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Review

Redox Regulation of Cardiomyocyte Survival and Death

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ABSTRACT

In this review, attempts were made to establish the role of reactive oxygen species as signaling molecules that regulate cardiomyocyte life and death during ischemia and reperfusion. Ischemia/reperfusion is a classical example because partial or mild ischemia can lead to simultaneous execution and repair of the cardiomyocytes, which is disrupted during severe ischemia leading to cell necrosis because of the lack of ATP. Apoptosis and repair processes are mediated by adaptive response in which oxygen free radicals function as typical signaling molecules through the activation of receptor tyrosine kinases, protein kinase C, and mitogen-activated protein kinases, as well as induction of redox-sensitive transcription factors and genes. Antioxid. Redox Signal. 3, 23–37.

INTRODUCTION

EVIDENCE IS RAPIDLY ACCUMULATING to support the role of reactive oxygen species as intracellular second messengers. The long-held view of oxygen free radicals being detrimental to the biological tissues was challenged after the recent discovery that these reactive species can function as signaling molecules (26). Involvement of oxygen free radicals in mitogenic stimulation of cell as well as growth factor- and cytokine-induced signal transduction strongly suggests that the reactive oxygen species function as second messengers (58). Moreover, the mitogenic signals mediated through the generation of reactive oxygen species activate many transcription factors, including nuclear transcription factor κB, (NFκB), and genes, including antioxidant enzymes and bcl-1 (9).

A large number of degenerative diseases, including coronary heart disease, have been linked to the overproduction of oxygen-derived free radicals. The results of many studies, including our own, have demonstrated that excessive production of reactive oxygen species in concert with drastic reduction of antioxidant reserve plays a crucial role in the pathophysiology of ischemic heart disease (13). Myocardial ischemia and reperfusion cause the cardiomyocytes to face conditions that shift their redox status to undergo a drastic change subjecting them to oxidative stress (48). Interventions with oxygen free radical scavengers or antioxidant therapy have been found to be cardioprotective against ischemic reperfusion injury (67).

Recent studies have documented that coronary heart diseases cause cardiomyocyte death not only by necrosis, but also by apoptosis (56). Reperfusion of ischemic myocardium results in apoptotic cell death and DNA fragmentation (45). In concert, ischemia/reperfusion is associated with the induction of a number of both pro- and anti-apoptotic genes and transcription factors (52). Ebselen, a gluthathione peroxidase (GSHPx) mimic, was found to reduce cardiomyocyte apoptosis (14). The hearts from the transgenic mice overexpressing GSHPx-1 gene

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were resistant to ischemia/reperfusion injury, whereas those from the GSHPx-1 knockout animals, devoid of any copy of GSHPx-1, were extremely vulnerable to the cellular injury as compared with wild-type controls (44).

The results mentioned above clearly indicate that cardiomyocyte death induced by ischemia/reperfusion is redox-regulated and that reactive oxygen species not only trigger the cell death, but also function as intracellular signaling molecules. This review will discuss the intracellular signaling pathways potentiated by the reactive oxygen species that lead to the induction of gene expression and apoptotic cell death.

MITOCHONDRIA—SAVIOR AND EXECUTIONER

A number of sources have been identified for the generation of oxygen-derived free radicals in the heart during ischemia and reperfusion, including catecholamine oxidation, xanthine dehydrogenase–xanthine oxidase conversion, microsomal respiratory chain via cytochrome P-450 monooxygenase system and mitochondrial uncoupling (15, 31). It appears that all of these sources actively take part in the process of free radical production, because inhibition of any of these sources can reduce the cellular injury to some extent. Mitochondria seem to play a crucial role in the process of production and propagation of reactive oxygen species as described below.

Life needs a continuous supply of energy, and cells depend on mitochondria for its production and supply. Hypoxia or ischemia causes rapid energy consumption by depleting ATP, which cannot be readily regenerated because of the absence of both glucose and oxygen. The mitochondrial respiratory chain is also a major source of reactive oxygen species. Upon reperfusion, ischemic heart mitochondria overproduce hydroxyl radicals ($OH^{\cdot}$), which can be reduced by pretreating the hearts with $OH^{\cdot}$, scavengers (12) (Fig. 1). Reduction of

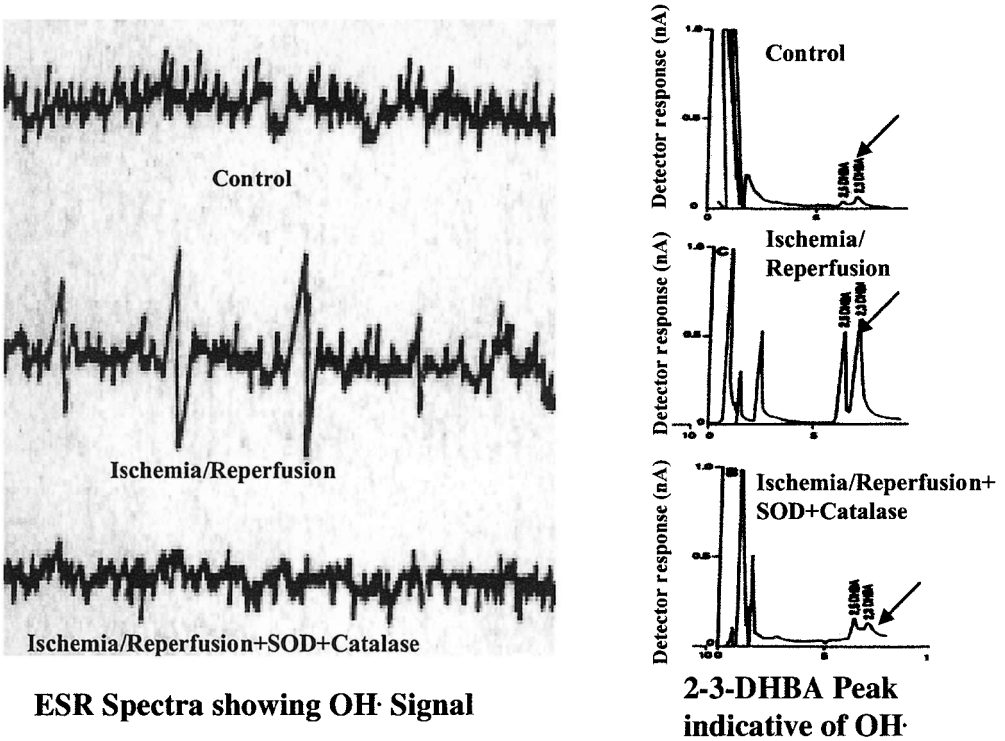

FIG. 1. Mitochondrial free radical generation during myocardial ischemia and reperfusion. Isolated perfused rat hearts were made ischemic for 30 min followed by reperfusion. Perfusate buffer was collected to measure the hydroxyl radicals by electron spin resonance (ESR) and HPLC techniques. Ischemic reperfused heart shows production of hydroxyl radicals by typical triplet when analyzed by ESR (**left**) and increased development of 2,3-dihydroxybenzoic acid (2,3-DHBA) (**right**; shown by arrow) when measured with HPLC. Perfusion of heart with SOD plus catalase abolished the formation of hydroxyl radical.

$OH^{\cdot}$ is associated with the amelioration of ischemic reperfusion injury, suggesting that mitochondrial free radical production plays a role in the pathophysiology of cellular injury. We now know that mitochondria play a major role in both necrotic and apoptotic cell death. Whereas partial ischemia or hypoxia causes both necrotic and apoptotic cell death, complete ischemia can cause only cell necrosis, because a certain amount of ATP is required to execute apoptosis. During reperfusion, the permeability transition pore in the inner mitochondrial membrane, which is normally closed, becomes opened, presumably due to the influx of Ca^{2+} and/or oxidants (23). Mitochondria rapidly lose their protonmotive force due to the leakage of protons through the mitochondrial permeability transition pore and rapidly hydrolyze cellular ATP resulting in swelling, bursting the outer mitochondrial membrane, and releasing cytochrome *c* and other intermembrane proteins, triggering apoptosis.

It should be clear from the above discussion that mitochondria serve as judge to decide whether a cell would live or die, and if die, whether the cell should die of necrosis or apoptosis. During partial or reversible ischemia, mitochondria of the cardiomyocytes can maintain a certain level of ATP, enough to execute apoptosis and simultaneously activate a cascade of enzymes responsible for the repair and prevention of cell death. Thus, there are two opposing forces existing in the ischemic mitochondria: one to execute that includes proteases, nucleases, and reactive oxygen species, and another to extricate that includes the repairing enzymes. During apoptosis, cytochrome *c* is released, activating caspase 3, which serves as the final executioner for apoptotic cell death. On the other hand, the anti-death gene, bcl-2, located on the outer mitochondrial membrane, tries to oppose the apoptotic process. In a recent study, our laboratory demonstrated that bcl-2 activity is down-regulated in the ischemic-reperfused myocardium when apoptotic cardiomyocytes appear (35). Myocardial adaptation to ischemia up-regulated bcl-2 and prevented apoptosis.

REDOX SIGNALING

Recent studies implicate that not only are reactive oxygen species the destructive elements for the cells, but also they are essential for the biological and physiological function of the cells. Biological cells including cardiomyocytes contain enzymes that can simultaneously generate reactive oxygen species and intracellular redox buffer in response to a specific stress. Depending on the amounts of antioxidant reserve and oxygen free radicals, the reactive oxygen species either are destroyed or persist. Thus, the oxygen free radicals fulfill the definition of a second messenger, which is either up-regulated or down-regulated after a physiologic stimulus like ischemia. A number of growth factors and cytokines have been found to induce oxidative stress and overproduce specific antioxidant enzymes (41, 62). Environmental stresses including heat stress, oxidative stress, and ischemia/reperfusion also produce oxidative stress, which is then translated into the induction of antioxidant enzymes (40).

Perhaps the finding that the production of reactive oxygen species during the agonist-induced activation NFκB provided the first concrete evidence for the role of reactive oxygen species as a second messenger. NFκB regulates the inducible expression of a number of genes involved in cell survival and execution. For example, NFκB has been found to control anti-apoptotic gene, bcl-2, and pro-apoptotic factors, bax and p53, in the ischemic/reperfused myocardium (5). Diverse extracellular signals from interleukin-1 (IL-1), tumor necrosis factor-α (TNFα, H_2O_2, etc. converge into development of oxidative stress, which leads to activation of NFκB (2). Such activation of NFκB can be blocked by antioxidants such as vitamin E (63) or α-lipoic acid (64). Antioxidants such as *N*-acetylcysteine can prevent NFκB activation by diverse stimuli, including H_2O_2 by supressing the generation of reactive oxygen species (60).

Role of tyrosine kinases

Receptor tyrosine kinase plays an important role in redox signaling by autophosphorylation of several tyrosines along their own intracellular tails. Recent studies from several laboratories including our own have implicated that

myocardial adaptation to ischemic stress occurs through the activation of several tyrosine kinases (10). Phosphorylation of tyrosine kinases has been shown to be linked to the activation of both phospholipase C and phospholipase D (PLD), leading to the activation of multiple kinases including protein kinase C (PKC) and mitogen-activated protein (MAP) kinases. PLD appears to play an important role in the intracellular signaling process. Cohen et al. also demonstrated that PLD is critical in ischemic adaptation (8). Activation of PLD was documented in ischemic reperfused hearts (51). PLD catalyzes the terminal diester bond of phosphatidylcholine, which results in the formation of choline and phosphatidic acid. Phosphatidic acid then serves as the substrate for diacylglycerol synthesis by the action of phosphatidic acid on phosphohydrolase. Diacylglycerol may itself serve as a second messenger by activating PKC. Work done by Eskildsen-Helmond et al. (20) also suggests a link between ischemic adaptation and activation of PLD, eventually resulting in potentiation of PKC isoenzymes. In a more recent study, Fryer et al. (21) demonstrated that pretreatment with tyrosine kinase inhibitors partially attenuated ischemic adaptation in the rat heart.

Role of MAP kinases

MAP kinases, a serine/threonine protein kinase family, play an essential role in mediating intracellular signal transduction events. In response to extracellular stimulation, MAP kinases are rapidly activated and in turn regulate cellular functions by inducing the phosphorylation of proteins, such as an oncogene product c-jun, S6 ribosomal protein kinase, and MAP kinase-activated protein (MAPKAP) kinase 2 (1, 61). MAPKAP kinase 2 has been implicated in a novel mammalian stress-activated signal transduction pathway initiated by a variety of mitogens, pro-inflammatory cytokines, or environmental stresses, where it regulates its substrate molecules by serine/threonine phosphorylation (7). In the case of rat heart, a MAP kinase cascade has already been identified (71). These authors have demonstrated that MAP kinase isoforms p42 MAP kinase and p44 MAP kinase and two peaks of MAP kinase kinase were activated by >10-fold in perfused hearts or ventricular myocytes exposed to phorbol 12-myristate 13-acetate for 5 min. In our own study, we identified the participation of MAP kinase cascades in the ischemically adapted rat hearts (42). The results of our study demonstrated that a kinase cascade involving tyrosine kinase–PLD–MAP kinases–MAPKAP kinase 2 is triggered after ischemic stress.

The intracellular signaling mechanisms that lead to adaptation require one or more members of MAP kinase cascades. Among the three distinct MAP kinase families, stress-activated protein kinase (SAPK), also known as c-JUN NH_2-terminal kinases (JNK), and p38 MAP kinase are known to be regulated by extracellular stresses, including environmental stress, oxidative stress, heat shock, and UV radiation (32). JNKs and p38 MAP kinase appear to be involved in distinct cellular functions, because they possess different cellular targets and are located on different signaling pathways. Thus, JNK kinases activate c-Jun, whereas p38 MAP kinase stimulates MAPKAP kinase. A recent study demonstrated that ischemic adaptation triggered a tyrosine kinase-regulated signaling pathway leading to the translocation and activation of p38 MAP kinase and MAPKAP kinase 2 (46).

The results of a recent study documented that 30 min of ischemia followed by 2 h of reperfusion increased the induction of JNK1, c-Jun, and p38 MAP kinase proteins (59). Ischemic adaptation also enhanced these kinases compared with control. However, subsequent ischemia/reperfusion-mediated increase in JNK1, p38, and c-Jun was blocked by this adaptation. Fifteen minutes of perfusion with anisomycin increased the amounts of both JNK1 and p38 MAP kinases as well as c-Jun in the heart, all of which were decreased in amount after subsequent ischemia/reperfusion. Curcumin, a JNK and c-Jun inhibitor, blocked the ischemia/reperfusion- and adaptation-mediated increase in JNK1 and c-Jun, whereas it had no effect on p38 MAP kinase. SB 203580, an inhibitor for p38 MAP kinase phosphorylation, on the other hand, was equally effective in reducing the amount of p38 MAP kinase, but exerted no effects on JNK1 and c-Jun. Is-

chemia/reperfusion-mediated increased myocardial infarction was reduced by treating the hearts with anisomycin, a dual activator of p38 MAP kinase and JNK. The cardioprotective effects of preconditioning were abolished by either curcumin or SB 203580. Thus, it appears that activation of SAPKs is obligatory for myocardial adaptation to ischemia, but the activation is only transient, and the activities rapidly come down to near baseline levels after subsequent ischemia and reperfusion (59).

Evidence suggests that MAPKAP kinase 2 is a crucial step leading to gene expression and myocyte adaptation resulting in adaptive cardioprotection. This unique protein kinase is highly expressed in heart muscle, suggesting that it also may be expressed and functioning in the myocardium in response to stress. MAPKAP kinase 2 also has been shown to have increased activity when subjected to oxidative stress as well as heart shock (46, 71). This increased activity of MAPKAP kinase 2 in association with heat-shock protein (HSP) gives rise to the hypothesis that this kinase may be one of the critical factors involved with ultimate transcription of proteins leading to adaptive protection of the heart. In cultured myocytes, the activity of MAPKAP kinase 2 was found to increase when the myocytes were subjected to oxidative stress as well as heat shock (71). HSPs are early targets of phosphorylation by a variety of stress conditions. HSP-27, HSP-32, and HSP-70 can be induced by oxidative stress and ischemic preconditioning (43). Not only have HSPs been found to be cardioprotective through reduction of infarct size, but evidence also suggests that they are important in delayed protection against infarction, the so-called "second window" of protection.

Role of PKC

As mentioned earlier, it has been demonstrated that cellular PKC activation is an important step in the mechanism of adaptive protection of heart (49). The PKC hypothesis received further support from the observations that any agent that can activate PKC can also precondition the heart. For example, phenylephrine, an α_1 agonist, angiotensin AT_1, and bradykinin B_2 receptors can activate PKC (19), and they can also precondition the hearts when infused prior to ischemia (49, 68). A variety of stress signals can also translocate and activate PKC. For example, mechanical stress induced by stretching can activate PKC in cultured myocytes (70). Immediately after stretching, activation of phosphatidylinositol turnover was observed suggesting a role of phospholipase C in PKC activation. Even a short-term ischemia or ischemia followed by reperfusion was previously shown to translocate and activate PKC. Furthermore, both α_1-receptor stimulation and Ca^{2+} can translocate and activate PKC (25).

A recent study from our laboratory demonstrated the inhibition of the enhanced tyrosine kinase phosphorylation during ischemic adaptation by dimethylthiourea (DMTU) (17). DMTU also inhibited adaptation-mediated increased phosphorylation of p38 MAP kinase and MAPKAP kinase 2 activity. However, DMTU had no effect on the translocation and activation of PKC resulting from adaptation. The cardioprotective effect of adaptation was abolished by both DMTU and SN-50. Ischemic adaptation resulted in the nuclear translocation and activation of NFκB. Increased NFκB binding was blocked by both DMTU and SN-50. The results of this study demonstrate that reactive oxygen species play a crucial role in signal transduction mediated by adaptation. This signaling process appears to be potentiated by tyrosine kinase phosphorylation resulting in the activation of p38 MAP kinase and MAPKAP kinase 2 leading to the activation of NFκB, suggesting a role of oxygen free radicals as second messenger. Free radical signaling seems to be independent of PKC, although PKC is activated during the adaptation process, suggesting the role of two separate signaling pathways in the adapted heart.

PHYSIOLOGICAL ROLE OF REDOX SIGNALING IN MAINTAINING MYOCARDIAL FUNCTION

Although ischemia/reperfusion has been found to cause cardiomyocyte death, the precise physiological role of redox signaling is not clear. Ischemia/reperfusion, especially intermittent ischemia commonly known as ischemic

preconditioning, leads to the activation of both G proteins and receptor tyrosine kinases, potentiating a signaling cascade resulting in the activation of multiple kinases that leads to the induction of the activation of several redox-sensing transcription factors and genes. Such intracellular events ultimately dictate the cells to survive or die. The changes in gene expression are likely to influence the physiologic function of the cardiomyocytes during postischemic survival.

A number of recent studies clearly demonstrated that redox signaling plays a physiological role in myocardial survival during the postischemic period. As mentioned earlier, oxygen free radicals are generated during ischemia/reperfusion. When the heart is adapted to ischemic stress by repeated short-term ischemia and reperfusion, the generation of the reactive oxygen species is rapidly increased, but does not increase at the same rate during subsequent ischemia and reperfusion (Fig. 2). The same pattern of the development of oxidative stress is observed when hearts are treated with endotoxin, IL-1 or TNFα. Interestingly, these interventions lead to the development of oxidative stress within a very short period, but reduce/inhibit subsequent oxidative stress development when hearts are subjected to ischemia/reperfusion. Additionally, such adaptive response is associated with the induction of the expression of a number of stress proteins, including HSP-27, HSP-70, and heme oxygenase and antioxidant enzymes that include manganese superoxide dismutase (Mn-SOD) and GSHPx (46). Such adaptive response is also associated with the increased binding activity of NFκB. Increased activity of NFκB and induction of the protective proteins can be blocked by pretreating the hearts with an oxygen free radical scavenger such as CMTU (17). More interestingly, an endotoxin-derived compound, monophosphoryl lipid A (MLA), was found to induce inducible nitric oxide synthase (iNOS) mRNA through a tyrosine kinase-dependent mechanism and protect the heart from ischemic reperfusion injury (Fig. 3).

Thus, it seems likely that one of the major functions of redox signaling in the ischemic myocardium is to synthesize stress-inducible proteins through the activation of transcription factors such as NFκB or by potentiating induction of other inducible proteins such as iNOS. However, more study is necessary to unveil

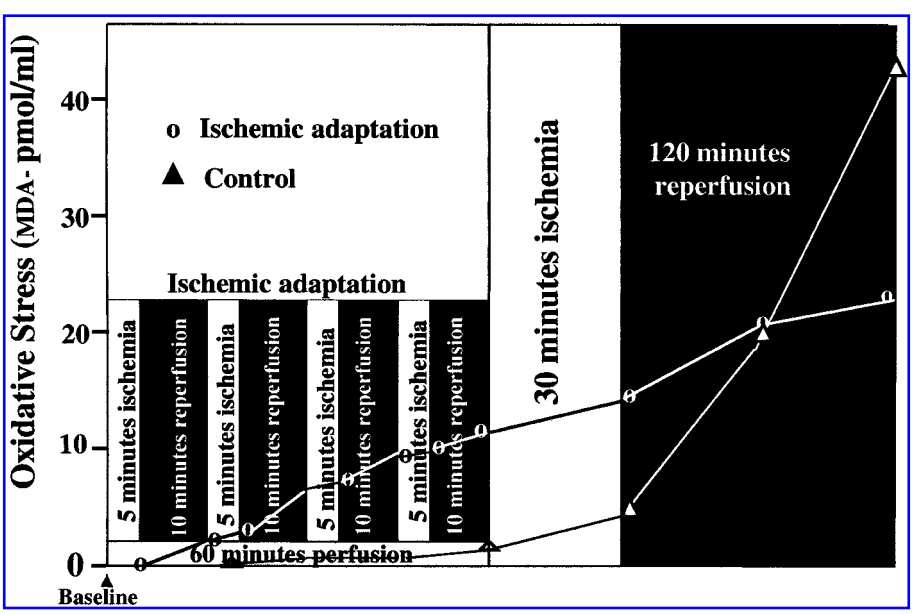

FIG. 2. Reduction of oxidative stress during myocardial adaptation to ischemia. Isolated rat hearts were either perfused with buffer for 60 min (△) (control) or adapted (○) by cyclic episodes of 5 min of ischemia each followed by 10 min of reperfusion. All hearts were then made globally ischemic for 30 min followed by 2 h of reperfusion. The results show decreased oxidative stress measured by malondialdehyde (MDA) formation during postischemic reperfusion in the adapted myocardium.

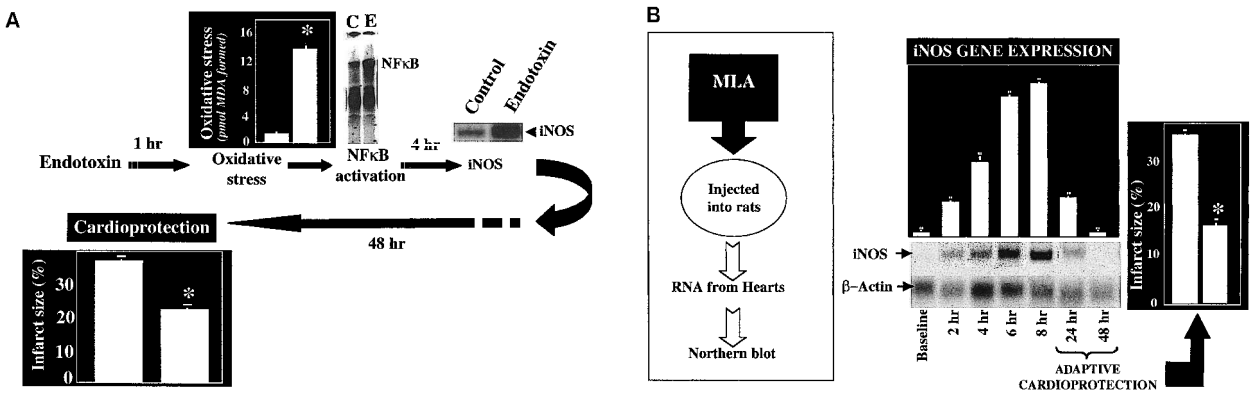

FIG. 3. Myocardial adaptation to oxidative stress with endotoxin (A) and MLA (B). As shown in the panels, endotoxin potentiates oxidative stress and triggers the activation of NFκB followed by iNOS induction. MLA also induces iNOS mRNA within a few hours. Both compounds reduce ischemia reperfusion injury as documented by reduction in infarct size.

completely the physiologic function of redox cycling.

REDOX-SENSING TRANSCRIPTION FACTORS

NFκB and activator potein-1 (AP-1) are two well-known redox-sensitive transcription factors. NFκB is a critical regulator for gene expression induced by diverse stress signals including mutagenic, oxidative, and ischemic stresses. Activation of NFκB is likely to be involved in the induction of gene expression associated with the ischemic adaptation. This nuclear transcription factor is a member of the Rel transcription factor family, which is involved in the regulation of stress defense mechanisms. A survey of the literature suggests that NFκB is an oxidative stress responsive transcription factor, and that reactive oxygen intermediates play a crucial role in the activation of the factor. Recent studies from our laboratory demonstrated that ischemic adaptation translocated and increased the binding of NFκB in the heart. NFκB binding activity is very low in nonischemic control hearts (36). Reperfusion of ischemic myocardium significantly increases the translocation of NFκB from cytosol to nucleus. Perfusion of the heart with DMTU inhibits NFκB translocation from the cytosol to the nucleus (17). NFκB binding activity is increased in the ischemically adapted hearts (Fig. 4).

AP-1 is another redox-sensitive signaling molecule that also plays an important regulatory role in cellular responses to stress induced by external factors, including UV radiation, phorbol esters, and TNFα (47). The binding site of AP-1 is recognized by Jun family member homodimers and Jun/Fos family member heterodimers. The balance between Jun and Fos is very critical for gene expression. Stress induced by ischemia/reperfusion was previ-

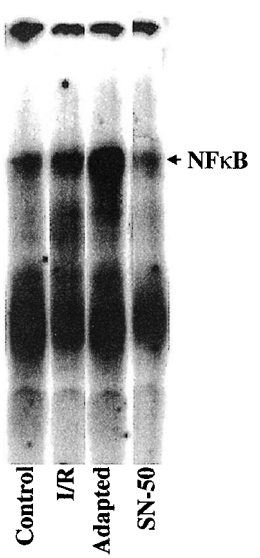

FIG. 4. Effects of ischemia/reperfusion (I/R), adaptation, SN-50, and DMTU on the binding activities of NFκB. Binding activity of NFκB is significantly increased in the adapted heart and is reduced by perfusing the hearts with SN-50, a NFκB blocker. Inhibition of NFκB blocks the cardioprotective effects of adaptation as shown in Fig. 5.

ously shown to induce the activation of c-Jun (30). AP-1 is a redox-sensitive signaling molecule that plays an important regulatory role in cellular responses to stress induced by external factors. The AP-1 transcription factor complex is composed of a group of proteins encoded by the Jun and Fos families that bind to the AP-1 consensus sequences. Regulation of AP-1 in response to external stimuli is mediated by members of the MAP kinase family. AP-1 regulates the activation of transcription of a variety of genes. Electrophoretic mobility shift assay indicated increased AP-1 binding activity in the ischemic/reperfused rat heart compared with the control perfused group (38). DMTU inhibited this binding activity significantly. Similar effects were also observed for the ischemically adapted group. Myocardial adaptation induced by cyclic episodes of short-term ischemia and reperfusion results in the activation of NFκB, but not AP-1.

In various cell lines, wild-type p53 (transcription factor, pro-apoptotic gene) induced by DNA damage has been shown to induce programmed cell death or apoptosis. It is reported that p53 protein functions as an active transcription factor in lesioned brain (28). How activation of p53 promotes apoptosis is unclear, but it might involve Bax, a series of p53-inducible genes, or signaling through Fas-related pathways (50). There are other p53 effectors, including caspases, that execute apoptotic cell death (66). Several studies on the master controller gene of apoptosis, p53, reveals conflicting results (6). Our recent study showed an increase in p53 activity in the ischemic/ reperfused myocardium (38). Such an increase in p53 activity was prevented by adapting the heart to ischemia.

REDOX-SENSING GENES

Bcl-2 is a well-known anti-death gene that functions as an intracellular antioxidant. Recent analysis of the bcl-2 gene family reveals complex network regulating apoptosis. Within this bcl-2 gene family, some of the candidates can supress apoptosis, whereas the others can induce apoptosis (69). Among the proteins coded by the genes of this family, Bcl-2 and Bcl-x_L act as cell death repressors (53), whereas Bax and an alternatively sliced Bcl-x product, Bcl-x_S, promote cell death (56). When in excess over Bcl-2, Bax counteracts the repressive action of Bcl-2 on apoptosis. Similarly, excess Bcl-x_S antagonizes the function of Bcl-x_L. Thus, a critical balance between the Bcl-2, Bax and Bcl-$x_{L/S}$ molecules may determine the fate of cells in response to cytotoxic agents or environmental stress. It has been reported that p53 activates the transcription of the Bax gene via p53-response elements while down regulating Bcl-2 expression at the same time (50).

Apoptosis initiated by various different stimuli can be blocked by overexpressing Bcl-2. For example, the activation of Bcl-2 was associated with the inhibition of apoptosis in the adapted myocardium (38). A down-regulation of this anti-death gene occurred in concert with the significant amount of apoptosis in the ischemic/reperfused myocardium.

APOPTOSIS

Programmed cell death or apoptosis is recognized as a physiological counterpart of cell replication and is the contributing cause of cardiomyocyte cell death during ischemia/reperfusion, myocardial infarction, and heart failure (33). Apoptosis is an energy-requiring process, needs *de novo* pro-apoptotic gene expression (p53, bax, etc.), and is directed by an inborn genetic program. The terminal result of this program is the fragmentation of nuclear DNA, which is associated with ultrastructural changes in cellular morphology, while the functional integrity of the cell membrane still remains intact. Two different mechanisms of cell death have been evaluated so far during myocardial infarction, i.e., necrosis and apoptosis. The important distinguishing features of cell necrosis are rupture of cell membrane, cell swelling, plasma membrane breakdown, clumping of nuclear chromatin, swelling and disruption of sarcoplasmic reticulum and mitochondria, and appearance of granular densities in the matrix of mitochondria. Due to rupture of the sarcoplasmic reticulum in the necrotic cardiomyocytes, Ca^{2+} overloading takes place, which causes disturbances in other

electrolytes. On the other hand, apoptosis occurs in the absence of membrane rupture and is characterized by internucleosomal cleavage of DNA by a Ca^{2+}- and Mg^{2+}-dependent endonuclease. Ultrastructural features of apoptosis include segregation of nuclear chromatin and condensation of the cytoplasm. The apoptotic cell surface develops protuberances known as apoptotic bodies, which are generally engulfed or digested by adjacent cells. Again apoptosis is an active, strongly regulated, energy-requiring process, whereas necrosis is a passive process and occurs in response to lethal injury. Untimely initiation of apoptotic cell death in the myocardium might also play an important role in the pathogenesis of various other myocardial diseases, such as heart failure, posttransplantation rejection, chemotherapy-induced cardiac dysfunction, and viral infections.

The common inducers of apoptosis include oxygen free radicals/oxidative stress and Ca^{2+}, which are also implicated in the pathogenesis of myocardial ischemic reperfusion injury. Cardiomyocytes exposed to hypoxia revealed apoptotic cell death as evidenced by DNA fragmentation in conjunction with the expression of Fas mRNA (65). In a recent study, apoptotic and necrotic myocyte cell deaths associated with ischemia/reperfusion were shown to be independent contributing variables of infarct size in rats (61). Another study has shown apoptosis to be a feature of human vascular pathology, including restenotic lesions and, to a lesser extent, atherosclerotic lesions, suggesting that apoptosis may modulate the cellularity of lesions that produce human vascular obstruction (29).

Apoptotic cell death is a function of the duration of reperfusion, and even up to 1 h of ischemia does not induce apoptosis (38). Apoptotic cells become apparent only in the 90- and 120-min reperfused hearts. None of the ischemic hearts showed any evidence of apoptosis. These results were corroborated with the findings of DNA fragmentation, which showed increased ladders of DNA bands only in the 120-min reperfused hearts. The presence of apoptotic cells and DNA fragmentation in the myocardium were completely abolished by pretreating the myocardium with ebselen, a GSHPx mimic that also reduced the ischemic reperfusion injury (45). In another related study, SOD plus catalase was found to ameliorate the apoptotic cell death (22).

Although cardiomyocyte apoptosis occurs only after prolonged reperfusion following an ischemic insult, the signal for apoptosis is initiated during ischemia. Translocation of phosphatidylserine and phosphatidylethanolamine, a hallmark for apoptosis, was found to occur during ischemia, but apoptosis did not become apparent until hearts were reperfused following an ischemic insult (37). In this study, isolated rat hearts subjected to 30 min of global ischemia followed by 2 hr of reperfusion showed apoptotic cardiomyocytes as expected. Assay of phosphatidylethanolamine and phosphatidylserine topography in cardiomyocytes using 2,4,6-trinitrobenzenesulfonate demonstrated that ~30% of total phosphatidylethanolamine and 5% of total phosphatidylserine were available for the 2,4,6-trinitrobenzenesulfonate derivative in the control hearts. The availability of both phosphatidylethanolamine and phosphatidylserine was significantly increased in cardiomyocytes from the hearts after 30 min of ischemia, and this level was maintained up to 2 h of reperfusion following ischemia. This suggests that ischemia results in a significant loss of normal asymmetric sarcolemmal phospholipid distribution, possibly with an outward migration of phosphatidylethanolamine and phosphatidylserine.

Apoptosis and redox signaling

There is increasing evidence that a variety of antioxidants and antioxidative enzymes play a major role in myocardial protection against acute stress, such as ischemia or hypoxia. Reactive oxygen species, including superoxide anions (O_2^-), hydroxyl radicals ($OH^{\cdot}$), and singlet oxygen ($^1O_2$) are believed to be the key factors causing reperfusion injury. To defend itself against free radical attack, the heart like many other organs is equipped with its own defense system (16). The first line of defense comprises antioxidants such as α-tocopherol, ascorbic acid, glutathione, and several antioxidant enzymes like SOD, catalase, and GSHPx.

SOD catalyzed the dismutation reaction, whereas catalase detoxifies H_2O_2 efficiently. A large number of studies exist in the literature to support a direct role of oxygen free radicals in apoptosis. An expression vector containing SOD was found to delay the event of apoptosis in cultured sympathetic neurons. This study also proved that if SOD was injected after the oxidative stress, it had no effect on apoptosis at all. As mentioned earlier, a seleno peroxide mimic, ebselen, could reduce the apoptotic cell death and DNA fragmentation in concern with the reduction of myocardial ischemic reperfusion injury (45). During the apoptotic changes, the activity of Cu/Zn-type SOD in the tadpole tail markedly increased with a concomitant inhibition in the catalase activity. The apoptotic process was found to be increased by the addition of H_2O_2 and aminotriazole, a potent catalase inhibitor (24). In another study, it was found that Cu/Zn-SOD, Mn-SOD, and catalase in cultured neutrophils were significantly effective in delaying the event of apoptosis, suggesting that reactive oxygen species play a role in the neutrophil apoptosis (55). Transgenic mice overexpressing the GSHPx-1 gene were resistant to, and knockout mice devoid of any copy of GSHPx-1 gene were susceptible to, myocardial ischemia reperfusion injury (44). In concert, overexpression of GSHPx-1 gene was associated with a significantly reduced number of apoptotic cardiomyocytes, and GSHPx-1 gene knockout mouse hearts had a significantly higher number of apoptotic cells compared with those found in the wild-type control.

Redox regulation of cardiomyocyte apoptosis in the ischemic reperfusion myocardium is further supported by the observation that ischemic adaptation-induced inhibition of cardiomyocyte apoptosis was blocked by a $OH^{\cdot}$ radical scavenger, DMTU, as well as a NFκB blocker, SN-50 peptide (17) (Fig. 5). In concert, NFκB and DMTU prevented the cardioprotective effects of myocardial adaptation to ischemia.

Execution by caspase activation

Caspase enzymes are a family of cysteine proteases that play a central role in apoptosis. In humans at least seven of the 10 currently known family members participate in one of two distinct signaling pathways: activation of proinflammatory cytokines and promotion of apoptotic cell death. As mentioned previously, during apoptosis, a loss of electrochemical gradient across the inner membrane occurs in mitochondria resulting in uncoupling of oxidative phosphorylation, generation of free radical, and Ca^{2+} overload into the cytosol. In addition, cytochrome *c* and possibly other proteins are released from mitochondria into the cytosol. Mitochondria play a very important role in apoptosis. The mechanism by which caspases are activated is still unknown. However, recent studies have established that activation of proximal caspases leads to the activation of the distal caspases in the process of apoptosis. (60). Caspase-3 (ced-3) activation begins when caspase-9 binds to Apaf-1 (ced-4 is homologous to the recently identified human protein, Apaf-1); this reaction is initiated by the release of cytochrome *c* in the cytosol. Caspase-9 is directly activated by Apaf-1 and cytochrome *c* (57). Significant accumulation of cytochrome *c* in the cytosol, over myofibrils, and near intercalated discs of cardiomyocytes in failing hearts is reported in a heart transplantation study. The cytochrome *c* was also associated with the activation of caspase-3 and cleavage of its substrate PKC-δ, but not poly(ADP-ribose) polymerase, whereas there was no accumulation of cytosolic cytochrome *c* or caspase-3 in the control hearts (54). In another study, rabbits subjected to 30 min of coronary artery occlusion followed by 3 h of reperfusion demonstrated significant activation of protein expression of caspases-2, -3, and -7. In addition, this study also successfully demonstrated selective cleavage of poly(ADP-ribose) polymerase into apoptotic fragments in ischemic/reperfused myocardium. Administration of broad-spectrum caspase inhibitor YVAD-cmk (4.8 mg/kg) partially blocked caspase activation and reduced apoptotic cells. This study indicated that caspases are critical mediators of myocardial injury induced by ischemia/reperfusion and that inhibition of caspases may be beneficial in myocardial infarction (27). Bialik et al. (4) reported that deprivation of serum and glucose, components of ischemia in vivo, induces apoptosis. This manifestation of apoptosis was blocked by

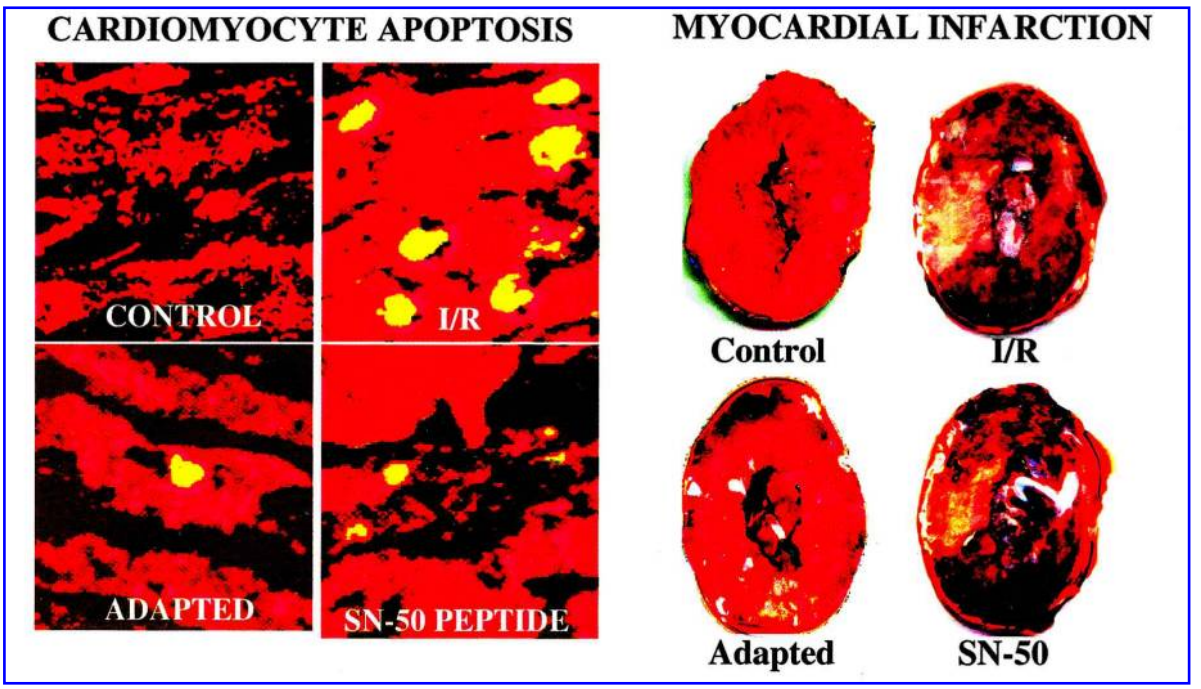

FIG. 5. Effects of SN-50 on cardioprotection achieved by ischemic adaptation. Apoptotic cardiomyocytes were revealed in the rat hearts subjected to 30 min of ischemia followed by 2 h of reperfusion (**left**). Apoptotic cells were significantly lower in the adapted heart, whereas more apoptotic cells were visible when hearts were treated with SN-50, a NFκB blocker, prior to adaptation. Increased infarction size (white area shown with 2,3,5-triphenyltetrazolium chloride staining) of the heart after ischemia/ reperfusion was significantly reduced when the hearts were adapted prior to ischemia/reperfusion. Infarct size lowering ability of adaptation was reduced when NFκB was blocked with SN-50 prior to adaptation.

zVAD-fmk, a peptide caspase inhibitor. In contrast to control cells, apoptotic cardiomyocytes revealed cytoplasmic accumulation of cytochrome *c* from mitochondria. Caspase processing was inhibited by the zVAD-fmk. During myocardial ischemia, cytochrome *c* is released from mitochondria, and this is one of the earliest events in ischemic myocardium. Therefore, the possible scenario of ischemia-induced myocardial apoptosis suggest elevation of cytosolic $Ca^{2}+$ concentration, release of cytochrome *c* from mitochondria, which can associate with Apaf-1 factor and pro-caspase-9, triggering the activation of caspase-3, and apoptosis.

MYOCARDIAL DEFENSE— ROLE OF ANTIOXIDANTS

Mammalian hearts are protected from the cellular injury by their own defense system, which includes various intracellular antioxidants, such as glutathione, α-tocopherol, ascorbic acid, and β-carotene, and antioxidant enzymes, including SOD, catalase, and GSHPx. These cellular compounds reduce/eliminate the oxidative stress by directly quenching the reactive oxygen species before they damage vital cellular components, and therefore, they can be considered as part of the first line of defense against the external stress. Often, because of the inadequacy of the intracellular antioxidants or due to the presence of increased amount of oxidative stress, the reactive oxygen species may reach their targets, which include nucleic acids, proteins, and lipids. This results in injury to the cellular components causing DNA strand breaks, protein degradation, and lipid peroxidation. Mammalian cells are also protected by a second line of defense system consisting of several lipolytic and proteolytic enzymes, proteases, phospholipases, etc., which are involved in the systematic recognition and removal of the injured cellular components (18). Recent studies from our laboratory indicated

that myocardial cells possess an inducible pathway for the antioxidant defense. The signal transduction pathways by which various stress signals are translated into oxidative stress leading to the modulation of antioxidants/antioxidant enzymes are different, but the patterns of the inducible enzymes are strikingly similar. The expression of the inducible genes in response to environmental stress has been shown to be the reflection of the ultimate adaptive responses and viewed as the third line of defense (16).

Although cells such as cardiomyocytes need sufficient antioxidant reserve to maintain a reducing environment for their own protection, these cells also need a certain redox state (defined as the ratio of the concentrations of oxidizing equivalents to the concentrations of reducing equivalents) to maintain normal hormonal and ionic homeostasis. GSH by functioning as the principal redox buffer protects the cells from external injury through redox signaling. A good example of redox cycling is the change in GSH/GSSG ratio in the cardiomyocytes as a consequence of ischemic or oxidative stress and restoration of the ratio after antioxidant therapy. Ischemia/reperfusion is also associated with a reduction of antioxidant reserve, which includes lowering of certain antioxidant enzymes. On the other hand, ischemic adaptation prior to a lethal ischemic episode prevents the ischemia/reperfusion-mediated lowering of antioxidant activities. For example, the activities of Mn-SOD and GSHPx are lowered after a prolonged reperfusion. Such lowering of the enzyme activities is prevented by subjecting the hearts to ischemic adaptation prior to prolonged ischemia/reperfusion. Cytokines such as IL-1 or TNFα as well as endotoxin also up-regulate these enzyme activities through the oxidative stress adaptation (43). Another antioxidant enzyme, heme oxygenase (also known as HSP-32), is also adaptively increased in response to ischemia/reperfusion (39). As mentioned earlier, the antioxidant and anti-death protein Bcl-2 is reduced in the ischemic myocardium, which is prevented by adapting the hearts to ischemia.

SUMMARY AND CONCLUSION

Survival and death of the cardiomyocytes depend critically on their redox state (Fig. 6). While sufficient antioxidant reserve warrants a reducing environment for the survival of the myocytes, the reserve is exhausted during ischemic heart disease leading to a change in redox state. Such changes in the redox state determine whether the cardiomyocytes should die, and if

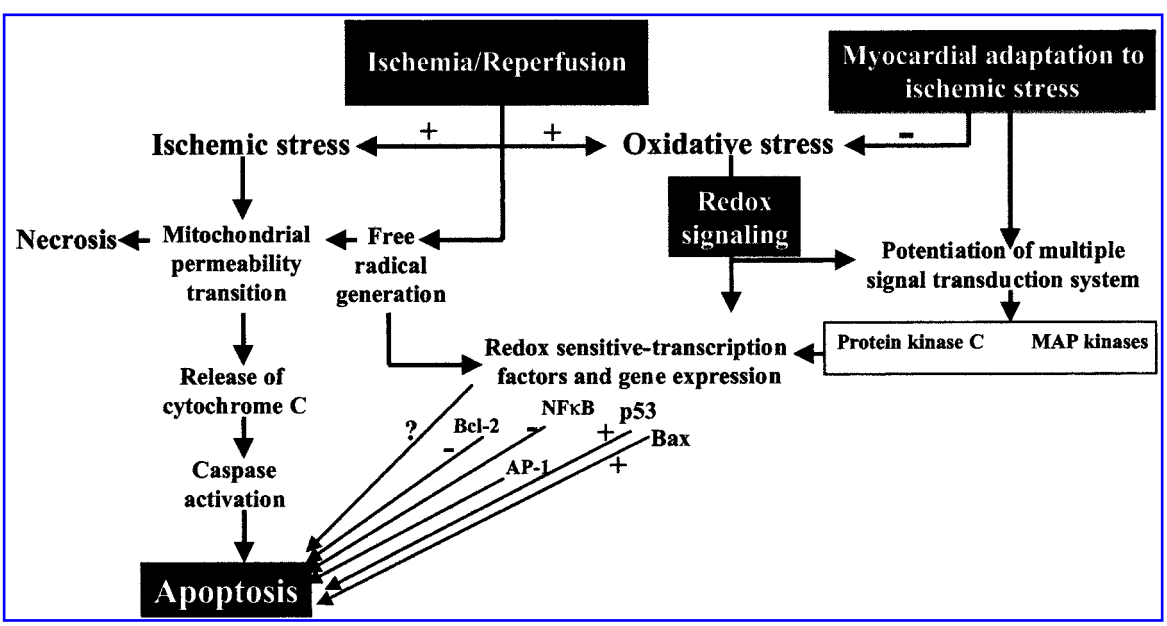

FIG. 6. Redox regulation of cardiomyocyte survival and death.

they die, whether they die of necrosis or apoptosis. Ultimate survival or death depends on the redox signaling and transcription regulation of several redox-sensing genes and DNA repair processes.

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ABBREVIATIONS

AP-1, activator protein-1; DMTU, dimethylthiorea; GSHPx, glutathione peroxidase; HSP, heat-shock protein; IL-1, interleukin-1; iNOS, inducible nitric oxide; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MAPKAP, mitogen-activated protein kinase-activated protein; MLA, monophosphoryl lipid A; NFκB, nuclear transcription factor κB; $OH^{\cdot}$, hydroxyl radical; PKC, protein kinase C; PLD, phospholipase D; SAPK, stress-activated protein kinase; SOD, superoxide dismutase; TNF-α, Tumor necrosis factor-α.

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