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Forum Review

Regulation of Hypertrophic and Apoptotic Signaling Pathways by Reactive Oxygen Species in Cardiac Myocytes

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ABSTRACT

Increasing evidence suggests that oxidative and nitrosative stress play an important role in regulation of cardiac myocyte growth and survival. The cardiovascular system is continuously exposed to both reactive oxygen species (ROS) and nitrogen species (RNS), collectively termed reactive inflammatory species (RIS), and imbalances between the enzymes that regulate their bioavailability are associated with cardiac hypertrophy and the pathogenesis of cardiomyopathies, myocardial infarction and heart failure. It is now clear that RIS act as critical regulators of cardiac myocyte hypertrophy and apoptosis through control of redox-sensitive signaling cascades, such as tyrosine kinases and phosphatases, protein kinase C, and mitogen-activated protein kinases. This review will focus on the mechanisms by which ROS/RNS modulate cardiac myocyte growth and apoptosis induced by neurohormones and cytokines, and will discuss evidence for a role in the pathophysiology of heart failure. *Antioxid. Redox Signal.* 5, 731–740.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS) and reactive nitrogen species (RNS) [collectively referred to as reactive inflammatory species (RIS)] play critical roles in modulating cardiac contractile function and metabolism during the pathogenesis of ischemia–reperfusion injury, drug-induced cardiomyopathy, and heart failure (39, 69, 78). Recent advances implicate RIS as coordinators of cardiac myocyte hypertrophy and apoptosis through control of redox-sensitive signaling cascades. This review will focus on the mechanisms by which RIS modulate cardiac myocyte growth and apoptosis induced by peptide hormones, adrenergic agonists, growth factors, and cytokines. The important effects of RIS on cardiac contractile function and metabolism are beyond the scope of the present discussion (for recent reviews, see 45, 69, 73).

RIS IN THE MYOCARDIUM

RIS exist in cells and tissues at low concentrations that are determined by the balance between their rates of production and rates of removal by antioxidant enzymes and compounds. Collectively, ROS refers to free radicals and oxidants derived from one-electron reduction of molecular oxygen. These are: superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$). $O_2^{\bullet -}$ is generated by xanthine oxidase, the mitochondrial respiratory chain, lipoxygenase, P450-based enzymes, and under certain circumstances, nitric oxide synthase (NOS) (62). Another important source of ROS production in the cardiovascular system is the phagocyte-type NADPH oxidase. Adult rat ventricular myocytes (ARVM) express all major components of this plasma membrane-bound oxidase, including the core heterodimer of $p22^{phox}$ and $gp91^{phox}$ subunits and the regulatory subunits $p47^{phox}$, $p67^{phox}$, and Rac1 (4, 42). This oxidase is an important mediator of G protein-coupled receptor (GPCR) signaling in vascular smooth muscle cells and cardiac myocytes (for a comprehensive review, see 26). Once formed, $O_2^{\bullet -}$ stimulates lipid and protein modifications, may interact with nitric oxide (NO) to form peroxynitrite ($ONOO^-$; see below), or can be converted by superoxide dismutase (SOD) into H_2O_2 and oxygen. In the heart, SOD is present as a mitochondrial Mn-SOD isoform and the cytosolic Cu/Zn-SOD isoform (13). H_2O_2 undergoes a Fe^{3+} Fenton reaction, to form

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•OH, or can be hydrolyzed by catalase and glutathione peroxidase.

RNS refers to nitrosative species formed from NO and $ONOO^-$. NO is synthesized from the conversion of arginine to citrulline by a family of NOS. Three isoforms of this flavoprotein have been purified and cloned from mammalian cells: neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS). nNOS and NOS isoforms are regulated by a variety of different stimuli in different cell types via Ca^{2+}/calmodulin activation, as well as *de novo* synthesis (17). In contrast, the activity of iNOS is regulated by expression in a Ca^{2+}-independent manner. Within the myocardium, eNOS is present in cardiac fibroblasts and vascular and endocardial endothelial cells (69). Although there is little basal cardiac iNOS, its expression in cardiac myocytes and fibroblasts is induced by a variety of cytokines. iNOS is also present in inflammatory cells that infiltrate the myocardium in response to injury or hemodynamic stress (3, 29). NO potently regulates guanylyl cyclase activity to increase cyclic GMP (cGMP). Higher concentrations of NO react with $O_2^{\bullet -}$ to form the oxidizing and nitrating species $ONOO^-$. $ONOO^-$ serves as a biological oxidizing and nitrating agent, resulting in lipid peroxidation and protein modifications.

Antioxidant enzymes are essential "turn-off" components that scavenge RIS. Cardiac myocytes express cytosolic SOD (Cu/Zn-SOD), mitochondrial SOD (Mn-SOD), the H_2O_2 scavenger catalase, glutathione peroxidase, as well as the nonenzymatic compounds thioredoxin (Trx) and glutathione (21). Up-regulation of these enzymes within the myocardium occurs during oxidative stress and in response to cytokine and growth factor activation (71). Increased myocardial SOD activity also occurs during chronic stable hypertrophy in rats (27). On the other hand, decreased expression of SOD and catalase has been documented in animals and humans with end-stage heart failure (19, 40, 53, 88).

A balance between ROS and RNS generating mechanisms and cellular antioxidants dictates the redox potential of the myocardium and tends to buffer transient changes in the redox status. However, changes in this equilibrium can occur in response to physiological and pathological conditions, including age, exercise, and hypertrophy (39). Increasing evidence suggests a role for intracellular RIS as mediators of normal and pathological signal transduction pathways within the myocardium. At the cellular level, RIS elicit a wide spectrum of responses ranging from proliferation and hypertrophy to sarcomeric disruption, intracellular Ca^{2+} overload, mitochondrial dysfunction (with subsequent depletion of internal energy stores), and cell death. The exact outcome can vary significantly with respect to the cellular source of the RIS, the type and amount released, interactions with neurohormonal and other growth factors, spatial localization of RIS generation within the cell and the duration of the response. For instance, Colucci's group recently demonstrated that H_2O_2, depending on the concentration, could promote either hypertrophy or apoptosis of ARVM (41). H_2O_2 at low concentrations (~10 μM) increased extracellular signal-regulated kinase (ERK1/2) mitogen-activated protein (MAP) kinase activation and protein synthesis. Higher concentrations of H_2O_2 (100–300 μM) induced p38, c-Jun N-terminal kinase (JNK), and ERK1/2 MAP kinases and Akt, resulting in apoptosis (measured by TUNEL and annexin V staining) (41). Thus, it appears that lower concentrations of RIS are involved in promoting or suppressing cardiac myocyte hypertrophy, whereas higher concentrations are associated with apoptosis and/or necrosis.

Oxidant-induced regulation of signaling cascades may occur indirectly by interrupting negative regulation or directly by activation of signaling components. These modifications of signaling molecules occur through oxidation of amino acid residues, most commonly tyrosine, cysteine, histidine, and tryptophan (21). Activation of src and protein kinase C (PKC) ϵ by tyrosine nitration is a notable example of this mechanism (5, 48, 49). On the other hand, RIS can also turn off signaling systems by disrupting protein-protein interactions, changing protein conformation, or increasing the susceptibility to proteolytic attack.

RIS participate in the control of cardiac hypertrophy, apoptosis, and remodeling through modulation of contractile proteins, metabolism, intracellular Ca^{2+} and ion homeostasis, apoptosis, and growth. Many of these effects appear to be mediated by activation of redox-sensitive kinase cascades, such as guanylyl cyclase, src, PYK2, PKC, MAP kinases, nuclear factor-κB (NF-κB), and phosphatidylinositol 3-kinase (PI3-kinase)/Akt (13, 21, 62, 78). In addition to direct effects on these kinases, RIS also modulate hypertrophic and apoptotic signaling by GPCR [*e.g.,* angiotensin type 1 receptor (AT_1R), α- and β-adrenergic receptors (AR)], growth factors, and cytokines.

In the following sections, we will review the role of RIS in regulating cardiac myocyte growth and apoptosis in cultured neonatal and adult cardiac myocytes, in the intact myocardium, and during the progression of heart failure. Both direct effects of RIS and modulation of receptor-mediated hypertrophic signaling will be considered.

ROLE OF RIS IN HYPERTROPHIC GROWTH OF CARDIAC MYOCYTES

Cardiac hypertrophy occurs in response to hemodynamic load related to both physiological and pathophysiological conditions. This growth can be an adaptive response to transient, intermittent increases in workload, such as exercise. On the other hand, hypertrophy associated with sustained hemodynamic load (*e.g.,* hypertension, aortic or mitral valve regurgitation), although initially adaptive, will eventually become detrimental and results in cardiac dysfunction, necrosis, and apoptosis. Characteristics of hypertrophic cardiac myocyte growth include an increase in myocyte cross-sectional area, increased mRNA content, and protein synthesis (67). At the molecular level, there is an induction of immediate early genes such as c-fos, c-jun, and erg-1, reexpression of fetal genes such as β-myosin heavy chain (β-MHC), α-skeletal muscle and α-smooth muscle actin, and atrial natriuretic factor (ANF), and increased GATA-4-dependent expression of brain natriuretic peptide (75). Among the extracellular stimuli that regulate cardiac myocyte growth are the following: peptide and amine hormones, such as angiotensin II (Ang II), norepinephrine (NE), endothelin-1, and thrombin, that activate GPCR; cytokines, such as interleukin-1β (IL-1β), interleukin-6, and tumor necro-

sis factor-α (TNF-α); and mitogens, such as fibroblast growth factor and epidermal growth factor. These agonists bind to cell-surface receptors that are coupled to multiple signal transduction cascades that link receptor activation to the regulation of hypertrophic growth, including tyrosine kinases (src, focal adhesion kinase), MAP kinases (ERK1/2, p38, JNK, ERK5), PKC, calcineurin, PI3-kinase)/Akt, and NF-κB (34, 74, 75). A complex balance between these signaling proteins dictates hypertrophic growth, whereas an imbalance between these systems may ultimately lead to myocyte death via apoptosis or necrosis. As mentioned above, it is now evident that RIS are capable of directly activating nearly all of these cascades. In neonatal rat ventricular myocytes (NRVM), H_2O_2 stimulates src, PKC, and p38, ERK1/2, and JNK MAP kinases (1, 63, 82), and PI3-kinase is required for H_2O_2 induced hypertrophy (79). In the following sections we will review the data identifying RIS as critical participants in the regulation of cardiac growth by hypertrophic agents.

RIS and hypertrophic growth induced by GPCR agonists

G proteins (GTP-binding proteins) are heterotrimeric protein complexesconsisting of α, β, and γ subunits. G proteins transducesignals from seven-transmembrane receptors to effector molecules. Within the myocardium, $G\alpha_i$ and $G\alpha_q$ appear to initiate many of the hypertrophic signaling pathways induced by Ang II, bradykinin, endothelin, and α-adrenergic stimulation. Although it is well known that GPCR activation can lead to ROS generation, a novel series of studies by Nishida *et al.* provide the first evidence that ROS can directly promote G-protein dissociation and activation (56, 57). In NRVM, the activation of ERK1/2 MAP kinase and Akt by H_2O_2 (≥300& μ*M*) required the Gβγ subunits of G_i and G_o, but was independent of ligand binding to GPCR (56). Subsequent studies indicated that H_2O_2 activates G_i and G_o by a •OH-dependent modification of two cysteine residues (Cys^{287}, Cys^{286}) that allows for dissociation of Gβγ and increased GTP binding to the α subunit (57). Thus, ROS appear to mediate hypertrophic growth signaling by direct activation of G proteins in NRVM that would allow the cardiac myocyte to detect and respond to changes in the redox potential of its environment in the absence of neurohormonal activation.

Ang II, ROS, and cardiac hypertrophy (Fig. 1).

Ang II acts as a hypertrophic factor involved in cardiac remodeling and has been shown to stimulate early growth signals. Most of the cellular actions of Ang II are mediated via interaction with the G_q-coupled AT_1R and subsequent activation of PKC, MAP kinases, tyrosine kinases and PI3-kinase (87). Recent studies suggest that many of the hypertrophic effects of Ang II are mediated through ROS generation. Nakamura *et al.* (52) first reportedthat Ang II induced ROS generation in cultured NRVM and that pretreatment with antioxidants led to the abolishment of Ang II–induced cardiac hypertrophy (cell enlargement and [^{3}H]-leucine incorporation). Shih *et al.* reported that Ang II-induced expression of the hypertrophic marker β-MHC was mediated by a $O_2^{\bullet -}$ and H_2O_2-dependent activation of the Ras/Raf/ERK MAP kinase pathway (70). Subsequent studies extended these observations and showed that ROS generation leads to the activation of the transcription factor, NF-κB, which resulted in cardiac hypertrophy (10, 33). Wenzel *et al.* convincingly showed that Ang II-induced activator protein-1 (AP-1) activity was dependent on the activation of p38 MAP kinase secondary to NADPH oxidase stimulation in ARVM (83). The mechanisms that link RIS generation to downstream signaling events in cultured cardiac myocytes have not been fully elucidated. Some studies report that cysteine residues within the regulatory domains of src and PKC are subject to oxidation and activation, and ROS may indirectly influence tyrosine kinase signaling cascades via inhibition of protein tyrosine phosphatases (54). Direct regulatory effects of ROS on transcription factor activities such as AP-1 and NF-κB have also been demonstrated in several cell types, including cardiac myocytes (10, 33, 66). Hirutani *et al.* have identified a novel signaling pathway involved in cardiac hypertrophy that is mediated by the redox-sensitive kinase apoptosis signal-regulating kinase-1 (ASK1), a MAP kinase kinase kinase that regulates JNK and p38 MAP kinases (33, 55) and is a mediator of TNF-α-induced apoptosis (25). Their results implicate a role for ASK1 in the activation of NF-κB and cardiac hypertrophy by Ang II, NE, and endothelin-1.

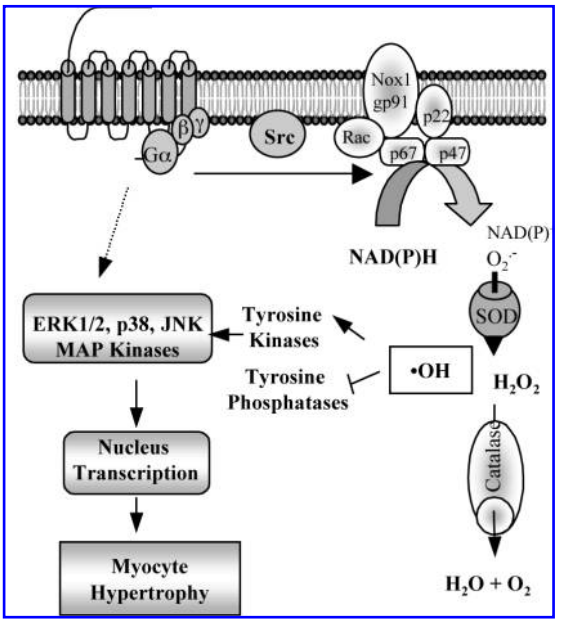

FIG. 1. Role of the NADPH oxidase in Ang II-induced cardiac myocyte hypertrophy.

Although these studies have identified important signaling intermediates downstream of ROS, much less is known about the mechanisms by which Ang II promotes NADPH oxidase activity and ROS generation. The small G proteins Rac and Rho are likely candidates because they have been linked to Ang II-induced ROS production and are upstream activators of p38 and JNK MAP kinases (76) and regulate the actin cytoskeleton (76), respectively. Treatments with the Rho inhibitor C3 exotoxin or overexpression of dominant-negative Rho and Rac completely inhibits Ang II-induced intracellular oxidation and ANF promoter activity (77). Furthermore, treatment of NRVM with simvastatin, an inhibitor of 3-hydroxy-3-

methylglutaryl-CoA reductase and blocker of Rho isoprenylation (and activation), decreased Ang II-induced protein content, [^{3}H]leucine uptake, and ANF promoter activity (77). These effects were associated with decreases in cell size, membrane Rho activity, $O_2^{\bullet-}$ production, and intracellular oxidation, and were reversed with L-mevalonate or geranylgeranylpyrophosphate, but not with farnesylpyrophosphate or cholesterol (77). These results suggest that Rho family members are one link between AT_1R and NADPH oxidase activation.

Recent *in vivo* studies have confirmed that NADPH oxidase-dependent ROS production is essential for Ang II-induced cardiac hypertrophy. Takemato *et al.* (77) extended their *in vitro* findings to show that simvastatin inhibited cardiac hypertrophy and decreased myocardial Rac1 activity and $O_2^{\bullet-}$ production in rats treated with Ang II infusion or subjected to transaortic constriction. Bendall *et al.* (7) provided further compelling evidence that Ang II-induced myocyte hypertrophy and interstitial fibrosis were markedly attenuated in $gp91^{phox-/-}$ mice. This study was particularly convincing because multiple markers for hypertrophy were assessed, including morphometric assessment of heart/body weight ratio, measurements of left ventricular (LV) myocyte cross-sectional area, and increased expression of ANF and β-MHC mRNA (7). However, the relative contributions of different cell types (*e.g.,* cardiomyocytes, endothelial cells, fibroblasts) to this Ang II-induced, $gp91^{phox}$-dependent hypertrophic response warrants further investigation. It is apparent from these *in vitro* and *in vivo* studies that NADPH oxidase-dependent ROS production is an important step in the hypertrophic signaling cascade induced by AT_1R activation Further studies are needed to determine additional mechanisms by which Ang II increases ROS formation and to identify the molecular targets of ROS that mediate hypertrophic growth responses.

ROS and the control of cardiac growth by adrenergic agonists. NE is a potent stimulus for the hypertrophic growth of ventricular myocytes that is associated with increased protein synthesis, the induction of protooncogene such as c-*fos* and c-*myc,* sarcomeric organization, and reexpression of fetal genes. NE interacts with α- and β-AR in the myocardium, and both receptor subtypes have been implicated in NE-induced ROS generation and hypertrophy. Luo *et al.* (46) demonstrated that blockade of both α- and β-AR was necessary to inhibit completely the NE-induced increase of ROS, beating, and protein content in NRVM. The H_20_2 scavenger catalase is able to block NE-induced increase in ROS and RNA and protein content in NRVM (46). Prolonged culture of ARVM is also associated with the development of a growth response to β_2-adrenergic stimulation, possibly due to phenotypic changes associated with long-term culture (60). On the other hand, most studies of short-term cultures of ARVM or NRVM have shown that NE causes hypertrophic growth entirely due to stimulation of α1-AR. (2, 84).

In ARVM, NE increased the level of intracellular ROS (as assessed by lucigenin chemiluminescence or cytochrome *c* reduction), and this effect was prevented by α1-AR blockade with prazosin, the SOD mimetic Mn(III)terakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), or the NADPH oxidase inhibitor diphenylene iodonium (85). The redox changes were associated with a hypertrophic growth phenotype characterized by an increase in [^{3}H]-leucine incorporation and protein accumulation, a sixfold induction of atrial natriuretic peptide mRNA, actin filament reorganization, and the induction of Mn-SOD mRNA (85).

ROS and cardiac myocyte hypertrophy in response to mechanical stretch. In addition to their role in receptor signaling mechanisms, ROS have emerged as important transducers of cardiac myocyte growth in response to mechanical strain. ROS can mediate either hypertrophy or apoptosis in cardiac myocytes in response to mechanical strain, depending on the type and amount of RIS produced. For example, AT_1R blockade inhibits ROS production and NRVM hypertrophy induced during cyclic stretch (58). This is consistent with prior work implicating paracrine release of angiotensin and endothelin in stretch-inducedhypertrophy (64) and apoptosis (14). Static stretch of isolated papillary muscles causes a similar amplitude-dependent increase in myocardial ROS production (14). However, not all of the effects of mechanical strain are mediated by Ang II. Yamamoto *et al.* demonstrated that the antioxidants *N*-acetyl-L-cysteine, catalase, and 1,2-dihydroxybenzene-3,5-disulfonate, but not AT_1R blockade, significantly inhibited NF-κB-dependent regulation of tenascin-c and brain natriuretic peptide (86).

The studies reviewed in this section provide compelling evidence for a fundamental role for ROS in the regulation of cardiac myocyte hypertrophy. Much less is known about the mechanisms that antagonize cardiac growth. The next section will review recent literature that supports the notion that low to moderate amounts of NO exert potent antihypertrophic effects to offset growth responses elicited by GPCR agonists and cytokines.

Role for RNS in cardiac myocyte hypertrophy (Fig. 2). Two experimental approaches have been used to define the role of NO in cardiac myocyte hypertrophy: addition of exogenous NO donors and correlation between NOS activity and hypertrophic agonists using pharmacological or genetic manipulation of NOS enzymes. Within the myocardium, NO is generated by eNOS, as well as iNOS. Cardiac myocytes express predominately iNOS, although a recent report suggested that NOS is located within the sarcoplasmic reticulum and may mediate nitration/nitrosylation of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) (68). In general, low to moderate levels of NO, produced by eNOS and nNOS, are associated with a suppression of hypertrophic growth.

Harding *et al.* (28) were among the first to demonstrate that exogenous NO and IL-1β-mediated NO production decreased α-AR-induced increases in protein content and brain natriuretic peptide expression in NRVM. Matsuoka *et al.* (50) reported that chronic treatment of spontaneously hypertensive rats with the NO precursor L-arginine attenuated cardiac hypertrophy *in vivo.* It appears that cGMP generation is responsible for the antihypertrophic actions of NO, because both the NO donor *S*-nitrosopenicillamine (SNAP) and 8-bromo-cGMP reduce NE-induced protein synthesis in NRVM (12).

In a recent study, Heinecke *et al.* (31) used gene microarray analysis to identify genes that are regulated by NO and are

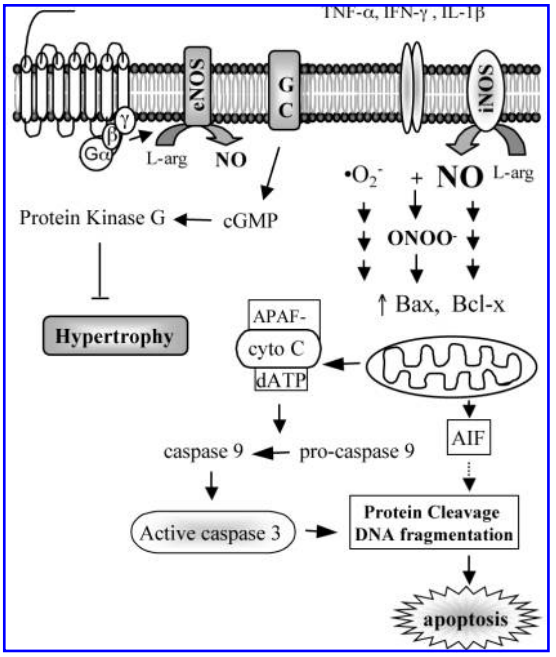

FIG. 2. Role of NO in cardiac myocyte hypertrophy and apoptosis. Low to moderate concentrations of NO produced by eNOS suppress cardiac myocyte hypertrophy in a cGMP-dependent manner. Higher concentrations of NO derived from iNOS mediate cytokine-induced apoptosis through $ONOO^-$ generation through the mitochondrial pathway. GC, guanylyl cyclase; Cyt c, cytochrome *c*; APAF-1, apoptotic protease activation factor 1; AIF, apoptosis-inducing factor.

functionally important in cardiac myocyte hypertrophy. NO was found to cause cGMP and $ONOO^-$-dependent modulation of a number of genes involved in endothelin-1-induced hypertrophy, including increased expression of glutathione-*S*-transferase and cyclin-dependent kinase inhibitor 1A, and decreased expression of the Na^+/H^+ exchanger-1 isoform, cyclin D, and muscle LIM protein (MLP). MLP is localized at the Z-disk, where it anchors the sarcomere to the plasma membrane, and is thought to facilitate sarcomeric assembly and cell enlargement during hypertrophy (22, 30). Interestingly, MLP suppression by either NO or antisense down-regulation inhibited endothelin-induced NRVM hypertrophy, and there was an inverse relationship between iNOS and MLP in failing human hearts (31).

RIS AND CARDIAC MYOCYTE APOPTOSIS

During the last few years, increasing evidence in both animal and human models suggests that apoptosis plays an important role during cardiac development and during the pathogenesis of dilated cardiomyopathy, congestive heart failure, hypertensive heart disease, myocardial infarction, and diabetic cardiomyopathy (24, 38). Apoptosis is a specialized form of programmed cell death that is characterized by nuclear chromatin condensation, DNA fragmentation, and cellular shrinking, followed by breakup of the nucleus and the formation of apoptotic bodies.

There are two major pathways through which apoptosis is induced: one involves death receptors and is exemplified by Fas-mediated caspase-8 activation, and the other is the stress- or mitochondria-mediated caspase-9 activation pathway. Both pathways converge on caspase-3 activation, resulting in nuclear degradation and cellular morphological change. The death receptor pathway in cardiac myocytes involves binding of Fas ligand (FasL) and TNF-α to their cognate receptors (Fas and TNFR-1) (20, 51). The mitochondrial pathway involves the release of cytochrome *c* into the cytosol, which is dependent on the opening of the mitochondrial permeability pore. Once released, cytochrome *c* forms an activation complex with apoptotic caspase 9 and triggers the apoptotic pathway. This process is tightly regulated by the balance between proapoptotic (Bax and Bcl-x_l) and antiapotic (Bcl-2) members of the Bcl-2 family.

In cardiac myocytes, the addition of exogenous ROS such as H_2O_2 causes apoptosis (16, 80). Studies in ARVM indicate that SOD inhibition results in ROS production and apoptosis. Finally, an increase in ROS and RNS is often associated with apoptotic processes triggered by β-adrenergic stimuli, TNF-α, and Fas (38). More recent work in ARVM also suggests that ROS are involved in β-AR-stimulated apoptosis. Coluccis's lab found that β-AR provoked cytochrome *c* release and decreased mitochondrial membrane potential, and DNA fragmentation was prevented by SOD/catalase mimetics (15, 72).

Collectively, these observations suggest that both mitochondria and ROS play an important role in β-AR-stimulated apoptosis. A later study from the same group also implicated a role for ROS-dependent JNK activation in β-AR-induced cytochrome *c* release (61). The source of ROS in these studies was not determined but may include the mitochondria, NADPH oxidase, or NOS. ROS production from the mitochondria is receiving increasing attention as an important mediator of redox-sensitive signaling in cardiac myocytes and is hypothesized to regulate hypertrophic growth responses (11).

Redox-sensitive cardiac myocyte apoptosis is also triggered by TNF and Fas ligand. These effects appear to be mediated by ASK1-induced, sustained activation of JNK. The activation of ASK1 by cytokines requires the association of the C-terminal domain of ASK1 to TRAF (TNF receptor-associated factor) receptors (44). The redox-sensitive enzyme Trx directly binds to ASK1 to inhibit its activity (65). TNF and ROS activate ASK1 in part by oxidizing Trx to release Trx from ASK1 (8, 65). However, Liu and Min recently reported that Trx could induce ASK1 ubiquitination/degradation in a redox-independent manner (44). Thus, it appears that ASK1 is important not only in RIS-induced apoptosis, but also in GPCR- and ROS-dependent hypertrophy (see above). These findings have led Bishopric and Webster to hypothesize that titration of ASK1 activity may therefore determine whether oxidative stress is interpreted as a survival or death signal (8).

There is conflicting evidence concerning the role of NO in apoptosis, with studies demonstrating both the proapoptotic and antiapoptotic effects. One source of the controversy results from the type of NO donor used *in vitro* to produce NO. Many studies use nitrosothiols as NO donors, but nitrosothiols are more reactive with biological thiols than NO, thereby

producing multiple products, including nitrosation and thiolation (6). In fact, *S*-nitrosation of the active sites of caspases may account for the reported antiapoptotic action of this class of NO (6). Other NO donors, such as DETA-NONOate, can directly release NO and may therefore provide more direct evidence for NO-dependent phenomena. The amount of NO also influences the effects on apoptosis. Higher concentrations of NO, which react with $O_2^{\bullet -}$ to form $ONOO^-$, are generally considered to be apoptotic (Fig. 2). For example, Ing *et al.* (36) reported that IL-1β, TNF-α, and interferon-γ (IFN-γ)-induced apoptosis was mediated by iNOS-dependent generation of NO that was unaffected by the protein kinase G inhibitor KT5823, but was completely inhibited by antioxidant scavengers. Moreover, the competitive iNOS-antagonist L-NMMA prevented NO production, DNA laddering, and expression of Bax and Bcl-x (36). Arstall *et al.* (3) demonstrated that IL-1β- and IFN-γ-induced apoptosis in NRVM was dependent on generation of high concentrations of NO by iNOS. NO increased the proapoptotic ratio of Bax to Bcl-2 (3). These effects were blocked by $ONOO^-$ scavengers, but not by ODQ, an inhibitor of soluble guanylyl cyclase (3). In ARVM, NO derived from SNAP or in response to cytokine-induced increases in iNOS, triggers apoptotic markers, including DNA fragmentation and increased expression of the p53 tumor suppression factor (59).

Virtually nothing is known about the cellular signaling cascades that participate in $ONOO^-$-induced cardiac myocyte apoptosis. Arstall *et al.* (3) demonstrated that $ONOO^-$ increases Bax levels in NRVM, but the molecular mechanisms that mediate this effect were not determined. Bers' laboratory reported that iNOS-derived NO/$ONOO^-$ suppressed β-AR-stimulated Ca^{2+} release in myocytes isolated from a failing human heart, but it is unclear if this decreased intracellular Ca^{2+} is involved in the β-AR-induced apoptosis (89). In other cell types, $ONOO^-$-induced apoptosis appears to involve p38, JNK, and ERK1/2 MAP kinases (37, 81). Further studies are required to identify signal transduction pathways involved in $ONOO^-$-induced cardiac apoptosis.

RIS AND HEART FAILURE

The involvement of RIS in ischemia–reperfusion injury and myocardial infarction has been well documented. Moreover, pioneering work from Bolli's laboratory has shown a cardioprotective role for eNOS- and iNOS-mediated NO production during ischemic preconditioning (9). Recent clinical and experimental studies have demonstrated that ROS are implicated in the transition from compensated hypertrophy to heart failure and in patients with congestive heart failure. In general, differences have been noted in RIS generation or antioxidant expression in patients with dilated cardiomyopathies and end-stage heart failure (40, 45). Decreased myocardial levels of SOD and catalase have been reported 6 weeks after myocardial infarction, whereas SOD activity and expression are increased during chronic stable heart hypertrophy (27). Dhalla *et al.* (19) demonstrated that an increase in oxidative stress during the transition of pressure overload hypertrophy to heart failure resulted in a profound decrease in LV function that was associated with significant disruption of sarcomeric integrity. The increased oxidative stress was related to a reduced expression of SOD and glutathione peroxidase activities (19). Increased activity of the phagocytic NADPH oxidase in cardiac myocytes has also been reported during the progression of pressure-overload LV hypertrophy (42), and increased myocardial NADPH oxidase activity was partly due to increased expression of phagocytic NADPH oxidase subunits ($p22^{phox}$, $gp91^{phox}$, $p67^{phox}$ and $p47^{phox}$) and increased signaling through ERK1/2, p38, and ERK5 MAP kinases. Mitochondria may also be a source of ROS, because a decrease in the activity of the NADH-ubiquinone-oxidoreductase complex (complex I) of the mitochondrial chain has been implicated as a source of ROS in failing myocardium (35).

Clinical studies from heart failure patients have also provided support for the role of RNS in heart failure progression. Increased levels of NOS and $^{\bullet}OH$ are critical mediators of TNF-α-induced heart failure (23, 43, 47). Increased iNOS expression is also observed in human dilated cardiomyopathy (18) and valvular heart disease (29). Yucel *et al.* (88) reported decreased plasma levels of glutathione and erythrocyte SOD in patients with dilated cardiomyopathic heart failure. Human end-stage heart failure is associated with increased expression of NOS and decreased Cu/Zn-SOD activity, inferring an $O_2^{\bullet -}$-dependent "inactivation" of NO and the consequent production of $ONOO^-$ (45). A general conclusion from these studies is that an imbalance between RIS generating system and antioxidant defenses is a prime for determinant of the overall redox state of the heart failure myocardium (32).

CONCLUSIONS

The work cited in this review supports a central role of RIS in the regulation of cardiac myocyte growth and survival in response to neurohormones and cytokines. The net result of RIS production is determined by the amount and type of RIS generated, the duration of the response, and the spatial localization of RIS production within the cell. Further investigation into the mechanisms that regulate RIS formation, the molecular targets of RIS, and the development of transgenic animal models to manipulate RIS levels within the myocardium will provide new insight into the regulation of cardiac myocyte growth and death. As increasing evidence points to the involvement of RIS in the pathogenesis and progression of cardiac disease, RIS may therefore represent a potential new target for therapeutic intervention for the treatment of heart failure.

ABBREVIATIONS

ANF, atrial natriuretic factor; Ang II, angiotensin II; AP-1, activator protein-1; AR, adrenergic receptor; ARVM, adult rat ventricular myocytes; ASK1, apoptosis signal-regulating kinase-1; AT_1R, angiotensin type 1 receptor; cGMP, cyclic GMP; eNODS, endothelial nitric oxide synthase; ERK1/2, extracellu-

lar signal-regulated kinase; GPCR, G protein-coupled receptor; H_2O_2, hydrogen peroxide; IFN-γ, interferon-γ; IL-1β, interleukin-1β; iNDS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LV, left ventricular; MAP kinase, mitogen-activated protein kinase; β-MHC, β-myosin heavy chain; MLP, muscle LIM protein; NE, norepinephrine; NF-κB, nuclear factor-κB; nNDS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NRVM, neonatal rat ventricular myocytes; $O_2^{\bullet -}$ superoxide; $^{\bullet}OH$, hydroxyl radical; $ONOO^-$, peroxynitrite; PI3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; RIS, reactive inflammatory species; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNAP, *S*-nitrosopenacillamine, SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; Trx, thioredoxin; TUNEL, terminal transferase mediated DNA nick-end labeling.

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