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Forum Mini Review

Oxidative Stress-Induced Signal Transduction Pathways in Cardiac Myocytes: Involvement of ROS in Heart Diseases

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ABSTRACT

Reactive oxygen species (ROS) are proposed to contribute to the deterioration of cardiac function in patients with heart diseases. It has been reported that ROS are increased in the failing heart and involved in atherosclerosis, myocardial ischemia/reperfusion injury, and heart failure. Antioxidant enzymes are decreased in the decompensated heart, depressing defense mechanisms against oxidative stress. A variety of proteins, including receptors, ionic channels, transporters, and components of signal transduction pathways, are substrates of oxidation by ROS. ROS also function as signal transduction intermediates to induce transcription factor activation, gene expression, cell growth, and apoptosis. Recently, the upstream and downstream molecules of ROS in signal transduction pathways have been the subjects of intense investigation. These molecules include the mitogen-activated protein kinase family, the Rho family of small GTP binding proteins, the Src family of tyrosine kinases, Ras, and cytokines. The modulation of oxidative stress-induced signaling pathways is effective for preventing the progression of heart diseases. *Antioxidant. Redox Signal.* 5, 789–794.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and superoxide anion (O_2^-), have been shown to be deleterious to various physiologically important molecules including proteins, lipids, and DNA (42). OH^- and O_2^- are free radicals, which means that they have at least one unpaired electron. H_2O_2 is not a radical but plays an important role in oxidative processes. In addition, nitric oxide (NO) can interact with O_2^-, forming peroxynitrite ($ONOO^-$). $ONOO^-$ reacts with cellular proteins generating nitrotyrosine, an end product of oxidative damage. NAD(P)H oxidases, which are membrane-associated enzymes that catalyze the reduction of oxygen by using NADH or NADPH as the electron donor, are major sources of O_2^- in vascular cells and cardiac myocytes (16). ROS are very unstable and highly reactive, and they tend to initiate chain reactions that result in irreversible chemical changes in proteins or lipids. These deleterious reactions can result in cellular dysfunction and cytotoxicity. A number of defense systems have evolved to counteract the accumulation of ROS. These include enzymatic scavengers such as catalase, glutathione peroxide, and superoxide dismutase (SOD). In heart, SOD is present in two isoforms: Mn-SOD, which is expressed in the mitochondrial matrix, and Cu/Zn-SOD, the cytosolic form. However, these defense mechanisms are not always adequate to counteract the production of ROS, resulting in what is termed a state of oxidative stress. Oxidative stress is implicated in a wide variety of diseases, including atherosclerosis, myocardial ischemia/reperfusion injury, and heart failure (28). Accumulating evidence has suggested that ROS function as signal transduction intermediates to induce transcription factor activation, gene expression, cell growth, and apoptosis (32, 42). It seems that understanding the molecular mechanisms leading to generation of ROS and endogenous antioxidant enzymes may provide new strategies for heart diseases. In this review, we discuss the major signaling pathways known to be involved in regulating the oxidative stress-induced heart diseases.

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OXIDATIVE STRESS-INDUCED SIGNALING PATHWAYS

ROS are generated by a variety of factors such as irradiation, ischemia/reperfusion, anti-cancer drugs, and inflammatory cytokines. Low levels of ROS are regularly produced during a process of physiological metabolism, and every cell contains several enzymes such as catalase, glutathione peroxidase, and SOD, which scavenge ROS from the cell. High levels of ROS are generated from a variety of sources, including xanthine oxidase, NADPH oxidase, the leakage of electrons from mitochondria, and the cyclooxygenase pathway of arachidonic acid metabolism, and induce a variety of tissue damages (32). In heart, it has been reported that ROS evoke many abnormalities, including cytotoxicity, cardiac stunning, arrhythmia, and reduction of contractility. Administration of oxygen free-radical scavengers such as SOD and catalase resulted in a significant decrease in infarct size after 90 min of coronary artery occlusion in canines (19). Furthermore, it has been shown that *N*-(2-mercaptopropionyl)-glycine, an endogenous antioxidant, markedly reduced cytotoxicity caused by H_2O_2 in cultured cardiac myocytes (18).

The mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases. A growing body of evidence has suggested that MAPKs play important roles in many cell functions, including proliferation and differentiation (32, 49). In particular, three subfamilies of MAPKs—extracellular signal-regulated kinase (ERK), c-jun NH_2-terminal protein kinase (JNK), and p38MAPK—have been extensively characterized. They are regulated by three distinctive signal transduction pathways and show different functions. ERKs are activated by a variety of growth factors, cytokines, and phorbol esters through several distinct classes of cell surface receptors such as receptor tyrosine kinases and G protein-coupled receptors, and play pivotal roles in proliferation and differentiation in many types of cells (10, 27). In cardiac myocytes, the activation of ERK has been reported to be critical for the development of hypertrophy and specific gene expression. It has been reported that angiotensin II (Ang II) activates the Src family of tyrosine kinases and Ras in cardiac myocytes through G protein-coupled Ang II type 1 receptor (37, 48). Activation of the Src family of tyrosine kinases and Ras is required for activation of ERK in smooth muscle cells, while protein kinase C, but not the Src family or Ras, is critical for ERK activation in cardiac myocytes (39, 48). The signal transduction pathways leading to activation of ERK seem to be different among cell types. JNK and p38MAPK are not activated efficiently by growth factors and phorbol esters but are preferentially activated by distinctive stimuli such as the proinflammatory cytokines, ultraviolet irradiation, ROS, and cellular stresses, including heat shock and osmotic stresses (10, 27). We have reported that the Src family of tyrosine kinases, Ras, and Raf-1 are critical for ERK activation by H_2O_2 and that activation of ERK plays an important role in protecting cardiac myocytes from apoptotic death following oxidative stress (3). Pretreatment with insulin significantly decreased the number of H_2O_2-induced terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL)-positive cardiac myocytes and DNA fragmentation induced by H_2O_2 (5). Insulin strongly activated both phosphatidylinositol 3-kinase (PI3K) and the downstream effector Akt. Moreover, a pro-apoptotic protein, Bad, was significantly phosphorylated and inactivated by insulin through the PI3K pathway. Pretreatment with a specific PI3K inhibitor, wortmannin, and overexpression of a dominant negative (d.n.) mutant of PI3K abolished the cytoprotective effect of insulin (5). These data suggest that insulin may protect cardiac myocytes from oxidative stress-induced apoptosis through the PI3K–Akt pathway.

ROS AND CARDIAC HYPERTROPHY

Mechanical stress is the most important stimulus for cardiac hypertrophy. Stretch of cardiomyocytes evokes various intracellular signals leading to cardiomyocyte hypertrophy (7, 46, 47, 49). We and others have developed an *in vitro* system by which cultured cardiac myocytes are subjected to mechanical stress and have demonstrated that mechanical stress induces a variety of hypertrophic responses such as activation of MAPKs, reprogramming of gene expressions, and an increase in protein synthesis (23, 36, 45). Although ERK was activated by mechanical stress partially through enhanced secretion of Ang II and endothelin-1, JNK is strongly activated by stretch independently of Ang II, suggesting that JNK might be directly activated by mechanical stress (24). p38MAPK is activated by various environmental stresses such as endotoxin, osmotic shock, metabolic inhibitors, or ROS (15). Although high levels of ROS induce cell injuries, including necrosis and apoptosis, low levels of ROS induce activation of p38MAPK and work as mediators of hypertrophic responses (3). It has been reported that p38MAPK α and β induce cardiomyocyte apoptosis and hypertrophy, respectively (44). It remains to be determined whether isoform-specific activation of p38MAPK depends on the levels of ROS in cardiac myocytes. It has been reported that cardiac hypertrophy is induced by p38MAPK but suppressed by JNK, and that SB203580, a specific p38MAPK inhibitor, inhibits the myofibrillar organization and hypertrophic cell profile in neonatal rat cardiomyocytes (11, 34). These results suggest that p38MAPK may play a critical role in the development of cardiac hypertrophy in response to mechanical stress.

The Rho family of small GTP-binding proteins (G proteins), consisting of the Rho, Rac, and Cdc42 subfamilies, plays pivotal roles in many aspects of cellular functions including cytoskeletal reorganization, growth, transformation, and differentiation (17, 41). Rac proteins have been reported to be involved in the development of cardiac hypertrophy (35). Moreover, it has been reported that Rac1 is essential for assembly of plasma membrane NADPH oxidase in some types of cells and that Rac1 regulates cell growth, migration, and cellular transformation by controlling the intracellular production of ROS (2, 16). Moreover, Rac1 is involved in a redox-dependent signal transduction pathway leading to activation of nuclear factor-κB (40). It has been reported that Rac1 is involved in the intracellular burst of ROS after reoxygenation and that JNK and p38MAPK are activated by a variety of cellular stresses, including ROS, in cardiac myocytes (6, 22). Previously, we have reported that Rho and Rac proteins play

critical roles in mechanical stress-induced hypertrophic responses such as activation of ERK, expression of fetal and immediate early response genes, and an increase in protein synthesis in cardiac myocytes (4). It has been reported that among small G proteins, Rac1 is involved in the activation of p38MAPK and in the induction of cardiac hypertrophy (12). We have demonstrated that stretch-induced activation of p38MAPK is significantly suppressed by expression of d.n. mutants of Rho family proteins in the following order: d.n. Rac1 > d.n. RhoA > d.n. Cdc42 (6). Conversely, overexpression of a constitutively active (c.a.) mutant of Rac1 strongly activated p38MAPK in cardiac myocytes. The p38MAPK activity was slightly increased by c.a. Cdc42 but not by c.a. RhoA. Overexpression of d.n. RhoA, d.n. Rac1, or d.n. Cdc42 did not have an effect on basal activity of p38MAPK in unstretched cardiomyocytes, and overexpression of d.n. and c.a. Rho family proteins did not change the expression levels of p38MAPK protein (6). These results suggest that Rho family proteins, especially Rac1, play an important role in mechanical stretch-induced activation of p38MAPK in cardiac myocytes. Rac proteins lead to the production of ROS in phagocytic cells. In nonphagocytic cells, Rac1 has a similar function and regulates cell growth and migration and cellular transformation by controlling intracellular ROS production.

N-acetyl-L-cysteine (NAC), a potent antioxidant, inhibited activation of p38MAPK induced by mechanical stretch in a concentration-dependent manner (6). Other antioxidants such as *N*-(2-mercaptopropionyl)-glycine and dimethyl sulfoxide also significantly suppressed stretch-induced p38MAPK activation. Stretch-induced activation of JNK was also abolished by the pretreatment with NAC, whereas the activation of ERK was only slightly suppressed by NAC. These results indicate that ROS are critically involved in stretch-induced activation of p38MAPK and JNK, but not of ERK, in cardiac myocytes. We examined whether mechanical stress induced an increase in ROS production in cardiac myocytes using 2-methyl-6-phenyl-3,7-dihydroimidazo-[1,2-a]pyrazin-3-one (CLA), which specifically detects the enzymatic formation of O_2^- in the xanthine/xanthine oxidase system (6). Mechanical stress increased CLA chemiluminescence signals as compared with unstretched cardiomyocytes, indicating that production of O_2^- is enhanced by mechanical stress. Stretch-induced production of superoxide was completely inhibited by overexpression of d.n. Rac1, while overexpression of d.n. RhoA or d.n. Cdc42 slightly attenuated the increase in superoxide generation induced by stretch. When either c.a. Rac1 or c.a. Cdc42 was expressed in cardiomyocytes, superoxide production was significantly increased as compared with unstretched cardiomyocytes, and this increase was abrogated by pretreatment with NAC. These results suggest that Rho family proteins, especially Rac1, play a pivotal role in stretch-induced production of ROS in cardiac myocytes. As previously reported, mechanical stress increases phenylalanine incorporation into cardiac myocytes compared with unstretched cardiomyocytes. The increase was significantly suppressed by the overexpression of d.n. Rac1 and by pretreatment with NAC and SB202190, an inhibitor of p38MAPK, suggesting that the Rac1–ROS–p38MAPK signaling pathway may play a critical role in stretch-induced cardiac hypertrophy (6) (Fig. 1).

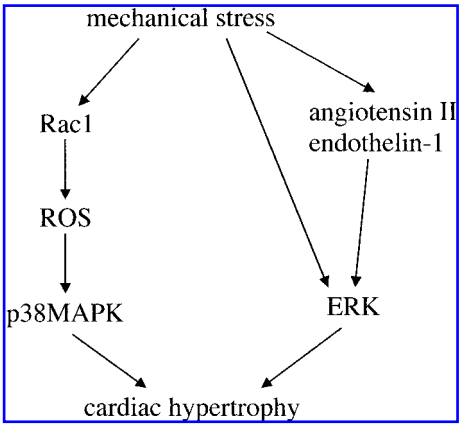

FIG. 1. Stretch-induced signal transduction pathways leading to cardiac hypertrophy in cardiac myocytes.

ROS AND CARDIOMYOCYTE APOPTOSIS

Recent studies reported that apoptosis plays an important role in cardiac development and diseases (9, 14). Although the precise mechanism of the cell death is yet unknown, it has been postulated that loss of cardiomyocytes by apoptosis causes heart failure (1, 21). Many studies have demonstrated that ischemia/reperfusion generates ROS and that ROS induce a variety of cardiomyocyte abnormalities, including cell death (20). We have previously reported that ROS strongly induce apoptosis in cultured cardiac myocytes (3). The plasma concentration of tumor necrosis factor-α (TNF-α) is elevated in many cardiac diseases, including heart failure, and TNF-α exerts a negative inotropic effect on the heart (29, 33, 43). Recently, basic and clinical studies have indicated that TNF-α plays a critical role in myocardial injury and development of heart failure. TNF-α can trigger apoptosis in many cell types, including adult rat cardiomyocytes. Moreover, a recent study has shown that transgenic mice with cardiac-specific overexpression of TNF-α develop dilated cardiomyopathy, and apoptosis is observed in the heart (26). These findings indicate that endogenous TNF-α contributes to apoptosis in cardiac cells.

We and others have reported that ROS induce production of TNF-α and cardiomyocyte apoptosis (8, 25). Cardiac myocytes cultured in serum-free media for 24 h are stained positive by the TUNEL method (~5%). When cardiac myocytes were incubated with 100 μ*M* H_2O_2 for 24 h, the number of TUNEL-positive cardiac myocytes was significantly increased (~30.5%) (8). However, pretreatment with anti–TNF-α antibody (100 μg/ml) for 3 h before addition of H_2O_2 significantly decreased the number of TUNEL-positive cardiomyocytes (~9.5%). Pretreatment with anti–TNF-α antibody alone did not have any effect on cardiac myocytes. When cardiac myocytes were incubated with TNF-α for 24 h, the number of TUNEL-positive cardiac myocytes was significantly increased (10 ng/ml TNF-α, ~8%; 100 ng/ml TNF-α, ~25.5%) (8). Our results suggest that TNF-α is involved in oxidative stress-induced apoptosis and that a high level of TNF-α induces

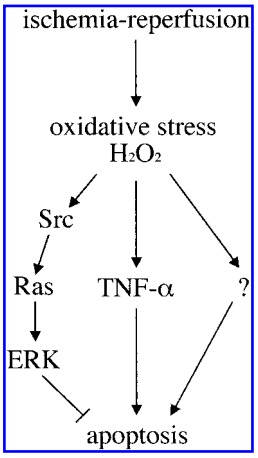

FIG. 2. Oxidative stress-induced signal transduction pathways leading to apoptosis in cardiac myocytes.

apoptosis in cardiac myocytes. The expression level of the TNF-α gene increased rapidly and transiently following H_2O_2 stimulation. The maximal increase in TNF-α mRNA was observed at 30 min after exposure to H_2O_2, and the level decreased thereafter. Although the concentration of TNF-α in culture media of untreated cardiomyocytes was under the detectable level from 10 min to 12 h, it increased slightly at 24 h (33 pg/ml). These data suggest that cultured cardiomyocytes constitutively secrete a small amount of TNF-α. When cardiomyocytes were incubated with H_2O_2, TNF-α was detectable in the culture medium from 6 h (~35 pg/ml), and the concentration of TNF-α was increased at 24 h (~106 pg/ml). Although we could not detect the expression of TNF-α in the medium within 6 h after H_2O_2 treatment, it is most likely that a small amount of TNF-α (<20 pg/ml) was not detectable by the enzyme-linked immunosorbent assay method used. It is also possible that posttranscriptional regulation caused the difference in the time course between mRNA and protein of TNF-α. The experiment using a neutralizing antibody suggested that secreted TNF-α plays an important role in H_2O_2-induced apoptosis in cardiac myocytes (8). Although H_2O_2 actually induces the production and secretion of TNF-α from cultured cardiac myocytes, there is a large discrepancy between the H_2O_2-induced concentration of TNF-α and the concentration of TNF-α that induces apoptosis in cardiac myocytes. Neither 1 μ*M* H_2O_2 nor 1 ng/ml TNF-α for 24 h induced apoptosis in cardiac myocytes; however, incubation with both 1 μ*M* H_2O_2 and 1 ng/ml TNF-α for 24 h significantly increased the number of TUNEL-positive cells compared with control (~11%) (8). Our data suggest that TNF-α and oxidative stress synergistically induce apoptosis in cultured cardiac myocytes (Fig. 2).

CONCLUSIONS

Mechanical stress is a pivotal stimulus for cells and evokes a wide variety of intracellular signals. It has been of great interest to elucidate how mechanical stress is converted into biochemical signals and transmitted to the nucleus. Previously, we and others have demonstrated that mechanical stress activates ERK partly through secreted vasoactive peptides such as Ang II and endothelin-1 in cardiomyocytes (38, 49). Our data suggested that stretch-induced activation of the ERK pathway is redox-insensitive. Thus, the Rac1–ROS–p38MAPK pathway is independent of the vasoactive peptides–ERK pathway. We also provided the first evidence indicating that p38MAPK is a critical component of the redox-sensitive signaling pathways activated by mechanical stress. However, the downstream targets of p38MAPK, which induce cardiac hypertrophy, remain to be clarified.

We have recently demonstrated that TNF-α significantly induces apoptosis in cardiomyocytes, indicating that ischemia/reperfusion-induced production of TNF-α may play an important role in myocardial damage (8). Although Krown *et al.* (25) have reported that TNF-α induces apoptosis of adult rat cardiomyocytes but not of neonatal rat cardiomyocytes, using comet analysis, we demonstrated that a high concentration of TNF-α induces apoptosis in neonatal rat cardiomyocytes by the TUNEL method. It is possible that they may use a low dose of TNF-α to examine apoptosis in neonatal rat cardiomyocytes. It has been reported that TNF-α induces the production of ROS in various types of cells, including cardiac myocytes (13, 31). Therefore, it is possible that ROS and TNF-α may mutually stimulate each production and synergistically induce cardiomyocyte apoptosis. Li *et al.* (30) reported that TNF-α enhances hypoxia–reoxygenation-mediated apoptosis in cultured human coronary artery endothelial cells. These results suggest that TNF-α cooperates with H_2O_2 for induction of apoptosis.

It seems that understanding the molecular mechanisms leading to generation of ROS and endogenous antioxidant enzymes may provide new strategies for treatment of heart diseases. Further investigations are necessary to elucidate the oxidative stress-induced signaling pathways in cardiac myocytes.

ABBREVIATIONS

Ang II, angiotensin II; CLA, 2-methyl-6-phenyl-3,7-dihydroimidazo-[1,2-a]pyrazin-3-one; c.a., constitutively active; d.n., dominant negative; ERK, extracellular signal-regulated kinase; G protein, GTP-binding protein; H_2O_2, hydrogen peroxide; JNK, c-jun NH_2-terminal protein kinase; MAPK, mitogen-activated protein kinase; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; O_2^-, superoxide anion; OH^-, hydroxyl radical; $ONOO^-$, peroxynitrite; PI3K, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling.

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