Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase

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Abstract

Objective: Erythropoietin (EPO), a cytokine best known for its ability to increase red blood cell mass, has recently been shown to protect cardiomyocytes from apoptotic cell death. The objective of the present study was to investigate the role of endothelial nitric oxide synthase (eNOS) in the anti-apoptotic effects of EPO in cardiomyocytes.

Methods and results: Neonatal mouse ventricular cardiomyocytes were isolated and cultured from wild-type and eNOS−/− mice. Treatment with EPO significantly reduced apoptosis induced by norepinephrine (NE) in the wild-type cardiomyocytes. The reduction of apoptosis was associated with significant increases in eNOS expression, phosphorylation and NO production. However, the anti-apoptotic effects of EPO were significantly decreased in wild-type cardiomyocytes treated with L-NAME, which inhibits nitric oxide synthase activity. The results were further confirmed using eNOS−/− cardiomyocytes. To investigate the in vivo significance of eNOS in mediating the anti-apoptotic effects of EPO, wild-type and eNOS−/− mice were subjected to myocardial ischemia and reperfusion. EPO decreased myocardial apoptosis and infarct size in wild-type mice. However, the protective effects of EPO were significantly diminished in eNOS−/− mice.

Conclusions: EPO increases eNOS expression and NO production in cardiomyocytes. The anti-apoptotic effects of EPO in cardiomyocytes are mediated by eNOS-derived NO production.

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This article is referred to in the Editorial by J. Fandrey (pages 1–2) in this issue.

1. Introduction

Erythropoietin (EPO) is a cytokine produced primarily in the kidney that is essential for red blood cell production [1]. In addition to its role in maintaining red blood cell mass, EPO has anti-apoptotic effects in a variety of cell types including neurons [2], endothelial cells [3], smooth muscle cells [4], and cardiomyocytes [5,6]. EPO exerts a significant anti-apoptotic effect in cardiac tissue after ischemic injury. In this regard, treatment with EPO inhibits hypoxia-induced apoptosis in cultured rat adult cardiomyocytes in vitro [5]. Furthermore, EPO reduces cardiomyocyte apoptosis following hypoxic injury in isolated hearts [7] and following myocardial infarction in rats [5]. Despite the well-characterized reductions in apoptosis after EPO treatment, the mechanisms mediating the effects of EPO are not fully understood.

Endothelial nitric oxide synthase (eNOS) is constitutively expressed in multiple tissues and produces low levels of nitric oxide (NO) that are involved in cellular signaling pathways. Under normal physiological conditions, eNOS is the predominant...
nitric oxide synthase (NOS) isoform expressed in the heart [8]. NO production from eNOS has been shown to protect cardiomyocytes from apoptosis [9]. The anti-apoptotic effects of NO are mediated by reducing oxidative stress via NADPH oxidase inhibition, modulating expression of protective genes such as heat shock protein 70 or Bcl-2, and by inhibiting caspase-3/caspase-8 activation through S-nitrosylation [9–11]. Activation of protein kinase B (Akt) upregulates eNOS expression and phosphorylation at serine 1177 leading to increased eNOS activity in endothelial cells [12,13]. We recently demonstrated that EPO increases eNOS expression in cardiomyocytes [14]. However, whether increased eNOS expression during EPO treatment is mediated by Akt in cardiomyocytes remains to be determined.

In the present study, we hypothesized that EPO increases eNOS expression and activity in cardiomyocytes via activation of Akt. We further hypothesized that the anti-apoptotic effects of EPO are mediated by NO production from eNOS. To test this hypothesis we employed both in vitro and in vivo models of cardiomyocyte death. Neonatal cardiomyocytes were cultured from wild-type and eNOS−/− mice, and apoptosis was stimulated by norepinephrine (NE). In animal studies, wild-type and eNOS−/− mice were subjected to myocardial ischemia and reperfusion resulting in myocardial apoptosis and infarction in the left ventricular myocardium. The effects of EPO on Akt activation and up-regulation of eNOS leading to inhibition of apoptosis were studied.

2. Methods

2.1. Experimental animals

Breeding pairs of C57BL/6 wild-type, and eNOS−/− (stock number 2684) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). A breeding program was carried out to produce neonates and adults for this study. Animals were provided food and water ad libitum in a 12/12-h light/dark cycle. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Experimental protocols were approved by the Animal Use Subcommittee at the University of Western Ontario, Canada.

2.2. Isolation and culture of neonatal mouse cardiomyocytes

Neonatal cardiomyocytes were prepared according to the methods we described previously [15]. Briefly, ventricular myocardial tissues from wild-type, or eNOS−/− mice born within 24 h were minced in a Ca2+- and Mg2+-free Hanks’ balanced solution. Cardiomyocytes were dispersed by 22.5 μg/ml liberase blendzyme IV at 37 °C for 40 min. The isolated cells were pre-plated for 90 min to remove adherent non-cardiomyocytes. The cardiomyocytes were plated in M199 medium containing 10% fetal bovine serum in 48 well polystyrene plates pre-coated with 1% gelatin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2. After 48 h of cell culture, cardiomyocytes were treated with recombinant human EPO (Eprex®, Ortho Biotech, Canada) and the indicated reagents.

2.3. Purity of cardiomyocyte culture

Cardiomyocytes were seeded on glass slides and cultured to subconfluence for 72 h, which was the duration of most experiments (48 h of cardiomyocyte culture plus 24 h of drug treatment) in the present study. Cells were fixed in 20% acetone/80% methanol and incubated overnight with a mouse monoclonal antibody to α-actinin (Sigma, 1:500). This was followed by incubation with a rhodamine-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Lab, 1:500) for 1 h. Cells were then incubated overnight with a mouse monoclonal antibody to eNOS (BD Transduction, 1:500) and followed by incubation with a FITC-conjugated anti-mouse secondary antibody (Invitrogen Molecular Probes, 1:500) for 1 h. Nuclei were stained with Hoechst 33342 (1 μg/mL). Slides were then mounted and visualized using a Zeiss Axiovert 200M microscope at 630× magnification. 30 fields (approximately 250 cells) were counted in triplicate. Cardiomyocytes were identified by characteristic striations stained by α-actinin at the sarcomeric Z-lines. The number of eNOS positive cells in the α-actinin positive population was also quantified.

2.4. Adenoviral infection

Cardiomyocytes were infected with an adenoviral construct containing a dominant negative mutant of Akt-1 (Adv-dnAkt1, purchased from Vector Biolabs, Philadelphia, PA) at a multiplicity of infection of 100 pfu/cell [16]. Adenovirus-mediated gene transfer was implemented by adding a minimal volume of the M199 with 2% fetal bovine serum containing Adv-dnAkt1. After culture for 2 h, the full volume of culture medium containing 10% fetal bovine serum was added. All experiments were performed after 24 h of adenoviral infection.

2.5. Measurement of NO production

Measurement of NO production in living cardiomyocytes was done using the membrane-permeable fluorescent indicator 4-aminomethylaminol-2′,7′-difluorofluorescein diacetate (DAF-FM, Invitrogen Molecular Probes). Cells were cultured as described above but plated on clear 96-well black polystyrene plates. After 48 h, the cells were incubated in the presence of 10 μM DAF-FM in M199 devoid of fetal bovine serum and phenol red (to prevent interference with fluorescence) for 1 h at 37 °C. After incubation with DAF-FM, the medium was replaced with fetal bovine serum/phenol red-free M199 and drugs were added. The fluorescence generated by NO production was measured using a Victor3 multilabel microplate reader (Wallac) using excitation and emission
performed as described previously [15, 17]. The oligonucleotide primers for eNOS (GenBank™ accession # U53142) were GAT GGC GAA GCG TGT GAA G (sense) and TCC TCC AGC CTT GTG TCC A (antisense). Primers for glyceraldehyde-3 phosphate dehydrogenase (GAPDH, GenBank™ accession # M17701) were: 5′ AAA GGG CAT CCT GGG CTA CA 3′ (sense) and 5′ CAG TGT TGG GGG CTG AGT TG 3′ (antisense). cDNAs were exponentially amplified for 36 cycles and 27 cycles for the eNOS and GAPDH genes, respectively. PCR products were separated on 1% agarose gel and imaged using a Fotodyne gel documentation system under ultraviolet light. Densitometric analysis was done using Gel Pro Analyzer 3.1 (Media Cybernetics). Data are expressed as the ratio of eNOS/GAPDH.

2.6. RNA Isolation and RT-PCR

Total RNA was isolated from cultured cardiomyocytes with TRIzol reagent (Invitrogen). Semi-quantitative RT-PCR was pressed as the percentage of untreated control.

2.7. Immunoprecipitation and western blot analysis

For analysis of phosphorylated eNOS protein levels (at serine 1177), immunoprecipitation was performed. Briefly, 1 mg protein lysate from cardiomyocytes was incubated with 2 μg of monoclonal eNOS antibody for 3 h. The immunocomplex was captured using Protein G–Agarose (Santa Cruz). Protein G–Agarose/eNOS immunocomplex was collected and denatured by heating to 95 °C for 5 min with gel loading buffer. The immunocomplex was separated by 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Both total and phosphorylated eNOS proteins were determined by Western blot analysis using anti-eNOS (Transduction laboratories), anti-P-eNOS (New England Biolabs), and anti-β-actin antibody (Chemicon). Data are expressed as the ratio of eNOS to β-actin protein expression or the ratio of P-eNOS to total eNOS levels where appropriate.

2.8. Caspase-3 activity measurement

Caspase-3 activity was measured using the BIOMOL QuantiZyme Assay System (AK-700) as we previously described [18]. Briefly, cells were lysed and protein concentration was determined. 50 μg samples were incubated at 37 °C for 16 h in the presence of caspase-3 substrate Ac-DEVD-AMC or Ac-DEVD-AMC plus inhibitor Ac-DEVD-CHO. Fluorescent intensity (excitation at 380, emission at 405) was measured using a Victor3 multilabel microplate reader (Wallac). Data are expressed as the amount of AMC substrate cleaved/μg protein.

2.9. Hoechst staining

Cardiomyocytes were seeded on glass slides and treated as indicated. Cells were fixed with 20% acetone/80% methanol and stained with Hoechst 33342 (10 μg/mL) for 10 min at room temperature. Thirty separate fields (approximately 1000 cells) were examined using a Leica DMR fluorescent microscope at 630× magnification to quantify the number of apoptotic nuclei. Data are expressed as the average percentage of apoptotic nuclei by the two independent observers.

2.10. 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. Terminal deoxynucleotidyl transferase d-UTP nick end labeling (TUNEL) staining was performed using an In Situ Cell Death Detection Kit, POD (Roche) as previously reported [18, 19]. Slides were counterstained with propidium iodide. Fifteen separate fields for the tissue sections and 30 separate fields for cultured cardiomyocytes were examined using fluorescence microscopy to quantify the number of TUNEL-positive nuclei. Data are expressed as the average percentage of TUNEL-positive nuclei by the two independent observers.

2.11. Myocardial ischemia and reperfusion

Male mice (2–3 months old) were pre-treated intravenously via a tail vein with 2500 U/kg of EPO (Eprex®, Ortho Biotech, Canada) or an equivalent volume of saline 24 h, as well as 30 min before induction of myocardial ischemia and reperfusion. Briefly, mice were anaesthetized with ketamine (50 mg/kg) and xylazine (12.5 mg/kg) cocktail. Animals were intubated and artificially ventilated with a respirator (SAR-830, CWE, Inc., 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 similar to our previous report [20]. After 45 min the chest was re-opened and the suture loosened to allow reperfusion for 3 h. The lungs were hyper-inflated using positive end-expiratory pressures (3 cm H2O) and the thorax closed.

2.12. Measurement of infarct size

Three hours after reperfusion the coronary artery was re-ligated using the same suture. Evans blue dye (1 mL of 2% w/v) 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 of approximately equal thickness from apex to base. The sections were stained with 1.5% triphenyltetrazolium chloride (TTC) for 30 min at room temperature. Since TTC stains viable tissue a deep red color, unstained tissue was presumed to be infarcted. Sections were weighed and each side of the section was photographed. The non-ischemic area (blue), the area at risk (red) and the infarct area (pale) were measured.
using SigmaScan Pro. Infarct size is expressed as a percentage of the weight of infarct area to the area at risk.

2.13. Statistical analysis

Data are expressed as means±SEM. Differences between groups were compared using the Student t-test, ANOVA, or two-way ANOVA where appropriate. Significant differences were further analyzed by the Student–Newman–Keuls test. A P value<0.05 was considered statistically significant.

3. Results

3.1. Erythropoietin treatment increases eNOS-derived NO production

Purity of cardiomyocytes was determined by α-actinin immunostaining with characteristic striation of cardiomyocytes. After 72 h of cell culture, 94±1% of cells were cardiomyocytes (n=4 independent wild-type cultures). Consistent with previous reports[8], all wild-type cardiomyocytes stained positive for eNOS. To determine if EPO treatment can increase eNOS protein levels in cardiomyocytes, cells were treated with 10–40 U/mL of EPO. Treatment with EPO increased eNOS mRNA expression (Fig. 1A) and eNOS protein expression (Fig. 1B) in a dose-dependent manner. The increase in mRNA and protein expression was maximal at 8 and 24 h, respectively (data not shown). The increase in eNOS expression was associated with a 50% increase in myocardial NO production after 24 h of EPO treatment (Fig. 1C). Phosphorylation of eNOS at serine 1177 is associated with an increase in eNOS activity[13]. To determine if EPO has the ability to phosphorylate eNOS at this residue in cardiomyocytes, the cells were treated with 20 U/mL of EPO. A 7-fold increase in eNOS phosphorylation at serine 1177 was observed within 1 h of EPO treatment (Fig. 1D).

3.2. eNOS expression/phosphorylation by PI3-kinase/Akt activation

To determine if Akt activation is involved in the increases in eNOS expression/phosphorylation seen after EPO treatment,
cardiomyocytes were incubated with EPO in the presence of LY294002 (a PI3 kinase inhibitor) or Adv-dnAkt1. While treatment with LY294002 or the Adv-dnAkt1 had little effect on basal eNOS expression and phosphorylation after 24 h (data not shown), the increase in eNOS protein expression after EPO treatment was completely blocked in the presence of LY294002 or Adv-dnAkt1 (Fig. 2A). Additionally, the increase in eNOS phosphorylation at serine 1177 seen after EPO treatment was also blocked (Fig. 2B) and treatment with either LY294002 or Adv-dnAkt1 attenuated EPO-mediated increases in NO production as measured by DAF-FM (Fig. 2C).

3.3. Inhibition of norepinephrine (NE)-induced apoptosis by a PI3-kinase/Akt dependent mechanism

NE has been shown to induce cardiomyocyte apoptosis [21,22]. To determine if the anti-apoptotic effects of EPO are mediated by PI3-kinase/Akt, cardiomyocytes were treated with NE (10 μM) in the presence or absence of EPO (20 U/mL) and LY294002 (10 μM). Cardiomyocyte apoptosis was measured by Hoechst and TUNEL staining, as well as caspase-3 activity. Treatment with NE resulted in a significant increase in apoptosis that was inhibited by concurrent treatment with EPO (P<0.05, Fig. 3A, B and C). The anti-apoptotic effects of EPO were completely blocked by concurrent treatment with
LY294002 (Fig. 3A, B and C). Furthermore, the effects of EPO on NE-induced caspase activity were abrogated by Adv-dnAkt1 (0.90±0.07 vs. 1.71±0.06 pmol/μg protein, P<0.05).

3.4. Effects of heat-inactivated EPO on eNOS expression, NO production and NE-induced apoptosis

In order to see if other components in the recombinant EPO preparation might have any effects observed in the present study, human recombinant EPO (Eprex®) was heated to 56 °C for 30 min to inactivate EPO as described by Yu et al [23]. Cardiomyocytes were treated with heat-inactivated EPO (20 U/mL) for 24 h. NO production and eNOS protein were determined by DAF-FM and western blot analysis, respectively. Heat-inactivated EPO had no effects on eNOS protein expression (99±11% of control) or NO production (93±4% of control, n=3 per group, P=n.s.). NE-induced apoptosis was assessed by caspase-3 activity. Heat-inactivated EPO did not have any significant effects on NE-induced caspase-3 activation (92±1% of NE-treated cells, n=3 per group, P=n.s.).

3.5. Effects of NOS inhibition on EPO-mediated anti-apoptotic effects

To clarify a potential role of NO in the anti-apoptotic effects of EPO, an NOS inhibitor L-NAME (300 μM), which inhibits NO production was added concurrently with EPO. L-NAME had little effect on basal or NE-induced apoptosis. However, when added with NE and EPO, L-NAME significantly inhibited the anti-apoptotic effects of EPO (Fig. 4A, B and C, P<0.05).

3.6. Effects of EPO on apoptosis in eNOS−/− cardiomyocytes

In addition to pharmacological inhibition of eNOS-derived NO production, neonatal cardiomyocytes were obtained from eNOS−/− mice to confirm the role of eNOS as a mediator of the anti-apoptotic effects of EPO (Fig. 4D, E and F). Compared to the wild-type counterparts, eNOS−/− cardiomyocytes had similar levels of basal and NE-induced apoptosis as measured by caspase-3 activity, Hoechst and TUNEL staining. However, in contrast to wild-type cells, treatment with EPO (20 U/mL)
EPO did not protect eNOS−/− cardiomyocytes from NE-induced apoptosis (Fig. 4D, E and F).

3.7. Effects of EPO on apoptosis and infarct size during myocardial ischemia and reperfusion

The role of eNOS in the anti-apoptotic effects of EPO was further investigated using an in vivo model of cardiomyocyte apoptosis induced by myocardial ischemia and reperfusion. Mice were subjected to 45 min of myocardial ischemia followed by 3 h of reperfusion. Pre-treatment with EPO (2500 U/kg, i.v.) for 24 h increased myocardial eNOS protein expression (data not shown), and significantly decreased caspase activity and TUNEL staining following myocardial ischemia and reperfusion in wild-type mice (Fig. 5A and B). However, EPO pre-treatment had no effects on myocardial apoptosis induced by myocardial ischemia and reperfusion in eNOS−/− mice (Fig. 5A and B). In addition, pre-treatment with EPO resulted in a significant reduction in infarct size in wild-type (P<0.05) but not in eNOS−/− mice (Fig. 5C). These results further support a role for eNOS in the cardioprotective effects of EPO in vivo.

4. Discussion

The major findings of this study are that treatment with EPO upregulates eNOS-derived NO production by increasing phosphorylation and expression of eNOS in cardiomyocytes. Increases in eNOS phosphorylation and expression are mediated by PI3-kinase/Akt. Furthermore, we demonstrate for the first time that the increase in eNOS-derived NO production is required for the EPO-mediated inhibition of apoptosis and reduction of infarct size following myocardial ischemia and reperfusion in vivo.

The overexpression of EPO has been associated with elevated levels of eNOS in the vasculature [24]. As well, EPO treatment increases eNOS phosphorylation in endothelial cells and prevents ischemia/hypoxia-induced apoptosis [25,26]. The present study demonstrated the increases in eNOS-derived NO production following EPO treatment in cardiomyocytes. This increase in NO production was accompanied by an elevation in eNOS mRNA, and protein expression, as well as an increase in phosphorylation of eNOS at serine 1177. These changes in eNOS expression were also seen in vivo following EPO treatment for 24 h. The results are consistent with our previous report that EPO increases eNOS expression in cardiomyocytes [14] and support the notion that EPO upregulates eNOS activity and NO production in cardiomyocytes.

Studies have shown that EPO activates PI3 kinase/Akt signaling pathway [27–29]. Activation of Akt is known to phosphorylate eNOS at serine 1177 in endothelial cells [13].
However, whether activation of Akt by EPO leads to eNOS expression and activity in cardiomyocytes was not known. In the present study, we demonstrated that EPO increased eNOS mRNA, protein and phosphorylation in cardiomyocytes. Furthermore, these effects of EPO were completely blocked by PI3 kinase inhibitor LY294002 or an adenoviral dominant negative Akt-1 construct. Our data suggests that EPO increases eNOS expression and phosphorylation via PI3 kinase/Akt signaling.

Excessive sympathetic activation is a characteristic feature of heart failure [30]. NE, the primary neurotransmitter of the sympathetic nervous system is able to induce cardiomyocyte apoptosis through activation of the beta-1 adrenoreceptor [31]. We sought to determine if EPO could attenuate NE-induced apoptosis in cardiomyocytes as a model for the excess sympathetic activation seen with heart failure. EPO has been demonstrated to protect the heart from ischemia/reperfusion injury [5], and from hypoxia-induced apoptosis [27]. The present study demonstrated that EPO also reduces NE-induced apoptosis in cardiomyocytes. The anti-apoptotic effect of EPO is dependent upon PI3-kinase/Akt activation because inhibition of PI3-kinase activation completely abrogated the protective effect of EPO. Furthermore, inhibition of eNOS-derived NO production either genetically, or pharmacologically with L-NAME, resulted in a markedly diminished anti-apoptotic response to EPO in cultured cardiomyocytes. These results were further confirmed by an in vivo model of myocardial ischemia/reperfusion using eNOS−/− mice. Our data demonstrates the significant contribution of eNOS to the protective effects of EPO. Interestingly, treatment with EPO also significantly reduced infarct size in wild-type mice. Studies have shown that myocyte apoptosis contributes to infarct size following myocardial infarction [32]. It is therefore possible that significant reductions in myocyte apoptosis by EPO within the area at risk resulted in a decrease in infarct size after myocardial ischemia/reperfusion. Furthermore, there is evidence that EPO activates protein kinase C and KATP potassium channels mediating immediate cardioprotective effects during ischemia [33]. Thus, it is likely that the cardioprotective actions of EPO are mediated through a combination of these mechanisms.

In summary, the present study demonstrated the pivotal role of eNOS-derived NO production in mediating the anti-apoptotic effects of EPO in cultured cardiomyocytes and in mice after myocardial ischemia/reperfusion. The reduction in apoptosis was dependent upon PI3-kinase/Akt activation (Fig. 6). The discovery that eNOS-derived NO production contributes to the anti-apoptotic effects of EPO offers insights into the signaling mechanisms required for EPO’s cardioprotective effects, which may help provide biological end-points for the clinical evaluation of EPO’s potential as a cardiovascular therapeutic.

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