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Erythropoietin prevents the acute myocardial inflammatory response induced by ischemia/reperfusion via induction of AP-1

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Abstract

Objective: Erythropoietin (EPO) prevents the myocardial dysfunction induced by ischemia/reperfusion (I/R). Since I/R-induced myocardial dysfunction is associated with an acute inflammatory response, we assessed the anti-inflammatory properties of EPO using in vitro and in vivo models of I/R.

Methods: Isolated cardiac myocytes were exposed to anoxia/reoxygenation (A/R; the in vitro counterpart to I/R). Hearts were challenged with I/R in situ.

Results: In vitro, A/R increased myocyte oxidant stress and converted the myocytes to a proinflammatory phenotype (these myocytes induced PMN transendothelial migration). Pretreatment of the myocytes with EPO prevented the A/R-induced proinflammatory effects. EPO increased myocyte (1) nuclear translocation of AP-1 (*c-fos/c-jun*), (2) eNOS, but not iNOS, protein expression, and (3) NO production. An AP-1 "decoy" oligonucleotide prevented the induction of eNOS by EPO and reversed the beneficial effect of EPO. An inhibitor of phosphatidylinostol 3 (PI3)-kinase prevented the nuclear translocation of AP-1 induced by EPO. In vivo, in wild type mice, I/R induced an increase in myocardial MPO activity (indicative of PMN infiltration); an effect prevented by pretreatment of the mice with EPO. This anti-inflammatory effect of EPO was not observed in cardiac specific *c-fos*$^{-/-}$ mice.

Conclusions: Collectively, these findings indicate that EPO can ameliorate the myocardial inflammatory response in both in vitro and in vivo models of I/R. This beneficial effect of EPO is mediated by eNOS-derived NO via a PI3-kinase-dependent activation of AP-1.

Keywords: Myocardial, ischemia/reperfusion; AP-1; eNOS

1. Introduction

Reperfusion of previously ischemic myocardium results in detrimental effects to the heart characterized by inflammation, infarction, and contractile impairments [1]. In vivo studies of myocardial ischemia/reperfusion (I/R) injury indicate that polymorphonuclear neutrophil (PMN) infiltration of the affected tissue plays a pivotal role in I/R-induced

Abbreviations: AP-1, Activation protein-1; I/R, ischemia/reperfusion; A/R, anoxia/reoxygenation; EPO, erythropoietin; EPO-R, erythropoietin receptor; NOS, nitric oxide synthase; PMN, polymorphonuclear leukocytes; DHR, dihydrorhodamine; DAF-FM, 4-amino-5-methylamino-2′ -7′ -difluorofluorescein; NFκB, nuclear factor-kappa B; MPO, myeloperoxidase; PI3-kinase, phosphatidylinositol 3 kinase

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cardiac dysfunction [1–3]. Indeed, the severity of the I/R-induced myocardial injury is directly related to the extent of PMN accumulation within the heart [4]. Furthermore, interfering with PMN infiltration into the myocardium (immunoneutralization or genetic deletion of adhesion molecules) affords protection against I/R-induced injury (reviewed in Ref. [5]). In vitro studies indicate that although cardiac myocytes are not injured by a simulated I/R challenge, they are converted to a proinflammatory phenotype capable of promoting PMN transendothelial migration [5,6]. Collectively, the in vivo and in vitro studies of I/R have provided compelling evidence to indicate that an acute inflammatory response in the heart is a major contributor to the I/R-induced cardiac dysfunction.

Erythropoietin (EPO) is a glycoprotein produced by the adult kidney that regulates hematopoiesis [7]. It is released in response to hypoxia and interacts with EPO receptors (EPO-R) on erythroid progenitor cells in the bone marrow to decrease apoptosis and promote their differentiation [7]. The resultant increase in red blood cell mass serves to maintain adequate tissue oxygenation in the face of the hypoxic stress. In addition to erythroid progenitor cells, it has become apparent that EPO also interacts with EPO-R on other cell types, e.g., neuronal cells [8,9], endothelial cells [10], vascular smooth muscle cells [11,12], and cardiac myocytes [13]. Some of the intracellular signals elicited by these latter interactions appear to play an important role in protecting organs/tissues from the deleterious effects of I/R or hypoxia. Much of the work dealing with this non-hematopoeitic function of EPO has focused on the brain and/or neurons [9,14,15]. More recently, it has been shown that EPO has a protective effect against the deleterious consequences of I/R (in vivo) or oxidant stress (in vitro) in the heart [16–20] and kidney [21].

The limited number of studies addressing the mechanism(s) by which EPO exerts its protective effect have focused on the inhibitory effect on I/R or oxidant stress-induced apoptosis [9,20,21]. Despite the fact that EPO can exert anti-inflammatory effects in the brain, i.e., reduced cytokine production and leukocyte recruitment [22], there is no information on the anti-inflammatory effects of EPO in the heart. Thus, the major objective of the present study was to assess whether EPO could ameliorate the myocardial inflammation induced by I/R and address some of the potential mechanisms involved. Using both in vitro and in vivo models of I/R, we provide evidence that the nuclear transcription factor, AP-1, plays an important role in the anti-inflammatory effects of EPO.

2. Materials and methods

This investigation conforms with the Guide for the care and use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23).

2.1. EPO receptor

EPO receptor in cardiac myocytes and heart tissue was assessed by Western blot as previously described [6,23]. Briefly, 5 μg protein of total cell or heart tissue lysates were resolved on 12.5% SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat milk, the membranes were blotted with an anti-EPO-R antibody (rabbit polyclonal IgG, SC-697; Santa Cruz). The specific bands were visualized by ECL plus Western blotting detection system (Amersham Biosciences) and quantified by densitometry. EPO-R was detected in cultured mouse neonatal cardiac myocytes, as well as, in whole tissue homogenates of adult mouse hearts (Fig. 1). These observations are consistent with previous studies noting the presence of EPO-R in rat neonatal [13] and adult [24] cardiac myocytes and adult rat hearts [24].

2.2. In vitro studies

2.2.1. Cells

Neonatal mouse cardiac myocytes, adult myocardial endothelial cells, and adult mouse PMN were isolated from wild type mice (C57BL6) as previously described [5]. For some experiments, neonatal cardiac myocytes were isolated from eNOS or iNOS deficient mice ($eNOS^{-/-}$ or $iNOS^{-/-}$ mice; Jackson Laboratory). The $eNOS^{-/-}$ and $iNOS^{-/-}$ mice were backcrossed for 8–10 generations.

2.2.2. Anoxia/Reoxygenation (A/R) protocol

The in vitro model of A/R used in the present study was similar to that described previously [5,6]. Briefly, confluent beating mouse cardiac myocyte monolayers were exposed to anoxia for 30 min in phenol red and glucose-free DMEM and then reoxygenated (anoxia/reoxygenation, A/R). Anoxic conditions were obtained by perfusion of a small humidified plexiglass chamber containing the myocytes with 95% N_2 and 5% CO_2 via a catalytic deoxygenator apparatus (Engelhard, NJ) and confirmed by measuring chamber pO_2. As a control, cardiac myocytes were exposed to normoxia (room air) rather than anoxia (normoxia/reoxygenation, N/R). The cardiac myocytes are not injured by this A/R protocol [6] and this A/R model mimics some of the key features of I/R models in vivo [25–27]. In some

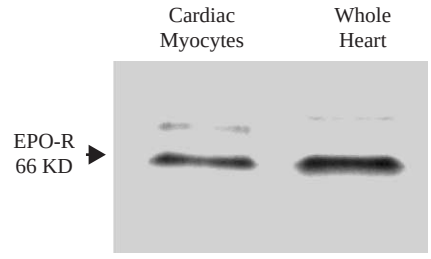


Fig. 1. Western blot demonstrating the presence of erythropoietin receptor (EPO-R) in isolated cardiac myocytes and whole heart. Representative of 2 experiments.

experiments, myocytes were pretreated with EPO using a previously published dosing regimen [5,6,23]. To this end the myocytes were incubated for 4 h either with M199 containing 5 IU/ml of recombinant mouse EPO (rmEPO; Roche) or just M199. Subsequently, the EPO and M199 were removed, and the myocytes were incubated with M199 containing 10% fetal calf serum for another 20 h prior to subjecting them to the A/R protocol.

2.2.3. Oxidant stress

Oxidant production within cardiac myocytes was assessed by measuring the oxidation of intracellular dihydrorhodamine 123 (DHR 123; Molecular Probe), an oxidant-sensitive fluorochrome, as described previously [5,6,23]. Briefly, the cells were treated with DHR 123 (5 μmol/L) for 1 h before being subjected to A/R or N/R. Thirty min after reoxygenation, the cells were washed with PBS, lysed, and DHR 123 oxidation was assessed spectrophotometrically at excitation and emission wavelengths of 502 and 523 nm, respectively.

2.2.4. PMN transendothelial migration

PMN transendothelial migration was assessed 30 min after reoxygenation as previously described [5,23,28]. Briefly, cardiac endothelial cells were grown to confluence on fibronectin-coated cell culture inserts (3-μm diameter pores). ^{51}Cr-labeled PMN in M199 were added to the apical aspect of the endothelial cell monolayers (PMN: endothelial cell ratio of 10:1) and co-incubated for 90 min with supernatants from A/R- or N/R-conditioned myocytes introduced into the basal compartment. The percentage of added neutrophils that migrated from the apical to the basal aspects of the insert membranes was quantified.

2.2.5. Activation protein-1 (AP-1)

Nuclear extracts were obtained from cardiac myocytes for an electrophoretic mobility shift assay (EMSA) as previously described [23]. A double-stranded oligonucleotide containing consensus binding sites for AP-1 (synthesized by ID Labs) was labeled with γ-^{32}P [ATP (Amersham)] by using T4 polynucleotide kinase (MBI Fermentas). The sequence of the AP-1 oligonucleotide is 5′ -cgc ttg atg agt cag ccg gaa-3′ . One picomole of the labeled oligonucleotide was incubated with 5 μg of nuclear protein for 30 min, loaded onto native 5% polyacrylamide gel, and electrophoresed at 250 V in 0.5× Tris-borate EDTA buffer. Dried gels were exposed to X-ray film (KODAK) for 16 h in cassettes with intensifying screens.

To assess the role of PI3-kinase in the regulation of AP-1 translocation to cardiomyocyte nuclei, an inhibitor (LY 294002; Sigma) was added to the myocytes (25 μmol/L) for 1 h prior to EPO treatment. Subsequently, nuclear extracts were obtained for EMSA as described above.

The same oligonucleotide used for EMSA was also employed in functional studies as an intracellular "decoy" to interfere with AP-1 nuclear translocation. The cardiac myocytes were co-incubated with the oligonucleotide (2.5 μmol/L in M199) for 1 h before and during the 4 h EPO pretreatment regimen.

2.2.6. Nitric oxide

Cardiac myocyte eNOS protein was assessed by Western blot as described above, but with the following modification. Total cell protein (5 μg) was resolved on 8% SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking, the membranes were blotted with an anti-eNOS antibody (rabbit anti-eNOS pAb, Transduction Laboratories).

NO production by cardiac myocytes was assessed by measuring the fluorescence of 4-amino-5-methylamino-2′ , 7′ -difluorofluorescein diacetate (DAF-FM diacetate), a specific NO probe (Molecular Probe) [6]. Briefly, DAF-FM diacetate (10 μmol/L) in M199 (phenol red free) was incubated with the cardiac myocytes for 2 hrs prior to experimental interventions. Subsequently, the myocytes and supernatants were obtained and sonicated. The sonicates were analyzed spectrofluorometrically at excitation and emission wavelengths of 495 and 515 nm, respectively. Fluorescence intensity (emission) was normalized to myocyte protein and data expressed as percent of control.

Pharmacologic inhibition of nitric oxide synthase (NOS) was accomplished by incubating cardiac myocytes with L-NAME (100 μmol/L) prior to assessment of endpoints.

2.3. In vivo studies

In vivo studies were undertaken to confirm that AP-1 plays an important role in I/R-induced myocardial inflammation. AP-1 is a dimeric complex consisting of *c-fos* and *c-jun*. Targeted disruption of the AP-1 components is either lethal at the embryonic stage (*c-jun*) or the mice are viable, but display morphological abnormalities (*c-fos*) [29]. Thus, mice deficient in cardiomyocyte *c-fos* were used in these studies and wild-type littermates served as controls. To generate mice lacking cardiomyocyte *c-fos*, we crossed mice with the *lox*P-c-*fos*-*lox*P insertion [30] with α-MHC-*cre* transgenic mice [31]. Southern blotting identified mice carrying both the homozygous *lox*P-c-*fos*-*lox*P gene (Fig. 2A) and the α-MHC-*cre* transgene (Fig. 2B). To confirm decreased *c-fos* expression in the heart, *c-fos* mRNA was determined in wild-type and *c-fos*$^{-/-}$ mice. In response to LPS, myocardial *c-fos* mRNA levels in *c-fos*$^{-/-}$ mice were markedly decreased as compared to the response in wild-type mice (Fig. 2C).

2.3.1. Ischemia/reperfusion (I/R) protocol

The in vivo model of I/R used in the present study was a modification of one previously used to induce myocardial infarcts [32]. Briefly, mice were anesthetized (sodium

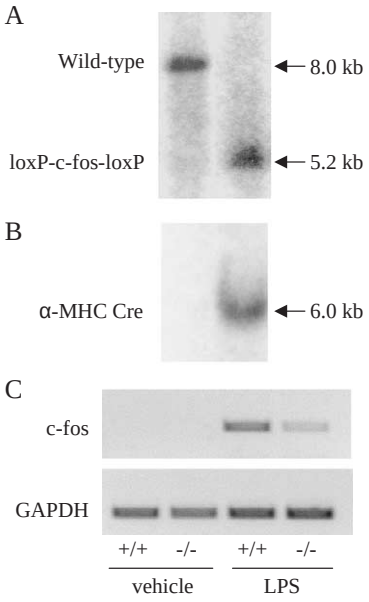


Fig. 2. Generation of mice deficient in cardiomyocyte *c-fos*. Mice with the *lox*P-c-*fos*-*lox*P insertion were crossed with α-MHC-*cre* transgenic mice. (A) Identification of the *lox*P-c-*fos*-*lox*P mice. Tail DNA was digested with *Bgl*II, electrophoretically separated, transferred onto membranes and hybridized with a 3′ probe for c-*fos*. (B) Identification of the α-MHC-*cre* transgene by Southern hybridization of the same mice as in (A), using a *cre*-specific probe. A mouse with both homozygous *lox*P-c-*fos*-*lox*P and α-MHC-*cre* transgene was identified (right lane of A and B). (C) Expression of *c-fos* mRNA in the heart. Adult mice were treated with lipopolysaccharide (LPS, 0.5 mg/kg, i.p.) or vehicle for 1 h. Hearts were isolated and RNA was extracted. Expression of *c-fos* and GAPDH mRNA was determined by RT-PCR using the same primers as used previously [30,32]. *c-fos* mRNA was not detected in vehicle treated wild-type (+/+) or cardiomyocyte *c-fos*$^{-/-}$ mice. LPS increased *c-fos* mRNA levels in the heart of a wild-type mouse. However, this response was markedly reduced in the *c-fos*$^{-/-}$ mouse. The low level of *c-fos* expression in *c-fos*$^{-/-}$ mouse is most likely due to vascular *c-fos*.

pentobarbital; 50 mg/kg i.p.), given atropine (0.05 mg s.c.) to reduce airway secretions, and artificially ventilated. After, a thoracotomy, the left coronary artery was occluded using a suture with a piece of tubing interposed between the artery and suture and the thorax closed. Thirty minutes later the thorax was reopened, the tubing removed and the suture cut. The thorax was closed again, the heart allowed to reperfuse for 2 h, harvested, and myocardial inflammation assessed. As a control, sham-operated mice underwent the same surgical procedure minus arterial occlusion. In some experiments, recombinant human EPO (rhEPO, 5000 U/kg; Ortho Biotech) was given i.p. 24 h prior to the I/R protocol. This dose of EPO has previously been used in analogous animal models of myocardial I/R [16,17,19].

2.3.2. Myocardial inflammation

As an index of PMN infiltration, myeloperoxidase (MPO) activity in the myocardium was determined as previously described [33]. Briefly, the hearts were excised, placed in phosphate buffer, and homogenized. A 1:10 dilution of the homogenate (10% wt/vol) was centrifuged at 6000×*g* for 20 min at 4 °C. The pellet was re-homogenized and sonicated for 10 s in 1 ml of 50 mM acetic acid (pH 6.0) containing 0.5% CETOH detergent. The prepared samples were used in reactions for MPO activity determined spectrophotometrically (650 nm) by measuring hydrogen peroxide-dependent oxidation of 3,3′, 5,5′ -tetramethylbenzidine.

2.3.3. Statistical analysis

All values are presented as mean+SEM. Statistical analysis was performed using ANOVA and student's t test (with a Bonferroni correction for multiple comparisons).

3. Results

3.1. A/R-induced myocyte conversion to a proinflammatory phenotype is ameliorated by EPO

In vitro studies were performed to address the potential mechanisms involved in the anti-inflammatory effects of EPO at the level of the myocyte. As shown in the upper panel of Fig. 3A, the A/R challenge (M199, A/R) significantly increased oxidative stress within the cardiac myocytes as compared to control myocytes (M199, N/R). Supernatants collected from A/R-challenged myocytes significantly increased PMN transendothelial migration, i.e., the myocytes were converted to a proinflammatory phenotype (lower panel of Fig. 3A). These observations are consistent with previous studies using this in vitro model of I/R [5,6,23]. When the myocytes were exposed to EPO 24 hrs prior to the A/R challenge (EPO, A/R), the A/R-induced myocyte oxidant stress and their conversion to a proinflammatory phenotype were prevented (Fig. 3A).

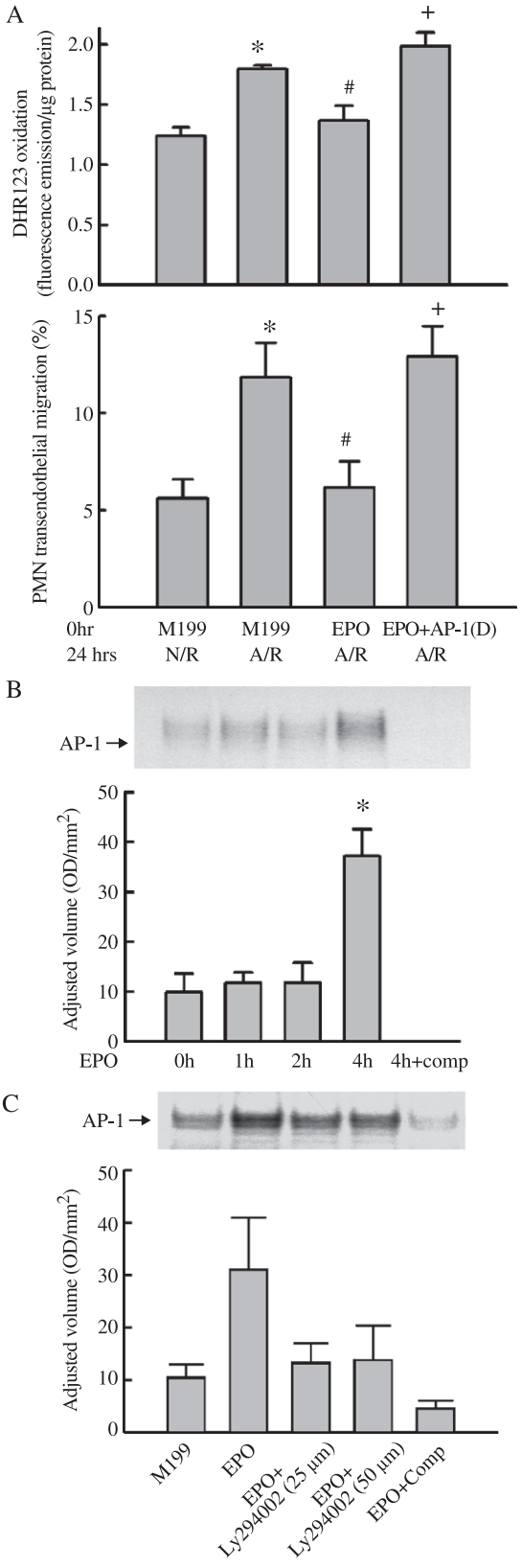
3.1.1. Role of AP-1

EPO has been shown to activate the nuclear transcription factors, NF-κB and AP-1 in cerebrocortical and erythroid cells [34,35]. In the present study, we focused on the potential role of AP-1 in mediating the anti-inflammatory effects of EPO after myocardial I/R. As shown in Fig. 3B, within 4 h after applying EPO to cardiac myocytes there was an increase in the binding of AP-1 to nuclear protein. The kinetics of AP-1 binding to nuclear proteins is consistent with previous studies [34,35].

Pretreatment of the cardiac myocytes with an AP-1 decoy (double stranded oligonucleotide containing AP-1 binding sites) prevented the beneficial effects of EPO with respect to the A/R-induced oxidant stress and conversion of myocytes to a proinflammatory phenotype (Fig. 3A).

Phosphatidylinositol 3 (PI3)-kinase has been implicated in 1) the induction of *c-fos* mRNA in vascular smooth muscle and [36] the EPO-mediated protection against I/R-induced myocardial apoptosis [20]. Thus, we assessed whether an inhibitor of PI3-kinase (LY 294002) could modulate the EPO-mediated nuclear translocation of AP-1.

As shown in Fig. 3C, the increase in AP-1 binding to nuclear protein induced by EPO was prevented by pretreatment of the myocytes with LY 294002.



3.1.2. Role of NO

As shown in Fig. 4A (upper panel), 24 h after EPO treatment there was an increase in cardiac myocyte eNOS protein as compared to control (M199 treated) myocytes. There was no detectable iNOS protein in control or EPO treated myocytes (data not shown). In addition, the cardiac myocytes produced significantly more NO (Fig. 4A, lower panel). The EPO-induced increase in NO production was abolished by L-NAME (data not shown). Pretreatment of the cardiac myocytes with the AP-1 decoy prevented both the EPO-induced increase in eNOS protein and NO production (Fig. 4A).

To assess the role of eNOS-derived NO in the EPO-mediated beneficial effects we used both pharmacologic blockade and mice deficient in the eNOS ($eNOS^{-/-}$) or iNOS ($iNOS^{-/-}$) isoforms. Since the A/R-induced myocyte oxidant stress is the critical initiating event in the development of a proinflammatory phenotype [5,6], in these experiments we focused on oxidant stress as the endpoint. As shown in Fig. 4B (upper panel), the A/R-induced increase in myocyte oxidative stress was not affected by pharmacologic inhibition of NOS with L-NAME. However, L-NAME prevented the inhibitory effect of EPO on the A/R-induced oxidant stress (compare to Fig. 3A). To assess the role of eNOS in EPO-induced myocyte protection, we used cardiac myocytes derived from $eNOS^{-/-}$ mice. As shown in the middle panel of Fig. 4B, A/R challenge of $eNOS^{-/-}$ myocytes resulted in an increase in oxidant stress. However, the inhibitory effect of EPO pretreatment was no longer apparent in $eNOS^{-/-}$ myocytes (EPO, A/R). By contrast, in $iNOS^{-/-}$ cardiac myocytes, the A/R-induced increase in oxidant stress was ameliorated by EPO pretreatment. Collectively, these results indicate that the inhibitory effect of EPO on the A/R-induced cardiomyocyte oxidant stress is mediated by eNOS-derived NO.

3.2. I/R-induced myocardial inflammation is ameliorated by EPO

To confirm the relevance of salient findings of the in vitro studies, an in vivo murine model of myocardial I/R was used. In wild type mice (Fig. 5A), I/R increased

Fig. 3. (A) An A/R challenge results in cardiac myocyte oxidant stress (DHR oxidation) and conversion to a proinflammatory phenotype (the myocytes induce PMN transendothelial migration); effects prevented by EPO pretreatment. An AP-1 decoy (AP-1 (D)) can reverse the EPO-mediated prevention of the A/R-induced oxidant stress and myocyte-mediated PMN transendothelial migration. *$p<0.05$ as compared to M199, N/R. $^{\#}p<0.05$ as compared to M199, A/R. $^{+}p<0.05$ as compared to EPO, A/R. $n=3$. (B) EPO increases the binding of AP-1 to cardiac myocyte nuclear protein. Upper panel is a representative EMSA and the histograms below are the densitometric analysis. *$p<0.05$ as compared to the 0 time point. 4 h+comp is the competition lane. $n=3$. (C) A PI3-kinase inhibitor, LY 294002 (25 and 50 μmol/L) prevents the EPO-induced increase in AP-1 binding to nuclear proteins in cardiomyocytes. Upper panel is a representative EMSA and the histograms below are the densitometric analysis of 2 separate experiments.

myocardial MPO activity as compared to sham operated controls (I/R vs. Sham). The increase in MPO activity indicates that I/R-induced an increase in PMN accumulation in the myocardium, i.e., an acute inflammatory response.

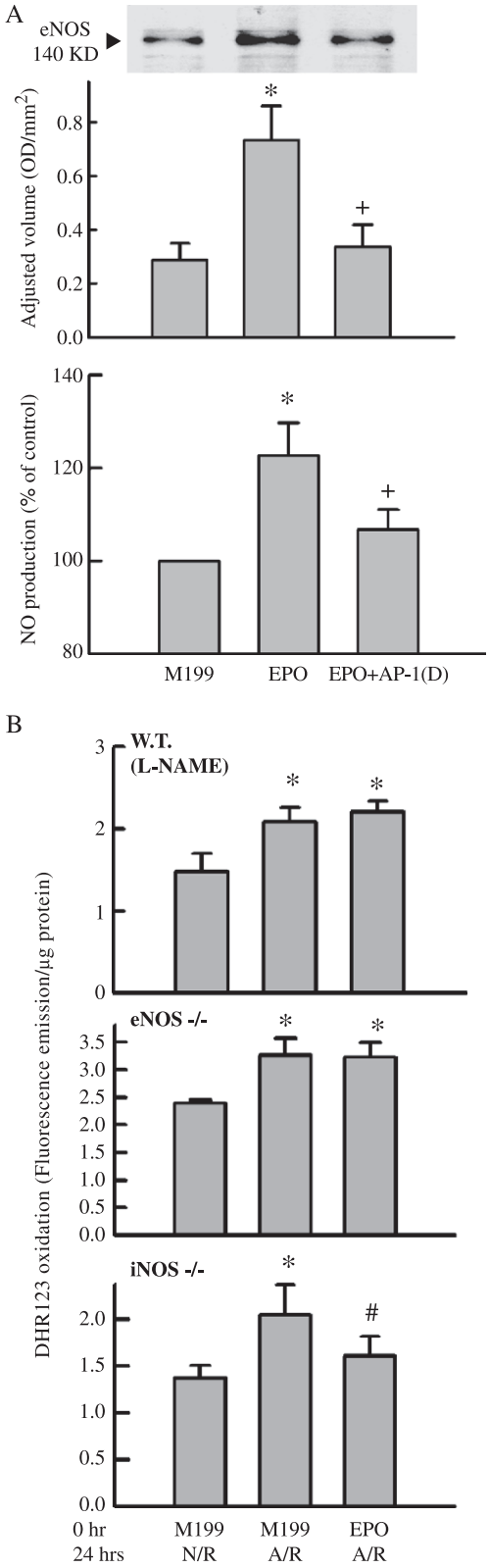


Fig. 4. (A) EPO induces eNOS protein expression and NO production by cardiac myocytes. In the upper panel a representative Western blot is shown above and densitometric analysis below. Control NO production was 5.68±0.46 fluorescence emission/µg protein. An AP-1 decoy (AP-1 (D)) prevents the increase in cardiac myocyte eNOS protein and NO production induced by EPO. *p<0.05 as compared to M199. ^{+}p<0.05 as compared to EPO. (B) EPO decreased the A/R-induced oxidant stress in cardiomyocytes derived from iNOS$^{-/-}$ mice, but not in (1) myocytes from wild type mice treated with L-NAME or (2) myocytes from eNOS$^{-/-}$ mice. *p<0.05 as compared to M199, N/R. $^{\#}p$<0.05 as compared to M199, A/R. n=3–4 for each experiment.

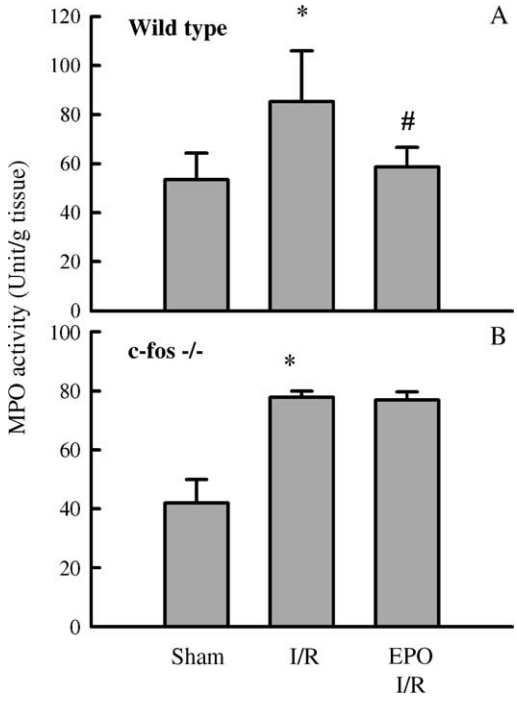


Fig. 5. EPO prevents the increase in the I/R-induced myocardial MPO activity in wild type littermate controls (A), but not in cardiomyocyte *c-fos*$^{-/-}$ mice (B). *p<0.05 as compared to Sham. #p<0.05 as compared to I/R. n=5 for wild type and n=3 for *c-fos*$^{-/-}$.

These observations are consistent with previous studies in cats [37]. If the mice were pretreated with EPO 24 hrs prior to the I/R challenge, the I/R-induced increase in myocardial MPO activity was prevented.

3.2.1. Role of AP-1

To address the role of AP-1 in the anti-inflammatory effects of EPO in the heart after an I/R challenge, we used mice deficient in cardiomyocyte *c-fos*. An I/R challenge to the hearts of these mice resulted in an increase in myocardial MPO activity as compared to sham operated controls (Fig. 5B). Thus, in these mice the inflammatory response to an I/R challenge was similar to that observed in wild type mice (compare to Fig. 5A). By contrast to observations in wild type mice, EPO did not prevent the increase in myocardial MPO activity induced by an I/R challenge to the hearts of *c-fos*$^{-/-}$ mice. This latter observation indicates that AP-1 plays an important role in the anti-inflammatory effects of EPO in myocardial I/R.

4. Discussion

Although EPO has recently been shown to ameliorate I/R-induced myocardial injury and/or dysfunction [16–18], its mechanism of action is not fully understood. An acute inflammatory response in the affected area plays a pivotal role in the I/R-induced cardiac dysfunction [1–3]. Thus, the major aims of the present study were to (1) determine whether EPO could prevent the I/R-induced acute inflammatory response and (2) address some of the potential mechanisms involved. Herein, we provide evidence that (1) EPO can prevent the I/R-induced myocardial inflammatory response in vivo and in vitro and (2) this anti-inflammatory effect is dependent on the activation/translocation of the nuclear transcription factor, AP-1.

Our approach involved the use of in vitro and in vivo models of I/R. The in vitro model targeted the myocytes. The myocytes were challenged with A/R to simulate I/R in vivo and some of the cellular mechanisms of the anti-inflammatory effects of EPO were addressed. This reductionist approach allowed for an interpretation of the findings unencumbered by the complications inherent in the more complex in vivo model. For example, we have previously noted that changes in NFκB in nuclear extacts obtained from whole tissue do not (1) identify the resident cells involved and (2) necessarily translate into functional events at a given target cell within that tissue [38]. Our in vitro studies served as a platform from which to launch in vivo studies of I/R in the heart to address salient features uncovered in isolated cardiomyocytes.

I/R induced a proinflammatory phenotype in cardiac myocytes in vitro (the myocytes induced PMN trans-endothelial migration) and an inflammatory response in vivo (an increase in myocardial MPO activity). EPO served an anti-inflammatory function in these two models by preventing the proinflammatory effects of I/R (Figs. 3A and 5A). These observations are consistent with studies in the brain, where EPO has been shown to inhibit inflammation in an experimental model of ischemic brain injury [22]. Based on in vitro experiments, the anti-inflammatory effects of EPO in the brain were attributed to its ability to decrease neuronal cell death. The in vitro model used in the present study (myocytes challenged with A/R) is not associated with myocyte death, either necrotic [6] or apoptotic (data not shown). Thus, the anti-inflammatory effects of EPO in our in vitro model cannot be attributed to myocyte death, but rather to intracellular events linked to EPO-R occupancy by EPO.

The intracellular signals that drive EPO-mediated cardiac protection are, at present, unclear. Previous studies in the brain indicate that EPO can signal intracellularly through pathways involving NF-κB or AP-1 [34,39]. Herein, we provide evidence that the nuclear transcription factor, AP-1, plays an important role in the anti-inflammatory effects of EPO in both in vitro and in vivo models of myocardial I/R. We show for the first time that EPO can increase the binding of AP-1 to nuclear proteins in cardiac myocytes. In addition, the AP-1 decoy prevents the beneficial effects of EPO with respect to A/R-induced conversion of myocytes to a proinflammatory phenotype (Fig. 3). Finally, the beneficial effects of EPO on the I/R-induced increase in myocardial MPO activity observed in wild type mice did not occur in cardiomyocyte *c-fos*$^{-/-}$ mice (Fig. 5). Taken together, these observations strongly suggest that AP-1 plays an important intracellular signaling role in the EPO-mediated anti-inflammatory effects in the myocardium after I/R.

In terms of the upstream regulators of AP-1, phosphatidylinositol 3 (PI3)-kinase appears to be important in the EPO-mediated activation of AP-1. Engagement of EPO-R in heart [20] or various isolated cells [21,36] results in PI3-kinase activation. However, PI3-kinase activation may [36,40,41] or may not [42] play a role in AP-1 activation/translocation. In the present study, inhibition of PI3-kinase prevented the EPO-induced AP-1 binding to nuclear proteins in cardiomyocytes (Fig. 3C); a prerequisite nuclear event for the manifestation of the anti-inflammatory effects of EPO in myocardial I/R (Fig. 5) or myocyte A/R (Fig. 3A). In a seemingly related study, inhibition of PI3-kinase prevented the EPO-induced protection against I/R-induced apoptosis in the heart [20]. Even though the PI3-kinase pathway is involved in both the EPO-induced anti-inflammatory (present study) and anti-apoptotic [20] effects in myocardial I/R, there most likely exist other different components in the signaling pathways of these two beneficial effects of EPO. This may explain why the anti-apoptotic effects of EPO can be noted within minutes of EPO administration [20], while the anti-inflammatory effects of EPO require hours (Fig. 3). Further studies are warranted to address these issues.

In terms of the effector mechanisms downstream of AP-1 activation/translocation which may be responsible for the anti-inflammatory effects of EPO, an increase in NO production appears likely. We have previously shown that increased NO production by cardiomyocytes is associated with an abrogation of the A/R-induced conversion of myocytes to a pro-inflammatory phenotype [6]. In the present study, EPO-treated myocytes increased their production of NO; an effect inhibited by the NOS inhibitor, L-NAME. Cardiac myocytes contain both eNOS and iNOS isoforms [43], both of which have been implicated as mediators of protection against A/R- or I/R-induced dysfunction [6,44]. Herein, we provide evidence that the eNOS isoform plays an important role in EPO-mediated cytoprotection (Fig. 4). We show that EPO can induce the expression of eNOS, but not iNOS, protein. Furthermore, EPO confers protection against the oxidant stress incurred as a result of an A/R challenge in iNOS deficient, but not eNOS deficient, cardiac myocytes. Finally, the AP-1 decoy prevented the EPO-induced increase in cardiomyocyte eNOS protein expression and NO production. These latter findings are consistent with the observation that the eNOS gene contains binding sites for AP-1 in its promoter region

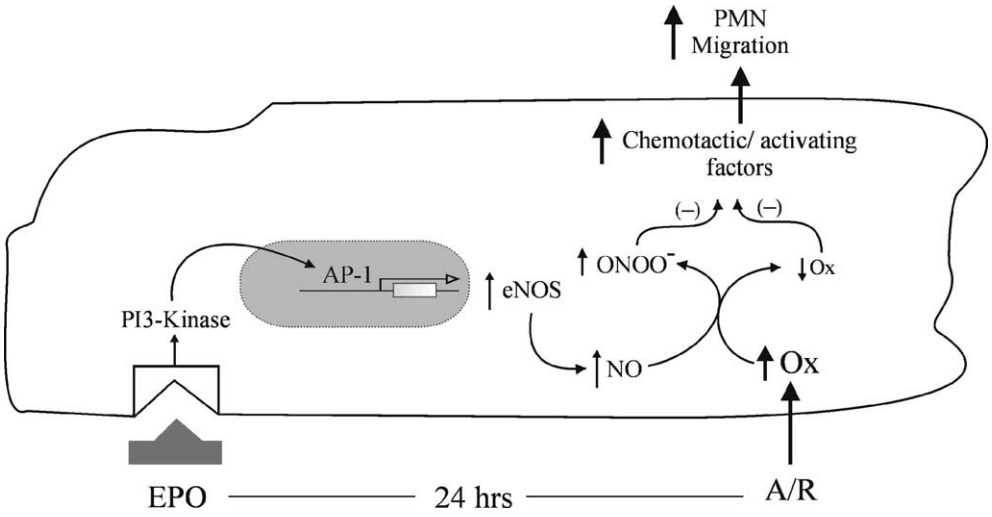


Fig. 6. A schematic of a working hypothesis illustrating the signaling pathways and effector mechanisms in the anti-inflammatory effects of EPO in cardiac myocytes. Ox, oxidant stress; $ONOO^-$, peroxynitrite.

[45]. To our knowledge, this is the first demonstration that EPO can induce eNOS in cardiac myocytes via an AP-1 dependent signaling pathway.

The results of the present study indicate that EPO can prevent the myocardial inflammatory response induced by I/R in in vivo (Fig. 5) and in vitro (Fig. 3A) models. The anti-inflammatory effects of EPO (1) required hours to be manifested as compared to the minutes required for the anti-apoptotic of EPO and (2) were independent of apoptosis. Thus, EPO may have a "multi-pronged" beneficial effect in I/R-induced myocardial dysfunction/injury in the clinical setting. Since the major objective of the present study was to address the potential mechanisms involved in the anti-inflammatory effects of EPO, we used doses of EPO that were similar to those used previously in experimental models of I/R. It must be pointed out that the dose used in the in vivo model is higher than the therapeutic doses used in humans. Thus, before EPO can be considered a potential therapeutic agent in humans, three major objectives must be accomplished: (1) a dose-efficacy relationship must be established in experimental models of I/R, (2) an effective dosing regimen must be determined (both pre and post I/R), and (3) a beneficial therapeutic approach proven to be effective must be tolerated by patients.

Fig. 6 schematically summarizes our current working hypothesis on the anti-inflammatory effects of EPO in cardiac myocytes. An A/R challenge to cardiac myocytes (1) increases intracellular oxidant stress and (2) converts the myocytes to a pro-inflammatory phenotype (right side, wide arrows). Exogenous administration of EPO results in protection against the A/R-induced inflammatory response (right side, small arrows). The anti-inflammatory effect of EPO is mediated by a signaling pathway involving PI3-kinase/AP-1 that leads to the induction of eNOS (left side). The resultant production of NO could modulate the inflammatory state of myocytes by inhibiting the generation/activity of chemotactic/activating factors [5,46] presumably due to either the generation of peroxynitrite or a reduction of oxidant stress, or both.

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