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Role of heme oxygenase-1 in the cardioprotective effects of erythropoietin during myocardial ischemia and reperfusion

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Burger D, Xiang F, Hammoud L, Lu X, Feng Q. Role of heme oxygenase-1 in the cardioprotective effects of erythropoietin during myocardial ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 296: H84–H93, 2009; doi:10.1152/ajpheart.00372.2008.—We have recently demonstrated that erythropoietin (EPO) protects cardiomyocytes from apoptosis during myocardial ischemia-reperfusion (I/R). The objective of the present study was to investigate the role of heme oxygenase (HO)-1 in the antiapoptotic effects of EPO. Primary cultures of neonatal mouse cardiomyocytes were subjected to anoxia-reoxygenation (A/R). Pretreatment with EPO significantly reduced apoptosis in A/R-treated cells. This reduction in apoptosis was preceded by an increase in the mRNA and protein expression of HO-1. Selective inhibition of HO-1 using chromium mesoporphyrin (CrMP) significantly diminished the ability of EPO to inhibit apoptosis. Cotreatment of EPO with SB-202190, an inhibitor of p38 activation, blocked the EPO-mediated HO-1 expression and antiapoptotic effects, suggesting a p38-dependent mechanism. The in vivo significance of p38 and HO-1 as mediators of EPO's cardioprotection was investigated in mice subjected to myocardial I/R. Pretreatment with EPO decreased infarct size as well as I/R-induced apoptosis in wild-type mice. However, these effects were significantly diminished in HO-$1^{-/-}$ mice. Furthermore, EPO given during ischemia reduced infarct size in mice subjected to I/R, and this effect was blocked by CrMP treatment in wild-type mice. Moreover, inhibition of p38 diminished the cardioprotective effects of EPO. We conclude that upregulation of HO-1 expression via p38 signaling contributes to EPO-mediated cardioprotection during myocardial I/R.

myocyte; apoptosis; myocardial infarction; p38

ERYTHROPOIETIN (EPO) has been shown to protect the heart from ischemia-reperfusion (I/R) injury (8, 11). After myocardial infarction, EPO improves cardiac function and reduces ventricular remodeling (41). Our laboratory has demonstrated an important role of protein kinase B (Akt) and endothelial nitric oxide (NO) synthase (eNOS)-derived NO production in the antiapoptotic effects of EPO during myocardial I/R (7). However, since EPO is known to activate multiple intracellular signaling pathways, it is possible that additional mechanisms are involved in EPO's cardioprotective effects.

Heme oxygenase (HO) is the rate-limiting enzyme responsible for the breakdown of heme into free ferrous iron, carbon monoxide (CO), and biliverdin, which is ultimately converted to bilirubin. There are three known isoforms of HO. HO-1 is expressed ubiquitously and is inducible. HO-2 is constitutively active and expressed primarily in the brain and testes, and HO-3 is constitutively active as well, but less efficient than the other two oxygenases (47). HO-1 and its byproducts play an important role in regulating cardiomyocyte survival. To this end, the cardiac-specific overexpression of HO-1 reduces oxidative stress and cell death in the heart after myocardial I/R (49). Additionally, HO-1 is known to have a protective role in atherosclerosis (55) and endotoxemia (51). The effects of HO-1 are mainly mediated by the production of CO and bilirubin, but roles for ferritin activation have also been identified (43).

The regulation of HO-1 expression is complex and multifactorial and may involve one or more kinase signaling cascades (47). Indeed, p38 MAPK, ERKs, and Akt have all been implicated in the control of HO-1 expression (16, 22, 52). Recently, EPO has been demonstrated to increase HO-1 expression in the kidney (25). Furthermore, increases in monocyte HO-1 mRNA were seen after EPO treatment in hemodialysis patients (10). However, whether EPO upregulates HO-1 expression in the heart is not known.

In the present study, we hypothesized that EPO increases HO-1 expression in cardiomyocytes. We further hypothesized that the antiapoptotic effects of EPO are mediated, in part, via the induction of HO-1 expression. Experiments used both in vivo and in vitro models of myocardial I/R. In vitro, cultured neonatal mouse ventricular cardiomyocytes were subjected to anoxia-reoxygenation (A/R) to induce apoptosis. In vivo, myocardial I/R was performed in wild-type (WT) and HO-$1^{-/-}$ mice. Our data showed that treatment with EPO resulted in HO-1 upregulation and reductions in infarct size and cardiomyocyte apoptosis after myocardial I/R.

METHODS

Experimental animals. HO-$1^{-/-}$ mice as well as their WT littermates were obtained from Dr. Raymond Regan (Thomas Jefferson University, Philadelphia, PA). A breeding program was carried out to produce neonates and adults for this study. Animals were provided food and water ad libitum on a 12:12-h light-dark cycle. This investigation conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996). Experimental protocols were approved by the Animal Use Subcommittee of the University of Western Ontario (London, ON, Canada).

Isolation and culture of neonatal cardiomyocytes. Neonatal cardiomyocytes were prepared according to methods we have previously described (7, 42). Briefly, ventricular myocardial tissues from WT mice born within 24 h were minced in a Ca^{2+}- and Mg^{2+}-free HBSS. Myocytes were then dispersed by 22.5 μg/ml liberase blendzyme IV (Roche, Mississauga, ON, Canada) at 37°C for 40 min. Isolated cells were preplated for 90 min to remove adherent noncardiomyocytes.

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Cardiomyocytes were then plated in medium 199 (M199) containing 10% FBS in 48-well polystyrene plates precoated with 1% gelatin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 48 h of cell culture, cardiomyocytes were treated with recombinant human EPO (Eprex, Ortho Biotech, Toronto, ON, Canada) and the indicated reagents.

Real-time RT-PCR. Total RNA was isolated from cultured cardiomyocytes with TRIzol reagent (Invitrogen) as previously described (20). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and random primers (Invitrogen, Burlington, ON, Canada). Real-time PCR was conducted using SYBR Green PCR Master Mix as per the manufacturer's instructions (Eurogentec, San Diego, CA) as previously described. The oligonucleotide primers for HO-1 were as follows: sense 5′-TCCAGACACCGCTCCTCCAG-3′ and antisense 5′-GGATTTGGGGCTGCTGGTTTC-3′. 28S rRNA was used as a loading control using the following oligonucleotide primers: sense 5′-TTGAAAATCCGGGGGAGAG-3′ and antisense 5′-ACATTGTTCCAACATGCCAG-3′. Samples were amplified for 35 cycles using a MJ Research Opticon Real-Time PCR machine. Levels of HO-1 were compared with that of 28S rRNA, and the relative expression HO-1 was obtained.

Western blot analysis. HO-1, phosphorylated/total p38, ERK1/2, and Akt protein levels in cardiomyocytes and heart tissues were measured by Western blot analysis. Briefly, 40 μg of protein were separated by SDS-PAGE using 10% gels. Proteins were then transferred to polyvinlyidene difluoride membranes, and blots were probed with antibodies against HO-1 (1:2,000, Stressgen, Victoria, BC, Canada), p38 (1:800, Cell Signaling, Danvers, MA), phosphorylated p38 (Thr^{180}/Tyr^{182}, 1:800, Cell Signaling), ERK1/2 (1:800, Cell Signaling), phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}, 1:800, Cell Signaling), Akt (1:800, ABM, Vancouver, BC, Canada), phosphorylated Akt (Ser^{473}, 1:800, ABM), or α-actinin (1:2,000, Sigma, Oakville, ON, Canada). Blots were then washed and probed with horseradish peroxidase-conjugated secondary antibodies (1:2,000, Bio-Rad, Hercules, CA), and detection was performed using an ECL detection method. Signals were determined by densitometry, and the ratios of HO-1 to α-actinin or phosphorylated kinase to total kinase were determined.

A/R protocol. Cardiomyocytes were cultured to subconfluence and subjected to A/R as previously described with modifications (45). Cardiomyocytes were exposed to anoxia in serum-free M199 for 90 min. Anoxic conditions were obtained by an incubation at 37°C in a small humidified Plexiglas chamber containing myocytes with 95% N_2-5% CO_2 and confirmed by measuring chamber P_{O_2}. After the anoxic period, media were replaced with fresh, serum-free M199, and cardiomyocytes were incubated for a further 90 min under normal incubation conditions (reoxygenation). As a control, cardiac myocytes were given the same media changes but incubated under normal incubation conditions (normoxia) rather than the A/R protocol.

Caspase activity. Caspase-3 and -9 activities were measured using the QuantiZyme Assay System (AK-700, BioMol, Plymouth Meeting, PA) as we have previously described (7). Cell (50 μg/sample) or tissue lysates from the ischemic region of the heart (200 μg/sample) were incubated at 37°C for 16 h in the presence of the caspase-3 substrate Ac-DEVD-AMC or Ac-DEVD-AMC plus the inhibitor Ac-DEVD-CHO. For caspase-9 activity, samples were incubated in the presence of the caspase-9 substrate Ac-LEHD-AMC or Ac-LEHD-AMC plus the inhibitor Ac-LEHD-CHO under the same conditions. Fluorescent intensity (excitation at 360 nm and emission at 460 nm) was measured using a SpectraMax M5 multilabel microplate reader (Molecular Devices, Sunnyvale, CA). Data are expressed as amounts of AMC substrate cleaved per microgram of protein.

TUNEL staining. For TUNEL staining in cultured cardiomyocytes, cells were seeded on glass slides and fixed with 20% acetone + 80% methanol. For TUNEL staining in myocardial tissues, hearts were fixed in 4% paraformaldehyde and embedded with paraffin. Heart sections (4 μm) were cut and mounted on glass slides. TUNEL staining was performed using an In Situ Cell Death Detection Kit Fluorescein (Roche) as previously described (17). Slides were counterstained with Hoechst 33342 (1 μg/ml). For in vitro TUNEL staining, data were examined by fluorescence microscopy (×630 magnification), and numbers of TUNEL-positive nuclei were quantified. For in vivo TUNEL staining, data were examined using fluorescence microscopy, and numbers of TUNEL-positive nuclei in the peri-infarcted area (the border zone of 200 μm adjacent to the infarcted region) of the heart were quantified. Twenty fields (~6,000 cells) were quantified and verified by a second blinded, independent observer. Data are expressed as TUNEL-positive nuclei as a percentage of total nuclei.

Immunohistochemistry. Hearts were isolated, fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections (5 μm) were transferred to positively charged slides, and a rabbit polyclonal antibody to HO-1 (Stressgen, 1:100) was used in conjunction with the Vectastain Elite Kit (Vector Laboratories) according to the manufacturer's instructions. The chromogen diaminobenzidine was used to detect HO-1 staining, and hematoxylin was used as a counterstain. Slides were imaged at ×400 magnification.

Myocardial I/R. For experiments involving EPO pretreatment, male mice (2–3 mo old) were treated with 2,500 U/kg iv of recombinant human EPO (Eprex, Ortho Biotech) or an equivalent volume of saline 24 h before ligation as well as 30 min before the induction of myocardial ischemia as per our previous report (7). For experiments involving EPO administration after ischemia, mice were treated with EPO (2,500 U/kg iv) 5 min after coronary artery ligation. Additionally, where indicated, inhibition of p38 in vivo was achieved through treatment with SB-202190 (2 mg/kg ip) for 24 h and 12 h before ligation, and HO-1 inhibition was achieved through treatment with chromium mesoporphyrin (CrMP; 10 μmol/kg ip) immediately before surgery.

I/R was induced as previously described (7). Briefly, mice were anesthetized with a ketamine (50 mg/kg) and xylazine (12.5 mg/kg) cocktail, and animals were intubated and artificially ventilated with a respirator (SAR-830, CWE, Ardmore, PA.). Subsequently, a left intercostal thoracotomy was performed, and the left side of the heart was exposed. The pericardium was opened, and the left coronary artery was occluded for 45 min by positioning a suture (8-0) around it. After 45 min, the chest was reopened, and the suture was loosened to allow reperfusion for 3 h (in the case of EPO pretreatment) or 6 h (in the case of EPO given after the onset of ischemia). The lungs were hyperinflated using positive end-expiratory pressures (3 cmH_2O), and the thorax was closed.

Measurement of infarct size. Three hours after reperfusion, the coronary artery was religated using the same suture. Evans blue dye [1 ml of 2% (wt/vol)] was injected into the left ventricle via the cannulated right carotid artery to distinguish between perfused and nonperfused areas of the heart. Hearts were excised and cut into four transverse slices from the apex to base. Sections were stained with 1.5% triphenyltetrazolium chloride (TTC) for 30 min at 37°C. Since TTC stains viable tissue a deep red color, unstained tissue was infarcted. Sections were weighed, and each side of the section was photographed. The nonischemic area (blue), area at risk (red), and infarct area (pale) were measured using SigmaScan Pro. Infarct size is expressed as a percentage of the weight of the infarct area to the area at risk.

Measurement of Hb. Hb levels were measured using a Hb assay kit (Pointe Scientific, Lincoln Park, MI) according to manufacturer's instructions. Briefly, blood was taken from the tail vein of mice and incubated with Hb reagent. After 5 min, absorbance at 540 nm was read using the SpectraMax M5 multilabel microplate reader and standardized to known concentrations of Hb.

Statistical analysis. Data are expressed as means ± SE. Differences between groups were compared using Student's unpaired *t*-test or one- or two-way ANOVA followed by the Bonferroni post test where

appropriate. P values of <0.05 were considered statistically significant.

RESULTS

EPO treatment increases HO-1 expression in cardiomyocytes. The effect of EPO on HO-1 expression in cardiomyocytes was examined in cultured neonatal cardiomyocytes using 20 U/ml EPO, which we found from our previous study (7) to be an optimal dose in the prevention of apoptosis in cardiomyocytes. Treatment with EPO rapidly increased HO-1 mRNA expression as measured by real-time RT-PCR (Fig. 1*A*). Furthermore, treatment with EPO resulted in increased HO-1 protein expression beginning at 6 h and peaking after 12 h (Fig. 1*B*).

Inhibition of HO-1 activity diminishes the antiapoptotic effects of EPO. To simulate myocardial I/R, an A/R protocol was employed to induce cardiomyocyte apoptosis. Cultured cardiomyocytes were treated with EPO (20 U/ml) for 24 h and subjected to A/R. EPO pretreatment significantly lowered A/R-induced apoptosis in cardiomyocytes as measured by TUNEL staining (Fig. 1*C*) and caspase-3 and -9 activities (Fig. 1, *D* and *E*), consistent with our previous observations. To determine if the antiapoptotic effects of EPO are mediated through the activation of HO-1, cardiomyocytes were cotreated with EPO and the HO inhibitor CrMP (10 μM). Treatment with CrMP significantly diminished the effects of EPO on caspase activity and TUNEL positivity after A/R (Fig. 1, *C–E*). Additionally, cotreatment with a separate HO-1 inhibitor, tin mesoporphyrin (10 μM), also attenuated the antiapoptotic effects of EPO on caspase-3 activity compared with EPO treatment alone (2.52 ± 0.17 vs. 1.57 ± 0.12 pmol AMC cleaved/μg protein, $P < 0.05$).

Role of CO in the antiapoptotic effects of HO-1. The antiapoptotic effects of HO-1 have been attributed to the production of CO (6, 26), bilirubin (26), and ferritin (3). To determine the mechanism by which EPO-mediated HO-1 expression protects cardiomyocytes from apoptosis, cells were treated with EPO (20 U/ml) in the presence or absence of the CO scavenger Hb (4 μg/ml). Cotreatment with Hb blocked EPO-mediated reductions in A/R-induced apoptosis ($P < 0.05$; Fig. 2*A*). However, as Hb is also known to bind NO (19), and we (7) have previously demonstrated that EPO-mediated cardioprotection is dependent on eNOS-derived NO, it is possible that Hb simply blocked the effects of eNOS-derived NO. Therefore, we compared the effects of Hb to an inhibitor of NO production, N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM). Whereas L-NAME partially blocked EPO-mediated reductions in A/R-induced apoptosis, Hb was significantly more effective in blocking the antiapoptotic ef-

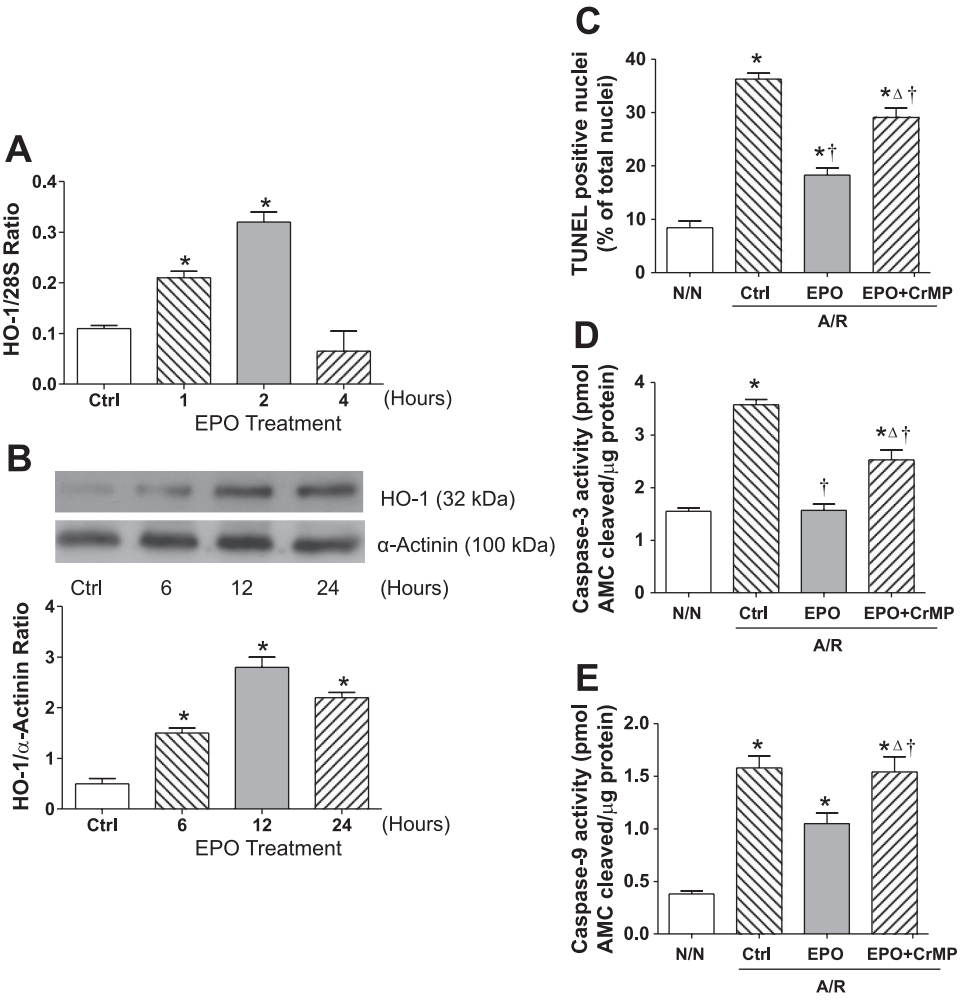

Fig. 1. Erythropoietin (EPO) protects cardiomyocytes from apoptosis via heme oxygenase (HO)-1. *A*: EPO (20 U/ml) increased HO-1 mRNA levels as measured by real-time RT-PCR in cardiomyocytes after 1 and 2 h of treatment. *B*, *bottom*: EPO increased HO-1 protein levels as measured by Western blot analysis in cultured cardiomyocytes after 6, 12, and 24 h of treatment. *Top*, representative blot. $n = 4–5$. $^*P < 0.05$ vs. control (Ctrl). Additionally, cardiomyocytes were pretreated with EPO (20 U/ml) for 24 h and subjected to anoxia-reoxygenation (A/R). Treatment with EPO significantly reduced A/R-induced apoptosis as measured by TUNEL staining (*C*), caspase-3 activity (*D*), and caspase-9 activity (*E*). These effects were diminished in cells cotreated with the HO-1 inhibitor chromium mesoporphyrin (CrMP). $n = 4–6$. $^*P < 0.05$ vs. normoxia (N/N), $\dagger P < 0.05$ vs. A/R Ctrl, and $\Delta P < 0.05$ vs. EPO treatment as determined by one-way (*A* and *B*) or two-way ANOVA (*C–E*) with the Bonferroni post test.

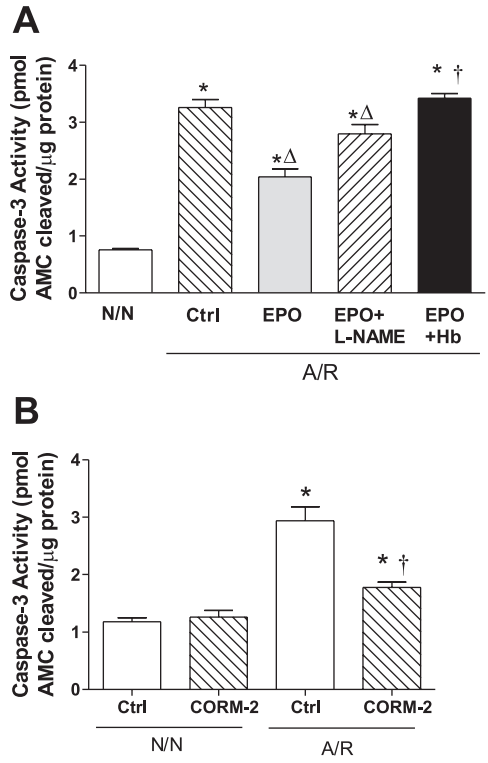

Fig. 2. Role of carbon monoxide (CO) in the antiapoptotic effects of EPO. *A*: treatment with EPO significantly reduced A/R-induced caspase-3 activation. This effect was diminished upon cotreatment with the nitric oxide (NO) synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) and further reduced upon cotreatment with Hb (4 μg/ml), which scavenges NO and CO. $n = 5–6$. *$P < 0.05$ vs. N/N, Δ$P < 0.05$ vs. A/R Ctrl, and †$P < 0.05$ vs. A/R + EPO + L-NAME as determined by one-way ANOVA with the Bonferroni post test. *B*: treatment with the CO-releasing molecule tricarbonyldichlororuthenium (II) dimer (CORM-2; 30 μM) 1 h before anoxia protected cardiomyocytes from A/R-induced caspase-3 activation in a manner similar to EPO. $n = 3$. *$P < 0.05$ vs. N/N and †$P < 0.05$ vs. A/R Ctrl as determined by two-way ANOVA with the Bonferroni post test.

fects of EPO ($P < 0.05$; Fig. 2*A*), indicating that, in addition to NO, CO contributes to the antiapoptotic effects of EPO.

To further confirm the role of CO in the antiapoptotic effects of EPO, cells were treated with a CO-releasing molecule, tricarbonyldichlororuthenium (II) dimer (CORM-2; 30 μM). The administration of CORM-2 at this concentration has been shown to release CO without significant cytotoxicity (36). Treatment with CORM-2 significantly reduced A/R-induced apoptosis in cardiomyocytes in a manner similar to that of EPO ($P < 0.05$; Fig. 2*B*).

EPO increases kinase phosphorylation in cultured cardiomyocytes. EPO is known to increase the phosphorylation of multiple kinases, including Akt, ERK1/2, and p38 (44). We examined the effects of EPO on the phosphorylation of p38, Akt, and ERK1/2 in cultured cardiomyocytes. After 30 min of EPO treatment (20 U/ml), phosphorylation of ERK1/2, p38, and Akt was significantly increased (Fig. 3).

EPO regulates HO-1 expression via a p38-dependent mechanism and inhibits apoptosis via p38 and Akt-dependent mechanisms. To determine how EPO regulates HO-1 expression, cardiomyocytes were pretreated with EPO (20 U/ml) in combination with LY-294002 (10 μM, upstream Akt inhibitor), U-0126 (10 μM, upstream ERK1/2 inhibitor), or SB-202190 (10 μM, p38 inhibitor). HO-1 protein levels were determined after 12 h of drug treatments. Pretreatment with SB-202190 (p38 inhibitor) but not LY-294002 (upstream Akt inhibitor) or U-0126 (ERK inhibitor) significantly inhibited the effect of EPO on HO-1 expression ($P < 0.05$; Fig. 4*A*), suggesting that p38 is responsible for the regulation of HO-1 expression by EPO.

To determine if p38 activation was involved in the antiapoptotic effects of EPO, cardiomyocytes were pretreated with EPO and SB-202190 for 24 h and subjected to A/R. TUNEL-positive nuclei and caspase-3 and -9 activities were decreased significantly by EPO treatment, and these effects of EPO on apoptosis were significantly diminished by inhibition of p38 with SB-202190 ($P < 0.05$; Fig. 4, *B–D*). Furthermore, the effects of EPO on A/R-induced caspase-3 activity were abrogated by cotreatment with LY-294002 (4.16 ± 0.32 vs. 2.16 ± 0.24 pmol AMC cleaved/μg protein, $P < 0.05$) but not U-0126 (2.32 ± 0.22 vs. 2.16 ± 0.24 pmol AMC cleaved/μg protein, P = not significant). Thus, p38 and Akt, but not ERK, are involved in EPO-mediated reductions in apoptosis.

Role of HO-1 in the cardioprotective effects of EPO during myocardial I/R. The role of HO-1 in the cardioprotective effects of EPO was investigated using an in vivo model of

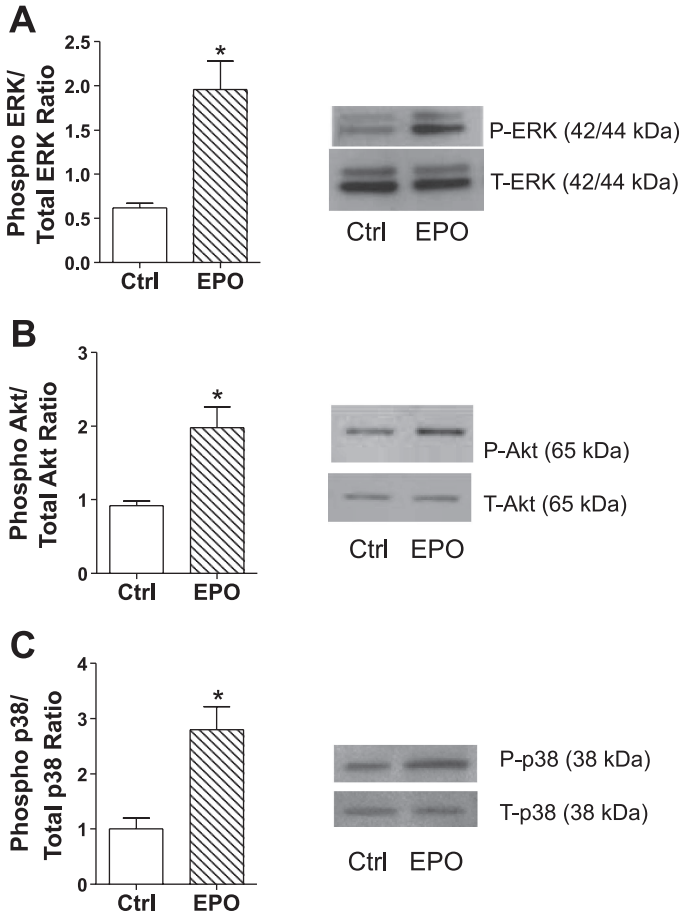

Fig. 3. EPO increases the phosphorylation of protein kinases in cardiomyocytes. Cardiomyocytes were treated with EPO (20 U/ml for 30 min). The phosphorylation of ERK1/2 (*A*), Akt (*B*), and p38 (*C*) protein kinases was measured by Western blot analysis. Phosphorylated (P)-to-total (T) protein kinase ratios were quantified by densitometry. Data are means ± SE of 4 independent experiments. *$P < 0.05$ vs. Ctrl as determined by Student's *t*-test.

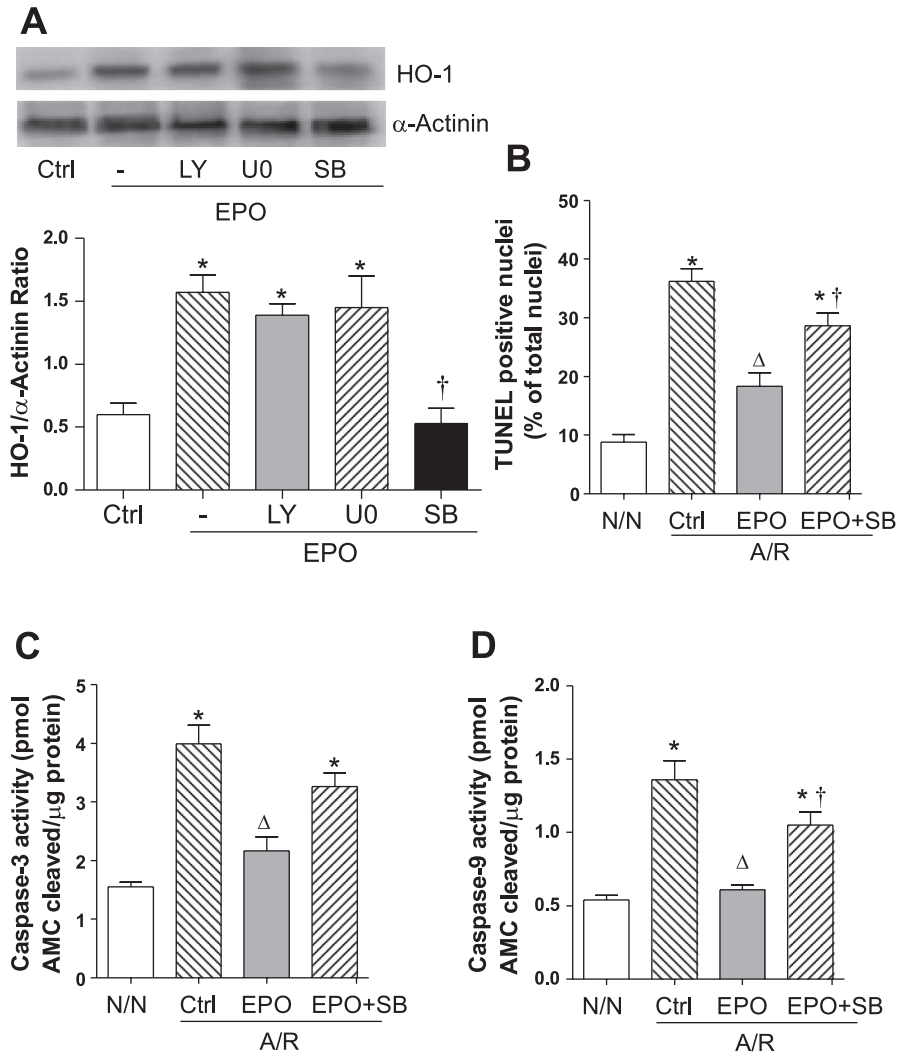

Fig. 4. EPO-mediated increases in HO-1 and decreases in apoptosis are dependent on p38 MAPK activation. *A*, *bottom*: HO-1 protein levels were determined by Western blot analysis in cardiomyocytes treated with EPO (20 U/ml) in the presence of LY-294002 (LY; 10 μM, upstream Akt inhibitor), U-0126 (U0; 10 μM, upstream ERK1/2 inhibitor), or SB-202190 (SB; 10 μM, p38 inhibitor) for 12 h. *Top*, representative blot. $n = 4–6$. *$P < 0.05$ vs. Ctrl and †$P < 0.05$ vs. EPO alone as determined by one-way ANOVA with the Bonferroni post-test. *B–D*: cardiomyocytes were treated with EPO (20 U/ml) in the presence or absence of SB for 24 h before A/R. Cotreatment with SB significantly diminished the antiapoptotic effects of EPO after A/R as measured by TUNEL staining (*B*), caspase-3 activity (*C*), and caspase-9 activity (*D*). $n = 4–6$. *$P < 0.05$ vs. N/N, Δ$P < 0.05$ vs. A/R Ctrl, and †$P < 0.05$ vs. A/R + EPO as determined by two-way ANOVA with the Bonferroni post test.

myocardial I/R. Mice were subjected to 45 min of myocardial ischemia followed by 3 h of reperfusion. Pretreatment with EPO (2,500 U/kg iv) for 24 h as well as 30 min before ligation had no effect on Hb levels (129.6 ± 1.7 vs. 129.5 ± 1.9 g/l) but increased myocardial HO-1 protein expression in WT mice ($P < 0.05$; Fig. 5*A*). Immunohistochemical staining showed that cardiomyocytes stained positive for HO-1 in mice treated with EPO (Fig. 5*B*). It has been proposed that HO-1 expression may be induced via NO (13, 15, 29). Given that we have previously reported EPO-mediated increases in eNOS-derived NO production (7), we examined the effects of EPO on HO-1 expression in eNOS$^{-/-}$ mice. Pretreatment with EPO (2,500 U/kg iv) for 24 h as well as 30 min before ligation increased myocardial HO-1 expression in eNOS$^{-/-}$ mice in a manner similar to that in WT mice (Fig. 5*A*). Thus, EPO-mediated increases in HO-1 appear to occur independent of eNOS-derived NO signaling.

To determine the role of HO-1 in EPO-mediated reductions in I/R-induced apoptosis and infarction, mice were subjected to ischemia for 45 min followed by reperfusion for 3 h. Pretreatment with EPO significantly decreased myocardial apoptosis as determined by TUNEL staining and caspase-3 and -9 activities in WT mice ($P < 0.05$; Fig. 5, *C–E*). However, these effects were not seen in HO-1$^{-/-}$ mice ($P < 0.05$; Fig. 5, *C–E*). Furthermore, EPO pretreatment resulted in a significant reduction in infarct size in WT mice but not in HO-1$^{-/-}$ mice (Fig. 6).

Pretreatment with EPO may be of limited clinical relevance. For example, in patients suffering from acute myocardial infarction, pharmacological intervention would typically occur after the onset of ischemia. To determine if EPO confers protection when administered after ischemia, mice were subjected to ischemia for 45 min followed by reperfusion for 6 h. This time window was chosen because upregulation of HO-1 protein expression in cardiomyocytes requires 6 h of EPO treatment (see Fig. 1*B*). Treatment with EPO (2,500 U/kg iv) 5 min after the onset of ischemia significantly reduced infarct size ($P < 0.05$; Fig. 6*B*). The protective effects of EPO given during ischemia were blocked by cotreatment with the HO-1 inhibitor CrMP.

Role of p38 in the cardioprotective effects of EPO during myocardial I/R. Our in vitro data suggested that EPO increases HO-1 expression via the activation of p38 MAPK in cultured cardiomyocytes. To determine if EPO treatment increases p38 phosphorylation, and to clarify the effects of I/R on p38 phosphorylation, mice were treated with EPO (2,500 U/kg iv)

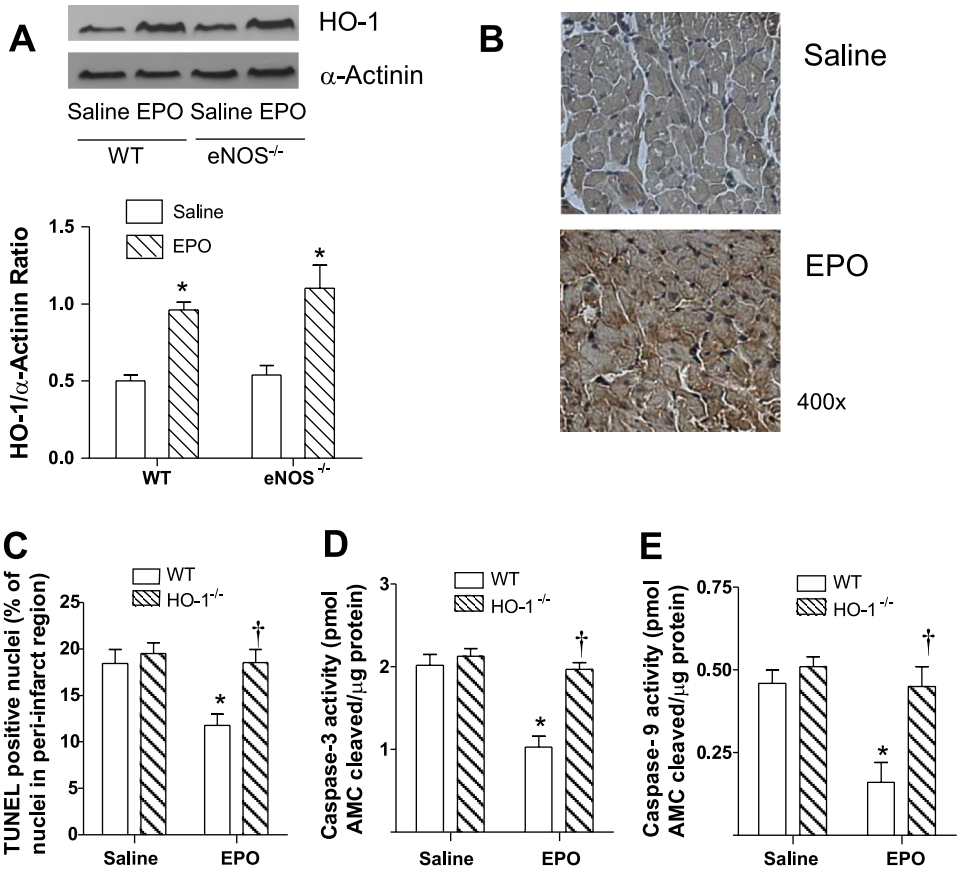

Fig. 5. Effects of EPO on HO-1 expression and myocardial apoptosis during myocardial ischemia-reperfusion (I/R). Wild-type (WT) and HO-$1^{-/-}$ mice were pretreated with saline or EPO (2,500 U/kg iv) for 24 h as well as 30 min before surgery. Myocardial ischemia was induced by occlusion of the coronary artery for 45 min followed by 3 h of reperfusion. *A*: EPO increased myocardial HO-1 expression in the noninfarcted region of WT and endothelial NOS (eNOS)$^{-/-}$ mice. *B*: representative immunohistochemical staining of HO-1 in saline- and EPO-treated WT hearts. *C–E*: EPO decreased myocardial apoptosis in WT mice but not HO-$1^{-/-}$ mice, as determined by TUNEL staining in the peri-infarcted region of the heart (*B*) and caspase-3 (*C*) and caspase-9 (*D*) activity in the ischemic region. $n = 5$–6 mice/group. *$P < 0.05$ vs. WT + saline and †$P < 0.05$ vs. WT + EPO as determined by Student's *t*-test (*A*) or two-way ANOVA followed by the Bonferroni post test (*C–E*).

for 24 h as well as 30 min before surgery. Phosphorylation of p38 was not increased by 45 min of ischemia followed by 3 h of reperfusion (I/R). However, EPO treatment significantly increased myocardial p38 phosphorylation in both sham-operated mice as well as mice subjected to I/R ($P < 0.05$; Fig. 7*A*).

To assess the role of p38 in the in vivo cardioprotective effects of EPO, mice were pretreated with the p38 inhibitor SB-202190 in the presence and absence of EPO. Inhibition of p38 alone had no significant effect on basal HO-1 expression and infarct size (Fig. 7, *B* and *C*) or myocardial apoptosis, as determined by TUNEL and caspase-3 and -9 activities (Fig. 8). However, SB-202190 significantly blocked EPO-mediated increases in HO-1 expression (Fig. 7*B*) and EPO-mediated decreases in infarct size (Fig. 7*C*) and myocardial apoptosis (Fig. 8). The results suggest that p38 activation contributes to HO-1 expression and cardioprotection by EPO treatment during myocardial I/R.

DISCUSSION

In the present study, we investigated the role of HO-1 in the cardioprotective effects of EPO during myocardial I/R. The major finding was that EPO upregulates HO-1 expression via the activation of p38 MAPK. Moreover, we demonstrated, for the first time, that the upregulation of HO-1 contributes to the antiapoptotic effects of EPO in cardiomyocytes in vitro and the cardioprotective effects of EPO treatment in myocardial I/R in vivo.

Recent studies (10, 25) have shown that EPO increases HO-1 expression in monocytes and renal endothelial cells. A novel finding from our study is that treatment with EPO increases HO-1 expression in cardiomyocytes as well as in the myocardium in vivo. We further demonstrated that HO-1 is important in the cardioprotective effects of EPO. Pharmacological inhibition of HO-1 resulted in a decreased antiapoptotic effect of EPO in vitro, and the genetic deletion of HO-1 resulted in a diminished antiapoptotic effect of EPO in vivo. Furthermore, reductions in infarct size after myocardial I/R were seen in WT mice but not in HO-$1^{-/-}$ mice pretreated with EPO. Additionally, we examined the effects of EPO administered during ischemia on infarct size in mice. Previous reports (21, 30, 40) have demonstrated that EPO reduces myocardial infarct size when administered after the onset of ischemia in rabbit, rodent, and canine models of I/R. Similar to these reports, EPO treatment significantly reduced infarct size when administered after the onset of ischemia in our model of I/R in mice. Moreover, this effect was blocked by cotreatment with the HO-1 inhibitor CrMP. Thus, our study provides strong evidence of a role for HO-1 in EPO-mediated cardioprotection.

The degradation of heme by HO-1 results in three principle reaction products: bilirubin, iron, and CO. Of note is the fact that all three products have been implicated in the protective effects of HO-1. Iron released by HO-1 may reduce apoptosis (14), possibly through increases in ferritin (3, 46). Additionally, bilirubin (and its precursor biliverdin) are known to reduce oxidative stress (53), inflammation (18), and apoptosis (26). Finally, CO production is known to be involved in the antiapoptotic (6, 26) and anti-inflammatory effects of HO-1 (38). In the present study, treatment with CORM-2 blocked

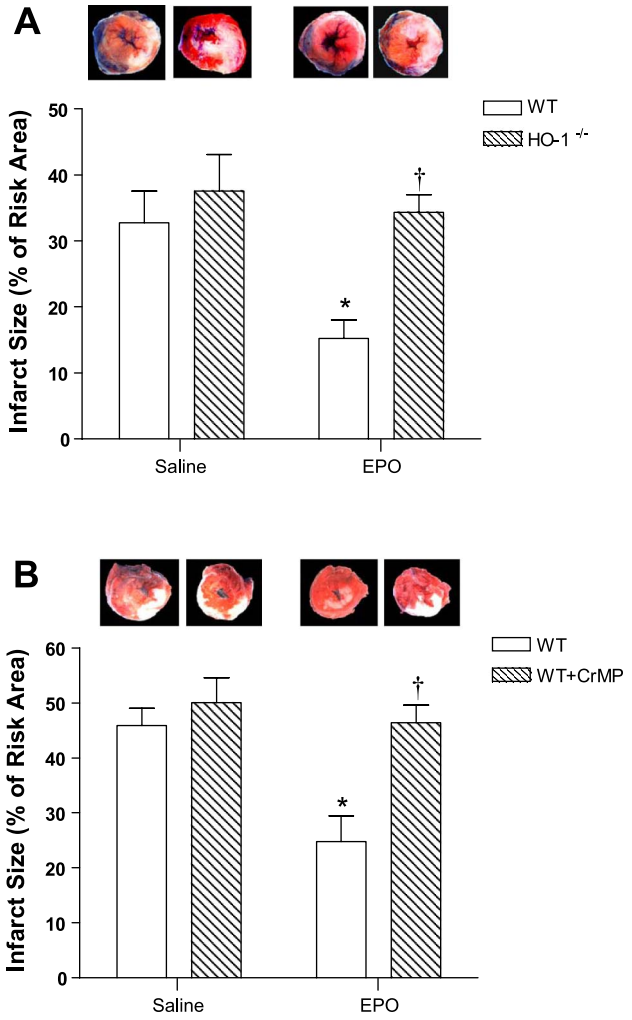

Fig. 6. Effects of EPO treatment on infarct size after myocardial I/R. *A*: WT and HO-1$^{-/-}$ mice were pretreated with saline or EPO (2,500 U/kg iv) for 24 h as well as 30 min before surgery. Myocardial ischemia was induced by occlusion of the coronary artery for 45 min followed by 3 h of reperfusion. EPO treatment significantly decreased infarct size in WT mice but not in HO-1$^{-/-}$ mice (n = 5–6). *B*: EPO (2,500 U/kg iv) treatment after ischemia reduced myocardial infarct size in WT mice. Myocardial ischemia was induced by occlusion of the coronary artery for 45 min followed by 6 h of reperfusion (n = 4). This effect was blocked by cotreatment with the HO-1 inhibitor CrMP (10 μmol/kg ip). *$P < 0.05$ vs. WT + saline and †$P < 0.05$ vs. WT + EPO as determined by two-way ANOVA followed by the Bonferroni post test. *Insets* are representative sections of triphenyltetrazolium chloride-stained hearts corresponding to each group.

A/R-induced apoptosis in a manner similar to EPO. Scavenging of CO with Hb completely reversed EPO-mediated reductions in apoptosis, an effect that was significantly greater than inhibition of NOS activity with L-NAME. These results suggest that the cardioprotective effects of EPO are mediated through the production of CO from HO-1 in addition to eNOS-derived NO.

The protein kinase p38 is known to be capable of inducing HO-1 expression in cardiomyocytes (22), whereas Akt and ERK1/2 seems to be important in the induction of HO-1 expression in several other cell types (24, 33, 54). Interestingly, EPO treatment has previously been shown to activate each of these kinase signaling pathways in isolated rabbit hearts (44). In our study, we also observed EPO-dependent increases in Akt, p38, and ERK1/2 phosphorylation in cultured cardiomyocytes and EPO-dependent increases in myocardial p38 phosphorylation in vivo. Furthermore, EPO-mediated increases in HO-1 expression were found to be dependent on p38 activation in isolated cardiomyocytes and in the heart. It is not surprising that EPO-mediated increases in HO-1 occur independent of ERK1/2 activation since, to our knowledge, ERK1/2 has not been implicated in the regulation of HO-1 in cardiomyocytes. Conversely, p38 is known to regulate HO-1

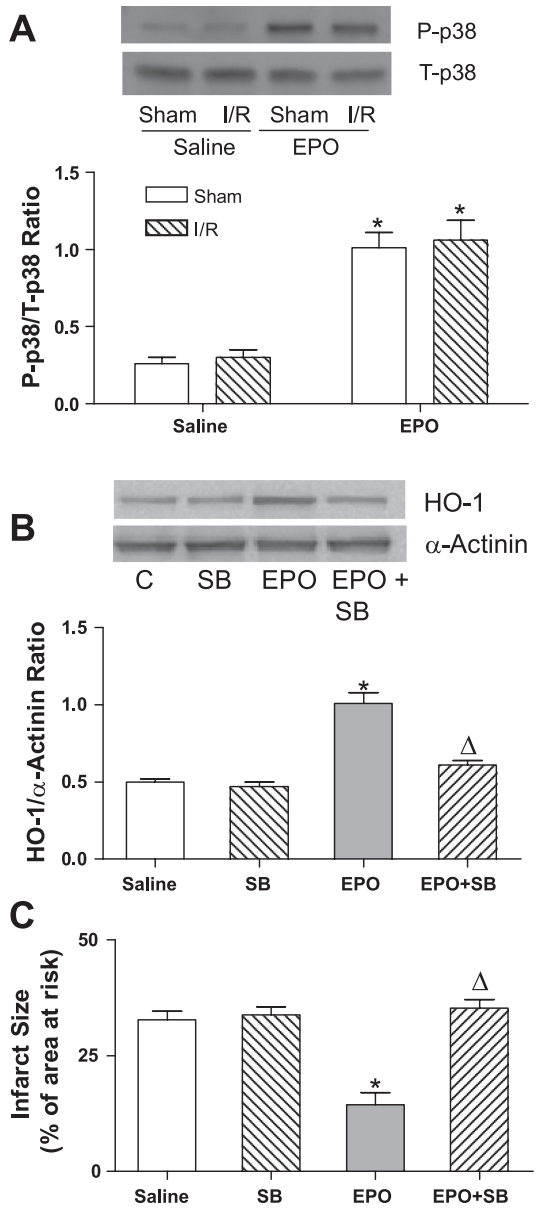

Fig. 7. Role of p38 in EPO-mediated HO-1 expression and reduction in infarct size after myocardial I/R. WT mice were pretreated with saline or EPO (2,500 U/kg iv) for 24 h as well as 30 min before surgery in the presence or absence of the p38 inhibitor SB (2 mg/kg ip). Myocardial ischemia was induced by occlusion of the coronary artery for 45 min followed by 3 h of reperfusion. *A*: myocardial p38 phosphorylation in the noninfarcted region of hearts of EPO-treated sham-operated and I/R mice. *B*, *bottom*: HO-1 protein expression in the noninfarcted region of the heart. *Top*, representative blot. C, Ctrl. *C*: infarct size after myocardial I/R. n = 4–5 mice/group. *$P < 0.05$ vs. saline and Δ$P < 0.05$ vs. EPO as determined by two-way ANOVA with the Bonferroni post test.

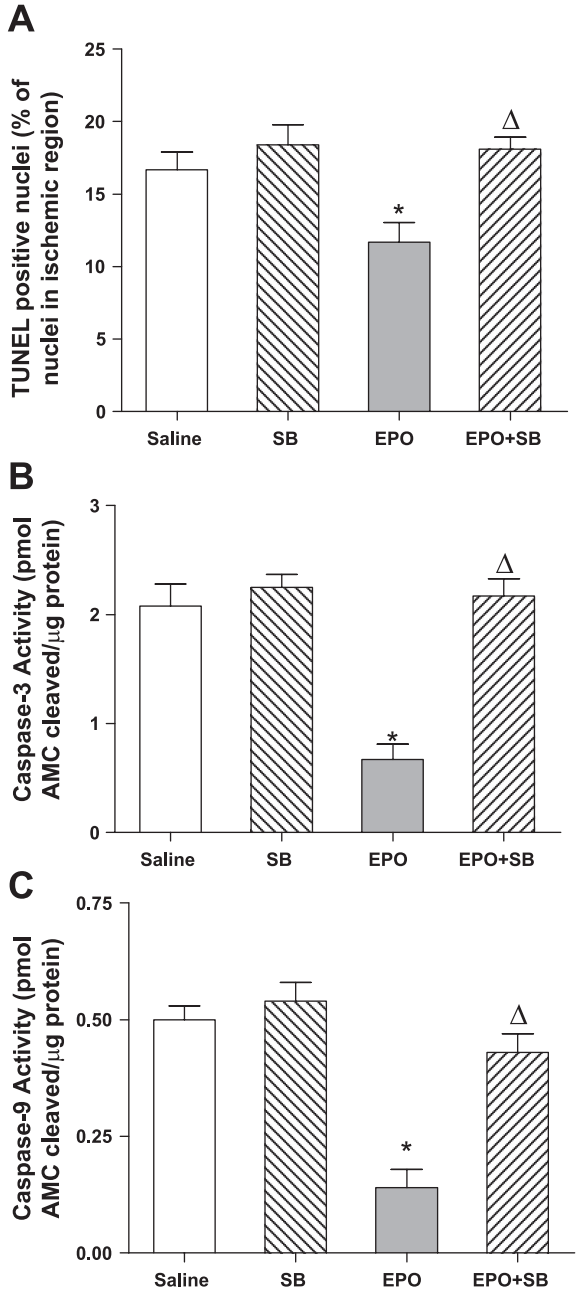

Fig. 8. Effects of p38 inhibition on EPO-mediated decreases in apoptosis after myocardial I/R. WT and HO-1$^{-/-}$ mice were pretreated with saline or EPO (2,500 U/kg iv) for 24 h as well as 30 min before surgery in the presence or absence of the p38 inhibitor SB (2 mg/kg ip). Myocardial ischemia was induced by occlusion of the coronary artery for 45 min followed by 3 h of reperfusion. Apoptosis was measured by TUNEL staining (*A*), caspase-3 activity (*B*), and caspase-9 activity (*C*). $n = 5$ mice/group. *$P < 0.05$ vs. saline and $\Delta P < 0.05$ vs. EPO as determined by two-way ANOVA with Bonferroni the post test.

expression in cardiomyocytes (22). Interestingly, p38 inhibition did not decrease HO-1 expression in the myocardium, suggesting that p38 does not play a major role in the regulation of HO-1 expression basally or during I/R. While both ischemia and reperfusion are known to increase p38 activation, p38 activation under these conditions is transient, and levels of phosphorylation return to baseline within 1 h (32). Since a 3-h reperfusion protocol was used in our study, it is likely that the transient p38 phosphorylation returned to the baseline. As such, we were not able to detect any significant p38 phosphorylation after myocardial I/R. However, pharmacological inhibition of p38 blocked EPO-mediated increases in HO-1. Thus, data from the present study support the notion that EPO-induced HO-1 expression is mediated by p38 in cardiomyocytes. While Akt activation is also known to be important in the induction of HO-1 expression (33), it does not appear to be involved in the induction of HO-1 expression after EPO treatment in our model. In addition to regulation at the transcriptional level, HO-1 can also be phosphorylated by Akt, resulting in an upregulation of HO-1 activity (48). It remains to be determined if EPO increases HO-1 activity via Akt-mediated phosphorylation in cardiomyocytes. Interestingly, HO-1 itself is known to increase Akt phosphorylation through increases in biliverdin reductase-dependent activation of phosphatidylinositol 3-kinase (PI3K) (39). However, in cultured cardiomyocytes, we found that Akt phosphorylation preceded HO-1 expression, suggesting that EPO is capable of activating Akt independent of HO-1.

We also examined the roles of Akt, ERK, and p38 in the antiapoptotic effects of EPO. Using pharmacological inhibitors, we demonstrated the important roles of p38 and Akt, but not ERK, in EPO-mediated protection of cardiomyocytes from A/R-induced apoptosis. Previous studies on EPO's antiapoptotic effects have focused largely on the roles of PI3K and Akt. Indeed, this pathway has been shown to be critical in the cardioprotective effects of EPO (7, 9, 28). However, the roles of p38 and ERK in the cardioprotective effects of EPO have not been adequately addressed. Inhibition of ERK activation had no effect on the antiapoptotic effects of EPO; however, p38 inhibition blocked EPO-mediated reductions in A/R-induced apoptosis in vitro and I/R-induced apoptosis in vivo. In the ischemic heart, p38 appears to have both protective and detrimental effects (2). For example, p38 has been shown to promote myocardial apoptosis (32). In contrast, p38 is also known to mediate the beneficial effects of preconditioning (35, 50). Our data suggest that, much like ischemic preconditioning, p38 activation after EPO treatment is beneficial and protective. Indeed, emerging evidence suggests that EPO administration mimics ischemic preconditioning in the heart (1).

Recently, we (7) demonstrated that EPO also inhibits apoptosis through an eNOS-dependent mechanism. Interestingly, NO production has previously been shown to increase HO-1 expression (13, 15). It is thus logical to predict that EPO increases HO-1 via eNOS. However, in this study, we found that EPO increases myocardial HO-1 expression in eNOS$^{-/-}$ mice. Moreover, while EPO increases eNOS via an Akt-dependent pathway (7, 45), EPO-mediated increases in HO-1 expression were found to be dependent on p38 and independent of Akt. Thus, our observations suggest that EPO activates eNOS and HO-1 via distinct pathways. Indeed, a recent report by Kim et al. (27) has brought into question the ability of NO to regulate HO-1 expression at all. They found that the mechanism by which the NO donor sodium nitroprusside increases HO-1 expression is via an increase in free iron release rather than NO production. NO itself played a negligible role in the induction of HO-1 expression (27).

Finally, in our study, the in vivo dose of 2,500 U/kg is ~10–15 times higher than the currently recommended clinical doses of 100–300 U/kg (12, 37), and the in vitro dose of 20 U/ml represents ~6–10 times the peak plasma levels achieved

with clinically relevant doses (23, 34). Recently, evidence has emerged that the tissue-protective effects of EPO are mediated by a heterodimeric receptor consisting of the EPO receptor (EPOR) and the β-chain of IL-3 and granulocyte-macrophage colony-stimulating factor receptors, also known as CD131 (4). It has been suggested that the affinity of the heterodimer is much lower than that of the homodimeric EPOR (5, 31). Thus, higher doses of EPO may be needed to achieve cardiac protection than are necessary for the correction of anemia. Importantly, given that our acute treatment with EPO did not alter Hb levels, the cardioprotective effects observed in this study are likely achieved through direct receptor-mediated signaling rather than an increase in oxygen delivery by red blood cells to the myocardium.

In summary, the present study identified an important role of HO-1 in the antiapoptotic and cardioprotective effects of EPO during myocardial I/R. The upregulation of HO-1 was dependent on p38 activation. These observations provide insights into the signaling mechanisms by which EPO exerts its potent cardioprotection in myocardial I/R.

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