycation end-products in turn activate NADPH oxidase and inhibit eNOS via

post-translational modification (129), whereas NADPH oxidase-mediated eNOS uncoupling further promotes oxidative stress (130). Insulin resistance, as found in type 2 diabetes, may inhibit GTP cyclohydrolase 1 (GTPCH1) activity and thus de novo $BH_4$ synthesis. Furthermore, the insulin-mediated activation of eNOS via the phosphoinositide 3 kinase (PI3K)/Akt pathway is disrupted in a state of insulin resistance. Increased levels of ATII found in patients with diabetes could contribute to NADPH oxidase activation and reduce $BH_4$ recycling from $BH_2$ by dihydrofolate reductase (131).

In hypercholesterolemia, NADPH oxidase and XO appear to be key sources of $O_2^-$ (68); in fact, uncoupling of eNOS is possibly secondary to oxidative stress induced by these enzymes (132,133), as observed in LDL cholesterol–treated ECs, *ApoE*$^{-/-}$ mice, and hypercholesterolemic humans (134,135). LDL cholesterol and, especially, oxidized LDL (oxLDL) cholesterol are known to promote generation of $O_2^-$ and $ONOO^-$, and thereby regulate monocyte adhesion, inflammatory vascular gene expression, and redox-sensitive transcription factors, such as NF-κB and activator protein-1. Cellular lipoxygenases (LOs) have been proposed as a possible enzymatic source of oxLDL cholesterol. Accordingly, elevated levels of 15-LO have been observed in atherosclerotic lesions of the human and rabbit aorta (136). Adding to the direct effects of LDL cholesterol on vascular ROS production, dyslipidemia may potentiate the effects of ATII via up-regulation of the AT1R (137).

In vascular aging, 2 characteristic ROS-related features have been defined, namely endothelial dysfunction and central arterial stiffness (138). Endothelial dysfunction is mediated by reduced NO bioavailability, either through decreased synthesis or increased degradation. Studies provide conflicting evidence on age-related changes in eNOS protein expression (139–141). However, more recent data are suggestive of an age-dependent eNOS uncoupling (142), favoring oxidative stress through decreased availability of NO and increased $O_2^-$ and $ONOO^-$ production. The perturbed redox balance promotes inflammation, which drives increased NADPH oxidase expression and $O_2^-$ production through mediators, such as blood-borne tumor necrosis factor α. In parallel, age-dependent activation of the renin-angiotensin-aldosterone system may further stimulate NADPH oxidases through ATII (143). However, age-dependent central arterial stiffness involves a loss of elastic fibers partly mediated by proteases with elastinolytic activity, including matrix metalloproteinases. These, in turn, have been linked to ROS through a ROS-induced activation of cyclophilin A, which mediates matrix metalloproteinase 9, tumor necrosis factor α, and IL-6 expression via the NF-κB and ERK pathways.

Components of cigarette smoke are also known to stimulate endothelial NADPH oxidase (144) and diminish mitochondrial function, thus inducing mitochondrial oxidative stress (145). Elevated $O_2^-$ and $ONOO^-$ formation mediate vascular DNA damage and inflammation, thereby accelerating vascular aging. Via oxidation of LDL cholesterol, cigarette smoke enhances its pro-oxidative potential (146). Through oxidative eNOS uncoupling, smokers suffer from endothelial dysfunction that may be attenuated by $BH_4$ supplementation (147).

## THE ANTIOXIDANT PARADOX: CLINICAL TRIALS AND THEIR SO FAR DISAPPOINTING OUTCOMES

The term *antioxidant paradox* refers to the fact that despite the crucial role ROS play in the pathogenesis of CVDs, supplementation of dietary antioxidants has shown little to no therapeutic effects (148).

A large meta-analysis showed that antioxidant supplements to exert no beneficial effects (vitamin C and selenium), or that they even potentially raise overall mortality (vitamin E, β-carotene) (149), although this inference was later repudiated because re-examination of data showed that the analyzed studies did not include mortality as a primary endpoint (150). A number of mechanisms could explain these unexpected clinical results. First, the antioxidants used may have been inappropriate. Indeed, at the time of initiation of clinical trials with natural antioxidants, no data from animal trials with these compounds were available. In fact, animal trials of that era had been carried out with more potent antioxidants, such as probucol or probucol analogues (151). Also, accessibility of the administered natural compounds to the exact site where ROS are produced (especially within cellular organelles such as mitochondria) may be limited. Additionally, $H_2O_2$ and hypochlorous acid (HOCl), which are strongly implicated in vascular damage, are not scavenged by antioxidant vitamins (152). Furthermore, any antioxidant would likely be oxidized rapidly to its inactive form, with little hope of reversal. Apart from the depletion of the active form, this could mediate an accumulation of the oxidized and potentially pro-oxidant form of the parent molecule (153,154). Moreover, the reaction of one type of ROS with a certain antioxidant could potentially generate another ROS, which the original antioxidant cannot scavenge. Due to the nature of ROS as signaling molecules, chronic antioxidant exposure might lead to compensatory up-regulation of ROS producing systems.

Regarding the dosages used, there may have been inconsistencies between different studies, as no information was made available on vascular antioxidant concentrations or efficacy (e.g., isoprostane levels as biomarkers for lipid peroxidation). Such information is critical, given the high rate constants of the reactions between endogenous molecules such as NO and ROS; indeed, for an antioxidant to work effectively, its reaction with ROS must outcompete that of the ROS with its target molecule. In line with this, vitamin E would have to exceed the concentration of NO 1 million-fold, which is in the millimolar range, which is unlikely to be achieved, even with the highest clinically administered doses (63).

Additionally, several clinical trials were performed on high-risk patients, in which vascular disease was advanced (151) and thus difficult to reverse. Also, several of these patients were already treated with acetylsalicylic acid (aspirin), making the treatment with an additional antioxidant less likely to be effective. Perhaps longer-term prospective studies with regular efficacy monitoring that are carried out in healthy individuals treated with a single antioxidant would achieve different results.

## POTENTIAL THERAPEUTIC TARGETS

The so far disappointing clinical results suggest a limited efficacy of conventional antioxidant treatments. Thus, novel strategies aimed at preventing ROS production or

stimulating endogenous antioxidant systems should be considered. In this context, enzymes of the NADPH oxidase family, especially those containing NOX1 or NOX2 catalytic subunits, are attractive targets, as they represent key sources of ROS in vascular disease states (63). The organizer subunit p47phox is a requisite for full activation of these isoforms. Without it, however, the enzymes can still be activated, albeit less efficiently. Interestingly, given the importance of NADPH oxidase activity for immune cell function, it potentially makes partial inhibition clinically more attractive than full blockade. Several animal studies on atherosclerosis, hypertension, and stroke have demonstrated the efficacy of p47phox inhibitors, such as Nox2ds-tat and apocynin (155–159). However, improvements are still needed in effectiveness, pharmacokinetics, and specificity before clinical testing of these compounds may be commenced.

Considerable advances have been made in delivering antioxidants to mitochondria. A well-established approach is conjugation of an antioxidant to a lipophilic cation such as triphenylphosphonium (TTP), which, due to its positive charge and the large negative potential gradient across the inner mitochondrial membrane, concentrates 100- to 500-fold higher within mitochondria than in the cytoplasm. The compound MitoQ follows this principle, harboring a quinone moiety, and diminishing radical production and mitochondrial damage without interfering with respiratory function. It was seen to blunt myocardial ischemia/reperfusion injury, nitrate-related endothelial damage, blood pressure, doxorubicin-induced heart damage, and sepsis-associated heart and liver injury (160). Two phase II trials on Parkinson's disease and hepatitis C have not reported any adverse effects, although benefits could only be shown against hepatitis C–induced liver damage. Further clinical studies focusing on cardiovascular endpoints will be needed. Other compounds relying on the same principle, such as MitoE (tocopherol), MitoTEMPOL (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl), SkQ (plastoquinone fused to TTP), Mito-Apocynin, Mito-Peroxidase, or MitoSOD have raised hopes in preclinical trials, but their clinical efficacy is yet to be demonstrated.

Experimental evidence has also highlighted gene therapy as a potential approach to attenuate vascular oxidative stress. Endothelial GTPCH1 overexpression increases $BH_4$ levels, and inhibits eNOS uncoupling and $O_2^-$ production in *ApoE*$^{-/-}$ mice (161). Gene therapy with SOD3 was shown to reduce ROS levels, stimulate endothelial regeneration, and inhibit in-stent restenosis in hyperlipidemic rabbits (162). Furthermore, overexpression of catalase in *ApoE*$^{-/-}$ mice was shown to decrease atherosclerosis, an effect further potentiated by SOD1 overexpression (87).

Novel therapeutic targets could also include the activator protein-1 transcription factor JunD, the mitochondrial adaptor p66$^{Shc}$, and SIRT1, a family-member of nicotinamide adenine dinucleotide (NAD)-dependent proteins. They are intimately involved in controlling ROS production/scavenging in diverse disease conditions. Whereas JunD and SIRT1 preserve endothelial function by attenuating oxidative stress, p66$^{Shc}$ promotes mitochondrial ROS production and apoptosis (138). All of these genes have been implicated in age-dependent vascular dysfunction; however, p66$^{Shc}$ has also been implicated in the vascular pathobiology triggered by various cardiovascular risk factors, including smoking, diabetes (127,163), and hypertension (125). Ongoing chemical library screening studies are aiming to reveal

compounds capable of enhancing JunD activity in the vasculature, whereas the SIRT1 activator SRT2104 has already been investigated in clinical trials (164).

The ER is intrinsically linked to free radicals, whereby oxidative stress mediates ER stress and vice versa. A major responsive signaling pathway in this respect is the unfolded protein response, mediating endothelial inflammation and dysfunction through regulation of eNOS expression/activity, as well as being implicated in resolving ER stress. When ER stress prevails, cells induce apoptosis via the unfolded protein response with the C/EBP homologous protein (CHOP) as a central proapoptotic factor. Apoptosis and CHOP are specifically implicated in plaque stability (165). Consequently, the ER folding capacity and mediators of the unfolded protein response are compelling therapeutic targets, with CHOP and its role in atherosclerosis being especially appealing (166,167).

### CONCLUSIONS REGARDING OXIDATIVE STRESS IN THE VASCULATURE

The vasculature is an important site of ROS and oxidative stress in human biology and disease. Although, as in cardiac myocytes, certain ROS can modulate physiological responses at low concentrations ROS become pathological, at higher levels. The failure of clinical antioxidants across several clinical trials has been disappointing, but there is realistic hope that better-targeted, and more specific and concentrated therapies may demonstrate efficacy.

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## ABBREVIATIONS AND ACRONYMs

| | |
|---|---|
| **ATII** | angiotensin II |
| **eNOS** | endothelial nitric oxide synthase |
| **$H_2O_2$** | hydrogen peroxide |
| **HF** | heart failure |
| **NADPH** | nicotinamide adenine dinucleotide phosphate |
| **NO** | nitric oxide |
| **NOX** | nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) |
| **$O_2^-$** | superoxide |

| | |
|---|---|
| **ROS** | reactive oxygen species |
| **SOD** | superoxide dismutase |

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## CONDENSED ABSTRACT

Although many different factors promote cardiac and vascular disease, most share an important pathological mechanism: oxidative stress. In the failing heart, oxidative stress occurs in the myocardium and correlates with left ventricular dysfunction. Reactive oxygen species (ROS) negatively affect myocardial calcium handling, cause arrhythmias, and contribute to adverse cardiac remodeling. Similarly, oxidative balance in the vasculature is tightly regulated by a wealth of pro- and anti-oxidant systems that orchestrate region-specific ROS production and removal. Although at low levels ROS regulate multiple physiological vascular cell functions, excessive ROS levels promote vascular disease by disruption of redox-dependent vascular signaling and oxidative damage.

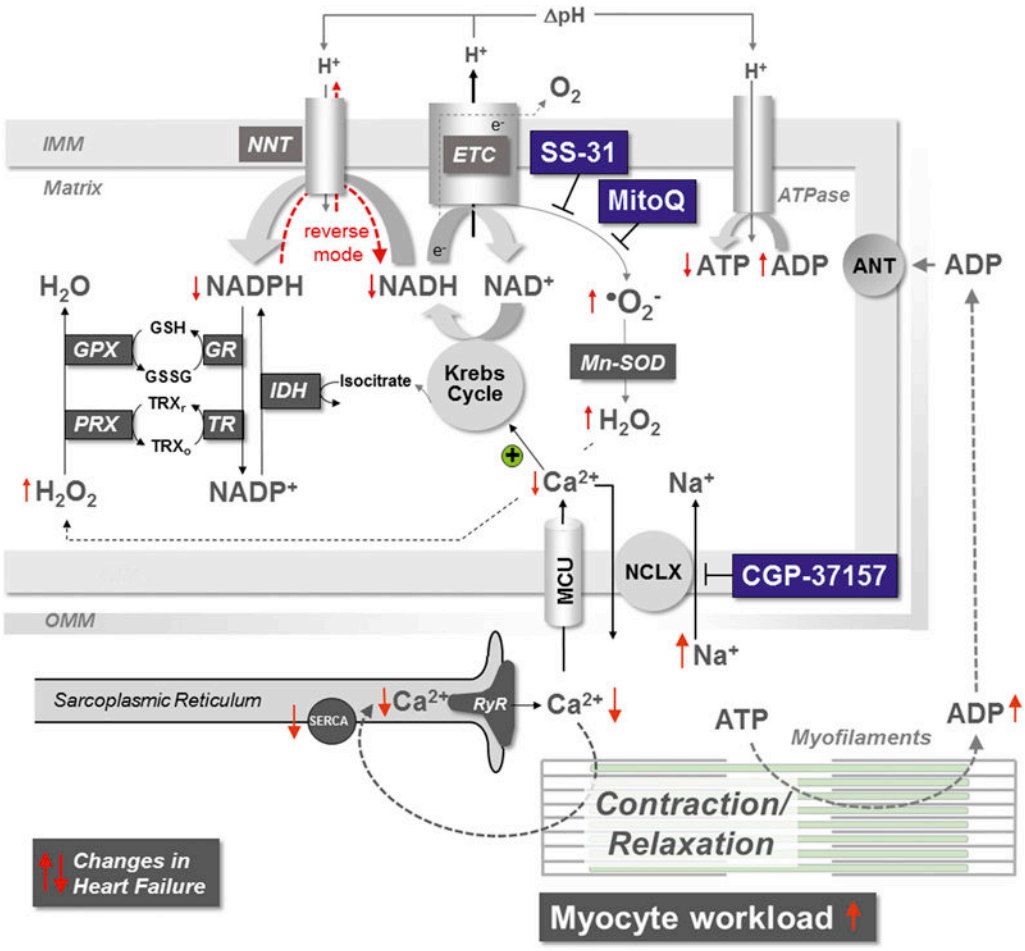



**Figure 1. Mitochondrial ROS Generation in HF**

In HF, the regulation of mitochondrial ROS emission is controlled by ion handling and mechanical workload. Increased myocyte workload and dysregulation of calcium ($Ca^{2+}$) and sodium ($Na^{+}$) handling reduce mitochondrial $Ca^{2+}$ accumulation, causing NADH and NADPH oxidation. NADPH depletion provokes $H_2O_2$ emission from mitochondria. ANT = adenine nucleotide translocator; ADP = adenosine diphosphate; ATP = adenosine triphosphate; ATPase = adenosine triphosphatase; GPX = glutathione peroxidase; GR = glutathione reductase; GSH/GSSG = reduced/oxidized glutathione; HF = heart failure; $H_2O_2$ = hydrogen peroxide; IDH = isocitrate dehydrogenase; IMM = inner mitochondrial membrane; MCU = mitochondrial calcium uniporter; NADH = nicotinamide adenine dinucleotide; NADPH = nicotinamide adenine dinucleotide phosphate; NCLX = mitochondrial sodium/calcium (and lithium) exchanger; NNT = nicotinamide nucleotide transhydrogenase; $O_2^-$ = superoxide; OMM = outer mitochondrial membrane; PRX = peroxiredoxin; ROS = reactive oxygen species; RyR = ryanodine receptor; SERCA = sarcoplasmic reticulum calcium adenosine triphosphatase; TR = thioredoxin reductase; TRXr/o = reduced/oxidized thioredoxin.

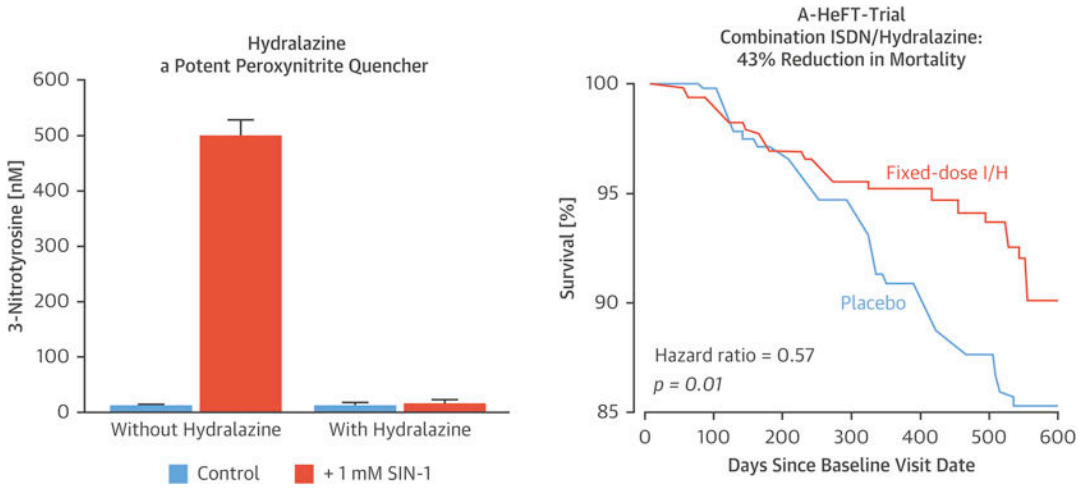



**Figure 2. Efficacy of Hydralazine and Nitrates in HF**

The combination of hydralazine and ISDN therapy is efficacious in the treatment of chronic HF with reduced ejection fraction. **(Left)** The powerful inhibitory effect of hydralazine on protein tyrosine nitration in rat smooth muscle cells by in situ generated $ONOO^-$ (caused by 3-morpholino sydnonimine [SIN-1] administration) is illustrated. Reprinted, with permission, from Daiber et al. (46). **(Right)** The marked effect of combined ISDN + hydralazine (I/H) on survival in patients with HF and reduced left ventricular ejection fraction is illustrated, as demonstrated in the African-American Heart Failure (A-HeFT) Trial. Reprinted, with permission, from Taylor et al. (50). ISDN = isosorbide dinitrate;

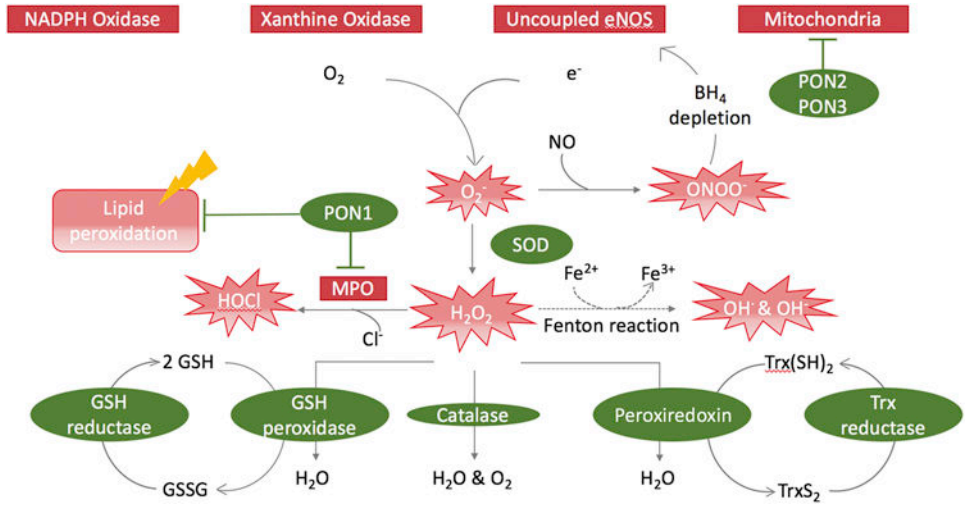



**Figure 3. Enzymes Determining Redox Balance Through Production or Scavenging of ROS**

In the vasculature, $O_2^-$ is generated by NADPH oxidase, xanthine oxidase, mitochondria, and uncoupled eNOS. Superoxide dismutase (SOD) converts $O_2^-$ to $H_2O_2$. Through the Fenton reaction, $H_2O_2$ can spontaneously convert to the hydroxyl radical $OH^{\bullet}$. Due to its extreme reactivity, $OH^{\bullet}$ can damage most cellular compartments. Immune cell–secreted myeloperoxidase (MPO) can mediate oxidation of chloride to hypochlorous acid (HOCl) using $H_2O_2$, and inactivate NO via oxidation to nitrite. By chlorination, HOCl inactivates multiple biomolecules, such as the eNOS substrate L-arginine and lipoproteins. The antioxidant enzymes glutathione (GSH) peroxidase, catalase, and peroxiredoxin convert $H_2O_2$ to oxygen and water. Paraoxonases (PON) 2 and 3 inhibit mitochondrial ROS production, whereas PON1 inhibits myeloperoxidase (MPO) and protects from oxidative stress mediated lipid-peroxidation. $BH_4$ = tetrahydrobiopterin; eNOS = endothelial nitric oxide synthase; NO = nitric oxide; Other abbreviations as in Figure 1.

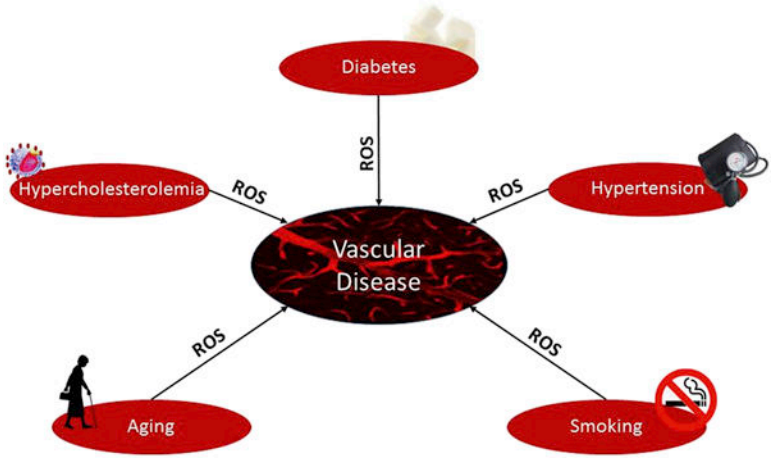



**Figure 4. ROS Mediate CVD in Response to Several Different Risk Factors**

Vascular disease relates to a variety of pathological states, and is almost invariably prompted by classical and nonclassical risk factors. ROS play a central role in mediating the noxious effects of classical risk factors, and thus represent attractive therapeutic and preventive targets. CVD = cardiovascular disease; ROS = reactive oxygen species.

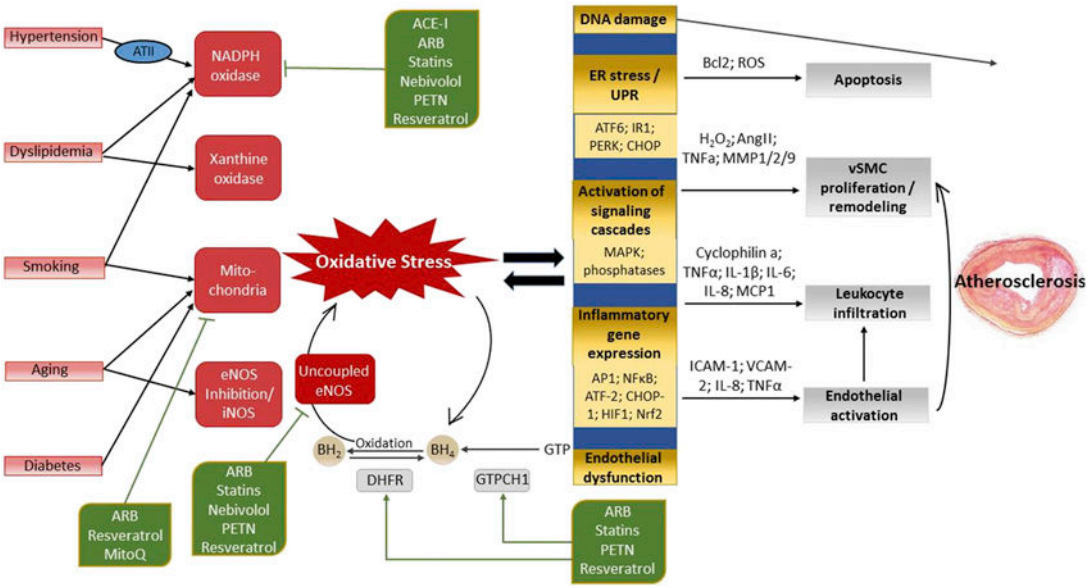
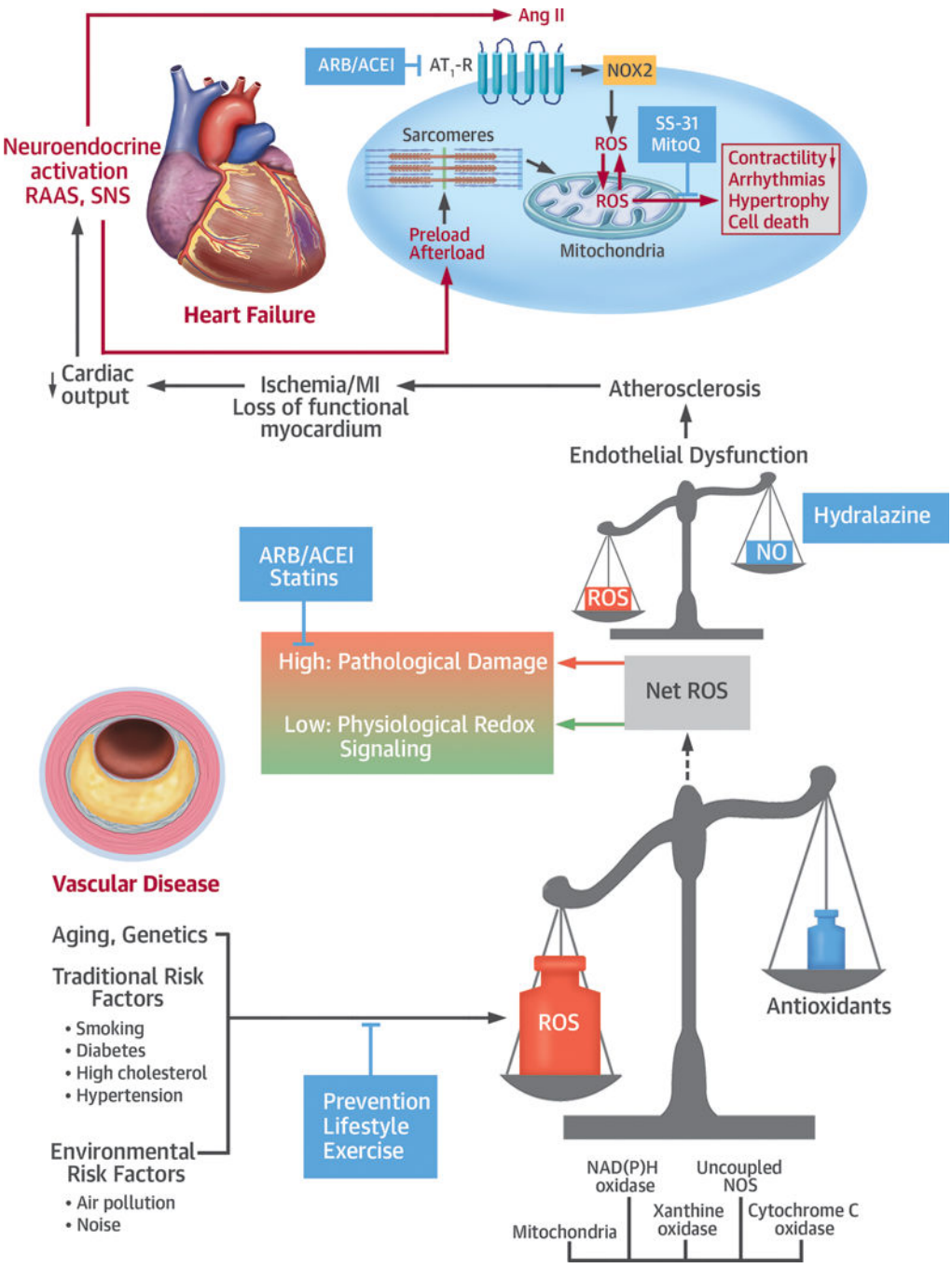



**Figure 5. Mechanisms Through Which Cardiovascular Risk Factors Mediate Atherogenesis and Available Pharmacological Tools for Targeting Crucial Pathogenic Factors**

Cardiovascular risk factors promote oxidative stress by stimulation of various pro-oxidant enzyme systems, tipping the redox balance in favor of oxidation, with $O_2^-$ as the key mediator. Hypertension activates NADPH oxidase via angiotensin II (ATII) and angiotensin 1 receptor (AT1R) signaling. The main ROS sources in states of hypercholesterolemia are NADPH oxidase and xanthine oxidase (activated by oxidized low-density lipoprotein), and the hypercholesterolemia-mediated up-regulation of AT1R. Hyperglycemia, as seen in diabetes, associates primarily with mitochondrial ROS production, which can secondarily activate NADPH oxidase. Compounds contained in cigarette smoke activate NADPH oxidase, which mediates mitochondrial dysfunction and thus ROS production. Aging is associated with increased mitochondrial dysfunction and reduced eNOS activity. Superoxide from all sources can inactivate vasoprotective NO by its reaction to $ONOO^-$, which, in turn, mediates uncoupling of eNOS-mediated oxygen reduction and $NO^-$ production by oxidation of the essential cofactor tetrahydrobiopterin ($BH_4$).

The enzymes dihydrofolate reductase (DHFR) and GTPCH1 counteract eNOS uncoupling by replenishing $BH_4$ levels via regeneration and de novo synthesis, respectively. ATII decreases $BH_4$ levels, not only by activating NADPH oxidase, but also by down-regulation of DHFR. NADPH oxidase has been seen to be inhibited by angiotensin-converting enzyme inhibitors (ACEIs), ATII receptor type 1 blockers (ARBs), 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins), the β-blocker nebivolol, the plant-derived polyphenol resveratrol, and the organic nitrate pentaerithrityl tetranitrate (PETN). The same compounds inhibit eNOS uncoupling by activating GTPCH1 and DHFR. Mitochondrial $O_2^-$ generation is decreased by ARBs, MitoQ and resveratrol. Other abbreviations as in Figures 1 and 3.




**Central Illustration. Mechanisms, Sources, and Implications of Oxidative Stress in Cardiovascular Disease and Heart Failure**

Aging, genetic predisposition, traditional risk factors, and environmental factors can induce oxidative stress, particularly in vessels, where nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and uncoupled nitric oxide synthase (NOS) are dominant sources. At physiological levels, a low increase in net reactive oxygen species (ROS) can induce protective effects through redox signaling mediating, for example, improved antioxidative capacity. If the generation of ROS outweighs antioxidative capacity, then at higher ROS levels cell damage and endothelial dysfunction arise, which contribute to the development of

atherosclerosis. Through ischemia in the context of myocardial infarction (MI), this can induce the loss of functional myocardium and, eventually, heart failure. Although heart failure also arises via other mechanisms, including diabetes and primary cardiomyopathic processes, ultimately neuroendocrine activation via the renin-angiotensin-aldosterone system (RAAS) and angiotensin II receptor type 1 ($AT_1$-R), and the sympathetic nervous system (SNS), combined with increased pre- and after-load, impose additional oxidative stress on the heart. Specific mechanisms leading to increased cardiac oxidative stress then include receptor-induced activation of NOX2 and mitochondrial redox mismatch. As a consequence, oxidation of mitochondrial NADPH gives rise to hydrogen peroxide ($H_2O_2$), which plays a causal role in contractile dysfunction, arrhythmia, and ultimately maladaptive cardiac remodeling through hypertrophy and cell death. Potential points of intervention are through lifestyle change, exercise, and medication to reduce risk factors and environmental stressors. Medical options include hydralazine to improve endothelial function, angiotensin-converting enzyme inhibitors (ACEI)/angiotensin receptor blockers (ARBs), and statins. More recently, drugs directly targeted to mitochondria have been developed, such as SS-31 or MitoQ, whose clinical efficacy is currently under evaluation. Ang II = angiotensin II; NO = nitric oxide.