Protection of Myocytes From Hypoxia-Reoxygenation Injury by Nitric Oxide Is Mediated by Modulation of Transforming Growth Factor-β1

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**Background**—Reoxygenation injury is a result of several complex events, including release of reactive oxygen species, protein kinase C (PKC) activation, and altered expression of transforming growth factor-β1 (TGF-β1). Nitric oxide (NO) generally protects tissues from reperfusion injury.

**Methods and Results**—We examined the modulation of TGF-β1 expression and activity and PKC activation in cultured rat heart myocytes exposed to a brief period of hypoxia-reoxygenation (H-R) by NO donor 3-morpholino-sydnonimine (SIN-1). H-R resulted in an increased expression of total TGF-β1 (mRNA and protein) but a decrease in the release of active TGF-β1. Myocyte PKC-α protein level was not altered by H-R, but its phosphorylation was augmented. Pretreatment of myocytes with SIN-1 diminished myocyte injury quantified as lactate dehydrogenase release. Simultaneously, release of active TGF-β1 increased and total TGF-β1 expression decreased (all P<0.05 versus H-R alone). PKC-α phosphorylation increased further in cells treated with SIN-1. The effects of SIN-1 were blocked by the NO scavenger phenyl-tetramethyl-imidazoline-oxyl-oxide as well as by the PKC inhibitor staurosporine. To examine if another NO donor would have a similar effect, cardiomyocytes were treated with nitroglycerin before H-R. With nitroglycerin treatment, similar to SIN-1 treatment, myocyte injury was diminished, TGF-β1 release increased, and total TGF-β1 expression decreased.

**Conclusions**—These observations suggest modulation of TGF-β1 expression as a novel mechanism of salutary effect of NO donors. PKC-α activation may play an important role in the protective effect of NO against H-R injury.

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Key Words: hypoxia ■ nitric oxide ■ proteins ■ growth substances

Most episodes of myocardial infarction result from occlusion of a narrowed coronary artery. As such, reperfusion therapy is often used in an attempt to salvage the ischemic tissues. However, reperfusion itself may, at least in the short term, lead to injury beyond that caused by ischemia alone. Reperfusion injury is caused by a number of factors, such as release of reactive oxygen species (ROS), cytokines, and other inflammatory mediators.1-3 Recognition of these mediators has led to development of strategies that combat the adverse effects of reperfusion.4-6 One of the approaches that has been used for limiting reperfusion injury in the experimental setting is the use of growth factors such as basic fibroblast growth factor,6,7 hepatocyte growth factor,8 and transforming growth factor-β1 (TGF-β1).9-13

TGF-β1, in particular, has been shown to protect ischemic-reperfused tissues by a variety of mechanisms that include reduction in the activity of tumor necrosis factor-α,9 production of superoxide anions,9 intracellular nitric oxide synthase (iNOS) expression,12 and adherence of neutrophils to endothelium.13 In isolated rat heart preparations, the cardioprotective effects of activated platelets during ischemia-reperfusion have been attributed to the release of TGF-β1.10 Isolated cultured rat myocytes, when exposed to 24 hours of hypoxia and 3 hours of reoxygenation (H-R), develop apoptosis and altered expression of related proteins, and incubation with recombinant TGF-β1 protects these cells against H-R-mediated apoptosis and injury.11 A recent study12 showed that H-R causes an increase in the expression of the gene for iNOS and phosphorylation of protein kinase B and that TGF-β1 attenuates these adverse effects of H-R.

Another strategy to protect cardiac tissues from H-R-mediated injury is treatment with NO donors.14-17 NO has been shown to inactivate ROS,18 which are considered cytotoxic.19

The present study was designed to study the alterations in TGF-β1 protein and activity in cultured rat myocytes during H-R. We also studied the effect of an NO donor, 3-morpholino-sydnonimine (SIN-1), on TGF-β1 expression...
and activity. In addition, we examined the role of protein kinase C (PKC-α), which is altered during H-R injury.

### Methods

#### Myocyte Isolation and Culture

The method for rat heart myocyte isolation and culture has been detailed earlier. All procedures were carried out under aseptic conditions.

Cells from each rat heart were divided into dishes containing 3 mL of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (~10⁴ cells in each dish) and cultured under 95% air and 5% CO₂ at 37°C. Culture medium was changed every 48 hours.

Cultured myocytes were divided into the following groups: (a) Control: Cells were kept in 95% air and 5% CO₂. (b) H-R: Cells were exposed to 24 hours of hypoxia (95% N₂-5% CO₂, P₀₂ ~30 mm Hg) followed by 3 hours of reoxygenation (95% air and 5% CO₂). (c) SIN-1+H-R: Cells were pretreated with 10 μmol/L of the NO donor SIN-1 (Sigma) for 1 hour followed by H-R. (d) SIN-1+H-R: Cells were pretreated with 100 μmol/L of SIN-1 for 1 hour followed by H-R. (e) PTIO+ SIN-1+H-R: Cells were pretreated with the NO scavenger 2-phenyl-4, 4, 5, 5-tetramethyl-imidazoline-1-oxyl 3-oxide (PTIO, 100 μmol/L, Sigma) for 1 hour along with SIN-1 followed by H-R. (f) Staurosporine+SIN-1+H-R: Cells were pretreated with the PKC inhibitor staurosporine (10 μmol/L, Sigma) for 1 hour along with SIN-1 followed by H-R.

Supernatants of myocytes were collected for determination of lactate dehydrogenase (LDH), nitrite, and active TGF-β₁ levels. Myocytes were harvested for examination of TGF-β₁ expression (protein and mRNA) and PKC-α expression and its phosphorylation.

To determine if the effect of SIN-1 was specific for SIN-1 or all NO donors, another set of myocytes was exposed to nitroglycerin (NTG) before H-R. Myocyte supernatant was used for measurement of LDH, nitrite, and active TGF-β₁ levels. Myocytes were used for measurement of TGF-β₁ expression.

#### Determination of LDH, Nitrite, and TGF-β₁ in Culture Medium

A spectrophotometric method based on the oxidation of lactate (Sigma) was used to measure LDH release. LDH activity was expressed as units per milliliter.

Nitrite levels were measured in the culture medium as index of NO generation by Greiss Reagent System (Promega), as described previously. Absorbance determination was made at 540 nm. Immunoreactive TGF-β₁ levels were measured by ELISA, with a kit from R & D Systems. This assay measures only active TGF-β₁. Absorbance determination was made at 462 nm.

#### Determination of Total TGF-β₁ Protein, and mRNA in Cultured Myocytes

Methodology for measurement of total TGF-β₁ protein and mRNA has been described earlier. The primer sequences for polymerase chain reaction amplification for TGF-β₁ mRNA were 3’-GAGCCTCAAAAGACAGGCCACT; 5’-GAAAGCCATCCTGGTGCCAGAT. The primers were designed to amplify a product of 461 bp. For Western analysis, the primary antibody to TGF-β₁ was obtained from Santa Cruz Biotechnology.

#### Immunoprecipitation and Western Analysis of PKC-α and Its Phosphorylation

Cell lysates (100 μg) from each experiment were immunoprecipitated and separated by 8% SDS-PAGE with a BioRad Mini-Protein cell and transferred to nitrocellulose membranes (Amersham). After incubation in blocking solution (5% nonfat milk, Sigma), membranes were incubated with 1:100 dilution primary polyclonal antibody to PKC-α (Santa Cruz Biotechnology) or monoclonal antibody to phospho-threonine (Santa Cruz Biotechnology) overnight at 4°C. Membranes were washed and incubated with 1:2000 dilution of secondary antibody (Santa Cruz Biotechnology) for 1 hour. The membranes were detected with the enhanced chemiluminescence system, as described earlier.

#### Data Analysis

All results are based on 5 separate experiments. Data are presented as mean±SEM. Statistical significance was determined in multiple comparisons among independent groups of data in which ANOVA testing indicated the presence of significant differences. A value of P<0.05 was considered statistically significant.

### Results

#### Effect of NO Donor SIN-1 on Myocyte Injury Induced by Hypoxia Reoxygenation

H-R caused a marked increase in LDH release in the supernatants, indicating myocyte injury (P<0.001 versus control group). Treatment of cultured myocytes with SIN-1 attenuated LDH release in response to H-R (P<0.05 versus H-R group). The decrease in LDH was more pronounced with higher concentration (100 μmol/L) than with lower concentration of SIN-1 (10 μmol/L) (P<0.05). The myocyte-protective effect of SIN-1 was blocked by the NO scavenger PTIO (P<0.05 versus SIN-1 100 μmol/L), indicating the specificity of the effect of SIN-1 (Figure 1).

H-R caused no significant change in nitrite levels in the supernatants of myocytes exposed to H-R. As expected, treatment of myocytes with SIN-1 increased the concentration of nitrite in the supernatants (P<0.05 versus control). The NO scavenger PTIO itself did not affect nitrite levels. These data are summarized in Figure 2.

#### TGF-β₁ Expression in Myocytes

As shown in Figure 3, TGF-β₁ expression (protein and mRNA) was upregulated in cultured myocytes exposed to H-R (P<0.01 versus control). Treatment of myocytes with SIN-1 significantly reduced this enhanced TGF-β₁ expression during H-R (P<0.05 versus H-R alone). The high concentration of SIN-1 (100 μmol/L) was more effective than the low concentration.
concentration (10 μmol/L) in this effect \(P<0.05\). The specificity of the effect of SIN-1 was confirmed by use of the NO scavenger PTIO, which reduced the effect of SIN-1 on TGF-\(\beta_1\) expression in myocytes \(P<0.01\) versus SIN-1 alone).

**Immunoreactive TGF-\(\beta_1\) Levels in Myocyte Supernatants**

H-R caused a marked reduction in active TGF-\(\beta_1\) levels in the supernatants of myocytes \(P<0.01\) versus control) (Figure 4), consistent with previous observations in the isolated rat heart exposed to H-R.\(^{10}\) It is important that treatment of myocytes with SIN-1 restored active TGF-\(\beta_1\) levels in the supernatants. Higher concentration of SIN-1 (100 μmol/L) was more effective than the lower concentration (10 μmol/L) in this effect \(P<0.05\). The scavenger of NO PTIO and PKC inhibitor staurosporine blocked this effect of SIN-1. Data from 5 separate experiments are expressed as mean±SEM.

**Role of PKC in H-R Injury and in the Effect of SIN-1**

H-R and the NO donor SIN-1 did not affect PKC-\(\alpha\) protein expression; however, the PKC inhibitor staurosporine decreased the PKC-\(\alpha\) protein level \(P<0.05\) versus SIN-1 alone) (Figure 5).

H-R increased phosphorylation of PKC-\(\alpha\) \(P<0.05\) versus control). Pretreatment of myocytes with the NO donor}

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**Figure 2.** Nitrite levels in the culture media. H-R caused a small increase in nitrite levels in the supernatants of myocytes \(P=0.07\) versus control). Treatment of myocytes with SIN-1 increased the concentration of nitrite in the supernatants. The NO scavenger PTIO did not affect nitrite levels. The PKC inhibitor staurosporine also did not affect nitrite levels in the supernatants. Data from 5 separate experiments are expressed as mean±SEM.

**Figure 3.** Representative gels of TGF-\(\beta_1\) protein and mRNA expression in cultured myocytes. H-R increased both total TGF-\(\beta_1\) protein and TGF-\(\beta_1\) mRNA expression. Incubation of myocytes with SIN-1 before hypoxia decreased the increase in TGF-\(\beta_1\) expression. Higher concentration of SIN-1 (100 μmol/L) was more potent in modulating TGF-\(\beta_1\) than the lower concentration (10 μmol/L). Both PTIO and staurosporine blocked the effect of SIN-1 on TGF-\(\beta_1\) expression. Data from 5 separate experiments are expressed as mean±SEM.

**Figure 4.** Immunoreactive (active) TGF-\(\beta_1\) levels in myocyte culture media. H-R caused a significant reduction in active TGF-\(\beta_1\) levels in the culture media of myocytes. Pretreatment of myocytes with SIN-1 restored active TGF-\(\beta_1\) levels in the supernatants. Both the scavenger of NO PTIO and PKC inhibitor staurosporine blocked this effect of SIN-1. Data from 5 separate experiments are expressed as mean±SEM.

**Figure 5.** Expression and phosphorylation of PKC\(\alpha\) in cultured myocytes determined by Western blot. H-R alone or treatment of myocytes with SIN-1 did not affect PKC-\(\alpha\) protein expression; however, the PKC inhibitor staurosporine decreased the PKC-\(\alpha\) protein level \(P<0.05\) versus SIN-1 alone) (Figure 5).

H-R increased phosphorylation of PKC-\(\alpha\) \(P<0.05\) versus control). Pretreatment of myocytes with the NO donor
SIN-1 caused a further small increase in phosphorylation of PKC-α (P<0.05 versus H-R alone). The specificity of the effect of NO was evident from experiments in which PTIO reduced PKC-α phosphorylation (P<0.05 versus SIN-1) (Figure 5).

The PKC inhibitor staurosporine also reduced the protective effect of SIN-1 on myocytes exposed to H-R, as measured by LDH release (Figure 1). The effects of SIN-1 on total TGF-β1 protein levels in cultured myocytes (Figure 3) and immunoreactive TGF-β1 levels in the supernatants of myocytes exposed to H-R (Figure 4) were also blocked by staurosporine (P<0.05 versus SIN-1 alone). It is noteworthy that the PKC inhibitor did not alter nitrite levels (Figure 2).

**NTG and Myocyte H-R Injury**

To determine if the effects of SIN-1 were specific for SIN-1 or attributable to all NO donors, another set of myocytes were pretreated with NTG for 1 hour before exposure to H-R. NTG pretreatment protected the myocytes from H-R injury measured as LDH release (Figure 1). The effects of SIN-1 on total TGF-β1, protein levels in cultured myocytes (Figure 3) and immunoreactive TGF-β1 levels in the supernatants of myocytes exposed to H-R (Figure 4) were also blocked by staurosporine (P<0.05 versus SIN-1 alone). It is noteworthy that the PKC inhibitor did not alter nitrite levels (Figure 2).

**Discussion**

This study confirms the results of previous studies showing reduction of H-R injury to myocytes by NO donors14–16 and the NO precursor l-arginine.17 In addition, the present study provides novel insight into the effects of NO in that the incubation of myocytes with SIN-1 or NTG restored the abnormality in TGF-β1 synthesis and activity. This study also shows that phosphorylation of PKC-α increased during H-R, and it was further augmented by SIN-1.

Growth factors and myocyte protection during H-R have been subjects of intense investigation in the recent past. In particular, the multifunctional polypeptide TGF-β1 has been shown to improve cardiac function in intact cat hearts, isolated rat hearts, and cultured rat myocytes exposed to H-R.9–13 TGF-β1 also modulates the expression of apoptotic proteins when myocytes are exposed to H-R.11 TGF-β1 has been thought to protect ischemic tissue by blocking the effect of tumor necrosis factor-α decreasing iNOS activity, and reducing the release of ROS.9,12,13

In studies in Langendorff rat hearts exposed to H-R,10 immunohistochemical evidence for depletion of active TGF-β1 in the myocytes was provided. Direct measurements in myocardial tissues exposed to H-R showed a 2-fold increase in latent TGF-β1 concentration at the transcriptional level and a 40% reduction in active TGF-β1 levels.10 In the present study in cultured rat myocytes, we confirmed that total TGF-β1 expression (mRNA and protein) increased markedly during H-R and active TGF-β1 concentration fell. The increase in TGF-β1 mRNA and protein may be a compensatory response to protect the myocytes from the deleterious effect of H-R. Alternatively, this observation of low active TGF-β1 levels despite elevated mRNA and protein may reflect an H-R–induced defect in the conversion of the latent form of TGF-β1 to its active form.

Endothelial NOS is generally downregulated and iNOS upregulated during H-R.12,22 Activation of iNOS results in
formation of large amounts of NO that will be seen as nitrite in the supernatants. Despite a small increase in nitrite levels in myocardial tissues, exogenous NO donors have been shown to protect myocardial tissues from H-R injury.\textsuperscript{1,4,15} The protective effect of exogenous NO donors may relate to chelation and neutralization of superoxide anions and other ROS,\textsuperscript{18,23} which are released during H-R. The present study clearly shows a myocyte-protective effect of two different NO donors, SIN-1 and NTG. The specificity of the myocyte-protective effect of SIN-1 became apparent in experiments in which the NO scavenger PTIO completely blocked the myocyte-protective effect of SIN-1. It is of note that PTIO did not decrease nitrite levels. Previous studies have shown that when this agent combines with NO released from SIN-1, the final product is still nitrite/nitrate.\textsuperscript{24} Two other aspects of the present study need comment. Nitrite levels in response to two different doses (10 and 100 μmol/L) of SIN-1 were not increased by a log-fold, suggesting that the conversion of SIN-1 to NO is a limited process. Second, we used PTIO only in combination with 100 μmol/L of SIN-1. This was done only to test the concept that PTIO would block the myocyte-protective effect of NO donors.

It is important to note that our study shows that NO donors can ameliorate the abnormality in TGF-β1 expression and activity during H-R. The increase in active TGF-β1 levels may be the basis of the myocyte-protective effect of SIN-1. This hypothesis gains support from previous studies showing that recombinant TGF-β1 markedly decreases H-R injury.\textsuperscript{11,12} Our recent work has shown that TGF-β1 inhibits the downregulation of endothelial NOS and the upregulation of iNOS.\textsuperscript{12}

We also examined the role of PKC-α in the effect of SIN-1. Previous studies have shown that H-R and shear stress activate several isoforms of PKC,\textsuperscript{25–28} which may be cardioprotective. PKC is also known to be involved in mediating NO-induced cardioprotection. Vondriska et al.\textsuperscript{29} have shown that NO-induced cardioprotection involves PKC-ε-Src module formation. As further evidence, inhibitors of PKC blocked cardioprotection in their study. Li et al.\textsuperscript{10} have shown that free radicals activate PKC-ε isoforms during H-R. In the present study, we observed that PKC-α activity (phosphorylation) increased during H-R, with further increase as the myocytes were treated with the NO donor SIN-1. It is important that the PKC inhibitor staurosporine blocked the cardioprotective effects of NO and simultaneously decreased PKC expression. It is of note that staurosporine did not affect nitrite levels because this compound only inhibits PKC activity. We suspect that staurosporine precludes PKC-α–Src module formation, as is the case with PKC-ε.\textsuperscript{29} These observations, taken together, suggest that PKC-α activation plays a signal transduction role in NO-induced cardioprotection. Since we did not measure other PKC isoforms, the role of other PKC isoforms in the effects of NO donors remains to be defined.

Although the cardioprotective effects of NO donors on cardiomyocytes have been largely attributed to their direct actions, this study suggests that modulation of TGF-β1 expression and activity may be important in the salutary effect of NO donors.

In summary, our observations of modulation of TGF-β1 expression and activity provide a novel mechanism of salutary action of NO donors on cardiac myocytes exposed to H-R. PKC-α activation may play a significant signaling role in mediating the salutary effects of exogenous NO during H-R.

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References


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