Nitroso–Redox Interactions in the Cardiovascular System

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Normal cardiovascular performance requires exquisite balancing of many complex biochemical processes. Perturbation of this balance may lead to myocardial dysfunction or may be a secondary result of structural heart disease such as myocardial infarction (MI) or cardiomyopathic processes. Altered signaling systems in turn contribute to the progression of myocardial dysfunction. The roles of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in normal and failing myocardium and vasculature have been the subject of intense investigation and continue to engage considerable debate. A disturbance in the oxidation–reduction state of the cell, in which ROS production exceeds antioxidant defenses, is called oxidative stress. By analogy, nitrosative stress is an impairment in nitric oxide (NO) signaling caused by increased amounts of RNS, which may be caused by or associated with a disturbance in the redox state. This review addresses the role of the redox state and nitroso–redox balance in determining cardiovascular function in health and disease.

The Chemistry of Nitroso–Redox Balance
Free radicals are highly reactive molecules with unpaired electrons. Free radical chemistry is the underpinning of 2 broad classes of signaling molecules in biological systems: ROS, which are reactive intermediates of oxygen metabolism, and a closely related group of RNS. The forms of ROS that are relevant in biological systems include the superoxide radical (O$_2^·$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH). RNS of biological importance include NO, low- and high-molecular-weight S-nitrosothiols, and peroxynitrite (ONOO$^-$) (Figure 1).

Superoxide, Peroxide, and Peroxynitrite
Superoxide is formed by the 1-electron reduction of molecular oxygen, resulting in a free radical. This reaction can be accomplished by a variety of oxidases, including xanthine oxidase (XO) and NADPH oxidases, or as a byproduct of oxidative phosphorylation. XO produces varying proportions of superoxide and hydrogen peroxide during reoxidation of the enzyme (Figure 2).

Peroxynitrite (ONOO$^-$) is a product of the reaction of NO with superoxide. At physiological pH, ONOO$^-$ is protonated to form peroxynitrous acid, which is a highly reactive species with a very short half-life in vivo. ONOO$^-$ is able to react with DNA, proteins, and lipids, potentially leading to cellular damage and cytotoxicity. ONOO$^-$ reaction with CO$_2$ is a physiologically relevant pathway, given the high rate constant for this reaction and the relatively high concentration of CO$_2$ in vivo. This reaction forms intermediates that provide a route to nitration and oxidation. Tyrosine nitration by ONOO$^-$ has been demonstrated in vitro and has long been suspected as a mechanism of protein inactivation. However, the mechanisms of protein inactivation by ONOO$^-$ are multifactorial and nonspecific with multiple targets, including thiols, methionine, and amines. In the case of the sarcoplasmic reticulum (SR) calcium ATPase (SERCA), for example, oxidation at multiple cysteine residues leads to loss of function.

Nitric Oxide
NO mediates signaling events through 2 important mechanisms. First, NO binds and affects the activity of enzymes with transition metal centers, leading to activation of soluble guanylyl cyclase and inhibition of cytochrome c. NO activates guanylyl cyclase by binding to its heme moiety, leading to the production of cGMP. cGMP then activates protein kinase G, leading to a series of signaling events with diverse consequences, including vascular smooth muscle relaxation. Second, NO exerts widespread signaling through the aforementioned covalent nitrosylation of sulphydryl groups on proteins and small molecules, which has been demonstrated in well over 100 proteins in multiple cells and tissues, including the heart.

Various proteins involved in the regulation of myocardial contractility are modified by protein S-nitrosylation. Cysteines susceptible to nitrosylation tend to be located between an acidic and a basic amino acid, leading to the proposal of a prototypic consensus acid-base motif for S-nitrosylation. This motif has been shown to be predictive in several examples in which the essential cysteines have been well characterized.

Biochemistry of Nitroso–Redox
ROS and RNS as Second Messengers
A key concept in understanding redox balance in the cardiovascular system is that the effects of ROS and RNS depend on the location, amount, and timing of their production. ROS are not intrinsically destructive; on the contrary, increasing...
evidence shows that they play necessary roles in normal signal transduction. In low concentrations, they are implicated as second messengers primarily through inhibition of phosphatases, acting downstream of effectors such as platelet-derived growth factor, epidermal growth factor, tumor necrosis factor-α, β-adrenergic agonists, and interleukin-1β. In higher concentrations, however, they take on pathophysiological roles. ROS affect the oxidative modification of diverse molecules, including DNA, proteins, lipids, and sugars, potentially leading to toxicity. This results not only in processes such as lipid peroxidation but also in the interruption of normal signaling pathways, which leads to organ malfunction, largely because of reversible and irreversible oxidative modification of proteins.

In terms of signaling specificity, there has been a recent explosion of data on the role of S-nitrosylation in biological systems. As such, increasing evidence indicates that RNS subserve a signaling system with diverse and widespread effects mediated through the nitrosylation of specific cysteine thiol residues or metal centers, which modifies protein activity.

**Figure 1.** Sources of ROS and RNS. Superoxide is produced by a variety of mechanisms, including the normal functioning of oxidase enzymes, hemoglobin, and mitochondria, as well as by uncoupled NOS. Superoxide dismutase catalyzes the dismutation of \( \text{O}_2^- \) to hydrogen peroxide and water. \( \text{H}_2\text{O}_2 \) can be converted by catalase or peroxidases to water and molecular oxygen. It also may be converted to the hydroxyl radical through the Fenton reaction, which requires \( \text{Fe}^{2+} \) as a cofactor. NO is produced primarily by 1 of the 3 forms of NOS. NO may interact with \( \text{O}_2^- \) to form the highly reactive peroxynitrite.

What Is the Nature of Oxidative and Nitrosative Modification?

To understand the idea of nitroso–redox modulation of protein function, it is important first to grasp how proteins are affected by oxidative and nitrosative modification. As mentioned, NO exerts diverse biological effects via posttranslational nitrosylation of specific cysteine thiol residues. This modification is responsible for a large part of the biological activity of NO and provides a mechanism for redox-based regulation of protein function. For example, in cardiac myocytes, S-nitrosylation modulates the function of ion channels that regulate excitation–contraction coupling and therefore normal systolic and diastolic function. The role of NO signaling in excitation–contraction coupling is discussed in more detail later.

Oxidative mechanisms may “compete” directly with NO for interaction with the same cysteine thiols. In general, nitrosylation is a highly reversible modification that lends itself more easily to roles in protein regulation, whereas oxidation represents a spectrum of modification ranging from reversible to irreversible change that entails some loss of control (Figure 3A). What is the nature of these oxidative modifications? In contrast to alcohols, thiols are less polar and are more easily oxidized. When occurring in close proximity to another thiol, oxidation of thiols can lead to formation of disulfide bonds. This may involve other cysteine moieties or can entail interactions with small-molecule thiols such as glutathione to form mixed disulfides. These further oxidation reactions involve formation of sulfenic acids (S-OH), the less reversible sulfenic (S-O2H) acids, and the irreversible formation of sulfonic (SO3H) acids (Figure 3B).

The targets of signaling by ROS are being increasingly appreciated, and there are multiple candidates. An important example is the tyrosine kinases, which, by virtue of a redox-sensitive cysteine residue in the region of the active site, represent a group of potential targets for ROS. In vitro experiments have shown that hydrogen peroxide inhibits protein phosphatase activity in a manner that is reversible by reducing agents. By similar modification of susceptible protein thiol groups, oxidants may regulate protein dimerization, protein-protein interactions, or modification by small molecules such as glutathione. In recent work, cardiac hypertrophy stimulated by α-adrenergic receptor stimulation has been shown to be associated with oxidative modification of redox-sensitive thiols on Ras.

**Figure 2.** A. XOR is reduced during the conversion of hypoxanthine to xanthine and of xanthine to uric acid. B. Reoxidation of fully reduced XOR involves electron transfer to oxygen, producing hydrogen peroxide and superoxide. Reproduced with permission from Berry and Hare. Copyright 2004, The Physiological Society.

**Redox Balance and Oxidative and Nitrosative Stress**

Both ROS and RNS are produced in most cell and tissue types, including those of the cardiovascular system. Production of these agents is counterbalanced in each case by specific mechanisms that help maintain local concentrations within physiological limits. Superoxide, first proposed by Fridovich and Handler in the 1960s as a biologically important reducing agent, was ultimately identified as a normal product of XO activity that led to cytochrome c...
Nitroso–Redox Balance and Imbalance

Nitroso–redox balance may be operationally defined by the idea that RNS and ROS work together in biological systems to achieve optimal signaling. The concept of imbalance arises because this signaling can be disrupted by either increased ROS or decreased RNS. Moreover, there is cross-talk between the enzymes that produce ROS and RNS, so NO deficiency can in some cases result in increased ROS production. Thus, the interactions between ROS and RNS are multifaceted and strike a balance that can be disrupted at both the cell and organ levels in cardiovascular disease states.

Sources of RNS and ROS

NO Synthases

NO is formed primarily by a family of enzymes known as NOSs, which oxidize the terminal guanidino nitrogen of L-arginine to form NO and the amino acid L-citrulline. There are 3 NOS isoforms, each with specific localization and function. NOS1 (neuronal NOS) and NOS3 (endothelial NOS) are found in a variety of cell types and are regulated by binding to calcium and calmodulin. NOS2 (inducible NOS), on the other hand, has very high baseline affinity for calcium and calmodulin; therefore, its activity is effectively independent of calcium concentration.

Sources of ROS

The activity of ROS depends on the amount and location of production and on temporal elements. For example, the neutrophil uses an NADPH oxidase to produce the respiratory burst that has a killing effect on target cells. The NADPH oxidase present in vascular cells is similar in structure to the neutrophil enzyme but produces superoxide in lesser amounts over longer periods of time. Thus, similar systems for production of ROS are used under normal circumstances to accomplish very different objectives. Another key oxidase is XO, which is involved in the final steps of purine degradation. XO is physiologically present in the heart, and increased XO activity and the resultant imbalance in redox activity have an important role in cardiac disease, including ischemia–reperfusion injury and heart failure. Superoxide and other ROS are produced in the mitochondria during oxidative phosphorylation as a normal byproduct of aerobic respiration, and agents such as ceramide are able to increase mitochondrial ROS production by inhibiting complex III respiration, resulting in increased peroxide formation. Hemoglobin can likewise serve as a significant producer of superoxide under hypoxic conditions, as can occur during myocardial ischemia. And the NOSs, when deprived of the substrate L-arginine and the cofactor (6R)-5,6,7,8-tetrahydro-L-arginino-

Figure 3. Potential oxidation fates of cysteine thiols. A, The progression from more to less reversible reactions entails a loss of modulatory control and a transition from signaling to toxicity. Thiols represent a ubiquitous target for modification by RNS and ROS. Protein thiols may undergo reversible reactions to form S-nitrosothiols or disulfides. S-nitrosothiol subserves a critical and widespread signaling mechanism and thus can be considered a posttranslational modification system akin to phosphorylation. Thiols are also susceptible to oxidation to sulfenic acids and to sulfenic and sulfonic acids, which are progressively less reversible reactions and therefore maladaptive to the extent that they block the more reversible and physiological regulation mediated by nitrosylation.
isoforms of gp91phox have been identified, so it is now studied the most. Over the past several years, multiple identified in vascular smooth muscle and colon carcinoma fibroblasts, and cardiomyocytes, whereas Nox1 has been diovascular system, Nox2 is found in endothelial cells, enzyme responsible for the respiratory burst. In the car-

which was originally described in neutrophils as the NADPH oxidases (Nox).

recognized to be a family of homologues that are called

p40phox. Of these proteins, the catalytic subunit gp91phox is the present in coronary arteries and is known to produce superoxide after stimulation with bradykinin (see the Figure 4).

Figure 4. The multisubunit NADPH oxidases are composed of the membrane-bound catalytic subunits NOX (gp91phox in the prototypical phagocyte oxidase) and p22phox, as well as the regulatory subunits p67phox, p47phox, and p40phox, and Rac. Enzymatic activity produces superoxide as a byproduct. Several gp91 isoforms have been isolated and characterized as the NOX family of proteins.

Biopterin (BH4), become uncoupled from NO production and produce superoxide. Other important oxidase pathways include lipoxygenase, auto-oxidation of catecholamines, and the cytochrome P450 class of enzymes, at least one of which is present in coronary arteries and is known to produce superoxide after stimulation with bradykinin (see the Table).

Mitochondria

O$_2^\cdot$ production occurs at a measurable rate during even normal oxidative phosphorylation, making mitochondria the greatest cellular source of ROS. The majority of O$_2^\cdot$ produced in the mitochondria has a relatively short half-life; it is acted on by manganese superoxide dismutase in the mitochondrial matrix or by copper/zinc superoxide dismutase in the intermembrane space. Nonetheless, mitochondrial ROS production increases in stress states, including heart failure. NOS1 localization to the mitochondria provides an additional mechanism for cross-talk between ROS and RNS. See elsewhere for reviews of mitochondrial ROS.

Xanthine Oxidoreductase

Xanthine oxidoreductase (XOR) is a member of the molybdoenzyme family that includes enzymes such as aldehyde oxidase and sulfite oxidase. XOR is encoded by a single gene, but the protein exists as a homodimer in 2 potentially interconvertible forms, xanthine dehydrogenase (XDH) and XO. XO catalyzes the final 2 steps of the purine degradation pathway, converting hypoxanthine to xanthine and xanthine to uric acid. XO is best known to most clinicians for this activity and as the target for the antigout drug allopurinol, which efficiently blocks XO activity. XDH can be converted to XO either by a reversible process involving thiol oxidation or by an irreversible process through proteolytic cleavage.

XOR is reduced during the reaction of xanthine to form uric acid. During the reoxidation process, 2 electrons are transferred to oxygen in each of the first 2 steps, thus generating hydrogen peroxide. In the final steps, the remaining 2 electrons are transferred to oxygen in separate steps to yield superoxide (Figure 2). Thus, each cycle of XOR activity produces 2 molecules of hydrogen peroxide and 2 molecules of superoxide.

Between the 2 forms of XOR, XO appears to be responsible for most of the ROS production. Although XO readily transfers electrons to molecular oxygen, XDH prefers NAD$^+$ as an electron acceptor. It is important to note, however, that once NAD$^+$ has been reduced to NADH, XDH is able to act as an NADH oxidase. This process ultimately leads to generation of superoxide and is not inhibited by allopurinol.
XOR thus produces both superoxide and hydrogen peroxide in the course of its normal activity.

The location of XOR expression has been the subject of some controversy, specifically whether it is expressed in the heart. The highest levels of XOR in mammals occur in liver, small intestine, and mammary gland. Early studies showed that XOR also is present in significant quantities in endothelial cells and serum, but identification in the heart has been confirmed by most but not all investigations. Current evidence supports XOR expression as a low-abundance protein in the SR of cardiac myocytes. In addition to its well-known activities, XO also has the capacity to act as a GSNO reductase. This has given rise to speculation that XO is present in the SR to regulate the S-nitrosylation of the ryanodine receptor (RyR) and other SR proteins.

**Interactions Between ROS and RNS**

As previously mentioned, there is linkage between the systems regulating ROS production and NO signalling. RNS–ROS cross-talk is evident at multiple levels. Interactions of RNS with ROS can take several forms, depending on the respective concentrations and rates of formation. NO can quench superoxide or combine with it to form peroxynitrite. Superoxide production by XOR or NO and ROS compete for the same thiols on target proteins.

In the failing heart, the beneficial effects of XO inhibition are dependent on NOS function. In an animal model of heart failure, treatment with NOS inhibitors abolishes the effects of allopurinol on contractility and myocardial efficiency without affecting basal function. XOR and NOS1 interact in the cardiac SR, and NOS regulates XO activity so that a deficiency in NOS1 translates to increased XOR activity (Figure 5). Furthermore, XOR not only is present in the SR but coinmunoprecipitates with NOS1. Within the SR, then, the calcium channel proteins essential to excitation–contraction coupling (RyR2 and SERCA2a) localize with enzymes that affect their function (Figure 6). NOS1 may lead to NO-based modifications, as discussed above. XOR may act not only through production of ROS but also potentially through its activity as a GSNO reductase, thus regulating the nitrosylation state of these important target proteins.

Some authors have suggested that XO also may contribute to the generation of NO. For example, in hypoxic states, NOSs are unable to produce NO, and XOR reduction of nitrates has been suggested as a mechanism of NO production; however, the ability of XOR to metabolize nitrates at physiological levels has not been demonstrated. In hypoxic rat heart, NO production continues even in the presence of NOS inhibitors, but the addition of allopurinol stops NO generation, presumably through inhibition of XOR. Interestingly, XOR is able to catalyze the reduction of nitrates to NO under experimental conditions, which has led to the suggestion that XOR may be involved in the vasodilator activity of nitrate drugs, including isosorbide dinitrate and nitroglycerin. However, this has not been demonstrated under physiological conditions. Further cross-talk between these 2 systems is suggested by the observation that NO may regulate XOR activity.

**NO-Redox in Excitation–Contraction Coupling**

Depolarization of the cardiac myocyte plasma membrane triggers a cascade of events leading to a rapid increase in cytosolic calcium and resulting muscle contraction. The initial entry of calcium, via the plasmalemmal L-type calcium channel, leads to a larger release of calcium from the SR through the coupled RyR channel, a process known as calcium-induced calcium release (Figure 6). Relaxation of the myocytes requires diastolic calcium removal from the cytoplasm, which is mediated by the SR reuptake via the calcium ATPase (SERCA2a) and the sarcosomal extrusion via the sodium–calcium exchanger (NCX). NO and the cellular redox state affect excitation–contraction coupling through interactions with calcium-handling proteins (primarily the L-type Ca2+ channel and the RyR), the contractile apparatus, and respiratory complexes. Both cGMP-dependent and -independent mechanisms are involved in NO effects on myocardial contractility.

Taking the interactions with plasma membrane and SR calcium channels as a prototype for this type of interaction, we know that NO may affect L-type Ca2+ channel opening either by cGMP formation or by nitrosylation of the channel protein. In the case of the L-type channel, cGMP inhibits channel activity, whereas S-nitrosylation and oxidation of the protein have a biphasic effect that is stimulatory at low concentration and inhibitory at high concentration.

Similarly, the SR Ca2+-release channel (RyR) is regulated by target cysteine nitrosylation or oxidation (Figure 6). The cardiac RyR2 has low-level basal S-NO. S-nitrosylation of additional cysteines leads to further...
activation of the channel. This is a highly reversible modification that can occur on a time scale commensurate with excitation–contraction coupling. In contrast, oxidation of multiple cysteine residues on RyR ultimately leads to irreversible activation of the channel (Figure 3A), a situation that favors SR leak and thus SR calcium depletion (Figure 6D). In the case of the skeletal muscle RyR1, the calcium-sensitizing effects of NO are mediated by specific nitrosylation of a particular cysteine (cysteine 3635) located within the calmodulin-binding domain of each subunit in the tetrameric receptor complex. By analogy, it is likely that a similar situation based on a single target cysteine regulates the cardiac-specific RyR, RyR2 (Figure 6).
Some investigators have proposed other variations of the nitroso-redox posttranslational modifications concept. As noted, the range of oxidative modifications also includes the formation of mixed disulfides with small molecules such as glutathione. The resulting modification, S-glutathionylation, has been demonstrated for these calcium-handling proteins in experimental models. In the case of RyR, treatment with GSNO results in S-nitrosylation of 2 cysteine residues and glutathionylation of 2 additional cysteine thiols. Similar modification also has been characterized for SERCA. Whether S-glutathionylation represents a true posttranslational modification system influencing physiology remains highly controversial, and it remains possible that it occurs as a byproduct of S-nitrosylation. More work is required to further characterize the precise role of protein S-glutathionylation in signaling and in oxidative and nitrosative stress.

**Regulation of NO Activity Involves Tight Spatial Localization of NO Production**

The paradigm of NO as a freely diffusible molecule with promiscuous effects is called into question by study of NO activity in organ systems, including the heart. In the cardiac myocyte, NO activity is determined in large part by its site of production, which in turn is controlled by spatial localization of the NOS enzymes. NOS3 is localized primarily to caveolae of the sarcolemma and t tubules, where its function is regulated by interaction with caveolin-3 and is linked to multiple cell surface receptors, including muscarinic, β-adrenergic, and bradykinin receptors. Activation of NOS3 cGMP signaling results in negative inotropy and chronotropy. The negative inotropic effects of NOS3 activation are more pronounced in heart that is stimulated by β-adrenergic activation, which has suggested that NO acts as a negative feedback mechanism over contractile reserve. Indeed, adrenergic activation in myocytes has been shown to stimulate NO production. NOS1, on the other hand, has been localized to the SR, where it influences SR calcium cycling; in this regard, immunoprecipitation studies have demonstrated NOS1 binding to the SR calcium-release channel, RyR. In contrast to NOS3, NOS1 has primarily positive inotropic effects in the heart.

**Dysregulation of NOSs in Heart Disease Involves Altered Spatial Localization**

NOS activity is profoundly altered in the myocardium after ischemic insult and in heart failure. After MI, NOS1 expression in the heart is increased. Perhaps more important, however, is that fact that the subcellular localization of the enzyme changes from the SR to the sarcolemma; in this way, the normal case of tightly regulated temporal and substrate specificity of the NOS enzymes is lost. Similar translocation of NOS1 has been described in end-stage human cardiomyopathy. These changes account for very different effects of NO signaling in the post-MI and failing heart.

**Oxidase Enzymes in Cardiovascular Disease**

**NADPH Oxidases in Cardiovascular Disease**

There is substantial evidence that NADPH oxidase activity is increased in multiple cardiac disease states. In an animal model of pressure-overload left ventricular hypertrophy, for example, NADPH oxidase subunit expression and activity are increased in both cardiomyocytes and endothelial cells. Expression of NADPH subunits, including NOX2, is increased after MI in both animal models and humans. Studies of explanted hearts from end-stage heart failure patients have found evidence of increased NADPH oxidase activity. Relevant information also has been gathered from mouse knockout models of NOX subunits of the NADPH oxidase. Mice deficient in NOX2, which, along with NOX4, is present in cardiac myocytes and endothelial cells, have an altered response to angiotensin II compared with wild-type mice. Although wild-type animals exposed to angiotensin II for 7 to 14 days show increased NADPH oxidase activity, ventricular hypertrophy, and atrial natriuretic peptide expression, NOX2−/− mice show a significantly blunted response. The development of interstitial fibrosis in these mice also is abrogated. Similarly, although NOX2−/− mice develop hypertrophy in response to aortic banding–induced pressure overload (likely in part as a result of upregulation of NOX4), systolic function as measured by echocardiography and pressure-volume analysis is relatively preserved in knockouts compared with wild-type mice. Likewise, decreased NADPH oxidase activity in the knockout mice correlates with decreased cardiac interstitial fibrosis.

Diabetes mellitus is a well-described risk factor for atherosclerotic coronary disease, but it also has been associated with the development of a cardiomyopathy that is independent of ischemic disease. Diabetics have more left ventricular hypertrophy, microvascular constrictive, interstitial fibrosis, and edema than control subjects, and these differences appear to be independent of hypertension. Some studies have suggested that hyperglycemia directly causes cardiac damage. A leading hypothesis to explain diabetes-associated myocardial dysfunction is the development of oxidative stress through accumulation of both early glycation products (which include hemoglobin A1C and glycate albumin) and advanced glycation end products. Prior studies have shown increases in both superoxide production and NOS levels in endothelial cells exposed to high glucose. Zhang et al recently studied this phenomenon in cultured rat cardiomyocytes, demonstrating an increase in ROS in response to treatment with glycated proteins. The mechanism of ROS production proved to be activation of a Nox2-containing NADPH oxidase, confirming the importance of this system for cardiac redox balance in multiple disease states.

**XOR in Cardiovascular Disease**

**XOR in Ischemia Reperfusion Injury**

Considerable evidence suggests that the activity of XOR is increased in the heart in multiple disease states. Ischemia–reperfusion injury is the prototypic example of a situation in which ROS are produced in amounts far exceeding those that cells and tissues can handle without damage. As early as 1981, the involvement of XOR-generated ROS was seen in ischemia–reperfusion injury in the intestine. The role of XOR in ischemic injury has subsequently been demonstrated...
in human aortic endothelial cells and in several heart models of ischemia–reperfusion.

In the setting of ischemia, ATP degradation leads to the accumulation of XOR substrates. ATP is the most abundant high-energy phosphate in the heart under normal conditions. After 30 minutes of induced ischemia in heart preparations, ATP degrades to AMP, with resulting significant accumulation of hypoxanthine and xanthine. XOR abundance also increases marginally. The concentrations of substrate decline rapidly during reperfusion, presumably as a result of XO-mediated reactions that liberate superoxide and hydrogen peroxide in large quantities. These changes match the time course of observed ROS generation. This suggests that the oxidative stress produced by XO during ischemia–reperfusion injury is largely accounted for by substrate availability. This has been seen in human patients also. Serum xanthine and hypoxanthine levels increase in patients after acute MI compared with healthy subjects or individuals with stable angina or cerebral insult. Similarly, increased levels of urinary xanthine and hypoxanthine were seen in a study comparing patients with acute coronary syndromes with healthy age-matched control subjects.

In addition to increased substrate, XOR activity also is increased after ischemia reperfusion injury. XOR is present not only in tissues but also in plasma. After ischemia–reperfusion, increases in XOR are consistently seen both in the myocardium and in the circulation. Although XO is the major producer of ROS, XDH also has the ability to produce superoxide, both via its normal activity when NAD$^+$ levels are low and through its NADH oxidase activity, which is not inhibited by allopurinol. Interestingly, recent work has shown that changes in endothelial cell levels of XO in response to shear stress are dependent on ROS produced by the NADPH oxidase. The NADPH oxidases, which are activated under circumstances similar to XOR, may play a role in the conversion of XDH to XO.

One question that has arisen is whether oxidative stress after reperfusion comes from oxidases present locally in the tissues or from neutrophils in the circulation. Reperfusion injury has been observed in systems lacking granulocytes. Neutrophil recruitment is itself stimulated by ROS because the introduction of superoxide has been seen to cause chemotaxis without preceding inflammation. This suggests that inflammatory cells are recruited by XOR-produced ROS. Indeed, this has been supported by experiments showing that allopurinol pretreatment partially blocks neutrophil infiltration and adhesion to endothelial cells.

Thus, oxidative stress after reperfusion injury is most likely attributable to a combination of local XO activity and recruited neutrophil action.

Clinical Studies of XOR Inhibition in Ischemia–Reperfusion

In a study of XO inhibition in ischemia–reperfusion injury, 22 patients undergoing routine CABG (on-pump bypass) were administered allopurinol during surgery. Compared with a control group, those who received allopurinol had less evidence of peroxide production in the myocardium and were significantly less likely to require postoperative inotropic support. In a study of 38 patients with acute MI, patients were randomly assigned to receive allopurinol or not before primary angioplasty. Not only were slow-flow phenomena less frequent in the allopurinol group, but cardiac index measured immediately after PTCA and ejection fraction at 6 months were significantly greater in treated patients.

XOR in Heart Failure

Heart failure is the result of a host of complex interactions involving the myocardium, vasculature, and neurohormonal systems. Oxidative stress is greatly increased in heart failure, but whether this is a cause or an effect has continued to undergo considerable scrutiny. With regard to XOR, both protein abundance and enzyme activity are elevated in the failing heart.

The availability of allopurinol and its active metabolite oxypurinol as safe, well-tested drugs for XO inhibition has greatly aided the study of the role of XOR in heart failure. In mouse models of experimental MI, XO inhibition leads to greater survival, improved left ventricular function, and enhanced mechanoenergetic coupling. Similar results have been seen in a truncated troponin I mouse model of heart failure and in a rat model of chronic heart failure in which allopurinol or oxypurinol attenuates left ventricular remodeling and dysfunction. In dogs with pacing-induced heart failure, intravenous allopurinol treatment acutely decreases myocardial oxygen consumption while improving mechanical energetics. Further work with chronic as opposed to acute allopurinol treatment in this canine model has shown improved contractility, reduced afterload with unchanged preload, and improved ventricular vascular coupling as well as enhanced responsiveness to dobutamine and exercise.

In support of an antioxidant mechanism of allopurinol in heart failure, we have shown that treatment with the nonspecific antioxidant ascorbate has beneficial effects similar to those of allopurinol in paced dogs. In fact, allopurinol had no additive effect beyond that seen with ascorbate. Treatment with NOS inhibitors abrogates the response to both allopurinol and ascorbate, suggesting that ROS produced by XOR interferes with NO signaling in the heart. In support of this, NOS inhibition in normal dogs produced a depression in myocardial energetics that was reversed by either allopurinol or ascorbate.

Given that XO has a low basal level of activity in normal hearts, treatment with XO inhibitors such as oxypurinol would be expected to have some effect in normal myocardium. Indeed, infusion with oxypurinol increases systolic twitch tension in isolated normal and failing rat myocardium, although the magnitude of this effect is significantly greater in heart failure tissue. In the whole animal in vivo, however, XOR inhibition has no measurable effect on myocardial function. The extent of inotropic improvement correlates with the increased XO activity in heart failure tissue; as a result, the inotropy seen with XO inhibition is relatively selective for heart failure.

Preclinical data have raised theoretical issues surrounding the advantages of allopurinol and oxypurinol treatment and their potential to stimulate cardiac performance and
ventricular–vascular coupling while enhancing mecha-
noenergetic coupling and actually decreasing myocardial oxygen consumption. This contrasts sharply with the inotropes currently used in clinical practice, which of course achieve enhanced myocardial systolic function at the expense of increased mechanical and oxidative stress.

**XOR Inhibition and Cardiac Remodeling**

To the extent that oxidative stress contributes to cardiac remodeling in the wake of both ischemic and nonischemic myocardial injury, we would expect to be able to modulate this remodeling process by limiting ROS generation. In this regard, we have recently studied the role of oxidative stress and XOR inhibition in the spontaneously hypertensive heart failure rat. In the spontaneously hypertensive heart failure model, experimental animals display hypertension that progresses to left ventricular hypertrophy, dysfunction, and dilation, with ventricular and biochemical alterations, including increased XOR expression and activity, that parallel those seen in humans with nonischemic cardiomyopathy. After 4 weeks of treatment with oxypurinol, spontaneously hypertensive heart failure rats with already-established heart failure showed decreased left ventricular XOR activity and superoxide production. This correlated with improved left ventricular volumes, ejection fraction, and measured end-systolic pressure–volume relation. Importantly, treated animals showed evidence of reverse remodeling of the left ventricle, with left ventricular volumes and myocyte width that reverted to values similar to those in control subjects. Oxypurinol had previously been shown to lower blood pressure in spontaneously hypertensive rats but not normal rats. Blood pressure lowering alone, however, is insufficient to account for the reverse remodeling observed.

**XO Inhibition in Endothelial Dysfunction**

Endothelial dysfunction describes abnormal vascular responses to stress and is thought to be an early marker of atherosclerotic disease. This local regulation of vascular tone is dependent on release of vasodilator signals, including signaling by NO, as the prototypic endothelium-derived relaxing factor. Oxidative stress is a major contributor to endothelial dysfunction, insofar as superoxide and other ROS have the ability to interfere with NO activity. As we have discussed, XOR is a major source of endothelial ROS. XOR shows high-affinity binding to endothelial cells, and bound enzyme both retains full production of superoxide and inhibits NO-dependent cGMP production. It is unsurprising then that XO inhibition has been shown to improve endothelial function in multiple studies. In studies of smokers and diabetics, both acute and chronic allopurinol treatment has resulted in improvements in parameters of endothelial function.

Similar results are seen in patients with heart failure, who also show impaired endothelium-dependent vasodilation compared with control subjects. This appears to be due, at least in part, to increased oxidative stress. Heart failure patients show a 2-fold increase in endothelium-bound XO, whereas endothelium-associated superoxide dismutase activity is diminished. In these patients as well, treatment with allopurinol results in an improvement in measured endothelial function that correlates with changes in uric acid levels.

**Clinical Trials of XOR Inhibition in Heart Failure**

Despite the wealth of promising preclinical data on the impact of XO inhibition on cardiac performance, energetics, and remodeling, the small clinical trials performed thus far have not fulfilled expectations in the treatment of heart failure. Gavin and Struthers randomized 50 congestive heart failure class II or III patients to 3 months’ treatment with allopurinol or placebo. Although B-type natriuretic peptide levels were lower in the treatment group, results of a treadmill test and 6-minute walking distance were unchanged. The Controlled Efficacy and Safety Study of Oxypurinol Added to Standard Therapy in Patients With New York Heart Association class III to IV Congestive Heart Failure (OPT-CHF) trial randomized 405 patients to oxypurinol treatment or placebo for 24 weeks. Preliminary results of the OPT-CHF trial indicate that oxypurinol treatment had no effect on the primary composite end point, which included functional class and clinical outcomes. Detailed analyses of the results of this trial are not yet published. This and other future studies will answer the question of whether XOR inhibition is able to effectively add to the current medical armamentarium of heart failure treatment, fulfilling the promise suggested by the wealth of preclinical data.

**Hemoglobin as a Reservoir for ROS and NO Signaling**

**Hemoglobin as a Carrier of NO Bioactivity**

Hemoglobin is the largest reservoir for both O₂ and NO in the body. In particular, NO is carried both by binding to hemes in a manner similar to O₂ and by S-nitrosylation of thiol groups (notably β-cys93) on the globin proteins. Thiocitrulates, including S-nitrosylated proteins and low-molecular-weight nitrosothiol derivatives of cysteine and glutathione, are potent vasodilators, as well as NO itself. Hemoglobin exists as a tetramer of 2 alpha and 2 beta chains that exhibits cooperative binding of oxygen. Binding of O₂, with coincident conversion of hemoglobin from the deoxy (T structure) to the oxy (R structure) form, promotes the generation of S-nitroso hemoglobin. Similarly, conversion to the deoxy T form promotes release of NO and S-nitrosothiols, with accompanying vasodilatory activity. This occurs by transfer of S-nitrosothiols from hemoglobin to band III in red cell membranes; this transfer can be blocked by oxidation of the recipient thiol, another example of nitroso–redox imbalance. Red blood cells therefore act as oxygen-sensing carriers of NO that release NO bioactivity in response to reduced oxygen saturation. Thus, red blood cells vasodilate blood vessels in response to low oxygen tension in a manner that relies on the physiological O₂ gradient. In heart failure, NO delivery from hemoglobin may be impaired by NO/redox disequilibrium.

**Hemoglobin as a Source of ROS**

Hemoglobin itself has a potent heme-oxidase activity that results in superoxide production. Heme oxidation occurs preferentially in the deoxy hemoglobin form, favoring ROS...
Redox Activity of Current Heart Disease Therapies

Heart Failure Therapies
Several drugs currently in use for treatment of heart disease have known redox modulatory activity. A better understanding of these mechanisms not only offers insight into the activity of current therapies but also may guide the development of new therapeutic agents. Increased signaling by angiotensin II, for example, is known to increase oxidative stress via the NADPH oxidase and to contribute to the heart failure phenotype. Standard therapy with ACE inhibitors has the expected effect of limiting oxidative stress, as do angiotensin II receptor blockers. Aldosterone contributes to the redox imbalance and long-term deleterious effects of chronic angiotensin II overexposure, and aldosterone itself has been implicated in increasing ROS production through activation of NADPH oxidases. Blockade of aldosterone activity with spironolactone ameliorates the oxidant and profibrotic effects in the heart. Similarly, restoration of redox balance provides a plausible mechanism for the clinical benefits of spironolactone in heart failure.

The combination of hydralazine and isosorbide dinitrate has recently gained new evidence for efficacy in heart failure. Evidence available to date suggests a redox mechanism for these drugs, although the precise mechanism of action of hydralazine remains elusive. Both the production and delivery of NO in the form of S-nitrosohemoglobin are impaired in the setting of heart failure, and it is not surprising that NO donors should have a beneficial effect. In addition, chronic nitrate treatment activates a membrane-bound NADH oxidase, which produces superoxide. Hydralazine has been suggested to inhibit this activity, both lowering superoxide levels and reducing nitrate tolerance. In addition, recent data have demonstrated that hydralazine may have free radical scavenging activity.

Statins as Modulators of NADPH Oxidase Activity
The statin drugs, inhibitors of 3-hydroxy 3-methyl glutaryl coenzyme A reductase, have shown great benefits in the treatment of atherosclerotic coronary disease. Their efficacy in reducing levels of LDL cholesterol is well known, but much has also been made of the so-called “pleiotropic” effects of statins. These include atherosclerotic plaque stabilization, improved bioavailability of NO, improvement of endothelial dysfunction, and attenuation of inflammatory reactions. Antioxidant properties of statins have been put forth as a possible unifying mechanism for statin pleiotropic effects. Evidence is accumulating that modulation of NADPH oxidase activity by these drugs may be responsible for this activity. As previously discussed, the GTP binding protein Rac1 is a principal regulatory subunit of the myocardial NADPH oxidase. Statins inhibit the isoprenylation and activation of Rac1 and other members of the Rho family of proteins. NADPH oxidase activity is likewise reduced through statin inhibition of Rac1.

In addition to their activity through NADPH oxidase, statins appear to have direct antioxidant effects on lipids. In this regard, the hydroxyl metabolites of atorvastatin (but not the parent compound) have been shown to inhibit oxidation of LDL, VLDL, and HDL. These same metabolites demonstrate free radical scavenging activity that may be responsible for the inhibition of lipoprotein oxidation.

Treatment with statins has been shown to reduce endothelial dysfunction with both short- and long-term treatment. A randomized, controlled trial of simvastatin in human subjects with elevated cholesterol showed an improvement in forearm vasodilator response to acetylcholine after only 4 weeks of treatment. Another recent trial measured brachial artery vasoactivity in postmenopausal women with hyperlipidemia treated with atorvastatin. Significant improvement was seen in the atorvastatin group as early as 2 weeks after treatment was started compared with control subjects. Statins also have been shown to reduce C-reactive protein levels independently of their lipid-lowering effects. Whether statin-mediated reductions in C-reactive protein and other inflammatory markers are due to their effects on redox balance remains to be investigated.

Conclusions
Signaling by RNS and modulation by ROS are a central part of normal myocardial and vascular function. NO and redox-based signaling involves production of second messengers such as cGMP, as well as posttranslational modification of proteins through S-nitrosylation and oxidation of specific cysteine residues. These mechanisms are important in such diverse processes as tissue oxygen delivery, regulation of vascular tone and function, and excitation–contraction coupling. Cross-talk between ROS- and RNS-generating systems strikes a tightly regulated balance that is disrupted in a wide array of cardiovascular disease, including hypertension, atherosclerosis, MI, and heart failure.

Disclosure
Dr Hare serves as a consultant to Cardiome Pharma Corp and Nitrox LLC. The terms of these arrangements are being managed by the Johns Hopkins University Committee on conflicts of interest. Dr Zimmet reports no disclosures.
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