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Role of ERK1/2 in the anti-apoptotic and cardioprotective effects of nitric oxide after myocardial ischemia and reperfusion*

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Objective: **Experimental results from cultured cells suggest that there is cross-talk between nitric oxide (NO) and extracellular signal-regulated kinase (ERK) in their anti-apoptotic effect. However, the cross-talk between these two molecules in either direction has not been confirmed in the whole organ or whole animal level. The aim of the present study was to determine whether ERK may play a role in the anti-apoptotic and cardioprotective effects of NO in myocardial ischemia/reperfusion (MI/R).** ***Methods:*** **Isolated perfused mouse hearts were subjected to 20 min of global ischemia and 120 min of reperfusion and treated with vehicle or an NO donor (SNAP, 10 μM) during reperfusion. To determine the role of ERK1/2 in the anti-apoptotic and cardioprotective effects of NO, hearts were pre-treated (10 min before ischemia) with U0126, a selective MEK1/2 inhibitor (1 μM).** ***Results:*** **Treatment with SNAP exerted significant cardioprotective effects as evidenced by reduced cardiac apoptosis (TUNEL and caspase 3 activity, $p < 0.01$), and improved cardiac functional recovery ($p < 0.01$). In addition, treatment with SNAP resulted in a 2.5-fold increase in ERK activation when compared with heart receiving vehicle. Pre-treatment with U0126 slightly increased post-ischemic myocardial apoptosis but had no significant effect on cardiac functional recovery in this isolated perfused heart model. However, treatment with U0126 completely blocked SNAP-induced ERK activation and markedly, although not completely, inhibited the cardioprotection exerted by SNAP.** ***Conclusion:*** **These results demonstrate that nitric oxide exerts its anti-apoptotic and cardioprotective effects, at least in part, by activation of ERK in ischemic/reperfused heart.**

Keywords: apoptosis; ERK1/2; myocardial ischemia/reperfusion; nitric oxide.

Ischemic heart disease is the single-most important cause of death in developed countries. Accumulating evidence indicates that apoptosis, a gene controlled programmed cell death pathway, contributes significantly to post-ischemic cardiomyocyte death, suggesting that therapeutic interventions that inhibit apoptotic cell death may attenuate ischemic/reperfusion-induced heart injury.[1]

Nitric oxide (NO), a ubiquitous molecule that is primarily generated by vascular endothelial cells under physiologic conditions, plays a critical role in cardiovascular homeostasis.[2,3] Substantial evidence exists that NO is an important cardioprotectant molecule, and NO donors, such as nitroglycerine, are used routinely in clinical practice to treat a variety of ischemic diseases. We have previously demonstrated that treatment with a nitric oxide donor at a sub-vasodilatory dose significantly reduced post-ischemic myocardial apoptosis *in vivo*.[4] However, the precise mechanisms responsible for inhibition of apoptosis by NO remain incompletely understood and are likely multi-factorial.

The mitogen-activated protein kinases (MAPKs) are a family of serine-threonine protein kinases that are activated in response to a variety of stimuli such as growth factors and cellular stresses.[5] Three major MAPK signaling pathways, including extracellular signal-related protein kinases (ERK1/2), p38 MAPK and c-Jun NH2-terminal protein kinases (JNKs)/stress-activated protein kinases (SAPKs) have been identified in mammalian cells.[6] Previous studies from our laboratory and other investigators have demonstrated that all three members of the MAPK family are activated by myocardial ischemia and reperfusion, and that the activations of ERK1/2 (beneficial) and p38 MAPKs-JNKs (deleterious) exert opposite effects on post-ischemic myocardial apoptosis and cardiac function recovery.[7,8]

Recent studies performed in cultured cells have demonstrated that NO may exert its anti-apoptotic effect via differential regulation of pro-apoptotic and anti-apoptotic MAPKs. NO has been reported to attenuate H_2O_2-induced apoptosis in H_9C_2 cardiomyoblasts by blocking H_2O_2-induced JNK activation[9] and protecting neonatal cardiomyocytes against simulated ischemia and reperfusion injury by inhibiting p38MAPK activation.[10] NO has also been reported to increase ERK1/2 activity by activation of its up-stream activator (*i.e.*, Ras)[11,12] or inactivation of its down-stream inactivator (*i.e.*, MKP-3),[13] thus reducing cytokine-induced apoptosis in endothelial cells. However, to date, direct evidence supporting the involvement of MAPK in NO's anti-apoptotic and cardioprotective effects

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in vivo or in an intact heart subjected to a real pathologic stimulation is lacking.

Therefore, the aims of the present study were (1) to determine whether administration of an NO donor at pharmacological concentration may activate ERK1/2 in an isolated perfused mouse heart and if so (2) to investigate its role in NO's anti-apoptotic and cardioprotective effects.

Materials and methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. S-Nitroso-N-acetyl-D,L-penacillamine (SNAP) and U0126 were freshly prepared daily. Male C57BL6 mice (20–25 g) fed on a standard laboratory diet were used in this study. The experiments were performed in adherence to National Institute of Health "Guidelines on the Use of Laboratory Animals" and were approved by Thomas Jefferson University Committee on Animal Care.

Heart preparation and measurement of functional parameters

Mice were anesthetized (sodium pentobarbital, 50 mg/kg, i.p.) and heparinized (sodium heparin, 5,000 U/kg, i.v.). Ten minutes after heparin injection, a midsternal thoracotomy was performed, and the heart was rapidly excised and placed into ice-cold Krebs-Henseleit (KH) buffer solution consisting of (in mM): NaCl, 118; KCl, 4.75; KH_2PO_4, 1.19; $MgSO_4 \cdot 7H_2O$, 1.19; $CaCl_2 \cdot 2H_2O$, 1.8; $NaHCO_3$, 25; EDTA, 0.5; and glucose, 11. All perfusion fluids were filtered through cellulose acetate membrane (pore size 2.0 μM).Within 30 s, the heart was mounted onto a non-recirculation Langendorff heart perfusion apparatus (Radnoti Glass Technology, Inc. Monrovia, CA). The heart was perfused in a retrograde fashion via the aorta at a constant pressure of 80 mm Hg with KH solution. The heart was electrically paced (420 bpm) by a pair of electrodes placed in the surface of the right ventricle. Coronary flow (CF) was measured via an in-line flow probe connected to an ultrasonic flow meter (Transonic Systems, Inc, Ithaca, NY).

To assess contractile function, a latex balloon was inserted into the left ventricular cavity through the mitral orifice and connected to a pressure transducer (Cobe CDXIII, Lakewood, CO). The balloon was initially inflated with water to produce an end-diastolic pressure of 8 to 10 mm Hg, which is on the plateau of the Starling curve for this preparation. Left ventricular pressure (LVP) and CF were continually recorded using a data acquisition system (PowerLab, ADInstruments, Inc., Milford, MA). The left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP = LVSP-LVEDP), and the maximal positive values of the instantaneous first derivative of LVP ($+dP/dt_{max}$) were obtained using computer algorithms and an interactive videographics program (PowerLab, Chart V5.0 for Windows).

Experiment protocol

After a 15-min equilibrium perfusion, the hearts were subjected to 20 min of global ischemia followed by 120 min of reperfusion (MI/R). At the time of reperfusion, they were randomized to receive one of the following treatments: (1) vehicle; (2) SNAP (an NO donor, 10 μM); (3) U0126 (a selective MEK1/2 inhibitor, 1 μM,); and (4) U0126 plus SNAP. The dose of SNAP was selected from preliminary experiments (1 to 30 μM, $n = 7$–9/dose) showing that the anti-apoptotic effect of SNAP plateaued at this concentration and further increasing SNAP concentration exerted direct negative inotropic effects (dP/dt_{max}: $-17.1 \pm 1.9\%$ after admission of 30 μM SNAP, $p < 0.01$). The dose of U0126 was determined from preliminary experiments (0.1 to 3 μM, $n = 5$–7/dose) showing that a >80 inhibition of ERK1/2 activation was achieved at this dose. The drugs were infused during the entire reperfusion period with a programmable Harvard Syringe Infusion Pump via a sidearm of the perfusion line just above the heart. The rate of infusion (1% of CF) was continuously adjusted based on the perfusate flow rate so that the desired target final concentration was achieved. Sham ischemic-reperfusion hearts (Sham MI) were perfused with KH solution for 140 min.

Measurement of ERK1/2 Phosphorylation and activation

At the completion of the perfusion protocol, the hearts were frozen in liquid nitrogen and analyzed within one week. The hearts were "freeze-clamped" using pre-cooled aluminum tongs and pulverized under liquid nitrogen. The powder was resuspended in ice-cold lysis buffer, and the protein content in the detergent-soluble supernatant fraction was measured. ERK1/2 phosphorylation was determined by Western blotting using an antibody against phosphorylated ERK1/2 and ERK1/2 activity was measured using a non-radioactive MAP kinase activity assay kit (Chemicon International, Inc.) by following the manufacturer's instruction. Results were expressed as a fold change over the sham MI/R group.

Determination of myocardial apoptosis with DNA ladder formation

Myocardial apoptosis was qualitatively analyzed by detection of DNA fragmentation (DNA ladders, $n = 5$–6/group), a hallmark of apoptosis as described previously.[14–16] In brief, at the end of the experiment, the free wall of left ventricle was isolated, minced while thawing and homogenized. The tissue was digested with proteinase K at 56°C overnight

and then incubated with DNA-free RNase at 37°C for an additional hour. Digested tissues were precipitated and centrifuged, and supernatants containing DNA were precipitated and centrifuged again. The resulting DNA pellets were washed and dissolved in DNA hydration solution. DNA electrophoresis was carried out at 60 V for 1–2 h. DNA ladder formation was visualized with a Kodak Image Station 400.

Determination of myocardial apoptosis by TUNEL

To determine myocardial apoptosis in a quantitative manner, the hearts were perfused with 4% paraformadehyde in PBS (pH 7.4) for 20 min. Four longitudinal sections from the free wall of the left ventricle were cut and further fixed in 4% paraformadehyde in PBS for 24 h at room temperature. Fixed tissues were embedded in a paraffin block and 2 slides at 4–5 μm thickness were cut from each tissue block. Immunohistochemical procedures for detecting apoptotic cardiomyocytes were performed by using an apoptosis detection kit (Boehringer Mannheim, Ridgefield, CT) according to the manufacturer's instructions.

Using a 20 X objective, the tissue slide was digitally photographed with a QICAM-Fast Digital Camera mounted onto an Olympus B X 51 Fluorescence Microscope. Total nuclei and the TUNEL positive nuclei in each field were counted by IP Lab Imaging Analysis Software (Version 3.5, Scanalytics, Fairfax, VA) with a custom-made script (By Mr. Ken Anderson, Bio Vision Technologies, North Exton, PA). The index of apoptosis (number of TUNEL positive nuclei/total number of nuclei × 100) was automatically calculated and exported to Microsoft Excel for further analysis. Results from different fields taken from the same animal were averaged and counted as 1 sample.

Determination of myocardial apoptosis by caspase 3 activation

Cardiac caspase 3 activity was performed by using caspase colorimetric assay kits (Chemicon International, Inc., Temecula, CA) as described in our previous study.[15] In brief, myocardial tissue was homogenized in ice-cold lysis buffer for 30 s using a PRO 200 homogenizer. The homogenates were centrifuged for 5 min at 10,000 × g at 4°C, supernatants were collected, and protein concentrations were measured by the bicinchoninic acid method (Pierce Chemical, Rockford, IL). To each well of a 96-well plate, supernatant containing 200 μg of protein was loaded and incubated with 25 μg Ac-DEVD-pNA at 37°C for 1.5 h. pNA was cleaved from DEVD and the free pNA was quantified using a SpectraMax-Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 405 nm.

Statistical analysis

All data in the text and figures are presented as means ± S.E. of n independent experiments. Hemodynamic data were analyzed by two-way ANOVA and all other data were analyzed with one-way ANOVA followed by the Bonferroni correction for post hoc t tests (GraphPad Prism, San Diego, CA). Probabilities of 0.05 or less with Bonferroni correction were considered to be statistically significant.

Results

Effect of NO on ischemia/reperfusion-induced ERK1/2 activation

There was no difference in the total ERK1/2 expression among the five groups studied, indicating that ischemia/reperfusion itself or treatments with NO donor/MEK1/2 inhibitor had no effect on ERK1/2 protein expression (Figure 1, upper panel). In contrast, ischemia/reperfusion significantly increased ERK1/2 phosphorylation (Figure 1, lower panel) and enhanced ERK1/2 activity (Figure 2). Most interestingly, treatment with SNAP further increased ERK1/2 phosphorylation (Figure 1) and resulted in a 2.4-fold increase in ERK1/2 activity when compared with vehicle (Figure 2). When the heart was pre-treated with 1 μM U0126, a selective MEK1/2 (the upstream kinase that activates ERK1/2) inhibitor, ERK1/2 phosphorylation was markedly inhibited (Figure 1) and ERK1/2 activity was reduced to a level comparable to that seen in the vehicle-treated group (Figure 2).

ERK1/2 Activation and anti-apoptotic signaling of nitric oxide

Having demonstrated that NO causes significant ERK1/2 phosphorylation and activation in the heart subjected to ischemia and reperfusion, we further investigated whether the ERK1/2 activation is required for NO's anti-apoptotic signaling. It is now well accepted that DNA ladder formation is highly specific for apoptotic cell death, but lacks of sensitivity and is difficult to quantify. In contrast, TUNEL staining

Figure 1. Representative Western blots showing that SNAP markedly enhanced MI/R-induced ERK1/2 phosphorylation, which was blocked by pre-treatment with U0126.

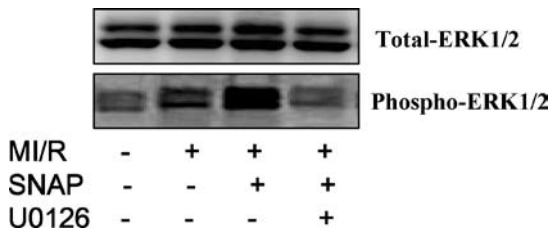


Figure 2. SNAP markedly enhanced MI/R-induced ERK1/2 activation, which was completely blocked by pre-treatment with U0126. $^{**}P <$ 0.01 vs. MI/R + Vehicle group, $^{++}P < 0.01$ vs. MI/R + SNAP group.

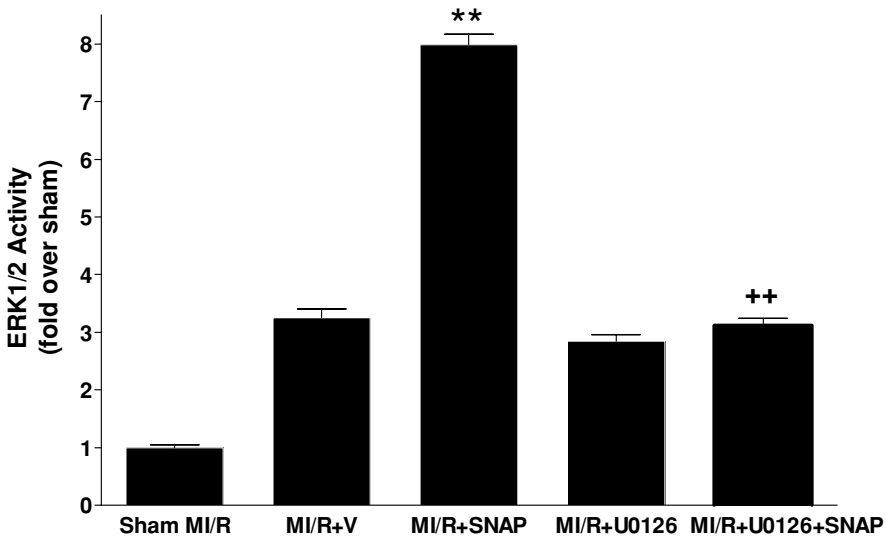


Figure 3. Representative photograph of electrophoretic analysis of internucleosomal DNA extracted from control or ischemic/reperfused heart with different treatments. *M* = molecular marker; 1 = sham MI/R; 2 = MI/R + Vehicle; 3 = MI/R + SNAP; 4 = MI/R + U0126 + SNAP.

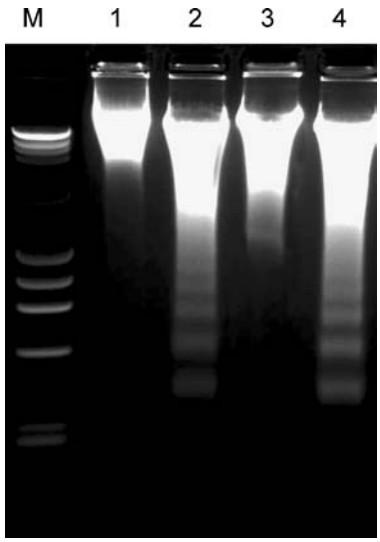
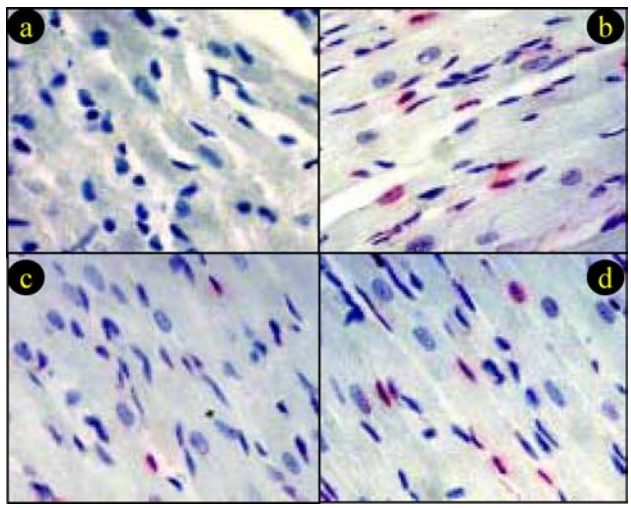


Figure 4. Representative photomicrographs of *in situ* detection of DNA fragments in heart tissue from control or ischemic/reperfused heart with different treatments. *a* = sham MI/R; *b* = MI/R + Vehicle; *c* = MI/R + SNAP; *d* = MI/R + U0126 + SNAP. The tissues were sectioned and stained using the TUNEL method, as described in "Materials and methods". Red staining represents positive nuclei.



of nuclei is extremely sensitive, but is less specific for apoptosis as some necrotic cells may stain positive. These two methods were thus used in combination to improve accuracy and reliability of our results. In myocardial tissue from sham MI hearts, no DNA ladder was detected (Figure 3, lane 1). In contrast, the formation of DNA nucleosome ladders was clearly detected in myocardial tissues obtained from MI/R hearts receiving only vehicle (Figure 3, lanes 2). In contrast, hearts treated with SNAP exhibited markedly decreased DNA fragmentation (Figure 3, lane 3). Most interestingly, the anti-apoptotic effect of NO was virtually abolished when the hearts were pre-treated with U0126 (Figure 3, lane 4).

In sham MI hearts that lacked DNA ladders, an extremely low level of TUNEL positive cells were observed (Figures 4a and 5). In contrast, TUNEL-positive nuclei were prevalent in tissues from ischemic-reperfused hearts receiving only vehicle (Figures 4b and 5). Treatment with SNAP reduced the numbers of cells stained positive by TUNEL from 7.2 ± 0.54% to 2.99 ± 0.61% (Figures 4c and 5). This

Figure 5. Percentage of nuclei staining positive for TUNEL in sham-operated control hearts or the hearts exposed to MI/R receiving different treatments. Four longitudinal sections from each heart were cut and embedded in a paraffin block, and two slides per block were evaluated histologically. Total nuclei and TUNEL positive nuclei were counted with an imagine analyzing software as described in the method section. Different fields taken from the same heart were averaged and counted as 1 sample. $n = 8$–10/group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MI/R + vehicle group,$^{++}P < 0.01$ vs. MI/R + SNAP group.

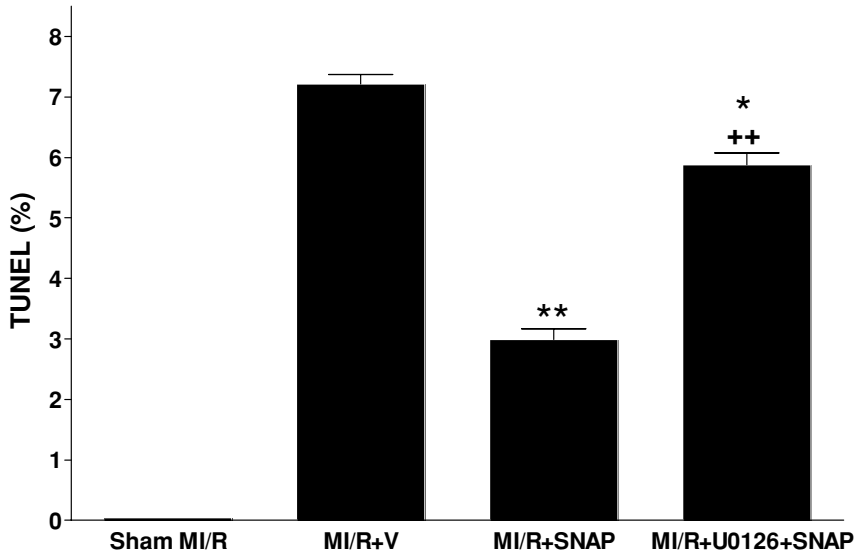
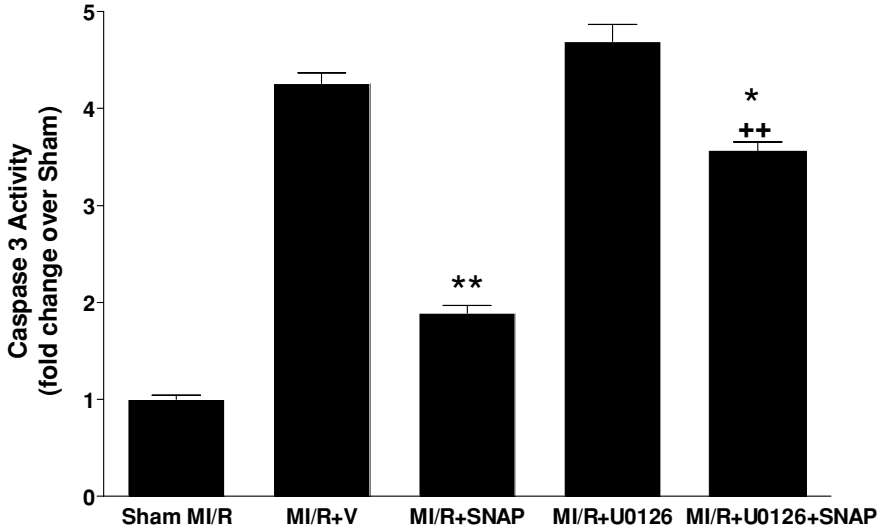


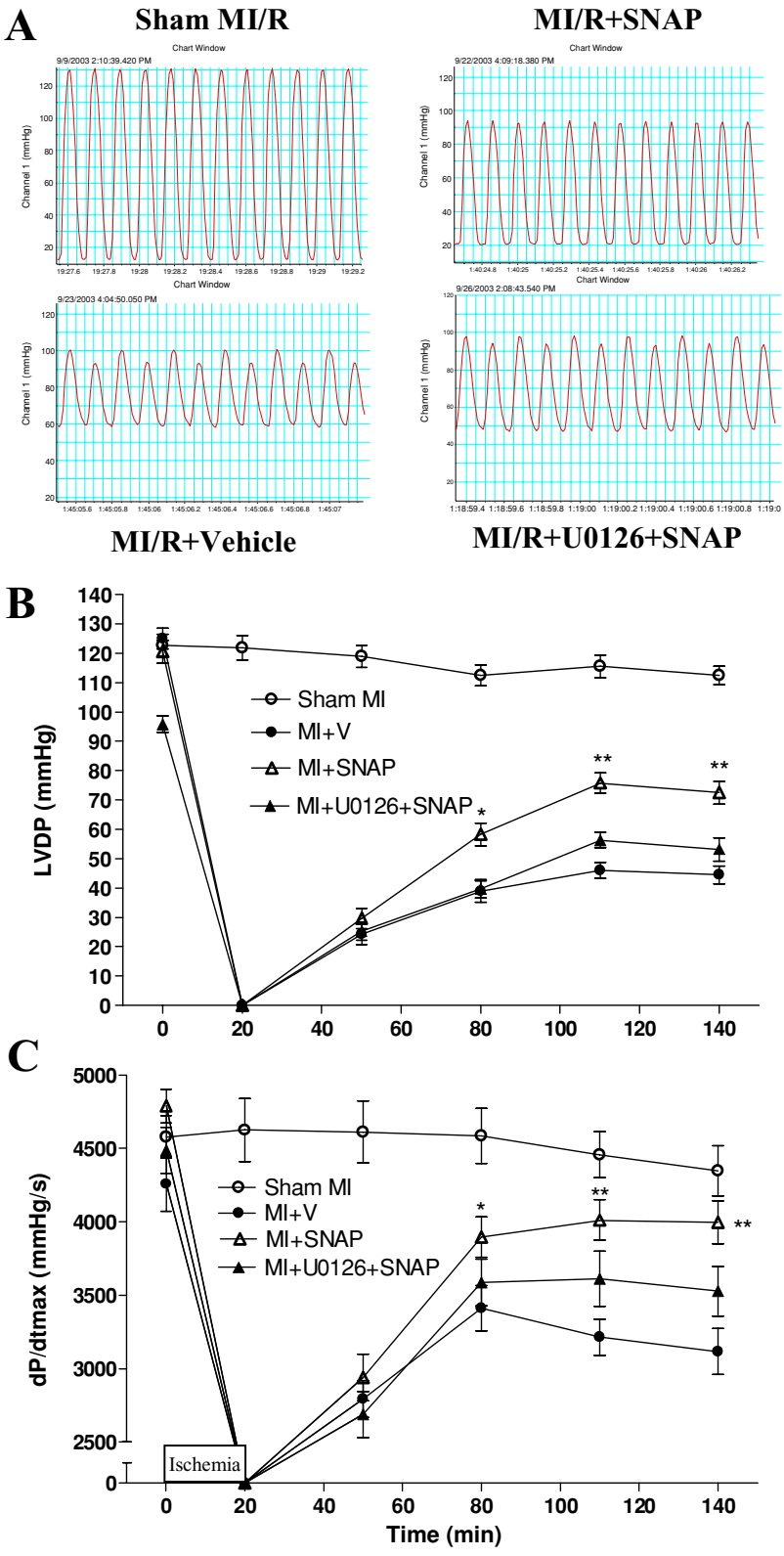
Figure 6. Myocardial caspase 3 activity expressed as fold change over sham MI/R. $n = 8$–12 hearts/group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MI/R + Vehicle group,$^{++}P < 0.01$ vs. MI/R + SNAP group.



anti-apoptotic effect of SNAP was markedly, although not completely blocked when the heart was pre-treated with U0126 (Figures 4d and 5).

Caspase 3 activation is the final common pathway via which a variety of pathologic stimuli induce caspase-dependent apoptosis. To obtain further evidence that ERK1/2 activation plays a critical role in anti-apoptotic signaling of NO, caspase 3 activity was determined in all 5 groups studied. As illustrated in Figure 6, treatment with SNAP markedly reduced ischemia/reperfusion-induced caspase 3 activation. Treatment with U0126 at the dose selected from our preliminary experiments (a dose that reduced ERK1/2 to a level comparable to that of vehicle) slightly increased caspase 3 activity ($P > 0.1$). However, pre-treatment with U0126 before administration of SNAP markedly, although not completely, inhibited caspase 3 suppression effect of NO in the ischemic/reperfused hearts. Taken together, these results indicate that the anti-apoptotic effect of nitric oxide in ischemic/reperfused heart is mediated, at least in part, by ERK1/2 activation.

Figure 7. ERK1/2 Inhibition blocked NO's cardioprotection. A: Representative recordings of left ventricular pressure (at 120 min reperfusion) in the isolated perfused mouse heart subjected to sham MI/R or MI/R with different treatments. B: Time course of LVDP changes in isolated perfused mouse hearts subjected to sham MI/R or MI/R with different treatments. C: Time course of left ventricular dP/dt_{max} in isolated perfused mouse hearts subjected to sham MI/R or MI/R with different treatments. $n = 10$–12 hearts/group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MI/R + Vehicle group.



Blockade of NO's cardioprotection by ERK1/2 inhibition

The results presented above demonstrated that the anti-apoptotic effect of NO is markedly blocked when ERK1/2 activation is inhibited. To further investigate whether the anti-apoptotic effect of NO may improve cardiac contractile functional recovery after ischemia/reperfusion and more importantly, to determine whether blockade of ERK1/2 activation may also inhibit NO's cardioprotective effect, left ventricular function was recorded. As summarized in Figure 7, treatment with SNAP significantly improved cardiac functional recovery after a 20-min global ischemia, which was markedly, although not completely, blocked by pre-treatment with U0126.

Discussion

Two novel observations have been made in the present study. First, we have provided direct evidence that treatment with a nitric oxide donor at a concentration that has no toxic effect and no functional impact on the heart resulted in significant ERK1/2 phosphorylation and activation. Second, we have demonstrated for the first time in a real pathologic model (*i.e.*, a functional heart subjected to ischemia/reperfusion) that activation of ERK1/2 plays a critical role in NO's anti-apoptotic and cardioprotective effects.

ERK1/2 is an established player in the anti-apoptotic defense network. ERK activates p90 ribosomal S6 kinase (p90RSK), thus resulting in transcriptional upregulation of anti-apoptotic molecules, such as cAMP-response element-binding (CREB).[17,18] Recent studies have demonstrated that ERK may also inhibit apoptotic signaling via post-transcriptional protein phosphorylation. Specifically, activation of ERK1/2 has been shown to result in BAD phosphorylation (Ser^{112}) both directly[19–23] and indirectly via ERK/p90RSK activation,[24,25] which specifically suppresses BAD-mediated apoptosis. These findings suggest that ERK promotes cell survival by both inhibiting components of the cell death machinery (*e.g.*, BAD) and increasing transcription of prosurvival genes (*e.g.*, CREB). Thus, the maintenance of ERK1/2 activation by increasing its activation or inhibiting its inactivation may be an effective intervention that can protect the heart from ischemia/reperfusion-induced cell death.

The effects of NO on myocardial ischemia and reperfusion injury have been extensively investigated in recent years. Numerous studies published by other investigators as well as our group have demonstrated that stimulation of endogenous NO production from eNOS or *in vivo* administration of pharmacological concentrations of NO markedly reduce myocardial ischemia/reperfusion injury.[4,26] However, *in vivo* cardioprotective effects NO have been mostly attributed to its anti-leukocyte/anti-platelet/anti-inflammatory property, and whether NO may directly activate prosurvival signals (*i.e.*, anti-apoptotic molecules) in the cardiomyocyte and thus attenuate post-ischemic cell death remain largely unknown. On the other hand, although the role of NO in the regulation of apoptotic signaling has been investigated in cultured cells, most, if not all, studies were performed in cell lines or mitogenic cells.[27,28] Since adult cardiomyocytes are post-mitogenic and their pro-survival/pro-apoptotic signaling machinery may differ from those mitogenic cells, signaling mechanisms by which NO confers its regulatory roles may also be different. In the present study, we have demonstrated that administration of NO at a concentration that had no direct inotropic/chronotropic/toxic effects in a functional adult mouse heart markedly activated ERK1/2, a pro-survival molecule that has been previously shown to reduce myocardial ischemia/reperfusion injury. Moreover, pre-treatment with U0126, a highly selective MEK1/2 inhibitor, completely blocked NO-induced ERK1/2 activation and markedly diminished the anti-apoptotic and cardioprotective effects of NO. These results provide clear evidence that the activation of ERK1/2 by NO is a novel mechanism that contributes to the protective effects of NO in heart subjected to ischemia and reperfusion.

NO activation of ERK1/2 has been reported in other cell types in culture. Mechanisms involving increased upstream activation and reduced downstream inactivation have been reported. In Jurkat cells, authentic NO and NO donors activates ERK1/2 by nitrosylative activation of Ras (Cys^{118}).[11,12] On the other hand, Dimmeler and colleagues[13] recently reported that NO increases ERK1/2 activity through preventing its inactivation by a downstream inactivator. Specifically, NO causes down-regulation of the cytosolic MAP kinase phosphatase-3 (MKP-3), which is known to dephosphorylate ERK1/2,[29] thus inhibiting the inactivation of ERK1/2. More interestingly, Callsen *et al*.[30] reported that in rat mesangial cells, NO results in a rapid and a delayed phase of ERK1/2 activation. Early and transient activation at 5 min was suppressed by the soluble guanylyl cyclase-blocking agent and mimicked by the lipophilic cyclic GMP (cGMP) analogue 8-bromo-cGMP. In contrast, NO-mediated activation achieved after several hours was unrelated to cGMP signaling. This late and persistent ERK1/2 activation induced by NO donors was found in association with inhibition of phosphatase activity. Based on these results, the authors concluded that NO may enhance ERK1/2 activity by activating an upstream molecule (early phase, occuring in minutes) and blocking a downstream inactivator (late phase, occurring after several hours). Since all of our observations were finished within 2 h after NO administration, it is unlikely that the mechanisms involved in the late phase ERK1/2 activation are involved in the current study. Moreover, our results demonstrated that NO-induced ERK1/2 activation was completely blocked by U0126, a highly selective MEK1/2 inhibitor, indicating that NO activates ERK1/2 by mechanisms involving activation of upstream molecules that activates ERK1/2.

In summary, we report here that the activation of ERK1/2 by NO is a novel mechanism that contributes to the protective effects of NO in heart subjected to ischemia and reperfusion. These results are scientifically important because it reveals a novel mechanism by which NO confers its anti-apoptotic and cardioprotective effects in the ischemic/reperfused heart.

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