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Nitric oxide preserves XIAP and reduces hypoxia/reoxygenation-induced cardiomyocytes apoptosis via ERK1/2 activation

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ABSTRACT

The signaling pathways that control the hypoxia/reoxygenation (H/R)-induced cardiomyocyte apoptosis have not been fully defined. In this study, we investigated whether extracellular signal-regulated kinase1/2 (ERK1/2) plays a role in NO's anti-apoptotic effect against H/R injury. Primary cultures of adult rat ventricular myocytes (ARVMs) were exposed to 3 h of hypoxia followed by 30, 60, 90 and 120 min of reoxygenation in presence of a vehicle, NO donor (GSNO, 50 μmol/L) and inhibitors of ERK1/2 (PD98059, 10 μmol/L). GSNO protected the cardiomyocyte from reoxygenation injury, as evidenced by decreased apoptosis, and this protective effect was inhibited by co-treatment with PD98059 during reoxygenation. Consistent with this, when administered with adenoviral vector encoding dominant negative ERK (Ad-dnERK), GSNO's effect was also blocked. Western blotting revealed that GSNO increased the ERK phosphorylation during reoxygenation. Furthermore, H/R-induced activation of caspase-3 and -9 were attenuated by GSNO. Interestingly, X-linked inhibitor of apoptosis protein (XIAP) protein levels decreased in myocytes subjected to reoxygenation, and ERK phosphorylation can improve XIAP expression, which involved inhibiting caspase-3, -7 and -9 activities. Overexpression experiment with adenoviral vector containing constitutively active ERK (Ad-caERK) alone acquired protection against apoptosis triggered by H/R injury and positively regulated XIAP expression compared with control adenovirus (Ad-LacZ). Our data demonstrated that, GSNO's antiapoptotic effect against reoxygenation injury involves ERK signaling pathway. The activation of ERK increased XIAP expression and led to decreased caspase activation.

1. Introduction

Apoptosis is thought to play a pivotal role in the progressive loss of cardiomyocytes and has been linked specifically to H/R induced myocardial injury [1]. However, the pathways involved in the H/R cardiomyocyte apoptosis remain largely unknown. Caspases play a central role in apoptosis, and classically, two pathways lead to their activation. Oligomerisation of transmembrane death receptors such as Fas or Tumor Necrosis Factor Receptor activate procaspase-8 through well characterized receptor complexes [2]. The other major pathway is non-receptor mediated and integrated by the mitochondrion [3]. Precaspase-9 released from the mitochondria is activated via interactions with Apaf-1 and cytochrome c in the presence of dATP. Once activated, the apical or regulator caspases caspase-8 or -9 cleave effector caspase-3, -6, or -7 to initiate caspase cascades that result in cleavage of a broad spectrum of cellular targets leading to apoptosis [4].

Caspase activity is blocked by the inhibitor of apoptosis protein (IAP) class of proteins which function by directly binding and inactivating processed effector and initiator caspases including caspase-3, -7, and -9. Thus, the IAP proteins play a key role in cell survival by modulating death-signaling pathways at a post-mitochondrial level. The IAP family is composed of XIAP, cIAP-1, cIAP-2, NIAP, Survivin and Bruce. They have the common structural baculoviral IAP repeat (BIR) domains, which serve as scaffolds for protein interactions, recruit caspases and inhibit their activity [5,6]. XIAP, the most potent suppressor of apoptosis among IAP family, contains three BIR domains, followed by a ubiquitin binding domain and a C-terminal RING finger domain which becomes highly ubiquitinated upon the induction of apoptosis, in part due to auto-ubiquitination, resulting in the rapid degradation [6–8]. XIAP activity is regulated by the SMAC/DIABLO and OMI/HtrA2 protein [6,9]. Recently, XIAP has been implicated in protecting cardiomyocytes from various apoptosis/stress stimuli [10,11]. However, the effects of XIAP in the heart H/R injury have not yet been well characterized.

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Nitric oxide (NO), a ubiquitous molecule, is one of the most important cardiovascular signaling molecules, with multiple regulatory effects on myocardial and vascular tissue as well as on other tissues and organ systems [12]. NO-donor drugs such as nitroglycerine and S-Nitrosoglutathione (GSNO) decompose spontaneously in the body, by a variety of mechanisms, to generate NO. Such drugs have been routinely used for many years in cardiovascular therapeutics. Previous studies showed that treatment with a NO donor significantly reduced post-ischemic myocardial apoptosis in vivo [13,14]. Although the role of NO in cardiomyocyte survival has already been investigated, the precise signaling pathways mediating the anti-apoptosis and in particular, the effect of several NO targets on these processes have not yet been established.

ERKs, one of the subfamily of mitogen-activated protein kinases (MAPKs), are most ideal candidates among the protein kinases that determine the cellular transcriptional activities, cell proliferation, differentiation, and cell survival [15]. ERK1/2 cascade has been shown to be involved in various cardiovascular disease and plays a critical role in myocardial protection against H/R injury [15]. Activation of ERK1/2 may result in phosphorylation and modulation of apoptosis related proteins such as Bad, Bcl-2, Bcl-xl in a variety of cell types, thus exerting its anti-apoptotic effect in a pre-mitochondrial manner [16,17]. However, the findings suggest that in human neutrophils, ERK activation protects cells against apoptotic cell death at a post-mitochondrial level, although the effectors involved have not been identified [18]. In this report, we demonstrate that H/R induced cardiomyocyte apoptosis is associated with reduction of the anti-apoptotic protein XIAP, which was attenuated by NO via activation of ERK1/2.

2. Materials and methods

2.1. Materials

Primary antibodies raised against ERK1/2 and phospho-ERK1/2 were purchased from Cell Signaling; anti-XIAP antibodies and S-Nitrosoglutathione (GSNO), Santa Cruz; Horseradish peroxidase-conjugated anti-rabbit and anti-goat IgG, Millipore; PD98059, Sigma, and caspase-3 and -9 colorimetric assay kit were from K&D system.

2.2. Isolation of ARVMs and cell culture

ARVMs were isolated from hearts of male Sprague–Dawley rats (300–350 g) as described previously [19]. Cells were plated in 35-mm dishes or glass cover slips precoated with laminin (1 μg/cm^2) at a density of 1×10^4/cm^2 in medium 199 supplemented with L-carnitine (2 mmol/L), *N*-2-mercaptopropionyl glycine (5 mmol/L), taurine (5 mmol/L), insulin (0.1 μmol/L), 2% FBS, and penicillin–streptomycin (100 IU/mL). Cells were maintained in tissue culture incubators at 37 °C under 5% CO_2 atmosphere.

2.3. Hypoxia-reoxygenation

Normal cell culture medium was replaced with N_2-saturated medium followed by hypoxia induced with a modular chamber (Modular Incubator; Billups-Rothenberg) perfused with 95% N_2/5% CO_2. The chamber was sealed and placed at 37 °C for indicated time periods for hypoxia. After hypoxia, cells were removed from the chamber and maintained in the regular incubator for reoxygenation periods. Control cultures were incubated under normoxic conditions for equivalent durations. Experimental samples were divided into three major groups, as follows: (1) normoxic control (C), (2) hypoxia alone (H/H), and (3) reoxygenation after 3 h of hypoxia (H/R). PD98059 (10 μmol/L) was added before reoxygenation.

2.4. Lactate dehydrogenase release assay

Cytotoxicity was quantified by a standard measurement of lactate dehydrogenase (LDH) release with the use of the LDH assay kit (Roche). The sum of the LDH release in the normoxia control sample was arbitrarily set to 1, and that in H/H or H/R was normalized to this value at the corresponding time point.

2.5. TUNNEL staining

Terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNNEL) was performed in cells plated on glass coverslips with the CardioTACS in situ Apoptosis Detection kit (R&D Systems) according to the manufacturer's instructions. For quantification of apoptosis, at least 400 randomly distributed cells were counted in each experiment, and the number of apoptotic cells was expressed as the percentage of total cell population.

2.6. Measurement of caspase-3 and -9 activity

Caspase activity was evaluated by use of caspase-3 and -9 colorimetric assay kits (R&D Systems) by following the manufacturer's protocol. Briefly, 100 μg total cell protein was added to 50 μl reaction buffer and 5 μl substrates of DEVD-pNA and LEHD-pNA for caspase-3 and -9, respectively. Samples were incubated at 37 °C for 2 h and the enzyme-catalyzed release of pNA was quantified at 405 nm with a SpectraMax-Plus microplate spectrophotometer (Molecular Devices). At each time points of study, the value of H/H or H/R treated samples were normalized to corresponding untreated controls allowing determination of the fold increase in caspase activity.

2.7. Western blot analysis

ARVMs were harvested in lysis buffer (Cell Signaling), and protein concentration was determined with the Bradford assay (Bio-Rad). Equal amounts of proteins were separated on SDS–PAGE and transferred to polyvinylidene difluoride membrane (Millipore) with a semidry transfer system (Bio-Rad). The membranes were probed with antibodies against phospho-ERK1/2 and XIAP, and β-actin. Immune complexes were visualized by ECL detection (Amersham) and relative of phospho-ERK1/2 was quantified by densitometry (ImageJ). All immunoblots were stripped in strip buffer (Piece) and then re-probed with anti-β-actin to confirm equal protein loading.

2.8. Generation of recombinant adenovirus and adenovirus infection

Adenovirus vector harboring dominant negative ERK (Ad-dnERK), constitutively active ERK (Ad-caERK) and β-galactosidase (Ad-LacZ) were constructed using AdenoX adenovirus construction kit (BD-Clontech). Recombinant plaques were isolated and propagated in 293 cells, and transgene expression and appropriate kinase activity were verified in cardiomyocytes. Viral titer was determined by plaque assay in 293 cells. Cardiomyocytes were transfected with adenoviral vectors at a multiplicity of infection (MOI) of 25–200.

2.9. Statistical analysis

The results were expressed as mean ± SEM from at least three independent experiments. Statistical analysis was performed with ANOVA followed by Bonferroni multiple-comparison test. Student's *t*-test was undertaken to compare the results between the

two groups. Values of $P < 0.05$ were considered as to be statistically significant.

3. Results

3.1. The deleterious effect of reoxygenation in our H/R model

We first sought to demonstrate that reoxygenation of hypoxic myocytes results in injury in our model. The cells were exposed to severe hypoxia alone (H/H) or to hypoxia followed by reoxygenation (H/R), and the supernatant was analyzed for LDH activity. As shown in Fig. 1, LDH releases from cells exposed to H/H or H/R were significantly higher than that from normoxic condition at each time points ($P < 0.05$). Cells that were exposed to H/H showed there was no significant increase in the release of LDH into the culture medium from 3 to 5 h. In contrast, when cardiomyocytes cells were exposed to 3 h of hypoxia followed by varying times of reoxygenation, cytotoxicity increased steadily over time. However, a much greater cumulative release of LDH occurred in reoxygenated cells (9.7-folds vs. control at 3 h hypoxia and 2 h reoxygenation) than in hypoxic cells alone (3.2-fold vs. control at 5 h). Taken together, these data indicate that in this model, myocytes injury occurred primarily during reoxygenation.

3.2. NO-donor GSNO during reoxygenation attenuates myocyte apoptosis

In the second series of isolated cell experiments, apoptosis was assessed by TUNNEL (Fig. 2A). After 5 h hypoxia alone, no significant increase in the percentage of cells with TUNNEL-positive nuclei. It was 5.2 ± 1.4%, 6.0 ± 1.5%, 6.1 ± 1.7%, 6.8 ± 2.1% and 6.7 ± 1.2% at 3, 3.5, 4, 4.5 and 5 h respectively (bottom line, Fig. 2A). However, the number of TUNNEL-positive cells under vehicle condition increased gradually during reoxygenation period of H/R and was up to 34.3 ± 3.7% at 2 h reoxygenation (top line, Fig. 2A). This value was significantly reduced to 18 ± 2.5% at 2 h reoxygenation after hypoxia in cells incubated with GSNO during reoxygenation (middle line, Fig. 2A).

3.3. Antiapoptotic effect of GSNO during reoxygenation requires phosphorylation of ERK

To investigate whether ERK was involved in NO signaling, we examined the activity of ERK during reoxygenation. As shown in Fig. 2B and C, western blot revealed that GSNO abruptly increased phosphorylation of ERK in ARVMs during reoxygenation (reoxygenation 30 min, 2.92 ± 0.23-fold over hypoxia alone, $P < 0.05$; Reoxygenation 60 min, 3.34 ± 0.23-fold over hypoxia alone, $P < 0.05$).

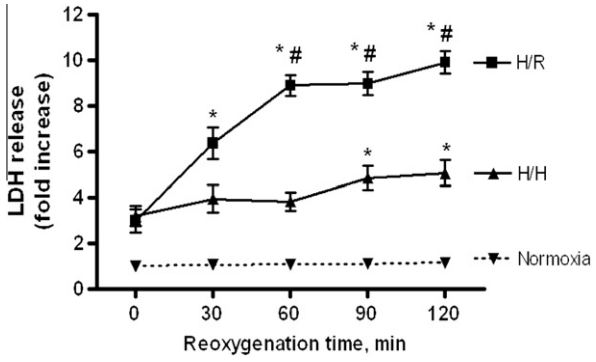

Fig. 1. Effect of H/R on LDH release. For the H/R group, cells were exposed to hypoxia for 3 h and then reoxygenated for indicated time, respectively. Samples were obtained at 0, 30, 60, 90 and 120 min after reoxygenation; For the H/H group, cells were exposed to hypoxia continuously for 5 h, and LDH release was measured at 3 h 30 min, 4 h, 4 h 30 min and 5 h (corresponding to each reoxygenation time point in H/R group). Compared to the H/H group and the normoxia group, LDH release was significantly greater in the H/R group ($^{*}P < 0.05$, vs. normoxia group; $^{\#}P < 0.05$, vs. H/H group. Data from three separate experiments are expressed as mean ± SEM).

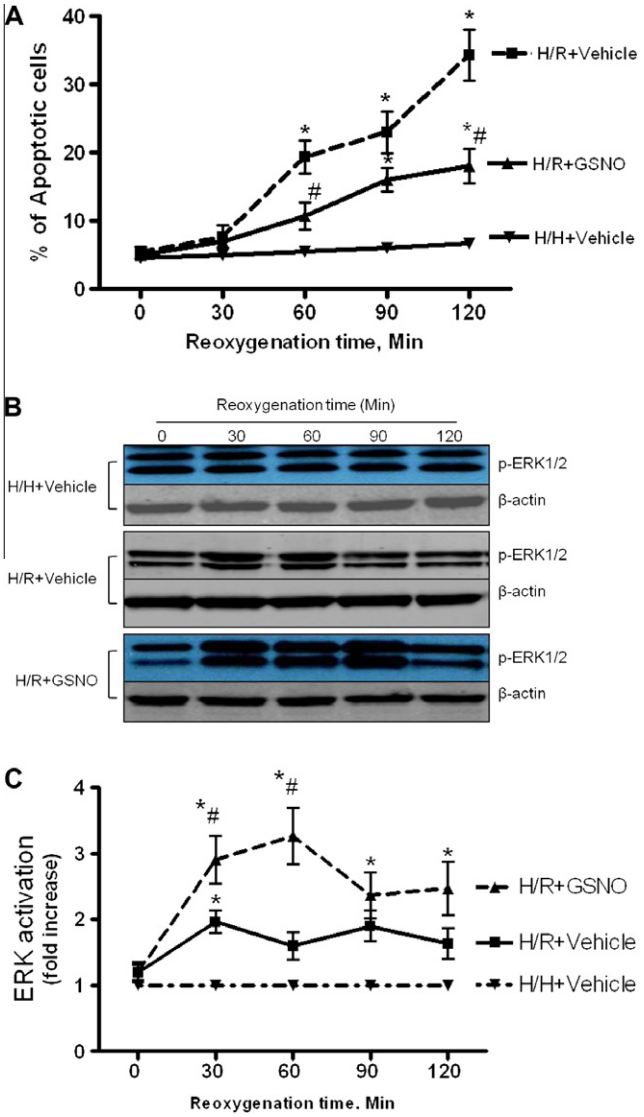

Fig. 2. Effect of H/R and GSNO on cardiomyocyte apoptosis as well as ERK phosphorylation. (A) Quantitation of myocytes undergoing apoptosis under indicated conditions, detected by TUNNEL staining. Compared to continuous hypoxia, H/R caused a marked increase in myocyte apoptosis after 2-h reoxygenation; Co-treatment with GSNO (20 μmol/L) during reoxygenation significantly attenuated the apoptosis process ($^{*}P < 0.05$ vs. H/H + vehicle, $^{\#}P < 0.05$ vs. H/R + vehicle). (B) Representative western blot showing levels of ERK1/2 phosphorylation under indicated conditions. β-Actin was used to ensure equal protein loading. Incubation of myocytes with GSNO during reoxygenation increased phosphorylation of ERK. (C) Densitometry analysis of p-ERK1/2 with NIH ImageJ. Data from four independent experiments were expressed as fold changes over the hypoxia alone (H/H), $^{*}P < 0.05$ vs. H/H, $^{\#}P < 0.05$ vs. H/R + vehicle, at respective time points.

3.4. ERK inhibitor abolished GSNO protection effect on reoxygenation injury

To investigate whether ERK was involved in NO signaling, we examined the role of ERK inhibitor PD98059 in cardiomyocyte apoptosis. As shown in Figs. 2A and 3A, PD98059 blocked GSNO-induced improvement in TUNNEL-positive ARVMs. Moreover,

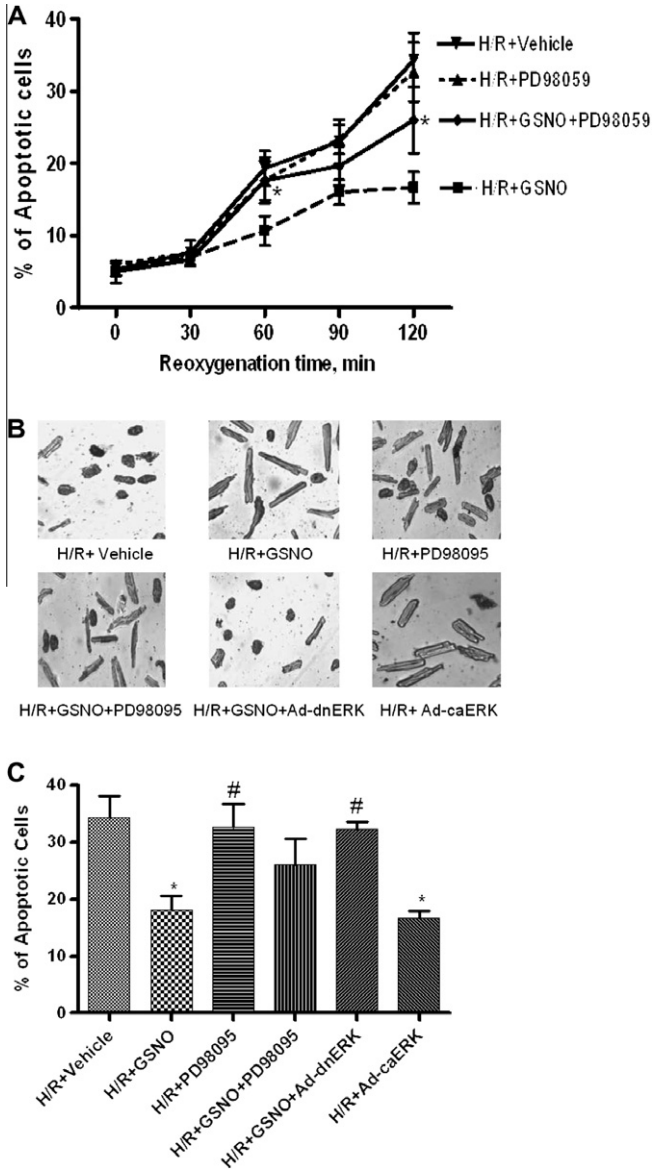

Fig. 3. The protective effect of GSNO depends on ERK1/2 activity. (A) The GSNO-induced improvement in cardiomyocyte apoptosis after reoxygenation was partially abolished by ERK inhibitor PD98059, $^{*}P < 0.05$ vs. H/R + GSNO group at respective time point. (B) Representative images of cardomyocyte apoptosis after 2-h reoxygenation, from 1 of the 4 independent experiments, determined by TUNNEL staining. (C) The percentage of apoptotic cardiomyocytes after 3-h hypoxia followed by 2-h reoxygenation, for indicated conditions. Data from four separate experiments, at least 400 randomly counted cells in each experiment, are expressed as mean ± SEM, $^{*}P < 0.05$ vs. H/R vehicle, $^{\#}P < 0.05$ vs. H/R + GSNO.

PD98059 by itself did not alter the percentage of TUNNEL-positive ARVMs during reoxygenation but abolished the reduction associated with GSNO treatment (at 120 min reoxygenation time point, 26.3 ± 6.9% in GSNO plus PD98059 group vs. 15.7 ± 4.2% in GSNO group, $P < 0.05$) (Fig. 3C). This observation suggests that GSNO at reoxygenation reduced the extent of apoptosis via p42/p44 MAPK activation.

To study the effect of ERK during reoxygenation, Ad-dnERK, Ad-caERK and control Ad-LacZ were used in our model. As shown in Fig. 3B and C, the Ad-dnERK abolished the protective effect of GSNO during reoxygenation similar to PD98095, When Ad-caERK alone was applied in H/R, there was a significant improvement in cellular viability (vehicle, 34 ± 5.2%; and Ad-caERK, 16 ± 6.2%; $P < 0.05$).

3.5. Reoxygenation causes activation of caspase-3 and -9 and caspase-3 and -9 inactivation is mediated by ERK phosphorlation

To determine whether the caspase was activated during reoxygenation, caspase-3 and -9 activities were measured using synthetic caspase substrates AcDEVD-pNa and AcLEHD-pNa, respectively. Significant increases in both caspase-3 and -9 activities were observed after reoxygenation that peaked at 3 h, (in fold changes), caspase-3 activity increased 3.2-fold vs. control ($P < 0.01$), and caspase-9 activity, increased 2.8-fold vs. control ($P < 0.01$), (data no shown). These data suggest that the mitochondria-mediated apoptosis pathway that involves caspase-3 and -9 is operative in reoxygenation-induced cardiomyocyte apoptosis.

To investigate the effect of ERK on caspase −3 and −9 activation during reoxygenation, GSNO, PD98059 and Ad-dnERK were used. As shown in Fig. 4A and B, the GSNO significantly decreased the caspase −3 and −9 activation (0.41-fold decrease vs. H/R vehicle and 0.38 decrease vs. H/R vehicle, respectively) during reoxygenation. The ERK inhibitor PD98059 and dnERK abolished this effect. These data indicate that ERK activation inhibits caspase-3 and -9 activity during reoxygenation.

3.6. ERK phosphorylation inhibits the decrease IAP protein levels during reoxygenation

To verify the involvement of XIAP in cardiomyocyte apoptosis and the ability of ERK to regulate its activity, the level of XIAP in myocytes subjected to H/R was analyzed. Western blotting indicated that the XIAP protein level steadily decreased with a prolonged reoxygenation period (data not shown). Cells exposed to H/R exhibited a marked decrease in XIAP levels compared to hypoxia alone (Fig. 4C). These data support the suggestion that the reoxygenation after hypoxia decreases the XIAP level, hence increasing the chance of cells going into apoptosis.

We further examined the effect of ERK phosphorylation on XIAP expression during reoxygenation, using GSNO, PD98059, Ad-dnERK and Ad-caERK. Fig. 4C demonstrated that GSNO caused XIAP protein level increase during reoxygenation (2.2-fold vs. H/R vehicle) or maintained XIAP levels at the H/H condition, while PD98059 and Ad-dnERK abolished this effect. Interestingly, the Ad-caERK alone increased XIAP level. These results suggest that XIAP protein up-regulation contributes to ERK's protective effect against reoxygenation-induced apoptosis.

4. Discussion

Building upon the adult cardiomyocyte apoptosis model, we demonstrated that NO improves cell survival during reoxygenation injury through ERK1/2 MAPK signaling pathway. PD98059 or selective knockdown of ERK phosphorylation using Ad-dnERK abolished the NO protective effect. Furthermore, XIAP protein level decreased in myocytes subjected to reoxygenation, whereas an enhanced p-ERK maintained or increased its levels. ERK signaling thus, is required for the induction of XIAP by NO in our system. Interestingly, Ad-ca ERK alone ameliorated the XIAP repression during H/R injury. These data suggest that ERK1/2 and its downstream target XIAP play an important role in the NO antiapoptotic signaling pathway. Additionally, in this study, NO attenuated H/R induced caspases activity and co-treatment with PD98059 or Ad-dnERK significantly reversed this effect, suggesting that the effect of NO signaling on caspase-3 and -9 activity was through preserving XIAP via activation of ERK. These findings are consistent with the previous findings in other cell lines [20,21], in which XIAP acts at the level of post mitochondria to protect cells from apoptosis. Therefore, ERK1/2 and XIAP signaling pathways play a critical role in the

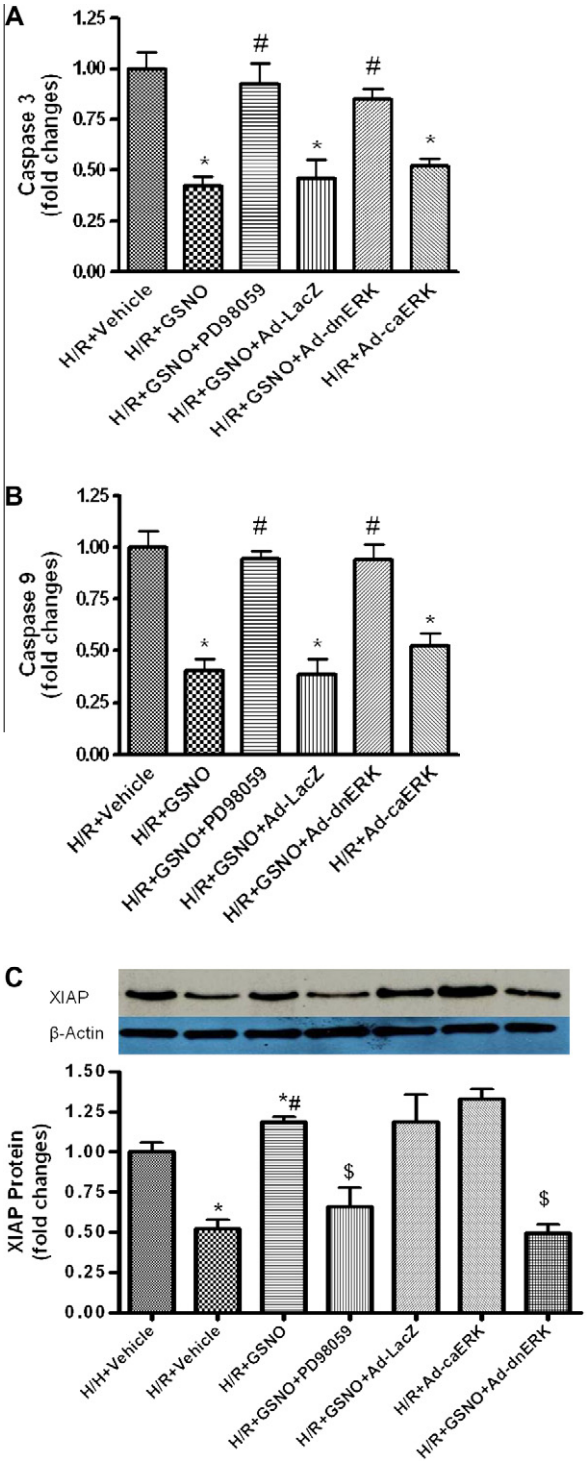

Fig. 4. Effect of H/R and GSNO on caspase-3 and -9 activation. (A and B) Quantitation of Elisa measurement of caspase-3 and -9 in cardiomyocytes exposed 3 h to hypoxia followed by 2-h reoxygenation. Cells were treated with vehicle, GSNO, GSNO plus PD98059, and Ad-dnERK or Ad-caERK, respectively. Results are expressed as fold changes relative to samples treated with the vehicle. $^{*}P < 0.05$ vs. H/R vehicle, $^{\#}P < 0.05$ vs. H/R + GSNO. (C) Representative western blot (top panel) and quantitative analysis (bottom panel) showing XIAP expression in the myocytes exposed for 3 h to hypoxia followed by 2-h reoxygenation under indicated treatment conditions. Data was shown as mean ± SEM from three independent experiments. $^{*}P < 0.05$ vs. H/H + vehicle, $^{\#}P < 0.05$ vs. H/R + vehicle, and $^{\$}P < 0.05$ vs. H/R + GSNO group.

prevention of apoptotic cell death by NO during H/R injury in rat adult myocytes.

Previous data suggested that apoptosis is a predominant mode of cell death during reoxygenation [1,22]. In our study we observed cardiomyocyte apoptosis increased dramatically after 3 h of hypoxia followed by 2 h of reoxygenation. This conclusion is further supported by activation of the mitochondrion-mediated apoptosis signaling pathway during reoxygenation [23]. Moreover, XIAP protein level decreased during reoxygenation in our experiment. Right now therapeutic reperfusion is performed to treat myocardial infarction without any measures to protect myocardium from apoptosis. Our results indicate that it may be possible to further limit the infarct size by preventing apoptosis, and ERK1/2 and XIAP might be promising targets for future drug development. In fact, it has been shown in a rat experimental myocardial infarction model that inhibition of caspases using pseudosubstrate acutely decreased the infarct size [23,24].

The interaction between pro-apoptotic and antiapoptotic members plays an important role in apoptosis initiation. We analyzed several members of IAP family, as the most likely candidates for antiapoptotic protein, in H/R myocytes. XIAP protein is highly expressed in the cardiovascular system [10,11]. Interestingly, XIAP levels were downregulated upon reoxygenation, whereas the other members of the IAP family, including cIAP 1, 2 did not change (data not shown). However, it was unclear whether XIAP is regulated by degradation or by pairing with Smac/Diablo during reoxygenation induced apoptosis [6,9]. It has been reported that both endogenous and exogenous oxidants reduced ERK1/2 activity [18]. In the present model, apoptosis predominantly occurs during reoxygenation where oxidant levels are elevated. These higher oxidant levels may inhibit ERK activation and lead to the rapid degradation of XIAP and enhanced apoptosis. As predicted, activation of ERK by NO increased or maintained XIAP levels during reoxygenation, and addition of ERK inhibitor or Ad-dnERK reversed this effect. Moreover, XIAP levels were restored when cardiomyocytes were transfected with virus encoding Ad-caERK. Previous data indicated that XIAP levels are regulated through a complex balance between proteosomal degradation and new synthesis [6–8]. In this study, ERK1/2 may help increase or maintain XIAP levels in one or two ways, either by preventing XIAP from ubiquitinylation, thereby inhibiting its degradation, or by promoting XIAP synthesis. However, the rapidity with which ERK activation appeared to exert its effect, within an hour, suggests that much of this regulation occurs at the level of ubiquitinylation resulting in reduced degradation.

One of the main mechanisms of caspase activation has been shown to involve the release of cytochrome *c* from mitochondria to cytosol. In cardiomyocytes by ischemia/reperfusion, there was activation of a mitochondrial pathway evidenced by the increase in cytosolic cytochrome *c* and the activation of caspase-3 and -9, but caspase inhibition alone prevented apoptosis without blocking cytochrome *c* release, which suggests that caspase inhibition occurs downstream of cytochrome *c* release [23]. Similarly, our results showed that XIAP blocked both caspase-3 and -9 activation during reoxygenation injury. These findings are consistent with the previous findings in other cell lines [18], in which XIAP acts at the level of post-mitochondria to inhibit caspase activity. It is notable that the induction of XIAP expression and inhibition of apoptosis by XIAP was not complete in our study. Thus, this could be explained by the contribution of the apoptotic pathways that may be independent of the XIAP effect.

The MAPK family has been studied intensively as a possible mediator for the generation of a pro-apoptotic protein in NO pathway [15,17]. However, the MAP kinase family is also implicated in the initiation of apoptosis in pathological states like ischemia and heart failure. Increasing evidence has shown that cardiomyocytes apoptosis is inhibited by PI3 kinase/Akt, which appears to block apoptosis at the level of mitochondrial changes and activation of effector caspases, and/or through ERK dependent processes

[25,26]. This latter pathway appears to act downstream of the mitochondria at the level of caspase inhibition. We demonstrate here that reoxygenation induced apoptosis was blocked by NO mediating ERK activation in adult rat myocytes.

We conclude that in the isolated adult rat cardiomyocyte model of H/R, NO and MAPK/ERK1/2 signaling pathways are required for the increased and stabilization of XIAP, which subsequently antagonize post-mitochondrial dysfunction of caspase activation, thereby antagonizing apoptosis. However, more work is necessary to understand the significance of apoptosis and the molecular mechanisms that govern these processes in ischemic heart disease.

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