

Reactive Oxygen Species in Heart Failure

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A constant supply of oxygen is indispensable for cardiac viability and function. However, oxygen is also central to the generation of reactive oxygen species (ROS). Indeed, it is estimated that up to 5% of the oxygen normally consumed by tissues can be transformed into ROS. Recent studies point to crucial roles of increased ROS in the pathophysiology of heart failure.¹

Generation and Counterbalancing of Reactive Oxygen Species

Reactive oxygen species, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^{\cdot-}$), and hydroxyl radicals ($\cdot OH$), are highly reactive molecules with unpaired electrons in their outer orbit. $O_2^{\cdot-}$ is produced by the one-electron reduction of molecular O_2 ; it has a half-life of only a few seconds and is rapidly dismutated to H_2O_2 . The diffusion capacity of $O_2^{\cdot-}$ is limited due to its poor membrane permeability, and therefore its actions are generally restricted to the intracellular compartment of production. In contrast, H_2O_2 is more stable and more cell membrane-permeable than $O_2^{\cdot-}$ and may therefore have the potential to act at more distant sites. The most reactive oxygen free radical is $\cdot OH$, which is formed from H_2O_2 via the Fenton or Haber-Weiss reactions. Normally, $\cdot OH$ is formed in negligible amounts, but in pathologic conditions (e.g., ischemia-reperfusion) it is generated in high amounts and contributes to oxidative stress-associated cellular damage. In settings where the level of the signaling molecule nitric oxide (NO) is in

the high nanomolar range, $O_2^{\cdot-}$ may react with NO to generate the potent oxidant $ONOO^{\cdot-}$, this reaction also resulting in inactivation of NO.

In health, these basally generated ROS are efficiently counterbalanced by several enzymatic and nonenzymatic pathways. Among the best-characterized endogenous antioxidant pathways are the superoxide dismutases (SODs), catalase, and glutathione peroxidase enzymes. The SOD isoenzymes efficiently convert $O_2^{\cdot-}$ to H_2O_2 in vivo, with manganese SOD (Mn SOD) present in high concentration in mitochondria, copper/zinc (Cu/Zn) SOD in the cytosol, and extracellular (SOD) at the plasma membrane or in the extracellular compartment. H_2O_2 levels are tightly regulated by cellular catalase and glutathione peroxidase, which scavenge H_2O_2 to water. In addition, thioredoxin and thioredoxin reductase can catalyze the regeneration of many antioxidant molecules, including ubiquinone (Q10), lipoic acid, and ascorbic acid, and as such constitute an important antioxidant defense against ROS. Nonenzymatic mechanisms include intracellular antioxidants such as the vitamins E, C, and β -carotene (a precursor to vitamin A), ubiquinone, lipoic acid, urate, and glutathione; the latter acts as a reducing substrate for the enzymatic activity of glutathione peroxidase.

The Biologic Significance of Reactive Oxygen Species

Reactive oxygen species can exert either potentially beneficial or detrimental effects that contribute to cardiac dysfunction and death. On the

one hand, when levels of ROS are elevated dramatically to overwhelm the cellular antioxidant defenses, they react directly with membrane lipids, proteins, and nucleic acid, causing cellular dysfunction and death (both through apoptosis and necrosis). The cellular production of one ROS may lead to the production of several others via radical chain reactions. For example, reactions between radicals and polyunsaturated fatty acids within cell membranes may result in a fatty acid peroxyl radical that can attack adjacent fatty acid side chains and initiate production of other lipid radicals. Lipid radicals produced in this chain reaction accumulate in the cell membrane and may have a myriad of effects on cellular function, including leakiness of the plasmalemma and dysfunction of membrane-bound receptors. Reactive oxygen species can contribute to mutagenesis of DNA by inducing strand breaks, purine oxidation, and protein-DNA cross-linking, which may significantly affect gene expression. Reactive oxygen species may also induce denaturation that renders proteins nonfunctional.

On the other hand, ROS can function as second messengers or regulatory mediators downstream of specific ligands, such as angiotensin II (AngII), endothelin, growth factors (fibroblast growth factor-2 [FGF-2], platelet-derived growth factor [PDGF]), cytokines (transforming growth factor- β 1 [TGF- β 1], tumor necrosis factor- α [TNF- α]), and many others (so-called redox signaling). Reactive oxygen species involved in signaling can activate various redox-sensitive protein kinases, inactivate protein tyrosine phosphatases, and modulate the activities of transcription factors such as activator protein 1 (AP-1), nuclear factor (NF)- κ B and hypoxia-inducible factor-1 (HIF-1), thereby inducing specific changes in gene expression and cell phenotype. Such redox-regulated effects underlie the essential roles of ROS in biologic processes such as normal cell proliferation and growth. They may also contribute to pathophysiologic processes, for example, the induction of cardiomyocyte hypertrophy through the activation of NF- κ B.

In cardiomyocytes, ROS may also exert specific direct effects on ion channels and membrane ion pumps, including L-type calcium channels, sodium channels, potassium channels, and the Na/Ca exchanger, which are critical for normal

cardiac excitation-contraction coupling and function. Reactive oxygen species may alter the activity of the sarcoplasmic reticulum Ca^{2+} -adenosine triphosphatase (ATPase) (SERCA2) as well as reduce myofilament calcium sensitivity, both of which are important determinants of myocardial contractility. Another major mode of ROS action is by affecting the function of proteins involved in energy metabolism, thereby inducing energetic deficit. It is also worth mentioning that ROS may promote autocrine/paracrine interactions by altering the secretion of bioactive agents. For example, fibroblasts stimulated by ROS increase their secretion of TGF- β , which may have significant effects on adjacent cardiomyocytes. Therefore, ROS has a host of potential actions within the myocardium.

Sources of Reactive Oxygen Species in Cardiac Cells

Potential sources of ROS include the mitochondrial respiratory chain, xanthine oxidase (XO), reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, lipoxygenase, cytochrome P-450s, NO synthases, peroxidases, and other hemoproteins. All these enzymes system are present in the three major cardiac cell types: cardiac myocytes, fibroblasts, and endothelial cells. Although their exact relative contributions to the generation of ROS is not known, it has become clear that mitochondria, XO, and NADPH oxidases are the predominant sources of ROS that may be involved in the pathophysiology of heart failure.

Mitochondrial Reactive Oxygen Species

Reactive oxygen species can be formed during oxidative phosphorylation in the mitochondria as a by-product of normal cellular aerobic metabolism.² Thus, the major process by which the heart derives energy can also result in the production of ROS. During the Krebs cycle, electrons derived from reduced nicotinamide adenine dinucleotide (NADH₂) and flavin adenine dinucleotide (reduced form; FADH₂) flow along the respiratory transport chain through a series of

cytochrome-based enzymes (complexes I, III, IV), which transport electrons finally to molecular O_2 . Normally, the high free energy of the electrons is gradually extracted and converted into adenosine triphosphate (ATP), and only 1% or less of electrons “leak” to form $O_2^{\cdot-}$. Most of this $O_2^{\cdot-}$ is rapidly scavenged by mitochondrial MnSOD. However, during hypoxia-reoxygenation or ischemia-reperfusion, the electron chain transfer is blocked at the level of complex I or III and electrons are inappropriately diverted directly to O_2 , resulting in a large amount of $O_2^{\cdot-}$ formation. The importance of the mitochondrial ROS in heart failure is emphasized by the finding that complete genetic deficiency of mitochondrial SOD in mice results in severe dilated cardiomyopathy and postnatal death.

Xanthine Oxidase

Xanthine oxidoreductase (XOR) is a molybdoenzyme capable of catalyzing the oxidation of hypoxanthine and xanthine in the process of purine metabolism. Xanthine oxidoreductase exists as one of two interconvertible yet functionally distinct forms, namely xanthine dehydrogenase (XD) or xanthine oxidase (XO). The former reduces the oxidized form of nicotinamide adenine dinucleotide (NAD^+), whereas the latter prefers molecular oxygen, leading to the production of both $O_2^{\cdot-}$ and H_2O_2 . The conversion of XD to XO occurs either through reversible thiol oxidation of sulfhydryl residues on XD or via rapid and irreversible proteolytic cleavage of a segment of XD during hypoxia, ischemia, or in the presence of various proinflammatory mediators. Although XO-mediated $O_2^{\cdot-}$ production (usually assessed by inhibition by allopurinol or oxypurinol) can be documented in several cardiac pathophysiologic settings, constitutive XOR activity is apparently very low. It has been suggested that endogenous XO synthesis in the heart may be low but that it may be released from XO-rich organs such as liver and intestine under pathophysiologic conditions and may subsequently bind to endothelial cells in situ in the heart.³

Many experimental studies support an important role for XO-derived ROS generation in myocardial ischemia-reperfusion injury, although there may be a relatively narrow window in which

this can be therapeutically targeted. During ischemia, irreversible proteolytic cleavage converts XD to XO, thereby priming the system for the triggering of microvascular inflammation by the generation of ROS upon the subsequent delivery of oxygen at reperfusion. The ROS thereby generated from XO could trigger the local accumulation and activation of neutrophils, leading to further bursts of ROS production and ultimately cardiac dysfunction. Increases in expression or activity of XO have been documented both in experimental canine heart failure and in end-stage failing human heart tissue, and treatment with the XO inhibitor allopurinol is reported to improve contractile function of failing hearts.

Nicotinamide Adenine Dinucleotide Phosphate Oxidases

Recently, a large body of evidence has indicated that an especially important source of ROS in cardiovascular system is a family of complex enzymes termed NADPH oxidases.⁴ The prototypic NADPH oxidase was first characterized in neutrophils, where it plays an essential role in nonspecific host defense against invading microbes during the process of phagocytosis. NADPH oxidase is a multimeric complex with a core membrane-bound cytochrome b_{558} that catalyzes electron transfer from NADPH to molecular oxygen, thereby generating $O_2^{\cdot-}$. The cytochrome is a heterodimer made up of a catalytic Nox (for NADPH oxidase) subunit and a $p22^{phox}$ subunit. Several Nox isoforms (Nox1-5) have recently been identified; endothelial cells, cardiomyocytes, and fibroblasts express Nox2 and Nox4, whereas vascular smooth muscle cells express mainly Nox4 and Nox1. Interestingly, Nox1 and Nox2 require cytosolic subunits (termed $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) and the small G-protein Rac1 for their activation, whereas current evidence suggests that Nox4 does not depend on these subunits. Reduced nicotinamide adenine dinucleotide phosphate oxidases in cardiovascular (and other nonphagocytic) cells continuously generate a low level of $O_2^{\cdot-}$; however, the level of $O_2^{\cdot-}$ production is significantly increased by several pathophysiologic stimuli, such as AngII, α -adrenergic agonists, endothelin-1, tumor necrosis factor- α , and mechanical stress. A

substantial proportion of the $O_2^{\cdot-}$ generated by NADPH oxidases in cardiovascular cells is produced intracellularly (in contrast to neutrophils) and is thought to be involved in redox signaling.⁴

Accumulating evidence suggests that NADPH oxidase-derived ROS play an important role in many cardiovascular diseases, including myocardial infarction and heart failure. Myocardial NADPH oxidase expression and activity are reported to be increased after acute myocardial infarction and in heart failure, both experimentally and in human disease.⁵ Interestingly, the upregulation of NADPH oxidase-mediated ROS production in the failing myocardium of patients with dilated cardiomyopathy and ischemic cardiomyopathy was associated with increased rac1 activity, which potentially can be inhibited by treatment with statins.

The Role of Reactive Oxygen Species in Heart Failure

Acute Myocardial Infarction

The production of large amounts of ROS during reperfusion has traditionally been suggested to contribute to reperfusion injury and cell death in the context of myocardial infarction (MI). For example, in ischemia-reperfused rat hearts, maximum oxidant production was detected with electron paramagnetic resonance (EPR) at 10 to 30 seconds after reperfusion. Similarly, in humans, EPR spin trapping has been used in conjunction with coronary artery bypass graft (CABG) surgery to show that ROSs are increased in the first 5 minutes after reperfusion. It is suggested that up to 60% of cardiac myocyte death in the early stages of reperfusion may be attributable to oxidative injury. These patients may have elevated serum markers of oxidative stress such as thiobarbituric acid reactive substances (TBARS), and a significant proportion may go on to develop heart failure.⁶

In experimental animals, pretreatment with antioxidants/enzymes (such as SOD) or the genetic overexpression of these enzymes affords protection against reperfusion injury. A frequently studied antioxidant agent is *N*-2-mercaptopyr-

onyl glycine (MPG), which is thought to work by directly reacting with free-radical species, promoting the resynthesis of glutathione, or acting as an alternative substrate for glutathione peroxidase, thereby limiting the cytotoxic effects of H_2O_2 and lipid peroxides. *N*-2-mercaptopyrponyl glycine has been shown to significantly reduce MI size for as long as 48 hours after reperfusion. Other antioxidant agents shown to exert some cardioprotective action in animal models of MI include *N*-acetylcysteine (NAC), dimethylthiourea, and desferrioxamine. In canines, a combination of SOD and catalase significantly reduced MI size after 90 minutes of coronary artery ischemia and 24 hours of reflow. Interestingly, administration of the XO inhibitor allopurinol in the setting of rat acute MI attenuated stunning and ameliorated excitation-contraction uncoupling. However, such therapies have not achieved clinical usage as yet.

The genetic overexpression of glutathione peroxidase (GSHPx) also protected against myocardial ischemia-reperfusion, whereas GSHPx knockout mice were more susceptible to myocardial reperfusion injury compared with their wild-type counterparts.

Postmyocardial Infarction Remodeling

Increased oxidative stress is recognized to promote adverse LV remodeling post-MI (i.e., chronic ventricular dilatation and contractile dysfunction), a major precursor to heart failure. Experimentally, treatment with the antioxidants probucol or dimethylthiourea can ameliorate adverse LV remodeling and improve contractile function. Consistent with these data, post-MI remodeling was prevented in mice overexpressing glutathione peroxidase. Recent data from our laboratory suggest that Nox2 contributes significantly to the adverse remodeling observed after MI, whereas another study implicates XO.

Reactive Oxygen Species in the Diabetic Heart

Diabetes is an established risk factor for cardiovascular events, and the mortality from ischemic heart disease of diabetic patients is three times higher than that of nondiabetics. There is an

increasing recognition that diabetic patients may have additional specific myocardial problems independent of coronary artery disease, age, hypertension, obesity, or hyperlipidemia, termed “diabetic cardiomyopathy.” Prominent functional consequences include diastolic and systolic dysfunction and heart failure, with an annual mortality of 15% to 20%. While the mechanisms underlying diabetic heart muscle disease are not fully understood, a considerable body of evidence implicates oxidative stress as an important pathogenic factor.⁷

In animal models of streptozotocin-induced type I diabetes, the production of ROS in the heart has been shown to be increased in association with a reduction in number of left ventricular cardiomyocytes. Of importance, the antioxidant NAC could prevent this reduction in myocyte number. In experimental type 2 diabetes, the impairment of cardiomyocyte contractility can be ameliorated by overexpression of the antioxidant protein metallothionein or the antioxidant enzyme catalase. Human patients with type 2 diabetes also have elevated levels of oxidative stress markers in the plasma, although the effects of antioxidants on cardiac function have not been defined.

The sources of ROS generation in diabetes are of interest. Hyperglycemia can stimulate ROS generation directly through effects on various cellular enzymes. In endothelial cells, the increased ROS production appears to be mitochondrial in origin, whereas in adult rat cardiomyocytes the ROS seem to be generated by NADPH oxidases, and this is inhibited by the oxidase inhibitor apocynin both in vitro and in vivo. Hyperglycemia also promotes the formation of glucose-modified proteins such as early glycated Amadori products and advanced glycation end-products (AGEs). The two main types of Amadori products in blood are hemoglobin A_{1c} (HbA_{1c}) and fructosamine, both of which are used to assess the degree of glucose control. There is a positive relationship between the levels of glycated products and diabetic heart disease, with each 1% increase in HbA_{1c} being associated with an 8% increase in the risk of heart failure or death. Interestingly, both early and late glycation end-products can stimulate ROS generation in several cell types. In endothelial cells and cardiomyocytes, a large proportion of this ROS production emanates from activated NADPH

oxidase. In the latter cell type, Nox2 NADPH oxidase-derived ROS led to NF- κ B activation and the upregulation of atrial natriuretic factor (ANF) mRNA.

Alcoholic Cardiomyopathy

Long-term misuse of alcohol or binge drinking is recognized to lead to cardiac dysfunction and failure, characterized as a unique type of dilated cardiomyopathy termed alcoholic cardiomyopathy. A cardinal feature of this cardiomyopathy is its precipitation by alcohol abuse and the significant rates of recovery following abstinence. Although several hypotheses have been postulated to mechanistically explain the occurrence of this condition, enhanced oxidative stress seems to be central in explaining the toxicity of ethanol and its metabolite acetaldehyde.⁸ Three main metabolic pathways for ethanol have so far been described in the human body, which involve alcohol dehydrogenase (ADH), microsomal ethanol oxidation system, and catalase. Each of these pathways is able to generate free radicals, including O₂⁻, hydroxyl, acetyl, and methyl radicals. Acetaldehyde is formed by the oxidation of ethanol by ADH and is subsequently oxidized to acetic acid mainly through ADH, a process accompanied by ROS generation. Most evidence supporting an etiologic role for oxidant stress in alcoholic cardiomyopathy comes from animal experiments. For example, a recent study showed that transgenic mice with cardiac overexpression of catalase displayed preserved cardiac function and improved intracellular Ca²⁺ handling against ethanol-induced damage. However, convincing data from human studies remain lacking.

Reactive Oxygen Species in Cardiac Hypertrophy

Prolonged cardiac hypertrophy is a common precursor to heart failure, while cardiomyocyte hypertrophy is also a prominent feature of adverse LV remodeling following MI. Cardiac hypertrophy occurs in response to diverse stimuli, including mechanical stretch and neurohormones, which subsequently trigger various downstream

signaling pathways such as the protein kinases C (PKCs), mitogen activated protein kinases (MAPKs), protein kinase B or Akt, calcineurin, and the transcription factors AP-1 and NF- κ B.

Extensive experimental studies support a role of oxidant signaling in the development of cardiac hypertrophy. Interestingly, recent studies suggest that NADPH oxidase-derived ROS are important in this regard. For example, the induction of cardiomyocyte hypertrophy by short-term infusion of angiotensin II *in vivo* has been shown to involve the activation of Nox2 NADPH oxidase, with mice deficient in Nox2 demonstrating substantially reduced angiotensin II-induced cardiac hypertrophy and interstitial fibrosis compared with wild-type animals.⁹

Clinical Utility of Antioxidants?

Despite the wealth of experimental data and the theoretical arguments regarding the involvement of increased oxidative stress in the pathogenesis of heart failure, the therapeutic potential of free radical-directed drugs has not yet been realized. Clinical trials of antioxidant therapy have been less than compelling. For example, recombinant human SOD failed to improve recovery of ventricular function in patients undergoing thrombolysis for anterior wall acute myocardial infarction. The heart outcomes prevention evaluation (HOPE) investigators found no protective effect of vitamin E in reducing death or cardiovascular events in at-risk patients over a 4.5-year period. However, several issues are worth noting with regard to the clinical potential of antioxidant therapies: (1) Many antioxidants that have been studied to date (e.g., vitamin E) are essentially scavengers of already-formed intracellular oxidants and therefore may be considered to be “symptomatic” rather than causal treatments. (2) The levels of relevant tissue oxidative stress in most patients included in clinical trials have been unquantified while the dosages of antioxidants have been arbitrary. (3) The relationship between ROS and heart failure is probably too complex to be addressed by a single nonspecific intervention. Interestingly, however, many current therapies that are effective in heart failure (such as angio-

tensin-converting enzyme inhibitors, angiotensin receptor antagonists, beta-blockers, and statins) have in common the property that they are all “antioxidant” in some way. These findings provide an opportunity to reconsider the therapeutic potential of antioxidant agents in patients with heart failure, perhaps by targeting specific sources of ROS generation rather than relatively blunt nonspecific approaches.¹⁰

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