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Forum Review

Thioredoxin Regulation of Ischemic Preconditioning

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ABSTRACT

Thioredoxins are a class of small redox-regulating proteins that appear to play a crucial role in many oxidative stress-inducible degenerative diseases. A recent study demonstrated a reduction of thioredoxin-1 (Trx1) protein in the ischemic reperfused myocardium. When the same heart was adapted to ischemic stress by preconditioning with repeated cyclic episodes of small duration of ischemia and reperfusion, there was an increased induction of Trx1 expression. Inhibition of Trx1 expression resulted in reduced postischemic ventricular recovery and increased myocardial infarct size in the preconditioned heart. Corroborating these findings, transgenic mouse hearts overexpressing Trx1 were resistant to ischemic reperfusion injury as compared with the hearts from wild-type mice. Thus, it appears that thioredoxin plays a crucial role in cardioprotection induced by preconditioning. *Antioxid. Redox Signal.* 6, 405–412.

INTRODUCTION

A GROWING BODY OF EVIDENCE is rapidly accumulating indicating that the cellular changes associated with myocardial ischemia and reperfusion are redox-regulated. Reactive oxygen species (ROS) that are generated in the ischemic reperfused myocardium affect the cellular stress response to virtually all cellular compartments at the regulatory level (10, 11). Ischemia and reperfusion render the heart in the oxidized environment maintained by the stabilizing disulfides present in the extracellular surface, whereas the intracellular environment is maintained in the reduced state with the help of free sulfhydryl groups. The principal disulfide reductase responsible for maintaining the inside of the cell in the reduced state is a small (12 kDa) ubiquitous protein with two redox-active half-cysteine residues in an exposed active center having an amino acid sequence as Cys-Gly-Pro-Cys, known as thioredoxin (Trx) (19–22, 46, 50). Trx is ubiquitously present in mammalian cells including hearts (4) and plays an important role in maintaining the redox environment of the cell.

Trx appears to play a crucial role in the redox regulation of the ROS signaling during and/or following ischemia and reperfusion. Trx1 is an important component of the cellular defense against cardiac injury (1, 41). Oxidized Trx was found to be released into plasma of patients undergoing cardiopulmonary bypass surgery (47). Endurance training by swimming, accompanied by a reduction of ischemia/reperfusion-induced oxidative stress with a concomitant increase in Trx reductase, resulted in a protection against myocardial ischemia/reperfusion injury (26). Human Trx attenuated hypoxia-reoxygenation injury of murine endothelial cells in a thiol-free condition, suggesting Trx protection of myocardial injury through a novel redox signaling pathway (49).

Trx is a stress-inducible protein. For example, oxidative stress can easily induce the expression of Trx1 (17, 51). A recent study showed that pretreatment of mice with a sublethal dose of lipopolysaccharides increased Trx concentrations that played an important role in the development of endotoxin tolerance via inhibition of ROS (55).

The present review focuses on the role of Trx in the myocardial ischemia/reperfusion injury and ischemic preconditioning (PC). Trx seems to be down-regulated in the ischemic reperfused myocardium and up-regulated in the preconditioned heart during ischemic stress adaptation. The adaptive protection was

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abolished by inhibition of Trx. These results were further supported by the observation that mouse hearts overexpressing Trx were resistant to ischemic reperfusion injury.

ISCHEMIC PRECONDITIONING

Hearts subjected to cyclic episodes of short duration of ischemia each followed by another small duration of reperfusion become resistant to subsequent lethal ischemic injury. This phenomenon is known as ischemic PC, which represents a state-of-the-art technique for myocardial preservation (9, 16, 30, 43). Stress PC of heart by repeated stunning can delay the onset of further irreversible injury (43, 52) or even reduce the subsequent postischemic ventricular dysfunction (8, 27, 31, 33, 36) and incidence of ventricular arrhythmias (64). Although PC remains to be one of the most powerful techniques for cardioprotection, the mechanisms of PC remain controversial.

Of many hypotheses, the most popular one has been the adenosine hypothesis. This received support from the observation that PC can be blocked by adenosine A1 antagonists, and that adenosine A1 agonists can limit infarct size (31). Recently, adenosine A3 receptor has also been implicated in cardioprotection (13). Although there is general agreement regarding the beneficial role of adenosine in ischemic tissue, the adenosine hypothesis remains controversial (66). A large number of studies exist in the literature to support the role of mitochondrial ATP-dependent potassium channel openers (K_{ATP}) in PC (18, 42).

Multiple kinases appear to be involved in PC induced by repeated ischemia and reperfusion. PC is associated with the translocation and activation of protein kinase C (PKC), and inhibition of PKC abolishes the cardioprotective abilities of PC (6, 25). PC also triggers a signaling pathway by potentiating tyrosine kinase phosphorylation leading to the activation of p38 mitogen-activated protein (MAP) kinase and MAP kinase-activated protein (MAPKAP) kinase 2 (34, 36, 70). More recently, protein kinase B, serine/threonine kinase (Akt), was also found to regulate the PC process (63).

A recent study demonstrated that 30 min of ischemia followed by 2 h of reperfusion increased the induction of c-Jun N-terminal kinase 1 (JNK1), c-Jun, and p38 MAP kinase proteins (56). PC also enhanced these kinases compared with control; however, subsequent ischemia/reperfusion-mediated increase in JNK1, p38, and c-Jun was blocked by PC. Thus, transient activation of proapoptotic factors such as JNK1, c-Jun, and p38 MAP kinase appears to be necessary for PC.

PRECONDITIONING AND REDOX SIGNALING

Recent studies suggest that ROS not only are destructive elements for cells, but also are essential for the biological and physiological function of the cells. Biological cells including cardiomyocytes contain enzymes that can simultaneously generate ROS and intracellular redox buffer in response to a specific stress. Depending on the amounts of antioxidant reserve and oxygen free radicals, the ROS are either destroyed or persist. Thus, the ROS fulfill the definition of messenger molecules, which are either up-regulated or down-regulated after physiologic stimuli like ischemia.

The cardioprotective abilities of PC seem to be linked to the redox signaling of ROS. Perhaps the finding that the production of ROS during the PC-induced activation of the nuclear transcription factor, nuclear factor-κB (NFκB), provided the first concrete evidence of the role of ROS as the signaling molecules (34). NFκB regulates the inducible expression of a number of genes involved in cell survival and execution. For example, NFκB can control antiapoptotic gene, bcl-2, and proapoptotic factors, bax and p53, in the ischemic/reperfused and preconditioned myocardium (32, 33, 35, 37–39).

Potentiation of the signaling cascade involving multiple kinases that leads to the induction of the activation of several redox-sensing transcription factors and genes ultimately dictates the cells to survive or die. Such changes in redox-sensitive gene expression are likely to influence the physiologic function of the cardiomyocytes during postischemic survival. Interestingly, the 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 dimethylthiourea or *N*-acetylcysteine (11). More interestingly, an endotoxin-derived compound, monophosphoryl lipid A, induced inducible nitric oxide synthase (iNOS) mRNA through a tyrosine kinase-dependent mechanism and protected the heart from ischemic reperfusion injury (68). 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, further supporting a role of oxygen free radicals as signaling molecules (36). Redox signaling seems to be independent of PKC although PKC is activated during the PC process, suggesting the role of two separate signaling pathways in PC (12). The major function of redox signaling in the ischemic myocardium appears to be 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.

THIOREDOXINS AND GLUTAREDOXINS, THE REDOX-SENSING PROTEINS

Trx and glutaredoxins (Grx) are the major cellular redox-sensing proteins that are ubiquitously present in mammalian tissues including heart (Fig. 1). These two redox-active sulfhydryl molecules are responsible for sensing the cellular redox status and maintaining the status in a nonequilibrium steady state. Both of them bind to apoptosis signal-regulating kinase 1 (ASK-1) and cooperatively regulate its activation. However, Trx directly binds to the N-terminal portion of ASK-1, whereas Grx binds to its C-terminal portion; but both of them inhibit apoptosis. Although both Trx1 and Grx1 exhibit activity in regeneration of oxidatively damaged proteins, recent studies have shown that they have different substrate preference. (To date, biochemical characterization of Trx and Grx has been almost exclusively conducted with Trx1 and Grx1.) Grx1 reactivates oxidized proteins containing mixed disulfides (Prot-SS and $Prot_1$-SS-$Prot_2$) more efficiently than does Trx1, whereas Trx preferentially regenerates protein sulfenic or sulfinic acid (56).

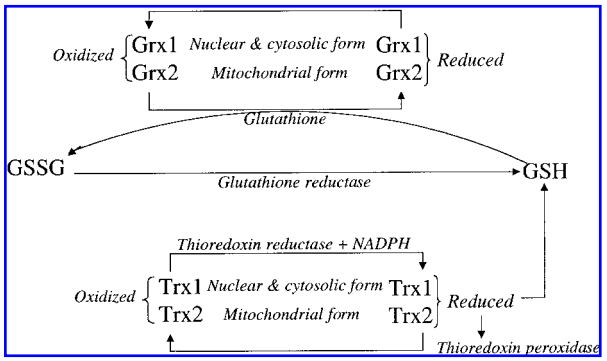

FIG. 1. Thioredoxin and glutaredoxin systems in mammalian heart.

This depicts the role of Grx1 in reductive cleavage of protein–glutathione (GSH) mixed disulfides (Prot-SS-G) (53).

Trx possess a dithiol/disulfide active site and can serve as electron donors for enzymes including Trx peroxidases and ribonucleotide reductases (5, 57). They are reduced by electrons from NADPH via Trx reductase, a member of the Trx family of proteins. In the mammalian system, the Trx protein family is composed of Trx, a 12-kDa thiol/disulfide oxidoreductase, Trx reductase, a member of a pyridine nucleotide disulfide oxidoreductase family of a homodimer of 56-kDa subunits, and Trx peroxidase consisting of two 25-kDa subunits. At least two Trx systems have been characterized, cytosolic (Trx1) and mitochondrial (Trx2) forms, although additional homologues of these proteins are also known to exist (19–22, 60).

Trx1 is the major Trx redox protein that is responsible for most of the biological signals for Trx, including the supply of reducing equivalents to Trx peroxidases and ribonucleotide reductase and the regulation of transcription factor activity. Trx1 is a 104-amino acid protein with a molecular mass of 12 kDa, which binds to a variety of cellular proteins including Ref-1, ASK-1, PKC, and NFκB and inhibits apoptosis (21, 22). Trx1 possesses free radical scavenging property (40), acts as a reducing cofactor (50), and can protect the cells from hydrogen peroxide-induced oxidative damage (62). Most of the antioxidant properties of Trx1 appear to be mediated through Trx peroxidase, which belongs to the antioxidant protein family, peroxiredoxins. The peroxiredoxins use thyl groups as reducing equivalents to scavenge ROS activity and the reduced form of Trx peroxidase then scavenges ROS (5, 28). These Trx peroxidases are present in red blood cells responsible for protecting them from oxidant injury. Both Trx peroxidases 1 and 2 possess antioxidant property, can scavenge ROS, and reduce apoptosis (28). Trx peroxidase is a novel inhibitor of apoptosis with a mechanism distinct from that of Bcl-2 (69).

Trx1 can be easily induced in tissues or cells subjected to a wide variety of stresses including oxidative stress, UV radiation, ionizing irradiation, heavy metals, and inflammation (19, 20). Trx1 is also induced in tissues subjected to ischemia and reperfusion (49, 65) or ischemic PC (65).

Trx1 appears to play an important role in a variety of degenerative diseases. For example, overexpression of Trx1 protects cancer cells from spontaneous apoptosis (3). Overexpression of Trx1 in human primary tumor causes increased cell proliferation, decreases apoptosis, and reduces patient survival (14, 15, 58). Trx1 has recently been found to regulate the expression of estrogen-metabolizing cytochromes P450, 1B1, and 1A1 in MCF human breast cancer cells (23). Trx1 can reduce myocardial ischemia/reperfusion injury and decrease the incidence of ventricular fibrillation (49, 59). In the case of the lung, recombinant human Trx1 improved lung function and reduced cellular injury of the lung subjected to ischemia and reperfusion (67). Transgenic mice overexpressing Trx1 in brain showed smaller infarct after cerebral artery occlusion (61). Brains from patients with Alzheimer's disease demonstrated reduced amount of Trx1 with a concomitant increase in Trx reductase (29). Increased Trx1 expression was reported for patients with HIV (45). Increased Trx1 expression was found in the epidermal cells of skin exposed to sun (53).

Grx, also known as thioltransferase, is a ubiquitous and heat-stable protein found in all prokaryotes and eukaryotes. Two Grx genes have been found in mammals. Grx1 was initially considered to be a cytosolic enzyme. However, recent results of immunolabeling experiments have shown that it is also present in the nucleus (52). The second Grx (*Grx2*) gene, which was reported recently, encodes two proteins as a result of alteration of RNA splicing. One of the Grx2 isoforms is located in the mitochondria and the other in the nucleus (54, 55). Similar to Trx, Grx also functions in catalyzing thiol/disulfide exchange (57). The active site of Grx [Cys-Pro-Tyr(Phe)-Cys] is oxidized during this process and is regenerated by using the reducing equivalents of GSH. The oxidized GSH is then recycled back to the reduced form of GSH by glutathione reductase. As GSH is the major intracellular nonprotein thiol, it forms the majority of mixed disulfides inside the cells under oxidative stress. Grx1 is, therefore, expected to play a predominant role in reversing this process.

In addition to its function in thiol/disulfide exchange, Grx1 also serves as an alternative electron donor to ribonucleotide reductase (59), participates in deiodination of thyroxin to triiodothyronine (58), and exhibits activity of dehydroascorbate reductase for regeneration of ascorbic acid (60). However, despite the potential role of Grx in the antioxidant defense mechanism, studies on its antioxidant function have been exceptionally limited. Our recent studies demonstrated that transgenic mice overexpressing Grx1 reduced the number of apoptotic cardiomyocytes in the ischemic reperfused heart. Another two studies published in recent years have shown that recombinant Grx protein of *E. coli* is capable of protecting cultured cerebellar granule neurons from dopamine-induced apoptosis, and the protection is mediated through activation of the Ras/phosphatidylinositol 3-kinase (PI 3-kinase)/Ref-1/Akt/NFκB pathway (61, 62). It is tempting to speculate that this Ras/PI 3-kinase/Ref-1/Akt/NFκB pathway may play an important role in the Trx1 and/or Grx redox regulation.

THIOREDOXINS AND HEART

Mammalian hearts contain two different Trx, mitochondrial and cytosolic of about 12.3 and 12 kDa, respectively (Fig. 1). They differ in their cysteine content: the mitochondrial Trx has two cysteine residues like the bacterial proteins, whereas the cytosolic Trx possesses six cysteines residues (19). Heart extracts were also found to contain an NADPH-specific Trx reductase of the known mammalian type.

Recent studies have indicated a possible association between plasma Trx concentrations and severity of heart failure (48). Trx immunoreactivity was none to trivial in control specimens. Positive Trx staining was found in the hearts of patients with active myocarditis and dilated cardiomyopathy. The positive staining was located in infiltrating cells and damaged myocytes in the perinecrotic lesions, suggesting that myocardial Trx was up-regulated in myocarditis and cardiomyopathies with active necrotic stage associated with DNA damage. In another related study, increased Trx expression was found in the skin biopsies (obtained during cardiac catheterization) of 29 of 35 patients with congestive heart failure, but none of the eight control subjects (48). Overexpression of Trx1 in the heart attenuated adriamycin-induced cardiotoxicity (59). Treatment with recombinant human Trx1 suppressed cardiomyocyte injury in adrenomycin-treated cardiomyocytes.

A crucial role of Trx has also been indicated in myocardial ischemic reperfusion injury. For example, measurements of plasma Trx in healthy volunteers and during open-heart surgery demonstrated elevated levels of Trx in arterial plasma during reperfusion of the postcardioplegic heart of the patients (47). Trx increased during surgical preparation for cardiopulmonary bypass, but decreased during the bypass due to the release of oxidized Trx into the plasma. In an isolated perfused rat heart model, Trx protected the hearts from reperfusion-induced arrhythmia (49). In the early reperfusion period, h-Trx reduced the incidence of ventricular fibrillation from 75% (untreated) to 42% and 25% at 0.01 μ*M* and 0.1 μ*M* concentrations, respectively. Interestingly, superoxide dismutase (SOD) was unable to protect these hearts from reperfusion-induced arrhythmias in this model.

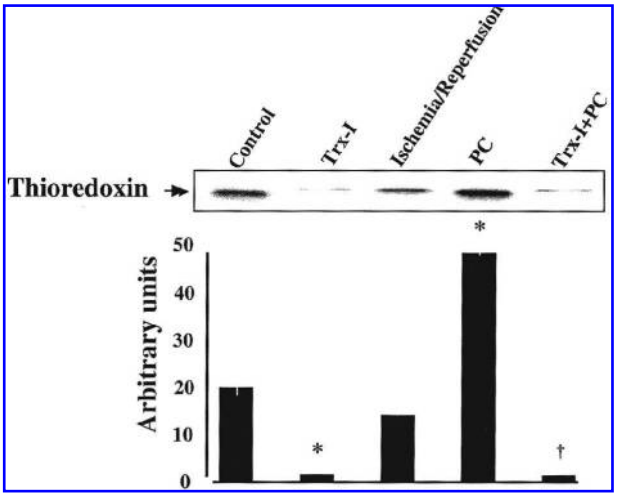

FIG. 2. Effects of ischemia/reperfusion and ischemic adaptation on the induction of the expression of Trx1 in the heart. Representative northern blots are shown at the top. The blots were densitometrically scanned and normalized against the housekeeping gene, β-actin, as described in Materials and Methods. The results indicate down-regulation of Trx1 in the ischemic-reperfused myocardium and its up-regulation in the preconditioned heart. Note the reduction of Trx1 expression when the hearts were preperfused with the inhibitor of Trx1. The bar graph represent the average of four blots per group (means ± SEM) for Trx1 expression. $^{*}p < 0.05$ versus control; $^{\dagger}p < 0.05$ versus adapted. Adapted from reference 65 with permission.

THIOREDOXIN IN PRECONDITIONED HEART

As mentioned earlier, hearts can be preconditioned by challenging them with sublethal duration of repeated ischemia and reperfusion that render them tolerant to subsequent lethal ischemia and reperfusion. Ischemia/reperfusion reduced the amount of Trx1, whereas PC induced the expression of this protein (65) (Fig. 2). Such induction of Trx1 is not surprising, because Trx1 is known to be induced by diverse stresses including oxidative stress (46). The increased expression of Trx1 is likely to be due to the adaptive response triggered by PC, because Trx1 overexpression was nicely corroborated with cardioprotective abilities of adaptation. These results are consistent with previous observations that Trx-1 is stress-inducible (46). Indeed, diverse stresses including UV radiation, *x*-ray irradiation, oxidative stress, and viral infection can induce Trx1 as a part of self-defense abilities of the cell. Consistent with these findings, the inhibition of Trx1 abolished the cardioprotective abilities of PC as evidenced by reduction of cardiomyocyte apoptosis and infarct size (Fig. 3).

One of the most salient features of PC is its ability to convert ischemia/reperfusion-induced death signal into survival signal (7, 56). PC thus induces the expression of anti-death gene Bcl-2, modulates the redox-sensitive transcription factors such as NFκB, activator protein-1 (AP-1), Stat 3 (signal transducers and activators of transcription), and Stat 5A, and abolishes cardiomyocyte death due to apoptosis (32–35, 37–39). Many redox-sensitive transcription factors including NFκB and AP-1 have been shown to be redox-regulated by Trx (2, 44). Trx thus plays a crucial role in thiol redox control of cell function through transcription regulation of target genes including NFκB that control numerous gene expressions. Studies from different laboratories including our own have provided evidence of an essential role of NFκB in the cardioprotective properties of ischemic PC (34, 38). Anti-death signal of PC was abolished with the inhibition of Trx1, and a significant number of apoptotic cardiomyocytes appeared in the preconditioned myocardium. These results were corroborated with the findings that Trx1 inhibition also abolished the oxidative stress-lowering ability of PC (41, 54), suggesting that Trx1 plays a crucial role in redox signaling mediated by PC (Fig. 4). Furthermore, these results implicate an antioxidant function of Trx1 in the heart. Indeed, Trx has been shown to function as an intracellular antioxidant by scavenging ROS and can protect the cells against oxidative stress. The results of our study clearly demonstrated an increased amount of malonaldehyde formation in hearts when Trx1 was inhibited. Trx can induce other antioxidants. For example, mRNA of MnSOD has been found to be induced by Trx1 (40). MnSOD, which plays a crucial role in myocardial defense against ischemic reperfusion injury, is induced when the heart is preconditioned to ischemic stress (9).

The role of Trx1 in myocardial ischemia/reperfusion injury and redox signaling was further substantiated by the observation that mouse hearts overexpressing Trx1 were resistant to

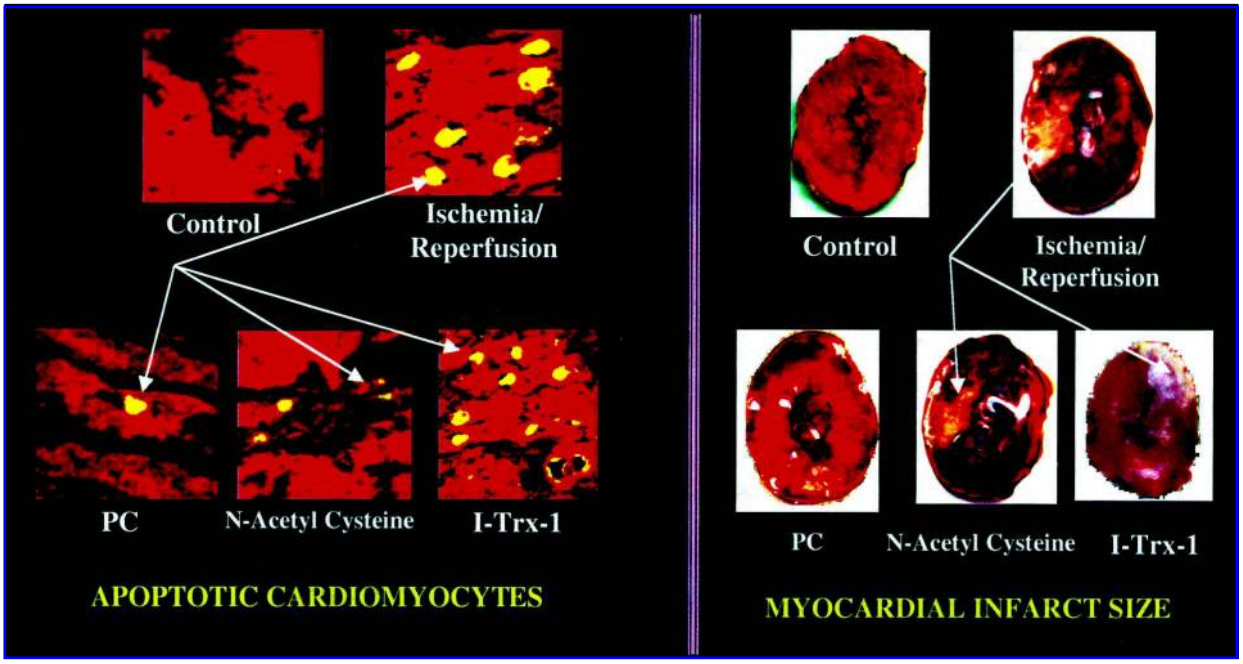

FIG. 3. Effects of ischemia/reperfusion and ischemic adaptation on myocardial infarct size (right) and cardiomyocyte apoptosis (left). Representative pictures are shown from each category. Infarct size was determined by TTC (2,3,5-triphenyl tetrazolium chloride) staining, whereas cardiomyocyte apoptosis was evaluated by TUNEL staining in conjunction with antibody against α-myosin heavy chain to specifically stain the apoptotic cardiomyocytes. The yellow-to-white regions indicate infarcted area, and apoptotic cardiomyocytes are shown in yellow circles. Note the reduction of apoptotic cardiomyocytes in the preconditioned myocardium. Preperfusion of the hearts with a ROS scavenger or a Trx1 inhibitor completely abolished the cardioprotective effects of PC, suggesting that ROS is functioning as signaling molecules in PC and Trx1 plays a crucial role in this process.

ischemic injury (65). The Trx1 transgene overexpression was associated with reduced myocardial infarct size and improved left ventricular performance in the postischemic myocardium compared with the wild-type hearts.

PC is a process of stress adaptation. Cyclic episodes of nonlethal ischemia and reperfusion create a combination of hypoxic and oxidative stress, which becomes instrumental in adapting the heart against subsequent lethal ischemic insult. It appears

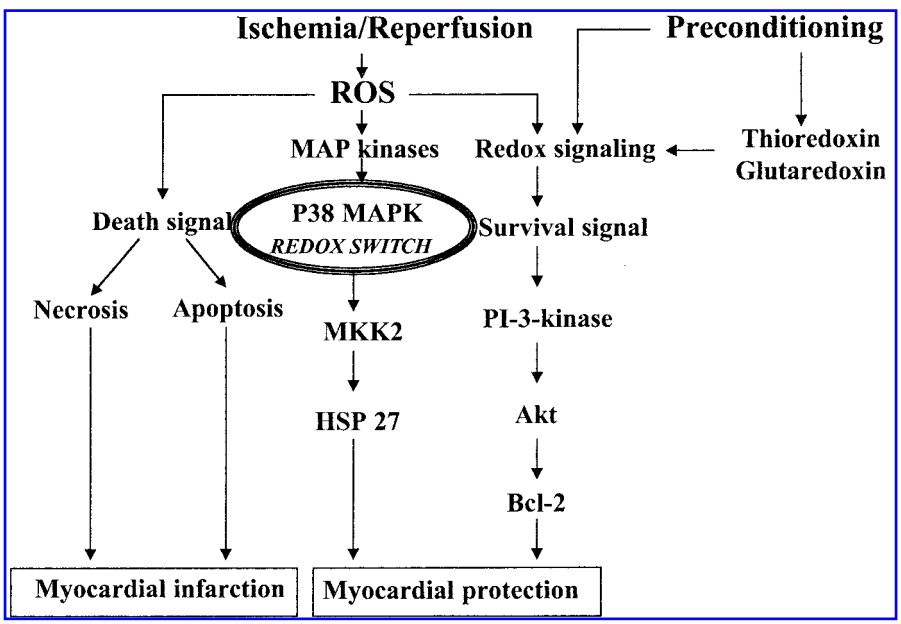

FIG. 4. Possible role of thioredoxin and glutaredoxin in PC-mediated redox signaling.

that PC occurs by transforming ischemia/reperfusion-induced death signal into a survival signal through the modulation of MAP kinases and a number of redox-regulated transcription factors and genes (Fig. 4). Trx1 may play an essential role in the process of adaptation, because Trx1 regulates transcription of many genes, which are also regulated by PC. Trx and Grx may regulate the "redox switch," which changes ischemia/reperfusion-induced "death signal" into PC-mediated "survival signal." Induction of the expression of Trx1 during PC is likely to be due to the adaptive response of PC that involves a process of harnessing the heart's own defense.

CLINICAL PERSPECTIVES

The mammalian Trx appears to play a role in myocardial ischemia/reperfusion injury. Reperfusion of the ischemic myocardium down-regulates Trx1 protein in the heart, whereas PC results in the induction of the expression of Trx1. Trx1 was found to be released from the hearts of patients undergoing cardiac surgery. Mouse hearts overexpressing Trx1 are resistant to ischemia/reperfusion injury. Inhibition of Trx1 abolishes the cardioprotective properties of PC, suggesting that Trx1 plays an essential role in the PC-mediated cardioprotection. It seems reasonable to speculate that an enhancement of Trx might protect the hearts from ischemia/reperfusion injury. Future studies using either Trx gene therapy or an intervention to increase Trx protein will reveal the usefulness of Trx in the clinical arena.

ACKNOWLEDGMENTS

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ABBREVIATIONS

AP-1, activator protein-1; ASK-1, apoptosis signal-regulating kinase 1; Grx, glutaredoxin(s); GSH, glutathione; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MAPKAP, MAP kinase-activated protein; NFκB, nuclear factor-κB; PC, preconditioning; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; Stat, signal transducers and activators of transcription; Trx, thioredoxin(s).

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