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Protection of peroxiredoxin II on oxidative stress-induced cardiomyocyte death and apoptosis

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Abstract

Peroxiredoxin II, a cytosolic isoform of the antioxidant enzyme family, has been implicated in cancer-associated cell death and apoptosis, but its functional role in the heart remains to be elucidated. Interestingly, the expression levels of peroxiredoxin II were decreased in mouse hearts upon ischemia-reperfusion, while they were elevated in two genetically modified hyperdynamic hearts with phospholamban ablation or protein phosphatase 1 inhibitor 1 overexpression. To delineate the functional significance of altered peroxiredoxin II expression, adenoviruses encoding sense or antisense peroxiredoxin II were generated; cardiomyocytes were infected, and then subjected to H₂O₂ treatment to mimic oxidative stress-induced cell death and apoptosis. H₂O₂ stimulation resulted in a significant decrease of endogenous peroxiredoxin II expression, along with reduced cell viability in control cells. However, overexpression of peroxiredoxin II significantly protected from H₂O₂-induced apoptosis and necrosis, while downregulation of this enzyme promoted the detrimental effects of oxidative stress in cardiomyocytes. The beneficial effects of peroxiredoxin II were associated with increased Bcl-2 expression, decreased expression of Bax and attenuated activity of caspases 3, 9 and 12. Furthermore, there were no significant alterations in the expression levels of the other five isoforms of peroxiredoxin, as well as active catalase or glutathione peroxidase-1 after ischemia-reperfusion or H₂O₂ treatment. These findings suggest that peroxiredoxin II may be a unique antioxidant in the cardiac system and may represent a potential target for cardiac protection from oxidative stress-induced injury.

Keywords

peroxiredoxin II; cardiomyocytes; protection; H₂O₂; apoptosis

Introduction

Recent experimental and clinical studies suggest that generation of reactive oxygen species (ROS) or oxidative stress is enhanced in heart failure [47], the leading cause of morbidity and mortality in the United States. ROS are intermediates of the reduction of O_2 to water and include superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2), which can cause the oxidation of membrane phospholipids, proteins and DNA. Growing evidence indicates that ROS play a critical role in many disorders of the cardiovascular system [4,19,46], such as ischemiareperfusion injury, myocardial stunning, apoptosis and arteriosclerosis [36]. Accumulation of ROS in mitochondria can lead to apoptotic cell death [55] and ROS may also have direct effects on cellular structure and function, including myocardial remodeling and failure [27,47]. Under physiological conditions, the toxic effects of ROS can be prevented by such scavenging enzymes as superoxide dismutase (SOD), glutathione peroxidase (GHPx) and catalase, as well as other non-enzymatic antioxidants. However, when the production of ROS becomes excessive, oxidative stress might have a harmful effect on the functional and structural integrity of the heart [40,47].

Recently, redox signaling and peroxiredoxin family enzymes have been suggested to play a role in mediating the antioxidant effects in the heart [11,25,37]. Ablation of peroxiredoxin VI, rendered the heart vulnerable to ischemia-reperfusion [32], while over-expression of the mitochondrial-specific peroxiredoxin III prevented left ventricular remodeling and failure after myocardial infarction in mice [29]. Interestingly, we have found that the expression levels of peroxiredoxin II, another member of the peroxiredoxin family, were increased in the hyperdynamic hearts of two genetically altered mouse models: phospholamban knockout (PLN KO) and protein phosphatase 1 inhibitor 1 overexpression (I-1 OE) [6,34]. Taken together, these studies prompt us to further investigate the functional significance of peroxiredoxin II in the heart.

Peroxiredoxin II is a member of an antioxidant enzyme family (other members are peroxiredoxin I, III, IV, V and VI), which has the ability to reduce H_2O_2 and hydroperoxides into water and alcohol, respectively. Most of the known mammalian peroxiredoxins, except the peroxiredoxin VI isoform, utilize thioredoxin as an immediate electron donor; hence they are known as thioredoxin peroxidases. Peroxiredoxin II is a 25 kDa protein, which has a high affinity for H_2O_2 (K_m for $H_2O_2 \leq 10 \mu M$), similar to GHPx (K_m for H_2O_2 is approximately 1 μM) and is abundant in the cytosol from a wide range of tissues, making it a major regulator of the H_2O_2 signal in the cell, compared to catalase (K_m for H_2O_2 around 1 mM) [49,51]. Peroxiredoxin II knockout mice exhibited hemolytic anemia, indicating that peroxiredoxin II plays a major role in protecting red blood cells from oxidative stress in mice [24]. Peroxiredoxin II deficiency also resulted in increased production of H_2O_2 , enhanced activation of platelet-derived growth factor (PDGF) receptor and phospholipase $C\gamma 1$, subsequently leading to increased cell proliferation and migration in response to PDGF [5]. Overexpression of peroxiredoxin II protected leukemia cells from apoptosis [54], while antisense peroxiredoxin II enhanced radiation-induced cancer cell death [33]. However, the functional significance of this isoform in the heart is not currently known. To determine whether peroxiredoxin II plays a role in cardiac oxidative stress, the levels of this enzyme were altered in isolated cardiomyocytes, which were then subjected to H_2O_2 treatment. Our results provide the first evidence that overexpression of peroxiredoxin II protects cardiomyocytes from oxidative stress-induced cell death and apoptosis, whereas downregulation of peroxiredoxin II greatly impairs these protective effects, suggesting a major role for this isoform in cardiac protection against oxidative stress.

Methods

Mouse models

Phospholamban (PLN) deficient and protein phosphatase 1 inhibitor 1 (I-1) overexpression mice were generated in our lab [26,35]. All experimental procedures and protocols used in this study were reviewed and approved by the University of Cincinnati Institutional animal care and use committee and are in accordance with the National Institutes of Health “*Guide for the care and use of laboratory animals*” (NIH publication No. 85–23, revised 1996).

Global ischemia-reperfusion injury ex vivo

Global ischemia-reperfusion injury was performed in isolated perfused hearts by Langendorff mode, as previously described [14]. After a 20-min equilibration period, the hearts were subjected to 40 min of no-flow global ischemia, followed by 60 min of reperfusion. Hearts were then snap frozen in liquid nitrogen and homogenized in 1× Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) supplemented with 1 mM PMSF and complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) for quantitative immunoblotting.

Isolation and culture of adult rat ventricular myocytes

Adult male Sprague-Dawley rats (8–10 weeks, 250–350 g) were anesthetized by pentobarbital (100 mg/kg) and heparinized (5,000 U/kg). Hearts were quickly removed and the aorta was cannulated by Langendorff mode and perfused with modified Krebs-Henseleit buffer (KHB: 118 mM NaCl, 4.8 mM KCl, 25 mM HEPES, 1.25 mM K_2HPO_4 , 1.25 mM $MgSO_4$, 11 mM Glucose, 5 mM Taurine and 10 mM Butane-dione Monoxime; pH 7.4) for 5–10 min [13]. Subsequently, hearts were perfused with an enzyme solution [KHB containing 0.7 mg/ml collagenase type II (278 U/mg), 0.2 mg/ml hyaluronidase, 0.1% BSA, and 25 μ M Ca] for 15 min. Then, 25 μ M Ca was added to the perfusion buffer, and hearts were perfused for another 5 min. The Ca concentration in the perfusion buffer was raised to 100 μ M and perfusion continued for 5 min. Finally, the hearts became flaccid and left ventricular tissue was excised, minced, pipette dissociated and filtered through a 240 μ m screen. Cells were quickly centrifuged at low speed (400 rpm/min), harvested and resuspended in 25 ml of 100 μ M CaKHB with 1% BSA. The cells were allowed to settle down automatically and Ca concentration was gradually raised to 1.0 mM. Afterwards, the cells were washed two times with DMEM supplemented with ATCC (2 mg/ml BSA, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 100 IU penicillin and 100 μ g/ml streptomycin). Cells were then counted and plated on laminin-precoated dishes.

H₂O₂ treatment and analysis of cell death and apoptosis

After 24 h of adenoviral infection, different doses of H₂O₂ (0, 50, 100 and 200 μ M) were added to cardiomyocytes and incubated for 2 h or for different time periods (0, 0.5, 1, 2, 4 to 8 h) [52,53], as indicated. Cardiomyocytes were then examined for the occurrence of apoptosis by Hoechst staining [13]. Cell viability was calculated by incubating cells with MTT for 2 h [12].

Adenoviral-mediated gene transfer

Peroxisome oxidoreductase II cDNA was purchased from Open Biosystem. Recombinant adenoviruses with sense or antisense of the peroxiredoxin II cDNA were generated, using the pAd.Track-CMV/pAdEasy-1 system [12]. The shuttle vector pAdTrack-CMV and the adenoviral backbone vector pAdEasy-1 were generously provided by Dr. Bert Vogelstein from the Johns Hopkins Oncology Center. Adenoviruses were amplified in HEK293 cells, purified with ViraKit from Virapur and titered according to the standard procedure of AdenoX™ rapid titer kit from Clontech. After 2 h of plating, cardiomyocytes were infected with sense or antisense

peroxiredoxin II adenoviruses (Ad.prxII or Ad.prxIIAS) or control Ad.GFP at a multiplicity of infection (MOI) of 200 for 2 h before the addition of a suitable volume of complete DMEM medium (DMEM containing 2 mg/ml BSA, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 100 IU/ml penicillin and 100 µg/ml streptomycin) [22]. The efficiency of adenoviral gene transfer was evaluated in cultured adult rat myocytes with the use of Ad.GFP. Nearly 100% of myocytes appeared infected at 200 MOI by 24 h. The cell phenotype and morphology remained similar among non-infected and adenoviral-infected groups after 24 h of infection. The myocytes were then treated with 50 µM H₂O₂ for 2 h, washed with PBS and harvested for quantitative immunoblotting, or used in the experiments outlined in the results.

Intracellular TBARS and LDH release

The content of cardiomyocyte thiobarbituric acid substances (TBARS) was determined, as previously described [15]. Cardiomyocyte plasma membrane integrity was assessed by measuring lactate dehydrogenase (LDH) release into the culture medium with an in vitro LDH assay kit (Sigma–Aldrich).

Quantitative immunoblotting

Cultured myocytes were harvested and lysed for 20 min at 4°C in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 0.4 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.5 mM DTT, and 2 µl/ml protease inhibitor cocktail). After 10 min of centrifugation at 10,000g, the supernatants were obtained and used in subsequent experiments. For each protein, equal amounts of samples (5–120 µg) were analyzed by SDS-PAGE. After transfer to membranes, quantitative immunoblotting analysis was performed with the corresponding primary antibodies [peroxiredoxins I to IV from Alexis Biochemicals (Lausen, Switzerland), peroxiredoxins V and VI from Abcam (Cambridge, MA), catalase and GHPx-1 from Santa Cruz Biotechnology (Santa Cruz, CA), Bcl-2 and Bax from Invitrogen (South San Francisco, CA); cleaved caspase 3 (Asp175), caspase 9, and caspase 12 from Cell Signaling (Danvers, MA); and calsequestrin (CSQ) from Affinity Bioreagents (Golden, CO)] at a 1:500 to 1:5,000 dilution. This was followed by incubation with a secondary antibody conjugated with horseradish peroxidase at a 1:5,000 dilution. Visualization was achieved using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) or ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). The intensities of the bands were determined by the AlphaEaseFC™ software. For each protein, the densitometric values from non-treatment controls were arbitrarily converted to 100%, and the values of samples from the other groups were normalized accordingly and expressed as percentile changes. CSQ was used as an internal standard.

Statistics

Data are expressed as mean ± SE. Comparisons between two groups were performed by Student *t* test (from Microsoft Office, Excel), while one-way ANOVA (from GraphPad Prism4) was used for multigroup comparison. Results were considered statistically significant at *P* < 0.05.

Results

Increased expression of peroxiredoxin II in the hyperdynamic hearts of two mouse models

Cardiac proteomics-based analysis of our two models with significantly enhanced cardiac function, the PLN KO and the protein phosphatase 1 inhibitor 1 overexpression (I-1 OE) mice, revealed increases in the levels of peroxiredoxin II [6,34]. Further quantitative immunoblotting showed that the levels of peroxiredoxin II expression were enhanced by 2.5-fold in the PLN-KO and by 2.4-fold in the I-1 OE, compared to age-matched wild types (Fig. 1a and 1b). These

results indicate that peroxiredoxin II, a relatively new antioxidant protein, may play an important functional role in the heart.

Alterations of cardiac peroxiredoxin II expression in ex vivo cardiac ischemia-reperfusion injury

It has been reported that ROS or oxidative stress are significantly increased upon cardiac ischemia-reperfusion injury. To investigate whether peroxiredoxin II expression is also altered, mouse hearts were perfused ex vivo in a Langendorff mode and subjected to 40 min of ischemia followed by 60 min of reperfusion. Interestingly, the peroxiredoxin II levels were significantly decreased to about 65% of pre-ischemic values, upon ischemia-reperfusion (Fig. 1c). These data suggest that decreased expression of peroxiredoxin II may contribute to the cardiac ischemic-reperfusion injury.

Dose-response and time-course of peroxiredoxin II expression upon H₂O₂ treatment of cardiomyocytes in vitro

The alterations of peroxiredoxin II expression in the hearts above promoted us to determine the functional significance of peroxiredoxin II, especially its antioxidant effects. To better understand this notion, H₂O₂ was chosen to treat isolated cardiomyocytes and mimic oxidative stress-induced cardiac cell injury. Briefly, cardiomyocytes were treated with different doses of H₂O₂ (0–200 μ M) for 2 h and the levels of peroxiredoxin II were determined by quantitative immunoblotting. Consistent with our ex vivo ischemia-reperfusion findings (Fig. 1c), there was a H₂O₂ dose-dependent decrease in peroxiredoxin II expression (Fig. 1d). These decreases appeared stable up to 8 h of H₂O₂ treatment. Notably, treatment of cardiomyocytes with 50 μ M H₂O₂ for 2 h resulted in a significant reduction of cell viability, compared to control non-treated myocytes (Fig. 1e). These results indicate that downregulation of peroxiredoxin II in cardiomyocytes may be associated with H₂O₂-induced cell injury.

Peroxiredoxin II overexpression protects myocytes from H₂O₂-induced cell death and apoptosis, while its downregulation eliminates this effect

To determine the role of peroxiredoxin II in cardiomyocyte death induced by H₂O₂, isolated cardiomyocytes were infected with Ad.GFP, Ad.prxII or Ad.prxII-AS for 24 h (Fig. 2a) and the levels of peroxiredoxin II were examined. In the cells infected with Ad.prxII, the level of peroxiredoxin II was increased by 1.5-fold, while this protein was decreased by 70% in the Ad.prxII-AS cells, compared to the Ad.GFP group (Fig. 2b). However, no apparent morphological alterations or differences in the number of adherent cells and rod-shaped cells were observed among the three groups. To determine the effect of peroxiredoxin II overexpression or downregulation on cell death and apoptosis, the infected cardiomyocytes were treated with H₂O₂ and cell viability as well as cell nuclear fragmentation were examined. Upon H₂O₂ treatment, cell viability was significantly decreased to the same extent in both control and Ad.GFP infected cells. More importantly, cell viability was restored upon overexpression of peroxiredoxin II. In contrast, downregulation of peroxiredoxin II significantly reduced the cell survival, compared to control cells (Fig. 2c). Accordingly, cell nuclear fragmentation was significantly increased by ~2.8-fold in both control and Ad.GFP infected cells. Overexpression of peroxiredoxin II reduced cell fragmentation to even lower levels than control or non-treated cells, while downregulation of peroxiredoxin II had opposite effects (Fig. 2d). These findings indicate that overexpression of peroxiredoxin II protects cardiomyocytes from H₂O₂-induced cell death and apoptosis, while downregulation of this protein eliminates the protective effect.

Peroxiredoxin II alters thiobarbituric acid substances (TBARS) and lactate dehydrogenase (LDH) in cardiomyocytes upon H₂O₂ treatment

To further determine the effect of overexpression or downregulation of peroxiredoxin II on cardiomyocyte membrane integrity before and after 50 μ M H₂O₂ treatment, the levels of TBARS were assessed. We found that overexpression or downregulation of peroxiredoxin II did not influence the intracellular TBARS under basal condition (data now shown). However, after H₂O₂ treatment, the levels of TBARS were increased by 14 or 15-fold in the control and Ad.GFP infected myocytes, compared to untreated cardiomyocytes (Fig. 3a). Overexpression of peroxiredoxin II significantly attenuated this increase, while downregulation of peroxiredoxin II resulted in further increases of TBARS (Fig. 3a). Accordingly, evaluation of the levels of lactate dehydrogenase (LDH) release, another parameter in evaluating cell membrane integrity, revealed similar results. Specifically, overexpression or downregulation of peroxiredoxin II did not play a role in LDH release under basal condition (data now shown). However, H₂O₂ treatment increased the LDH levels by threefold in control groups (Fig. 3b), and this effect was attenuated by overexpression of peroxiredoxin II, while downregulation of this enzyme resulted in increased LDH release (Fig. 3b). Thus, increases in peroxiredoxin II expression in cardiomyocytes decrease TBARS and LDH levels, while downregulation of peroxiredoxin II has opposite effects in H₂O₂-treated cardiomyocytes.

Peroxiredoxin II overexpression increases Bcl-2, inhibits Bax and decreases active caspases 3, 9 and 12 expression in cardiomyocytes

To further investigate the possible mechanisms underlying the protection of cell death and apoptosis by peroxiredoxin II overexpression, the expression levels of key apoptotic-related proteins were quantitatively assessed. H₂O₂ treatment was associated with significant decreases in the expression of the anti-apoptotic protein Bcl-2 in non-infected or Ad.GFP infected myocytes. However, overexpression of peroxiredoxin II prevented the Bcl-2 decrease, rather than further increased its levels. Accordingly, downregulation of peroxiredoxin II resulted in augmented decreases of Bcl-2, compared to the control treated groups (Fig. 4a, b). Moreover, the well-known pro-apoptotic proteins Bax, active caspases 3, 9 and 12 were significantly increased upon H₂O₂ treatment (Fig. 4a, b), which were prevented by peroxiredoxin II overexpression. Consequently, reduction in peroxiredoxin II levels had opposite results and promoted further increases in these proapoptotic proteins, compared to controls (Fig. 4a, b). These results suggest that the mechanisms underlying the protection of peroxiredoxin II are associated with increased expression of the anti-apoptotic protein Bcl-2, and decreased expression of the proapoptotic proteins Bax and active caspases 3, 9 and 12.

Expression of the other peroxiredoxin family members, catalase and glutathione peroxidase (GHPx-1)

To further determine the specificity of decreased expression of peroxiredoxin II in the heart upon ischemia-reperfusion injury or in the cardiomyocytes with H₂O₂ treatment, protein levels of the other five peroxiredoxin isoforms, including: peroxiredoxin I, III, IV, V and VI were examined. As shown in Fig. 5a–d, we did not find any significant differences in these proteins, except peroxiredoxin I, which was decreased to 80% in the hearts after ischemia-reperfusion, compared to pre-ischemia-reperfused hearts. Similarly, the peroxiredoxin I expression was downregulated to 72% in H₂O₂-treated cardiomyocytes, compared to controls. In addition, the active forms of two representative cytosolic house-keeping proteins, catalase and GHPx-1, were examined. There was no significant difference in the expression of catalase and GHPx-1 after ischemia-reperfusion injury. In cardiomyocytes treated with H₂O₂, the levels of catalase were not altered, while GHPx-1 levels were significantly depressed (Fig. 5c–d). Although it is not currently clear why the levels of GHPx-1 were not consistently decreased in isolated hearts

or isolated cardiomyocytes, these findings suggest that peroxiredoxin I and GHPx-1 may also play a role in cardiac ischemia-reperfusion or H₂O₂-induced injury.

We further evaluated the role of overexpression or downregulation of peroxiredoxin II on the expression of peroxiredoxin I, catalase and GHPx-1 in cardiomyocytes with or without H₂O₂ treatment. Under basal condition, these proteins' expression showed no significant difference among Ad.GFP, Ad.prxII and Ad.prxII-AS-infected cardiomyocytes (Fig. 6a, b). Furthermore, H₂O₂ treatment did not elicit any significant alterations in the levels of catalase, compared to control cells (Fig. 6a, b). In addition, the decreased expression levels of peroxiredoxin I and GHPx-1 remained the same among the cells infected with Ad.-prxII or Ad.prxII-AS, compared to controls (Fig. 6a, b). These data indicate that overexpression or downregulation of peroxiredoxin II does not alter the expression levels of peroxiredoxin I, catalase and GHPx-1 before and after H₂O₂ treatment, suggesting a specific effect of peroxiredoxin II in mediating oxidative-stress-induced cell injury.

Discussion

The present study provides the first evidence that peroxiredoxin II may play an important protective role in cardiac oxidative stress. Increases in the levels of peroxiredoxin II protected the cardiomyocytes against oxidative stress-induced apoptosis and cell death by H₂O₂, the major source of ROS in cardiomyocytes under stress conditions [23]. These beneficial effects of peroxiredoxin II in cardiomyocytes were associated with increases in the anti-apoptotic protein Bcl-2 and decreases in the pro-apoptotic protein Bax expression, as well as attenuation of the activity of active caspases 3, 9 and 12. Figure 7 summarizes the possible effects of peroxiredoxin II in oxidative-stress-induced cardiac injury. Consistent with our findings, overexpression of peroxiredoxin I and peroxiredoxin II protected thyroid cells from H₂O₂-induced apoptosis, which was also associated with reduced Bax levels [23]. Furthermore, overexpression of human peroxiredoxin II in Molt-4 leukemia cells inhibited release of cytochrome c from mitochondria to cytosol and lipid peroxidation, therefore protecting the cells from apoptosis in a similar manner as Bcl-2 [54]. Interestingly, peroxiredoxin II could also prevent H₂O₂ accumulation in these leukemic cells, suggesting that it may function upstream of Bcl-2 [54].

To further investigate the specificity of the loss of peroxiredoxin II during cardiac ischemia-reperfusion injury and in response to H₂O₂, we examined the expression levels of the other 5 isoforms of the peroxiredoxin family, as well as two representative cytosolic house keeping proteins: catalase and GHPx-1. Consistent with other previous reports [29,32,50], there were no significant differences in the expression levels of most of these proteins. Furthermore, overexpression or downregulation of peroxiredoxin II did not change the expression of the cytosolic house keeping proteins, catalase and GHPx-1, with or without oxidative stress stimulation. Interestingly, the expression levels of peroxiredoxin I were found to be significantly decreased after ischemia-reperfusion injury and after H₂O₂ treatment, indicating the potential effect of this protein involved in the process of oxidative stress-related cardiac injury. However, peroxiredoxin II overexpression or downregulation did not further influence the expression of peroxiredoxin I. These findings indicate that the antioxidant properties of peroxiredoxin II in the heart might be independent of other antioxidant enzymes, suggesting a unique effect of peroxiredoxin II in the process of cardiac ischemia-reperfusion injury or H₂O₂-induced cell death.

The decreased expression of peroxiredoxin II found in the hearts of ischemia-reperfusion injury or cardiomyocytes after H₂O₂ treatment may involve leakage of this enzyme into the extracellular fluid and then elimination during reperfusion of the hearts or culturing of the cells [49]. Although the exact mechanisms and interactions among various antioxidants are not fully

understood, it is possible that one anti-oxidant may equilibrate with another to establish a cellular redox potential and that all endogenous antioxidants may work in conjunction with one another to protect against oxidative stress [8]. It is also possible that certain antioxidants act as the first line of defense against oxidative stress in ischemiareperfusion, while other antioxidants may only act later during severe oxidative stress [8]. It is unclear if the loss of peroxiredoxin II is based on its oxidation by H_2O_2 or excessive ROS and subsequent degradation by the proteasome. However, it has been reported that silica, one of the fibrogenic agents, induces rapid degradation of peroxiredoxin I and peroxiredoxin II in Rat2 cells along with significant production of ROS in the cells. The degradation of peroxiredoxin enzymes was insensitive to the proteasome inhibitors: MG132 and lactacystin [43]. Another report showed opposite results, as cardiomyocytes treated with MG132 exhibited induced antioxidant protein expression, including peroxiredoxin I and SOD-1 [10]. Although experimental studies with proteasome inhibitor strongly suggest the role of proteasomes in the process of ischemia-reperfusion injury [1,2,16,39], there are still some controversial reports. One study has shown that reperfusion of isolated hearts with MG132 improves posthypoxic function of excised isolated papillary muscles [45]. However, this occurred after a prehypoxic delay of at least 30 minutes with demonstrable increases in heat shock proteins, but no determination of myocardial proteasome activity. The same group also showed that incubation of vascular smooth muscle cells with low concentrations of proteasome inhibitors results in upregulation of proteasome subunit transcription and translation [30], indicating the possibility that the previous results were related to increases in proteasome activity. Conversely, it was reported that preischemic treatment of isolated rat hearts with MG132 results in a dose-dependent decrease in postischemic function, but increased levels of ubiquitinated proteins [38]. In addition, pretreatment with the more specific inhibitor, lactacystin, according to a protocol that decreased preischemic proteasome activity by 40%, failed to have any effects on postischemic function [9]. Taken together, these studies indicate the complicated regulation for cardiac ischemia-reperfusion injury with multiple factors involved. Furthermore, several studies have demonstrated the complex modification of cardiac peroxiredoxins during oxidative stress. Schroder et al. [41] reported that H_2O_2 induces an array of changes in the myocardium, including formation of disulfide bonds that were intermolecular for peroxiredoxin I, II and III, but intramolecular within peroxiredoxin V. Peroxiredoxin oxidation was also associated with movement from the cytosol to the membrane and myofilament-enriched fraction. Jang et al. [21] suggested that two cytosolic yeast peroxiredoxins: peroxiredoxin I and peroxiredoxin II, which display diversity in structure and apparent molecular weight (MW), act alternatively as peroxidases and molecular chaperones. The peroxidase function predominates in the lower MW forms, whereas the chaperone function predominates in the higher MW complexes. Oxidative stress and heat shock exposure of yeast cause the protein structures of cytosolic peroxiredoxin I and II to shift from low MW species to high MW complexes. Moon et al. [31] also indicated that human peroxiredoxin II assumes a high MW complex structure, which has a highly efficient chaperone function upon exposure to oxidative stress. However, the subsequent removal of stressors induces the dissociation of this protein structure into low MW proteins and triggers a chaperone-to-peroxidase functional switch. These studies indicate a complicated intracellular molecular interaction, which needs to be further investigated.

It has been reported that short term exposure of cardiomyocytes to H_2O_2 results in cardiomyocyte death due to necrosis as well as apoptosis [28]. Cardiomyocyte necrosis induced by H_2O_2 is associated with cell membrane phospholipid peroxidation by ROS. Hydroxyl radicals generated from the decomposition of H_2O_2 through the iron dependent Fenton reaction initiate lipid peroxidation by reacting with methylene groups of polyunsaturated fatty acids [3]. Our results suggest that hydroxyl radical-mediated lipid peroxidation and LDH release, may contribute to H_2O_2 -induced cell necrosis. Increases in peroxiredoxin II appear to prevent cell necrosis by attenuating TBARS and LDH release, while reduction in peroxiredoxin II levels have opposite effects. The anti-apoptotic effects of peroxiredoxin II overexpression were

associated with increases in Bcl-2 and preserved Bax levels after H₂O₂ treatment, leading to inhibition of caspases 3 and 9 activation (Fig. 7). Consequently, there was reduced cell death and apoptosis, suggesting that the cardioprotective effects of peroxiredoxin II are at least partially associated with the mitochondrial signaling pathway. Furthermore, caspase 12 was inhibited in peroxiredoxin II-overexpressing cardiomyocytes, indicating that protection was also associated with reduced H₂O₂-triggered ER/SR stress [20] (Fig. 7).

The in vivo role of peroxiredoxin II in oxidative stress-induced cardiomyocyte death and apoptosis is currently unclear. Neonatal cardiomyocytes exposed to H₂O₂ for 2 hours exhibited increased transcripts of peroxiredoxin II and V, which shifted to lower pI values upon two-dimensional gel electrophoresis, suggesting complex regulation of these two enzymatic activities in cardiomyocytes [7]. It could be hypothesized that overexpression of peroxiredoxin II in the hyperdynamic PLN KO and protein phosphatase 1 inhibitor-1 overexpressing (I-1 OE) hearts may serve as an important cardioprotective mechanism, allowing for normal life-span in these models [44]. Interestingly, in vitro studies have shown that cotransfection of PLN and HAX-1, another anti-apoptotic protein, in HEK293 cells enhances protection from hypoxia-reoxygenation-induced cell death and apoptosis [48], which suggest a potential involvement of PLN in cardiomyocyte apoptosis. However, the potential association between peroxiredoxin II and PLN in the cardiac anti-apoptotic pathways needs to be further examined.

A major consequence of increased cardiac metabolism associated with PLN KO and I-1 OE mice is the significant increase in formation of oxidative radicals at the level of the cellular NAD(P)H oxidases in the mitochondrial electron transport chain [18], which represents a major source of free radical generation in the cell. The increased expression of peroxiredoxin II (the cytosolic form of the peroxiredoxin family) in these hyperdynamic mouse hearts may represent an essential adaptation to neutralize oxidative stress generated by increased oxide-reduction activity. This energetic adaptation may also be important in heart failure, where multiple anatomic and molecular factors converge to increase the net energetic demands on the heart. Growing evidence suggests an important role for increased oxidative stress in adverse left ventricular remodeling after myocardial infarction [17]. It has been shown that various antioxidant approaches (vitamin C, vitamin E, probucol, dimethylthiourea or genetic manipulation) can ameliorate this adverse remodeling. The beneficial effects of these experimental approaches extend to improved contractile function, reduced left ventricular dilatation and lower mortality, especially in a short term. However, long term effects of these treatments seem disappointing [42]. A better understanding of the precise roles of oxidative stress and redox signaling pathways may therefore provide the basis for new therapeutic strategies [42]. The protective effects of peroxiredoxin II from oxidative stress-induced cell injury observed in this study may yield new insights in the treatment of cardiac oxidative stress.

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Fig. 1.

Alterations of peroxiredoxin II expression in the hearts and isolated cardiomyocytes as well as cell viability upon H₂O₂ treatment. Hearts from phospholamban deficient (**a**: PLN KO) and protein phosphatase 1 inhibitor-1 overexpression (**b**: I-1 OE) mice were homogenized and processed for quantitative immunoblotting for the expression of peroxiredoxin II. **c** Wild type hearts were subjected to ex vivo Langendorff perfusion, consisting of 40 min ischemia (pre I/R) followed by 60 min reperfusion (post I/R) and the levels of peroxiredoxin II were determined; $n = 6$ hearts for each group. Values are mean $\pm$ SE, $*P < 0.05$, compared to pre I/R or wild type values. **d** Quantitative immunoblotting and relative expressions of peroxiredoxin II (prxII) in cultured cardiomyocytes (24 h) in response to treatment with various H₂O₂ doses for 2 h. Calsequestrin was used as a loading control ($n = 7$ hearts for each group). **e** Cardiomyocyte viability was analyzed by MTT assay after H₂O₂ (50 μ M) treatment for 2 h; $n = 6$ hearts for each group. Values are mean $\pm$ SE, $*P < 0.05$, compared to control.

Fig. 2.

Effects of peroxiredoxin II (PrxII) overexpression and downregulation on cardiomyocyte viability and nuclear fragmentation upon H₂O₂ treatment. **a** Cardiomyocytes infected with adenoviruses: Ad.GFP, Ad.prxII (sense) or Ad.prxII-AS (antisense), under light ($\times 40$, *left panel*) and fluorescence ($\times 40$, *right panel*) microscope. **b** Quantitative immunoblotting of peroxiredoxin II expression levels in infected cardiomyocytes. Calsequestrin was used as a loading control; $n = 8$ hearts for each group. Values are mean $\pm$ SE, $*P < 0.05$, Vs. Ad.GFP group. Cardiomyocytes were treated with H₂O₂ (50 μ M) for 2 h after 24 h viral infection, then **(c)** MTT assay was used to analyze the cell viability, and **(d)** cell nuclear fragmentation was determined by Hoechst staining. $n = 4$ hearts for each group. Values are mean $\pm$ SE, $*P < 0.05$, Vs. control (control without H₂O₂ treatment). ^Δ $P < 0.05$, Vs. Ad.GFP

Fig. 3.

Effects of peroxiredoxin II (prxII) overexpression and downregulation on intracellular lipid peroxidation (TBARS) and lactate dehydrogenase (LDH) release upon H₂O₂ treatment. Cardiomyocytes were isolated, infected with Ad.GFP, Ad.prxII and Ad.prxII-AS for 24 h, and then treated with 50 μ M H₂O₂ for 2 h. Cells were harvested, intracellular lipid peroxidation, TBARS (**a**) and LDH release (**b**) were determined; $n = 4$ hearts for each group. Values are mean $\pm$ SE, * $P < 0.05$, Vs. control (control without H₂O₂ treatment), ^ $P < 0.05$, Vs. Ad.GFP

Fig. 4.

Effects of peroxiredoxin II (prxII) overexpression and downregulation on apoptosis-related proteins in cardiomyocytes after H₂O₂ treatment. Cardiomyocytes were isolated, infected with Ad. GFP, Ad.prxII and Ad.prxII-AS for 24 h, and then treated with 50 μ M H₂O₂ for 2 h. Cells were harvested, lysed and processed for quantitative immunoblotting to determine the expression levels of Bcl-2, Bax, active caspase 3, active caspase 9 and active caspase 12. **a** Representative immunoblots for Bcl-2, Bax, active caspases 3, 9 and 12. **b** Quantitation of these proteins; $n = 4-6$ hearts for each group. Values are mean $\pm$ SE, * $P < 0.05$, Vs. control (control without H₂O₂ treatment), ^ $P < 0.05$, Vs. Ad.GFP

Fig. 5.

Effect of cardiac ischemia-reperfusion injury and H₂O₂ stimulation on the expression of peroxiredoxin family members, catalase and glutathione peroxidase (GHPx-1). **a** and **b** WT hearts were subjected to ex vivo Langendorff perfusion, as described in Fig. 1. The levels of the other five peroxiredoxin family members, including peroxiredoxin I, III, IV, V and VI, as well as two representative cytosolic house keeping proteins: catalase and GHPx-1 were determined. **c** and **d** Cardiomyocytes were isolated and cultured for 24 h, then treated with 50 μ M H₂O₂ for 2 h. Cells were harvested, lysed and processed for western blot to examine the expression levels of the same proteins as those in (**a**) and (**b**); $n = 4$ hearts for each group, Values are mean $\pm$ SE, * $P \leq 0.05$, Vs. per I/R or control cells

Fig. 6.

Effect of overexpression or downregulation of peroxiredoxin II on the expression levels of catalase, GHPx-1 and peroxiredoxin I. Cardiomyocytes were isolated, infected with Ad.GFP, Ad.prxII and Ad.prxII-AS for 24 h, and then some of the cells were treated with 50 μ M H₂O₂ for 2 h. Cells were then harvested, lysed and processed for western blot to examine the expression levels of catalase, GHPx-1 and peroxiredoxin I. **a** Representative immunoblots and **b** quantitation of the expressions of catalase, GHPx-1 and peroxiredoxin I; $n = 4$ hearts for each group. Values are mean $\pm$ SE

Fig. 7.
Proposed scheme for the effects of peroxiredoxin II (prx II) in H_2O_2 or oxidative stress-induced myocyte apoptosis and necrosis