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Forum Review

Role of Reversible, Thioredoxin-Sensitive Oxidative Protein Modifications in Cardiac Myocytes

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ABSTRACT

Reactive oxygen species (ROS) are important mediators of myocardial remodeling. However, the precise molecular mechanisms by which ROS exert their effects are incompletely understood. ROS induce oxidative posttranslational protein modifications that can regulate the function of structural, functional, and signaling proteins. For example, oxidative modification of free reactive thiols (*S*-thiolation) on the small G protein Ras increases Ras activity and thereby promotes ROS-dependent hypertrophic signaling in cardiac myocytes. By reducing thiols and restoring reversible thiol modifications, thioredoxin and glutaredoxin can act as regulators of ROS-mediated protein function. Understanding the regulation and functional relevance of oxidative protein modifications in myocardial remodeling may lead to new therapeutic strategies. *Antioxid. Redox Signal.* 8, 2153–2159.

INTRODUCTION

An increasing body of evidence suggests that reactive oxygen species (ROS) actively participate as signaling molecules in the process of myocardial remodeling (48). This hypothesis is supported by studies in which antioxidant treatment ameliorates adverse remodeling in response to a variety of pathological stimuli such as aortic constriction and myocardial infarction (14, 28, 50). Furthermore, *in vitro* ROS mimic many of the typical features observed in myocardial remodeling. We have recently shown that treatment of adult rat ventricular myocytes with hydrogen peroxide (H_2O_2) leads to differential, concentration-dependent activation of specific kinase signaling pathways, resulting in hypertrophy in response to low (<50 μ*M*) and apoptosis to high concentrations (≥100 μ*M*) of H_2O_2 (33). Accordingly, ROS have been implicated as mediators of hypertrophic and apoptotic signaling in response to various remodeling stimuli, such as mechanical strain (44), α-adrenergic receptor (αAR) stimulation (3, 53, 66), β-adrenergic receptor stimulation (47), TNF-α (43), angiotensin (43), and endothelin (53). However, the mechanisms by which ROS exert their effects are poorly understood.

Whereas ROS in very high levels may be associated with irreversible damage to proteins, lipids, and nucleic acids, much lower levels of ROS may induce more subtle, and often reversible, posttranslational modifications of target proteins that can regulate function, a process also referred to as redox regulation. In this review, we will describe the concept of posttranslational oxidative protein modifications as a mechanism of redox-mediated intracellular signaling. As an example of the functional relevance of oxidative modifications in the cardiac myocyte, we will discuss our studies of the small G protein Ras and the role of thioredoxin in regulating the oxidative modification of Ras and cardiac hypertrophy.

REACTIVE OXYGEN AND NITROGEN SPECIES THAT PARTICIPATE IN ROS-SIGNALING

Mitochondria, NAD(P)H oxidase, and nitric oxide synthase are major sources of ROS and reactive nitrogen species (RNS) (48). Superoxide anion ($O_2^{\bullet -}$), arising from mitochon-

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dria or NADPH oxidase, is rapidly dismutated into H_2O_2 (48) or may react with NO to form the more reactive peroxynitrite ($ONOO^-$) (6). H_2O_2 itself can be converted into highly reactive hydroxyl radicals (•OH) via the Fenton reaction (Fig. 1).

Under basal conditions, the production of ROS/RNS is counterbalanced by clearance via antioxidant enzymes/enzyme systems such as superoxide dismutases, catalase, and peroxidases. Superoxide dismutase degrades $O_2^{\bullet -}$ to H_2O_2, which is further metabolized to H_2O by catalase or a peroxidase (Fig. 1). H_2O_2 can be produced in response to physiologic stimuli such as Gq-coupled receptor activation. Only recently it has been shown that H_2O_2 is self-propagating and amplifies its own production via an NAD(P)H oxidase-dependent mechanism in endothelial cells (8). Our data suggest that H_2O_2, rather than $O_2^{\bullet -}$, is the major species in regulating myocyte phenotype in response to a variety of hypertrophic and apoptotic stimuli. For example, αAR-stimulated hypertrophy in adult cardiomyocytes can be inhibited by adenoviral overexpression of catalase but not superoxide dismutase (32). Likewise, hypertrophy of neonatal rat ventricular myocytes in response to mechanical strain is abolished by the adenoviral overexpression of catalase, whereas superoxide dismutase has no effect (45).

POSTTRANSLATIONAL OXIDATIVE (THIOL) MODIFICATIONS AND PROTEIN REGULATION

Both ROS and RNS can react with proteins, leading to posttranslational oxidative protein modifications. The nature of the oxidative modification a protein can undergo depends on a variety of factors, including the specific oxidant species, the concentration and/or time of exposure, and the intracellular localization (compartmentalization), as well as the structure of the target protein itself. Although the intracellular environment is maintained in a reduced state, some oxidative modifications may exist under basal conditions. When oxidative stress increases, a low and/or short-term oxidant burden can induce oxidative modifications that are readily reversible, lead to reversible activation or inhibition of specific target molecules (redox regulation), and thus qualify as signaling events. Conversely, with higher levels of oxidative stress, irreversible oxidative modifications may result in the loss of protein function, protein degradation, or the accumulation of defective protein. Irreversible modifications have been proposed to play a central role in the pathogenesis of neurodegenerative processes (10), as well as cardiovascular diseases associated with aging and atherosclerosis (2).

Cysteine thiols are particularly susceptible to oxidative modification. The two major determinants of the susceptibility of thiols to redox regulation are the accessibility of the thiol within the three-dimensional structure of the protein and the reactivity of the cysteine, which is influenced by the surrounding amino acids. Most thiol modifications are unstable and can easily be reversed or replaced by other, more stable modifications. Oxidative modifications of thiols include *S*-thiolation, *S*-nitrosylation, sulfenic/sulfinic/sulfonic acid formation, or inter- and intraprotein disulfide formation (13, 67) (Fig. 1). *S*-thiolation refers to the formation of a disulfide bond between a protein thiol and a low-molecular-mass thiol such as cysteine, homocysteine, glutathione, or other nonprotein thiols, and is also referred to as a mixed (*i.e.*, protein/nonprotein) disulfide. Because glutathione is the most abundant nonprotein thiol in the cell, *S*-glutathiolation is the most frequent form of oxidative thiol modification. Oxidative thiol modifications can be reversible, and thus are good candidates to participate in protein redox regulation. Furthermore, reversible thiol modifications may provide protection against irreversible modifications (*e.g.,* sulfonic acid formation) and protein damage in response to higher levels of oxidative stress. *S*-glutathiolation may also serve as a storage mechanism for glutathione to prevent loss of glutathione under prooxidant conditions, because oxidized glutathione (GSSG) would otherwise be extruded from the cell (51).

S-glutathiolation has recently been implicated in the redox regulation of a variety of proteins. By introducing the negative charges of GSH, *S*-glutathiolation inhibits the DNA-binding activity of the transcription factors c-Jun and NFκB (29, 46). The cytoskeletal proteins tubulin (36) and actin can be *S*-glutathiolated, whereby *S*-glutathiolation regulates actin polymerization (61). Regulatory effects of *S*-glutathiolation have also been described for metabolic enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and creatine kinase, in both cases resulting in inhibition of enzyme activity (30, 42). Other targets for *S*-glutathiolation include ion channels (5, 63), signaling molecules (4, 18), and mitochondrial proteins involved in the regulation of ROS generation and metabolism (18, 54).

We have studied the role of *S*-thiolation in the regulation of Ras (31, 45). Ras is a small G protein that plays a crucial role in the regulation of hypertrophic growth in cardiac myocytes in response to stimuli such as α-adrenergic receptor agonists and mechanical strain (62). *S*-thiolation and *S*-nitrosylation of reactive cysteines on Ras have been implicated in Ras regulation *in vitro* (34, 39).

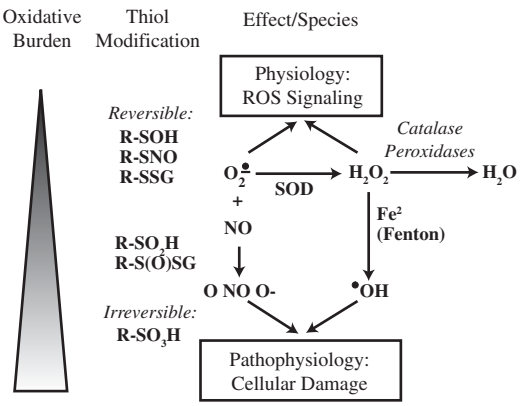

FIG. 1. Regulation of reactive oxygen/nitrogen species and oxidative thiol modifications. R-SOH, sulfenic acid formation; R-SNO, *S*-nitrosation; R-SSG, *S*-glutathiolation; R-SO_2H, sulfinic acid formation; R-S(O)SG, thiosulfinate formation; R-SO_3H, sulfonic acid formation. For details, see text.

REGULATION OF OXIDATIVE PROTEIN MODIFICATIONS VIA THIOREDOXIN AND GLUTAREDOXIN

For most types of protein regulation, it is desirable that the mechanism be reversible. Oxidative thiol modifications can be reversed via interaction with other thiol-containing reducing agents (*e.g.,* glutathione), via direct thiol disulfide exchange reactions, or by means of an enzymatically mediated reaction (21). Enzymes capable of reducing thiols (dethiolation) include the glutaredoxin (GRX)/GRX reductase system, the thioredoxin (TRX)/TRX reductase system, and protein disulfide isomerases (PDI) (24–27). These enzymes all have in common a redox-active Cys-X-X-Cys sequence. Although these enzymes can act bidirectionally (*i.e.*, as oxidant or reducing systems) (15, 16, 19), TRX/TRX reductase and GRX act primarily as antioxidants by reducing protein disulfides and mixed disulfides, as well as by restoring other reducing enzyme systems. In contrast, PDI may act predominantly as an oxidant by forming intrachain disulfide bonds that contribute to protein folding.

Upon reduction of disulfide bonds, GRX and TRX are oxidized and form an intramolecular disulfide bridge between the two cysteines of the Cys-X-X-Cys sequence. The oxidized TRX and GRX are subsequently restored by the action of TRX reductase and glutathione reductase, respectively, in an NADPH-dependent manner (Fig. 2). TRX also acts as a substrate for TRX peroxidases/peroxiredoxins and thus can be directly involved in the clearance of ROS, namely H_2O_2 (24). TRX has been shown to regenerate oxidized thiols and restore the consecutive functional changes of the target protein in various models (19, 38). Although GRX might be more effective in restoring glutathiolated protein thiols, TRX is capable of doing so as well (40, 55).

Interestingly, the activity of TRX itself can be regulated by *S*-glutathiolation or *S*-nitrosylation (69). TRX contains five cysteines: Cys^{32} and Cys^{35}, both located in the redox regulatory domain, as well as three additional cysteines in positions 62, 69, and 73. *S*-glutathiolation of TRX at Cys^{73} has been shown to inhibit the redox-regulatory activity of TRX (9), whereas *S*-nitrosylation of TRX at Cys^{69} increases its activity in human umbilical vascular endothelial cells (HUVECs) (23).

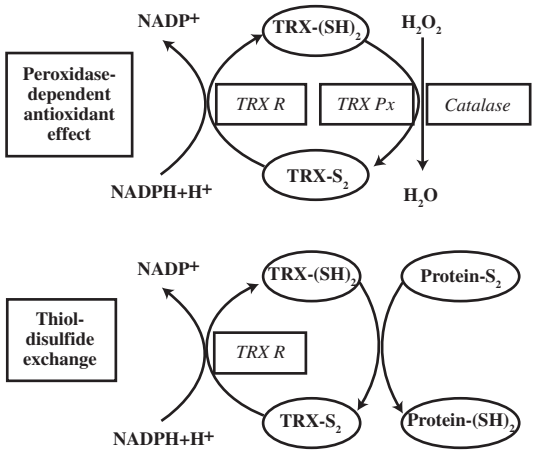

FIG. 2. Mechanism of action for thioredoxin. *Top panel:* Peroxidase-dependent antioxidant effect. *Bottom panel:* Thiol–disulfide exchange. TRX R, thioredoxin reductase; TRX Px, thioredoxin peroxidase. For details, see text.

POSTTRANSLATIONAL OXIDATIVE PROTEIN MODIFICATIONS IN THE HEART

Ischemia–reperfusion is a well-established, though complex, model of oxidative stress-induced cardiac injury. The complexity is due in part to the fact that ROS not only contribute to the resulting injury, but also mediate many of the cardioprotective effects observed in context with ischemic preconditioning. It appears likely that ROS-mediated cardiac injury and cardioprotection involve posttranslational oxidative modifications of structural, functional, and signaling proteins. Ischemia–reperfusion has been used to identify cardiac proteins that may represent a target for oxidative protein modifications.

Metabolic disturbances, ionic imbalance, necrotic and apoptotic cell death, and contractile dysfunction are all observed in the setting of ischemia–reperfusion. Furthermore, activation of a variety of signaling molecules contributes to both the adaptive and maladaptive responses to ischemia– reperfusion. Accordingly, it has been informative to study the oxidative modifications of proteins that occur in the setting of ischemia/reperfusion. Eaton *et al.* (18) found oxidative modifications of the metabolic enzymes GAPDH, triosephosphate isomerase, and aconitase, the structural protein actin, the chaperone protein HSP27, and the signaling molecules protein-tyrosine phosphatase 1B, protein kinase Cα, and the G protein Ras during reperfusion of the ischemic rat heart (18). Data from other experimental models has extended the list of oxidatively modified proteins relevant to the heart to include creatine kinase (7, 12, 41), the ion channels Na^+/K^+-ATPase (22), sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (2), and ryanodine receptor (52), and the structural proteins tropomyosin, tubulin, troponin I, myosin light and heavy chain, and desmin (7). It is likely that this is only a partial list of the proteins that undergo oxidation in the heart.

Consistent with the hypothesis that oxidative stress is an important mediator of ischemia–reperfusion injury, it has recently been shown that mice overexpressing TRX exhibit improved postischemic recovery and reduced myocardial infarct size as compared with wild-type mice (57). One explanation for the beneficial effect of TRX in this model could be the protection of reactive protein thiols from oxidative modification.

FUNCTIONAL RELEVANCE OF OXIDATIVE MODIFICATION OF RAS

ROS and RNS can activate a number of signaling pathways, such as mitogen-activated protein kinases (MAPKs),

the phosphatidyl inositol 3-kinase (PI3K)/Akt pathway, the NF-κB signaling system, p53, and the heat-shock response (20). All three MAPK pathways, extracellular signal–regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 MAPK, are activated by exogenous H_2O_2 (33) or by receptor-stimulated production of H_2O_2 in cardiomyocytes and various other cell types (56). However, the ROS-dependent step in the activation of MAPK signaling appears to be upstream of the MAPKs themselves, although the target of ROS and the mechanism of its activation have not clearly been identified. We have focused on Ras, a small regulatory G protein that plays a crucial role in the activation of the hypertrophic Raf-MEK1/2-ERK1/2 kinase pathway in cardiomyocytes.

In adult rat ventricular myocytes *in vitro*, αAR-stimulated hypertrophy is mediated via activation of the Ras-Raf-MEK1/2-ERK1/2 signaling pathway (62, 65). We and others have shown that αAR-mediated hypertrophic signaling and hypertrophy are mediated by ROS (3, 53, 66). Although Ras activation is both sufficient and necessary for αAR-stimulated protein synthesis in adult cardiomyocytes (62), data from our group show that αAR-stimulated activation of Ras can be inhibited by the catalase/superoxide dismutase mimetic Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), suggesting that the ROS-sensitive step in the activation of this pathway is located at or above the level of Ras (66). Ras has been described as a target for $NO^{\bullet}$ and H_2O_2, whereby oxidative modification by both may lead to its activation (17, 34, 35, 64). Ras contains four reactive cysteine residues, and *in vitro* work on purified Ras has shown that it can be oxidatively modified at all four cysteines by various oxidant species, including H_2O_2 (39). By using biotinylated iodoacetamide (BIAM) to label free reactive thiols, we showed that αAR stimulation of adult rat ventricular myocytes induces oxidative modification of reactive thiols on Ras, which is associated with a proportional increase in Ras activity (31). The decrease in free reactive thiols on Ras with αAR stimulation could be reversed by dithiothreitol and inhibited by adenoviral overexpression of TRX1 (the cytosolic form of TRX). TRX1 likewise prevented the increase in Ras activity and the hypertrophic response, suggesting that the oxidative modification observed on Ras was functionally relevant for αAR-mediated signal transduction and hypertrophy in adult cardiomyocytes.

In another model of ROS-mediated cardiac hypertrophy stimulated by mechanical strain, we used mass spectrometry to show that *S*-glutathiolation of Cys^{118} on Ras, a reactive thiol located in the GTP-binding region of Ras, is responsible for ROS-dependent hypertrophic signal transduction in neonatal rat ventricular myocytes exposed to mechanical strain (45): Cyclic mechanical strain induced hypertrophy was associated with glutathiolation of Cys^{118} of Ras, increased Ras activity, and phosphorylation of ERK. Importantly, mechanical strain-induced signaling and myocyte hypertrophy were inhibited by the overexpression of GRX1 (the cytosolic form of GRX) or overexpression of a mutated form of Ras in which Cys^{118} was substituted with serine, thus supporting the hypothesis that *S*-glutathiolation of Ras is functionally relevant for ROS-mediated signal transduction leading to cardiomyocyte hypertrophy (Fig. 3).

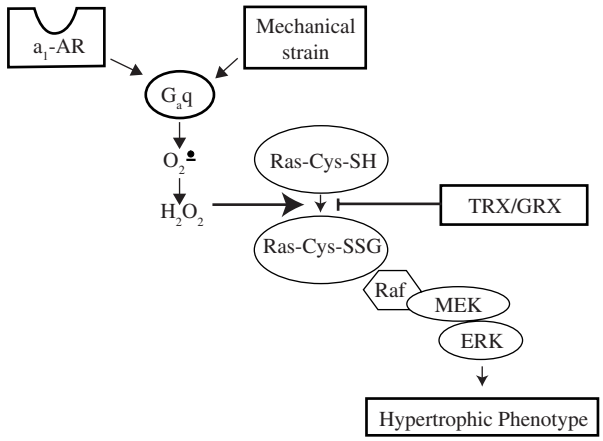

FIG. 3. Proposed model for ROS-dependent, Ras-mediated hypertrophic signaling in cardiomyocytes. NE/Prop, norepinephrine/propranolol; α_1-AR, alpha-1 adrenergic receptor; TRX, thioredoxin; GRX, glutaredoxin. For details, see text.

Angiotensin II–induced hypertrophy of vascular smooth muscle cells is another known ROS-dependent process that involves NADPH oxidase–dependent production of H_2O_2 (37, 60) and ROS-dependent activation of p38 and Akt (58, 59). Cohen's group (1) recently identified Ras as the upstream molecular target of ROS in this model. They could likewise show that *S*-glutathiolation of Cys^{118} on Ras was the critical step mediating angiotensin II–induced hypertrophy, because angiotensin II–induced signaling and hypertrophic response were inhibited by overexpression of GRX1 or the mutated C118S-Ras. Cohen's group further showed that recombinant Ras protein is activated by *S*-glutathiolation caused by the combination of peroxynitrite and GSH or by GSSG alone (11).

THIOREDOXIN AS REGULATOR OF OXIDATIVE MODIFICATION OF RAS AND CARDIAC HYPERTROPHY

Because activation of Ras plays a crucial role in hypertrophic signaling in cardiac myocytes, and oxidative modification of Ras thiols may be a mechanism of Ras activation in response to ROS-dependent hypertrophic stimuli, it may be reasoned that TRX would protect Ras thiols and thus exert an antihypertrophic effect in the heart. Yamamoto *et al*. (68) used a cardiac-specific transgenic mouse model overexpressing a dominant negative mutant of human TRX1 (dn-hTRX1) as well as wild-type hTRX1 to test the role of endogenous TRX1 in the regulation of cardiac growth and hypertrophy. They found that dn-hTRX1 mice exhibited a hypertrophic phenotype at baseline and an augmented hypertrophic response after aortic banding, as compared with nontransgenic mice. Conversely, cardiac-specific overexpression of wild-type hTRX1 inhibited pressure-overload–induced hypertrophy. They found increased activation of ERK, Raf-1, and Ras at baseline, and accentuated activation of ERK in response to

aortic banding in dn-hTRX1, as compared with nontransgenic hearts, suggesting that the Ras-Raf-MEK-ERK cascade was involved in mediating the hypertrophic phenotype. Furthermore, dn-hTRX1 was associated with enhanced *S*-thiolation of Ras in COS-7 cells, indicating in principle, that the prohypertrophic effect of dn-hTRX1 at baseline might be linked to increased oxidative modification of Ras thiols.

These *in vivo* results are supported by our observation that TRX1 inhibits Ras thiol oxidation in cardiac myocytes (31). Together, these data suggest that TRX can regulate myocyte hypertrophy in response to ROS-dependent, Ras-mediated hypertrophic stimuli, possibly through a mechanism involving protection of Ras thiols from oxidative modification. Given the multiple actions of TRX, it is important to note, however, that the effect of TRX on hypertrophy might differ depending on the mechanism of hypertrophic signaling. Accordingly, TRX has been shown to exhibit growth-factor–like properties and exert growth-promoting effects in tumor cells (69), vascular smooth muscle cells (49), as well as in the heart (70).

SUMMARY

Posttranslational oxidative protein modifications can be induced by reactive oxygen and nitrogen species. Reversible modifications participate in the regulation of protein function of a variety of structural, functional, and signaling proteins, and thereby mediate redox-sensitive signal transduction. *S*-glutathiolation of cysteine thiols, one of the most frequent and best investigated oxidative modifications, appears to play a major role in the regulation of a variety of cellular processes that regulate cellular structure and function. *S*-glutathiolation of Ras, for example, increases Ras activity and mediates ROS-dependent hypertrophic signaling in cardiomyocytes and vascular smooth muscle cells. Reversibly modified protein thiols can be restored and/or protected by GRX and TRX. For example, GRX and TRX can modulate ROS-dependent, Ras-mediated hypertrophy in cardiomyocytes and vascular smooth muscle cells. The identification of additional target molecules that undergo oxidative modifications and their functional relevance will lead to a better understanding of the complex role of TRX and GRX in cardiac remodeling and may present new therapeutic approaches to the treatment of cardiovascular disease.

ABBREVIATIONS

αAR, α-adrenergic receptor; BIAM, biotinylated iodoacetamide; ERK, extracellular signal–regulated kinase; GRX, glutaredoxin; GSH, glutathione; GADPH, glyceraldehyde 3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; JNK, c-jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MnTMPyP, Mn(III)tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride; NO, nitric oxide; GSSG, oxidized glutathione; $ONOO^-$, peroxynitrate; PI3K, phosphatidyl inositol 3-kinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide anion; TRX, thioredoxin.

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