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Overexpression of glutaredoxin protects cardiomyocytes against nitric oxide-induced apoptosis with suppressing the *S*-nitrosylation of proteins and nuclear translocation of GAPDH

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ABSTRACT

There is increasing evidence demonstrating that glutaredoxin 1 (GRX1), a cytosolic enzyme responsible for the catalysis of protein deglutathionylation, plays distinct roles in inflammation and apoptosis by inducing changes in the cellular redox system. In this study, we investigated whether and how the overexpression of GRX1 protects cardiomyocytes against nitric oxide (NO)-induced apoptosis. Cardiomyocytes (H9c2 cells) were transfected with the expression vector for mouse GRX1 cDNA, and mock-transfected cells were used as a control. Compared with the mock-transfected cells, the GRX1-transfected cells were more resistant to NO-induced apoptosis. Stimulation with NO significantly increased the nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a pro-apoptotic protein, in the mock-transfected cells, but did not change GAPDH localization in the GRX1-transfected cells. Furthermore, we found that NO stimulation clearly induced the oxidative modification of GAPDH in the mock-transfected cells, whereas less modification of GAPDH was observed in the GRX1-transfected cells. These data suggest that the overexpression of GRX1 could protect cardiomyocytes against NO-induced apoptosis, likely through the inhibition of the oxidative modification and the nuclear translocation of GAPDH.

1. Introduction

It is well documented that oxidative stress induces DNA damage and cell apoptosis, thereby contributing to accelerated aging and the development of various diseases [1,2]. In general, living cells use two major systems to maintain their cellular thiol-disulfide redox status under reducing conditions, namely, the thioredoxin (TRX)/thioredoxin reductase (TRXR) system and the glutathione (GSH)/glutaredoxin (GRX) system [3].

GRX proteins, which belong to the TRX superfamily and are also known as thioltransferases [4], help defend cells from oxidative stresses by mediating reversible electron transfers between reduced/oxidized glutathione (GSH/GSSG) and target proteins within cells [4–7]. In mammalian cells, GRX proteins are known to have two dithiol isoforms, GRX1 and GRX2. GRX1 is predominantly localized to the cytoplasm but has also been detected in the nucleus by immunohistochemical studies. Increasing evidence has demonstrated that GRX1 plays distinct roles in inflammation, apoptosis, and tumor progression by inducing changes in the cellular redox system [4,8]. We have previously found that GRX1 protects cells from apoptosis by regulating the redox state of Akt/protein kinase B (Akt) [9]. It has also recently been reported that GRX1 regulates apoptosis in cardiomyocytes via nuclear factor-kappa B (NFκB) [10]. However, the molecular mechanisms used by GRX1 to prevent nitric oxide (NO)-induced apoptosis are not yet fully understood.

S-nitrosylation, a redox-based reaction in which NO is used to convert proteins or low-molecular-weight thiols into *S*-nitrosothiols,

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TRX, thioredoxin; GRX, glutaredoxin; GSH, reduced form of glutathione; GSSG, oxidized glutathione; NO, nitric oxide; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; AMS, 4-acetamide-4′-maleimydylstilbene-2,2′-disulfonic acid; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine.

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has been found to play a role in enzymatic activity [11], the regulation of gene transcription [12], protein nuclear translocation [13], and apoptosis [14]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is well known for its function as a critical compound for glycolysis. It has recently been reported that GAPDH is also involved in responding to oxidative and nitrosative stress and that the nuclear translocation of *S*-nitrosylated GAPDH is implicated in the process of NO-induced apoptosis [15].

In this study, we investigated whether GRX1 plays a role in protecting cells against the NO-induced apoptosis of myocardial H9c2 cells. We also focused on the effect of NO on the oxidative modification and nuclear translocation of GAPDH in these cells.

2. Materials and methods

2.1. Reagents

The anti-GRX1 antibody was purchased from R&D Systems (Minneapolis, MN, USA). The GSH, GSSG, nicotinamide adenine dinucleotide phosphate (NADPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and anti-FLAG M2 mouse monoclonal antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). The anti-GAPDH antibody was purchased from Merck Millipore (Billerica, MA, USA), and the 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) was purchased from Life Technologies (Carlsbad, CA, USA). Rabbit GAPDH and GSSG reductase were purchased from Roche Applied Science (Penzberg, Germany). *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) was purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Cell culture and GRX1 overexpression

H9c2 cells, a clonal line derived from embryonic rat heart cells, were obtained from the American Type Culture Collection (CRL-1446). The overexpression of GRX1 in H9c2 cells was accomplished using previously established methods [9]. Briefly, H9c2 cells were transfected with vectors that constitutively overexpress FLAG-tagged mouse GRX1 protein, using the vector pTRE2Hyg-GRX [9]. We successfully obtained three clones, and one of these clones (H9c2-GRX30) was used in this study. Mock-transfected H9c2 cells were used as a control (H9c2-Vector). H9c2-GRX30 cells had greater thioltransferase activity than either the parent H9c2 cells or the H9c2-Vector cells (data not shown) [9]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) that had been supplemented with 10% fetal calf serum (FCS), 75 μg/ml G418, and 75 μg/ml hygromycin B in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.3. Immunoblot analysis

Cultured cells were harvested and lysed in lysis buffer (20 mM Tris–HCl [pH 7.2], 130 mM NaCl, and 1% Nonidet P-40 that included protease inhibitors [20 μM phenylmethylsulfonyl fluoride, 50 μM pepstatin, and 50 μM leupeptin]). Protein samples were electrophoresed on 10% or 12.5% SDS–polyacrylamide gels (SDS–PAGE) under reducing and non-reducing conditions and then transferred to nitrocellulose membranes using previously established methods [16]. The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS; 10 mM Tris–HCl [pH 7.2] and 150 mM NaCl) and then incubated at 4 °C overnight with the primary antibody in TBS that contained 0.1% Tween 20. The blots were coupled with the peroxidase-conjugated secondary antibodies, washed, and then developed using a commercially available ECL detection kit (GE Healthcare, Little Chalfont, UK) in accordance with the manufacturer's instructions.

2.4. MTT assay

The proliferative activity of cultured cells was evaluated using an MTT assay in accordance with previously established methods [17]. Cells were placed into 96-well plates (3×10^3 in 100 μl of medium per well) and cultured overnight. After treatment with or without SNAP for 24 h, 10 μl of 0.5% MTT solution was added, and the cells were then incubated for an additional 4 h. The reaction was stopped by adding 100 μl of lysis buffer (10 mM HCl and 10% SDS), and the proliferative activity of cells was evaluated by measuring the absorbance at 570 nm using a microplate reader.

2.5. TUNEL assay

Apoptotic cells were first detected using the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) method with the ApopTag Plus In Situ Apoptosis Detection Kit (Merck Millipore) and then measured by flow cytometry (Cytomics FC500, Beckman Coulter, Fullerton, CA, USA) using methods that have been described previously [18].

2.6. Fluorescence microscopy

To identify the intracellular localization of GAPDH, cells were grown on glass coverslips in a six-well culture plate (Nalge Nunc International, Naperville, IL, USA) for 24 h. The cells on the glass coverslips were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2) and permeabilized for 10 min with PBS containing 1% Triton X-100. The cells were then blocked with 3% bovine serum albumin (BSA) in PBS, incubated with an anti-GAPDH antibody for 1 h, and washed with PBS. The primary antibody was subsequently visualized using FITC-conjugated anti-mouse immunoglobulins (DAKO A/S, Glostrup, Denmark). The nuclei were visualized by staining with Hoechst 33342. The distribution of GAPDH in cells was directly observed under fluorescence microscopy (LSM5Pascal, Carl Zeiss, Jena, Germany).

2.7. Determination of redox states

The redox states of proteins were assessed by modifying free thiols with AMS in accordance with previously published methods [9,19]. Briefly, after incubation with or without SNAP, cell lysates or proteins were treated with trichloroacetic acid at a final concentration of 7.5% to denature and precipitate the proteins and to avoid any subsequent redox reactions. The protein precipitates were collected by centrifugation at 12,000*g* for 10 min at 4 °C, washed with acetone twice, and dissolved in a buffer containing 50 mM Tris–HCl, 1% SDS, and 15 mM AMS. The proteins were then separated by 10% SDS-PAGE without using any reducing agents and blotted onto a nitrocellulose membrane. The proteins in the membranes were then visualized by immunoblotting, as described above.

2.8. Protein purification

FLAG-tagged mouse GRX1 was expressed as a glutathione *S*-transferase (GST)-fusion protein in bacterial expression systems (pGEX-6p-1, GE Healthcare) and purified in accordance with previously established methods [9]. In brief, competent *E. coli* strain BL-21 (DE3) cells were transformed with pGEX-GRX, and expression was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. GST-fused GRX (GST-GRX) was affinity purified from cell lysates using glutathione-Sepharose 4B and digested with PreScission Protease. The cleaved GST was removed with glutathione-Sepharose 4B.

3. Results

3.1. GRX1 overexpression protected cells from NO-induced apoptosis

Immunoblot assays confirmed that the expression of GRX1 increased in H9c2-GRX30 cells, a clone of H9c2 cells that constitutively overexpressed mouse GRX1 [9] (Fig. 1A). The expression of GRX1 was immunologically undetectable in either mock-transfected H9c2 cells (H9c2-Vector cells) or untransfected H9c2 cells, although the transcript of GRX1 was detected in the parental H9c2 cells [20].

To examine the effect of GRX1 overexpression in protecting cells against NO-induced stress, H9c2-Vector and H9c2-GRX30 cells were treated with SNAP in the concentrations indicated in Fig. 1. The MTT assay revealed that SNAP dramatically inhibited the proliferative activity of H9c2-Vector cells, even at concentrations as low as 200 μM (Fig. 1B). The proliferative activity was observed to be significantly greater in the H9c2-GRX30 cells than in the H9c2-Vector cells, although SNAP also inhibited the proliferative activity of H9c2-GRX30 cells in a dose-dependent manner. Furthermore, the TUNEL assay demonstrated that after 72-h treatment with 200 μM SNAP, cell apoptosis was clearly induced in H9c2-Vector cells but did not appear to occur in H9c2-GRX30 cells (Fig. 1C).

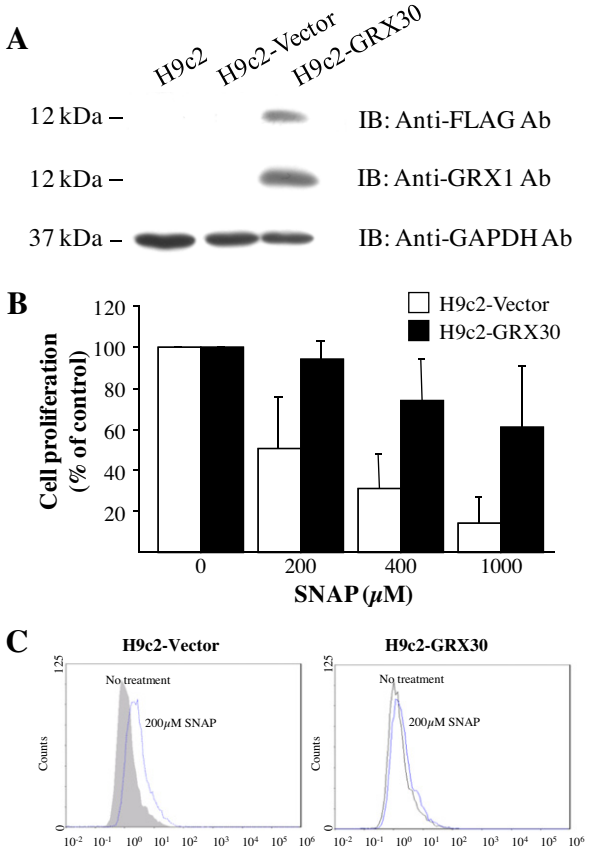

Fig. 1. GRX1 overexpression and NO-induced cell damage in H9c2 cells. (A) The characterization of cells overexpressing the GRX1 gene. (B) Mock- (H9c2-Vector) and GRX1-transfected (H9c2-GRX30) cells were treated with the indicated concentrations of SNAP for 24 h. Cell proliferative activity was then evaluated by an MTT assay, as described in the Section 2. The data represent the mean ± SD from each of three independent experiments. (C) After being treated either with or without 200 μM SNAP for 72 h, apoptotic cells were detected by the TUNEL method and then evaluated by flow cytometry.

3.2. GRX1 overexpression suppressed the NO-induced nuclear translocation of GAPDH

Next, we focused on GAPDH because it plays a functional role in the process of NO-induced apoptosis [15]. As GAPDH is known to translocate to the nucleus during apoptosis [15], we observed the subcellular localization of GAPDH under immunofluorescence microscopy after treating cells with 200 μM SNAP for 24 h. We categorized these cells with respect to their subcellular distribution of GAPDH [21]; in particular, we classified the examined GAPDH distributions as predominantly nuclear (N > C), predominantly cytoplasmic (N < C), or diffuse (N=C) (Fig. 2B). The percentage of cells demonstrating predominantly nuclear GAPDH expression was calculated to evaluate the nuclear translocation. As shown in Fig. 2A, the nuclear expression of GAPDH was more frequently observed in the H9c2-Vector cells than in the H9c2-GRX30 cells, both before and after treatment with SNAP. Interestingly, treatment with SNAP increased the proportion of H9c2-Vector cells that evinced predominantly nuclear GAPDH expression but did not significantly change this ratio in H9c2-GRX30 cells. This result suggests that the resistance of H9c2-GRX30 cells to NO-induced apoptosis might be related to the inhibition of nuclear translocation of GAPDH.

3.3. GRX1 overexpression suppressed the NO-induced oxidative modification of GAPDH

Recent studies have revealed that the TRX system mediates Cys denitrosylation [22,23]. Although GRX1 can reduce molecular disulfide bonds and thereby decrease the *S*-glutathionylation of specific protein thiols [24,25], it is not known whether GRX1 can reverse the *S*-nitrosylation of protein thiols.

To investigate whether GRX1 overexpression affects protein *S*-nitrosylation, we examined the *S*-nitrosylation of proteins by immunoblot analysis using a specific antibody in the cells both before and after the treatment with SNAP. In H9c2-Vector cells, an *S*-nitrosylated protein of 37 kDa was detected and the intensity was slightly increased with SNAP under nonreducing conditions (Fig. 3A, left). The immunoreactive bands were diminished under reducing conditions with 2-mercaptoethanol, suggesting redox-sensitive *S*-nitrosylation of the 37-kDa protein. In contrast, the *S*-nitrosylated protein band was barely observed in H9c2-GRX30 cells, even after treatment with SNAP (Fig. 3A, right). These results indicate that the overexpression of GRX1 suppresses the oxidative modification of proteins in cells under the NO-induced stress conditions.

To further examine the redox state of GAPDH, we utilized AMS alkylation methods [19]. As shown in Fig. 3B, the oxidatively modified form of GAPDH was induced in H9c2-Vector cells in a time-dependent manner after stimulation with 200 μM SNAP. However, this modified form of GAPDH appeared to be either unchanged or reduced in quantity in the H9c2-GRX30 cells at 30 min after SNAP stimulation, and was only very slightly increased at 60 min after SNAP stimulation. These results suggest that GRX1 overexpression suppresses the NO-induced oxidation of GAPDH in H9c2 cells.

3.4. GRX system regulated redox state of GAPDH

To elucidate the involvement of the GRX system in the redox regulation of GAPDH under the NO-induced stress conditions, we examined whether GRX1 could protect GAPDH from NO-induced modifications *in vitro*. For this assay, mouse GRX1 was expressed in bacterial expression systems and purified as described in the Section 2 of this paper. The GRX1 was confirmed to possess thiol-transferase activity (data not shown). Rabbit GAPDH (0.5 μg) was desalted by gel filtration on NAP-5 columns (GE Healthcare) and reduced with 20 mM DTT. After gel filtration, the reduced GAPDH

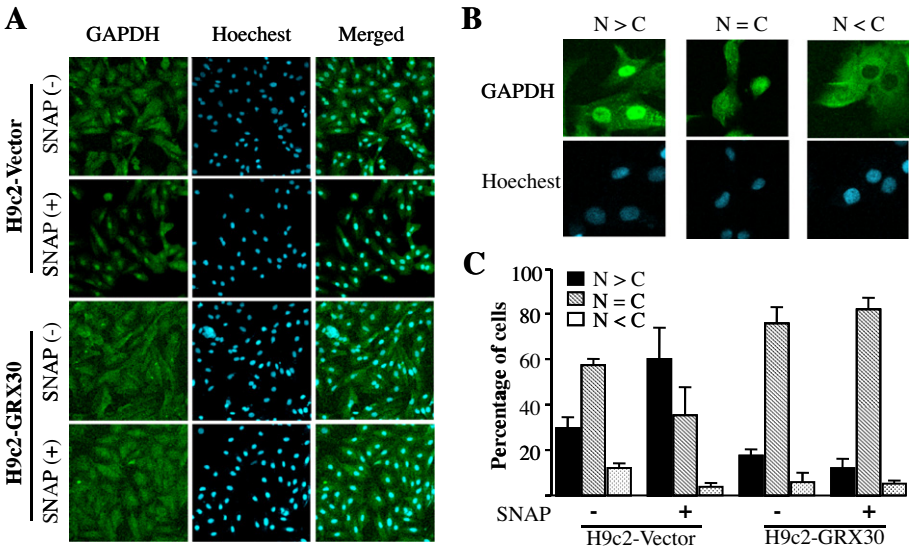

Fig. 2. The immunostaining assay for intracellular GAPDH localization in mock- and GRX1-transfected cells (H9c2-Vector and H9c2-GRX30 cells, respectively) treated with SNAP. The subcellular localization of GAPDH was detected by immunofluorescence microscopy, as described in the Section 2. (A) Representative images of GAPDH expression in cells treated either with or without 200 μM SNAP for 24 h. (B) These example images indicate different patterns of intracellular localization of GAPDH in the cells. N > C: cells with GAPDH predominantly detected in the nuclei. N = C: cells with GAPDH detected non-specifically and evenly throughout cells. N < C: cells with GAPDH predominantly detected in the cytoplasm. (C) More than 100 cells were counted to determine the percentage of cells that express GAPDH with nuclear, diffuse, and cytoplasmic distributions. The graphs represent the mean ± SD for each of three independent experiments.

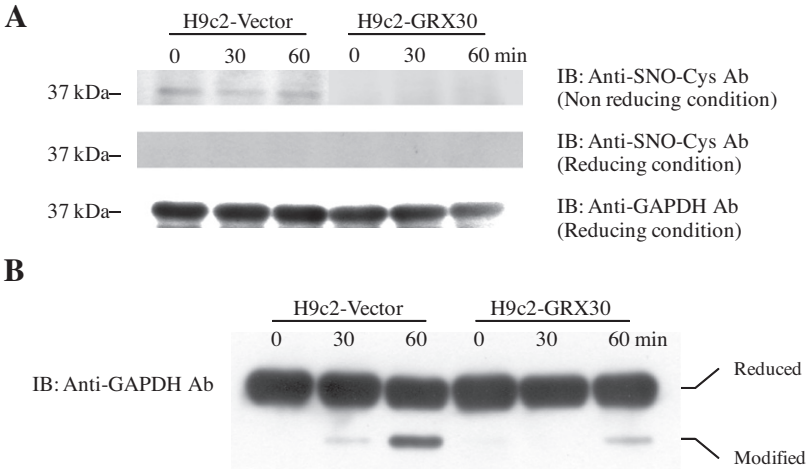

Fig. 3. Immunoblot assay for *S*-nitrosylated proteins and oxidatively modified GAPDH in the cells treated with SNAP. (A) H9c2-Vector and H9c2-GRX30 cells were treated with 200 μM SNAP for the period indicated. Protein samples from the cells were subjected to nonreducing 10% SDS–PAGE and transferred to a nitrocellulose membrane. The *S*-nitrosylated proteins were detected with the anti-SNO-Cys antibody (nonreducing condition). After treatment with 2-mercaptoethanol, the samples were analyzed again as described above (reducing condition). (B) Cells were treated with 200 μM SNAP for the period indicated. Proteins from the cells were denatured and modified with AMS as described in the Section 2. The redox state of GAPDH was assessed based on the mobility shifts of the protein in immunoblot analysis using the anti-GAPDH antibody. The positions of reduced and modified proteins are indicated.

was incubated with 400 μM SNAP for 1 h on ice. After removal of SNAP by another gel filtration, the GAPDH was incubated in a buffer containing the components of the redox system (as indicated in the figure legends) at room temperature. After modification with AMS, the redox state of GAPDH was examined by immunoblot analysis. As shown in Fig. 4, rabbit GAPDH existed in its fully reduced form (lane 1). The GSH-regenerating system (GSH/GSSG, NADPH/GSSG reductase) did not affect the basal redox state of GAPDH (lane 2). Without GRX1, the NO-induced modification of GAPDH was observed even in the presence of GSH/GSSG and NADPH/GSSG reductase (lane 3). By contrast, the NO-induced modification was reduced with GRX1 in a time-dependent manner (lane 4-6). The results of the *in vitro* assay clearly indicate that GRX1 regulates the redox state of GAPDH in concert with GSH/GSSG and NADPH/GSSG reductase.

4. Discussion

In the present study, we have demonstrated that the overexpression of GRX1, an enzyme responsible for maintaining a constant cellular redox state, could effectively protect cardiomyocytes against NO-induced apoptosis, and that this effect would result from the inhibition of both the oxidative modification and nuclear translocation of GAPDH.

Recent studies have found that GAPDH, an enzyme that is generally known for its function in glycolysis and gluconeogenesis,

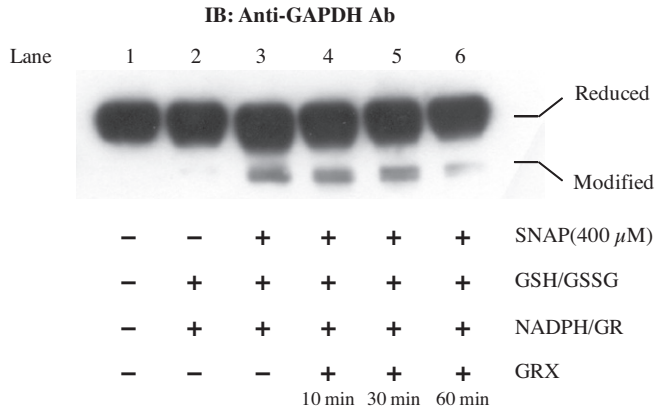

Fig. 4. The redox regulation of GAPDH by the GRX system *in vitro*. The redox state of GAPDH in the presence or absence of SNAP in buffer containing components of the GRX system. Rabbit GAPDH (0.5 μg) was desalted and reduced with 20 mM DTT. After gel filtration, the reduced GAPDH was incubated with 400 μM SNAP for 1 h on ice. After removal of SNAP by another gel filtration, the GAPDH was incubated at room temperature in a buffer containing the components of the GRX system. GSH/GSSG: 1 mM GSH and 0.05 mM GSSG; NADPH/GR, 1 mM NADPH and 1.2 units of GR; GRX, 3.2 μg purified mouse GRX1. The redox state of GAPDH was examined by immunoblot analysis, as described above.

displays many diverse non-glycolytic functions, including nuclear RNA export [26], DNA repair [27,28], membrane fusion [29,30], and microtubule bundling [31]. One of the most intriguing functions of GAPDH is its involvement in the initiation of apoptosis [32,33]. Furthermore, it has been reported that the nuclear translocation of GAPDH is likely related to the *S*-nitrosylation of GAPDH at a catalytic cysteine (Cys150) and the augmentation of its binding to Siah1 [15].

The results of this study were generally in agreement with the findings of these previously published studies, as the nuclear translocation of GAPDH after the treatment with SNAP was observed in H9c2-Vector cells but not in H9c2-GRX30 cells. The results suggest that the overexpression of GRX1 protects H9c2 cells from NO-induced apoptosis, and that this protection is at least partially mediated through the inhibition of the nuclear translocation of GAPDH. However, we could not observe the involvement of Siah1 in the nuclear translocation of GAPDH in the cells treated with NO (data not shown). This lack of evidence for Siah1 involvement might be caused by the difference in the cells used for different studies and could imply that a different nuclear localization signal is used for GAPDH in H9c2 cells. Further investigations are required to clarify the precise mechanism by which GRX1 suppresses the nuclear translocation of GAPDH.

S-nitrosylation, a redox-based reaction that uses NO to convert protein or low-molecular-weight thiols into *S*-nitrosothiols, is known as a common mechanism by which NO modulates diverse cellular functions. GRX can catalyze the reduction of protein disulfide bonds through a disulfide exchange reaction that utilizes the active site Cys-Pro-Tyr-Cys and a dithiol mechanism involving both active site thiols. Furthermore, GRX has a unique ability to reduce the mixed disulfide bonds formed between GSH and either proteins or low-molecular-weight thiols (deglutathionylation) or to participate in the formation of these bonds (glutathionylation) through a monothiol mechanism that requires only the more NH_2-terminal active-site Cys [24,25]. Oxidized GRX is selectively recycled to the reduced form by GSH, which causes the formation of GSSG. The regeneration of GSH occurs through coupling with NADPH and GSSG reductase. GRX partially shares its function as a redox sensor with TRX, which favors intramolecular disulfide substrates and is turned over by NADPH and TRXR.

The modifications of the cysteine thiols of GAPDH that are induced by an NO-donor are hypothesized to be *S*-nitrosylation [15,34], *S*-glutathionylation [35,36] and intermolecular disulfide bond formation [37]. In the present study, the SNAP-induced *S*-nitrosylation of GAPDH might occur in H9c2-Vector cells but not in GRX1-overexpressing H9c2 cells, as detected by immunoblotting using the anti-*S*-nitroso cysteine antibody. In addition, we showed that GRX1 reduced the NO-induced modifications of GAPDH both *in vivo* and *in vitro* (Figs. 3B and 4), which suggests that the GRX system also mediates Cys denitrosylation. Taken together, we have demonstrated that overexpression of GRX1 effectively protects cardiomyocytes from NO-induced apoptosis by regulating the redox state of GAPDH, which provides a novel approach for the prevention and treatment of oxidative-stress-related disorders.

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