0022-3565/09/3293-900–907$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 329:900–907, 2009

Vol. 329, No. 3
150896/3476412
Printed in U.S.A.

Erythropoietin Protects the Heart from Ventricular Arrhythmia during Ischemia and Reperfusion via Neuronal Nitric-Oxide Synthase

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Received January 13, 2009; accepted March 20, 2009

ABSTRACT

Erythropoietin (EPO) is a potent cardioprotective agent in models of myocardial ischemia and reperfusion (I/R). It has been suggested recently that EPO may also reduce ventricular arrhythmia after I/R. The present study investigated the role of neuronal nitric oxide synthase (nNOS) on the antiarrhythmic effects of EPO. EPO treatment increased nNOS expression in isolated neonatal mouse ventricular myocytes. Cotreatment with the phosphatidylinositol 3 (PI3)-kinase inhibitor, LY294002 [2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride], or treatment of cardiomyocytes infected with a dominant negative adenovirus targeted to Akt1 (ADV-dnAkt1) blocked the effects of EPO on nNOS expression, suggesting that EPO regulates nNOS expression via PI3-kinase and Akt. To examine the in vivo antiarrhythmic effects of EPO, wild-type (WT) and nNOS(−/−) mice were anesthetized and, after a baseline measurement, subjected to myocardial I/R to provoke ventricular arrhythmias. Pretreatment with EPO 24 h before ischemia increased nNOS expression and significantly reduced the number of premature ventricular contractions (PVCs) and the incidence of ventricular tachycardia (VT) in WT mice. In contrast, treatment with EPO had no effect on PVCs or the incidence of VT in nNOS(−/−) mice. Furthermore, EPO treatment after ischemia significantly reduced the threshold dose of cesium chloride (CsCl) to induce VT. We conclude that EPO via nNOS protects the heart from spontaneous and CsCl-induced ventricular arrhythmia during myocardial I/R.

Erythropoietin (EPO) is a 30-kDa glycoprotein that is produced in the kidney and is best known for its ability to regulate red cell production (Fisher, 2003). There is growing evidence that EPO exerts tissue protective effects beyond its traditional role in hematopoiesis. EPO has been shown to act in a number of nonhematopoietic tissues, including the brain (Bernaudin et al., 1999), kidney (Katavetin et al., 2007), and heart (Calvillo et al., 2003). In the heart, EPO is protective in models of myocardial infarction (MI) (Nishiya et al., 2006), ischemia and reperfusion (I/R) injury (Calvillo et al., 2003; Burger et al., 2006), and cardiomyopathy (Kim et al., 2008). EPO treatment also increases survival in a rodent model of doxorubicin-induced cardiomyopathy (Hamed et al., 2006). Several mechanisms have been implicated in the cardioprotective effects of EPO, including reductions in infarct size (Hanlon et al., 2005), apoptosis (Burger et al., 2006), and inflammation (Rui et al., 2005; Li et al., 2006) and the promotion of angiogenesis (Nishiya et al., 2006). A report by Hirata et al. (2005) suggested that EPO may also reduce I/R-induced ventricular arrhythmia via protein kinase B (Akt) activation.

Studies have shown that nNOS, a Ca^{2+}-dependent enzyme originally identified in the brain, is also expressed in cardiomyocytes, where it localizes to the sarcoplasmic reticulum (Xu et al., 1999). In cardiomyocytes, nNOS is believed to play an important role in Ca^{2+} handling and the regulation of contractility (Xu et al., 1999; Sears et al., 2003). Deletion of nNOS leads to increased intracellular Ca^{2+} and cell shortening in cardiomyocytes (Sears et al., 2003). The increases in intracellular Ca^{2+} are associated with increases in L-type Ca^{2+} channel activity (Sears et al., 2003; Heaton et al., 2006;

This work was supported by the Heart and Stroke Foundation of Ontario [Grant T-6040]; and the Canadian Institutes of Health Research (Grant MOP-64395). D.E.B. was supported by an Ontario Graduate Scholarship Award. Q.P.F. is a Heart and Stroke Foundation of Ontario Career Investigator.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.109.150896.

ABBREVIATIONS: EPO, erythropoietin; MI, myocardial infarction; I/R, ischemia and reperfusion; nNOS, neuronal nitric-oxide synthase; eNOS, endothelial nitric oxide synthase; WT, wild type; PCR, polymerase chain reaction; ADV-dnAkt1, dominant negative adenovirus to Akt1; ECG, electrocardiogram; CsCl, cesium chloride; VT, ventricular tachycardia; PVC, premature ventricular contraction; TTC, triphenyltetrazolium chloride; PI3, phosphatidylinositol 3; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride.

Burkard et al., 2007) and a decrease in sarcoplasmic reticulum Ca^{2+} ATPase function (Burkard et al., 2007). A recent report by Gonzalez et al. (2007) found that deficiency in nNOS leads to spontaneous Ca^{2+} waves in isolated cardiomyocytes. These spontaneous waves were associated with deficient nitrosylation of ryanodine receptors and are thought to be proarrhythmogenic. It is interesting that inhibition of nitric oxide production has been shown to be proarrhythmogenic in isolated rat and rabbit hearts, suggesting that nNOS-derived nitric oxide production may play an important antiarrhythmic role in the heart (Pabla and Curtis, 1995, 1996).

Although previous studies by our laboratory (Burger et al., 2006) and others (Rui et al., 2005; Lee et al., 2006) have demonstrated EPO-mediated increases in endothelial nitric oxide synthase (eNOS) expression and phosphorylation, the ability of EPO to regulate nNOS has not been investigated. Moreover the role of nNOS in the antiarrhythmic effects of EPO is not known. In the present study, we hypothesized that EPO increases nNOS expression in cardiomyocytes and that EPO-mediated increases in nNOS protect the heart from ventricular arrhythmia. To test our hypothesis, we examined cardiac electrophysiology in EPO-treated wild-type (WT) and nNOS(−/−) mice subjected to I/R. Our results demonstrate for the first time that EPO reduces ventricular arrhythmia during I/R through up-regulation of nNOS in cardiomyocytes.

Materials and Methods

Animals. WT and nNOS(−/−) mice of C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME), and a breeding program was implemented at the University of Western Ontario animal care facilities. Animals were provided with water and food ad libitum and housed in a temperature- and humidity-controlled facility with 12-h light/dark cycles. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH, 1996), and animal handling was approved by the Animal Use Subcommittee at the University of Western Ontario, Canada.

Isolation and Culture of Neonatal Mouse Ventricular Myocytes. Neonatal cardiomyocytes were prepared according to the methods previously described (Song et al., 2000). Ventricular myocardial tissues from WT mice born within 24 h were minced in Hanks' balanced salt solution, and cardiomyocytes were dispersed by 22.5 μg/ml liberase blendzyme IV at 37°C for 30 min. Isolated cells were preplated on polystyrene plates for 60 min to allow for the removal of noncardiomyocytes. Cardiomyocytes were then plated in medium 199 containing 10% fetal calf serum on polystyrene plates coated with 1% gelatin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h, after which they were treated with recombinant human EPO (Eprex; Ortho Biotech Products, Bridgewater, NJ) and/or the indicated reagents.

Real-Time Reverse Transcriptase-PCR. Total RNA was isolated from cultured cardiomyocytes with TRIzol reagent (Invitrogen) as described previously (Song et al., 2000; Hammoud et al., 2007). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and random primers (Invitrogen Canada Inc., Burlington, ON, Canada). Real-time PCR was conducted using SYBR Green PCR Master Mix as per the manufacturer's instructions (Eurogentec, Seraing, Belgium). The oligonucleotide primers for nNOS were: sense, 5′-AAG TAC CCG GAA CCC TTG C-3′; and antisense, 5′-GCT CCT GTG TTG GCT GTC AC-3′. 28S rRNA was used as a loading control with oligonucleotide primers for sense, 5′-TTG AAA ATC CGG GGG AGA G-3′; and antisense, 5′-ACA TTG TTC CAA CAT GCC AG-3′. Samples were amplified for 35 cycles using a MJ Research Opticon Real-Time PCR machine (MJ Research, Watertown, MA). The levels of nNOS were compared with those of 28S rRNA, and the relative expression of nNOS was obtained.

Western Blot Analysis. Total nNOS protein levels in cardiomyocytes and heart tissues (nonischemic region of the left ventricular myocardium) were measured by Western blot analysis. In brief, 40 μg of protein was separated by SDS-polyacrylamide gel electrophoresis using 10% gels. Proteins were then transferred to nitrocellulose membranes, and blots were probed with antibodies against nNOS (1:2000; Sigma Diagnostics Canada, Mississauga, ON, Canada) and β-actin (1:2000; Sigma Diagnostics Canada). Blots were then washed and probed with horseradish peroxidase-conjugated secondary antibodies (1:2000; Bio-Rad, Hercules, CA), and detection was performed using an enhanced chemiluminescent detection method and exposed to Kodak X-ray film (Eastman Kodak, Rochester, NY) until all bands were clearly visible without detectable saturation. The developed film was scanned, and the respective band intensities were quantified using FluorChem 8000 (Alpha Innotech, San Leandro, CA). Densitometric values of the bands were corrected for background. The densitometric ratio of nNOS/β-actin is presented.

Adenoviral Infection. Cardiomyocytes were infected with an adenoviral construct containing a dominant negative mutant of Akt-1 (ADV-dnAkt1; Vector Biolabs, Philadelphia, PA) at a multiplicity of infection of 100 plaque-forming units/cell. Adenovirus-mediated gene transfer was implemented by adding a minimal volume of medium 199 with 2% fetal bovine serum containing ADV-dnAkt1. Viral constructs containing green fluorescent protein served as negative controls. 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.

In Vivo Erythropoietin Treatment. For in vivo studies, animals were subjected to one of two dosing protocols. Protocol 1 consisted of EPO administration in advance of ischemia; EPO (2500 U/kg) was injected intravenously 24 h before surgery and then a second time 30 min before surgery. Protocol 2 consisted of EPO administration after the onset of ischemia; EPO (2500 U/kg) was injected intravenously in a single dose immediately after the ligation of the coronary artery.

Ischemia/Reperfusion. Adult nNOS(−/−) mice and their WT littermates (males, 2–3 months old) were subjected to I/R through ligation of the left coronary artery. Mice were anesthetized with ketamine (50 mg/kg) and xylazine (12.5 mg/kg), intubated, and mechanically ventilated. A left intercostal thoracotomy was performed, and the left coronary artery was ligated by placing a suture (8-0) around it. The lungs were then hyperinflated using positive end-expiratory pressures (3 cm of H_2O), and the thorax was closed. The artery was occluded for 45 min to induce ischemia, after which the suture was loosened to allow reperfusion for 45 min.

Monitoring of electrocardiogram (ECG) during I/R was performed in anesthetized mice using limb lead I with needle electrodes inserted subcutaneously. ECG was recorded 5-min before (for baseline measurements) and throughout the entire I/R protocol using PowerLab Chart 5.0 (ADInstruments Ltd., Chalgrove, Oxfordshire, UK).

Cesium Chloride Infusion. After 6 h of reperfusion, mice were re-anesthetized and the jugular vein was cannulated and cesium chloride (CsCl) was infused using a syringe pump (Sage Instruments, Freedom, CA). CsCl has been reported previously to induce ventricular tachycardia (VT) in dogs (Jones et al., 2001). ECG was monitored using limb lead I with needle electrodes and recorded using PowerLab. The minimum dose of CsCl required to induce VT was determined.

ECG Analysis. Ventricular arrhythmias were analyzed off-line according to the Lambeth Convention guidelines for the analysis of experimental arrhythmias (Walker et al., 1988). Premature ventricular contractions (PVCs) were defined as single premature QRS complexes in relation to the P-wave, and VT was defined as a run of three or more premature QRS complexes. The number of singlet and doublet PVCs and the incidence of VT were quantified.

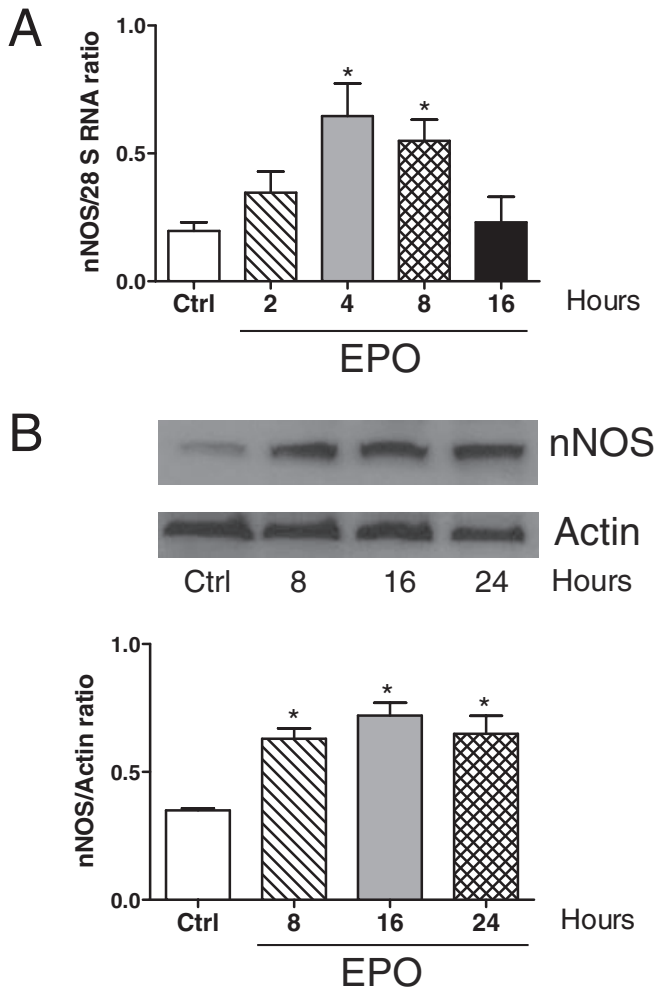

Fig. 1. EPO increases nNOS expression in neonatal mouse ventricular cardiomyocytes. A, EPO (20 U/ml) increased nNOS mRNA levels as measured by real time reverse transcriptase-PCR in cardiomyocytes after 4 and 8 h of treatment. B, EPO increased nNOS protein levels as measured by Western blot in cultured cardiomyocytes after 8, 16, and 24 h of treatment. Data are mean ± S.E.M. from four to six independent experiments. *, $P < 0.05$ versus control (Ctrl).

Measurement of Infarct Size. Infarct size was measured as described previously (Burger et al., 2006). In brief, after reperfusion, the coronary artery was re-ligated using the same suture. Evans blue dye 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 triphenyltetrazolium chloride (TTC) for 30 min at room temperature. Because 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 nonischemic area (blue), the area at risk (red), and the infarct area (pale) were measured as a percentage of overall area using Sigma-Scan Pro (SPSS Inc., Chicago, IL) and normalized according to weight of the individual sections. Infarct size is expressed as a percentage of the weight of infarct area to the weight of the ischemic area at risk.

Statistical Analysis. All data are expressed as mean ± S.E.M. Statistical significance was assessed using the Student's *t* test, analysis of variance, or two-way analysis of variance followed by the Bonferroni post hoc test where appropriate using Prism 4.0 (GraphPad Software Inc., San Diego, CA). Differences were considered significant at the level of $P < 0.05$.

Results

Erythropoietin Treatment Increases nNOS Expression in Cardiomyocytes. To determine whether EPO treatment can increase nNOS expression levels in cardiomyocytes, cells were treated with 20 U/ml EPO. This dose was previously found to up-regulate eNOS in cultured cardiomyocytes (Burger et al., 2006). Treatment with EPO significantly increased nNOS mRNA expression peaking at 4 h (Fig. 1A, $P < 0.05$) and nNOS protein expression peaking at 16 h (Fig. 1B, $P < 0.05$).

Role of PI3-Kinase/Akt in EPO-Mediated Increases in nNOS Expression. To determine whether Akt activation is responsible for the increases in nNOS expression seen after EPO treatment, cardiomyocytes were incubated with EPO in the presence of LY294002 (a PI3-kinase inhibitor) or ADV-dnAkt1. Akt has been shown previously to be important in EPO-mediated cardioprotection (Burger et al., 2006) and in the regulation of nNOS expression (Han et al., 2007; Nakata et al., 2007). Treatment with LY294002 or the ADV-dnAkt1 had little effect on basal nNOS expression after 24 h;

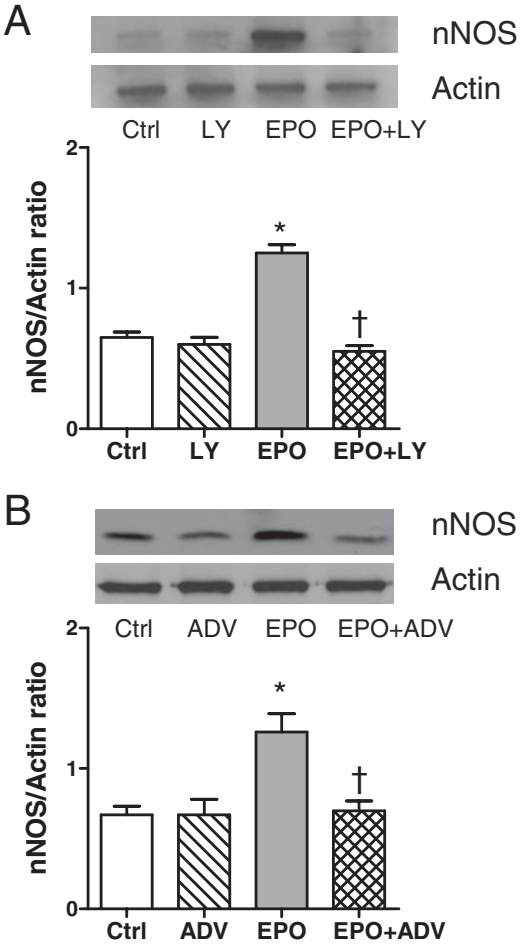

Fig. 2. Inhibition of PI3-kinase/Akt-1 decreases EPO-mediated nNOS expression. Cultured cardiomyocytes were treated with 20 U/ml EPO alone or in combination with 10 μM of the PI3-kinase inhibitor LY294002 (LY, A) or 100 plaque-forming units/cell of an adenoviral dominant negative Akt-1 construct (ADV, B). nNOS protein levels were measured by Western blot analysis after 24 h of EPO treatment and are expressed as the ratio of nNOS/actin. Inserts, representative blots. Data are mean ± S.E.M. from three to four independent experiments. *, $P < 0.05$ versus control (Ctrl); †, $P < 0.05$ versus EPO treatment alone.

however, EPO-mediated increases in nNOS protein expression were blocked in the presence of LY294002 or ADV-dnAkt1 (Fig. 2, A and B).

EPO Treatment Prevents I/R-Induced Ventricular Arrhythmia. The role of nNOS in the antiarrhythmic effects of EPO was examined using an in vivo model of I/R-induced ventricular arrhythmia with 45 min of ischemia followed by 45 min of reperfusion. Pretreatment with EPO using protocol 1 increased myocardial nNOS expression (Fig. 3A). The increases in nNOS expression were associated with reductions in the number of I/R-induced PVCs (Fig. 3B) and the incidence of VT (Fig. 3C) in WT mice. However, EPO pretreatment had no effect on I/R-induced PVCs or the incidence of VT in nNOS(−/−) mice (Fig. 3, B and C). No changes were observed in heart rate among any treatment groups (Fig. 3D). Representative tracings are shown in Fig. 4. Our results suggest that EPO reduces I/R-induced arrhythmia via nNOS.

EPO Treatment Reduces Infarct Size in WT and nNOS(−/−) Mice. It has been reported by a number of labs, including ours, that EPO treatment protects the heart from I/R-induced injuries (Hirata et al., 2005; Burger et al., 2006; Liu et al., 2006). However the role of nNOS in EPO-mediated reductions in infarct size is unclear. In addition to ventricular arrhythmia, we examined the effects of EPO pretreatment on infarct size in WT and nNOS(−/−) mice. EPO pretreatment using protocol 1 significantly reduced infarct size to a similar extent in both WT and nNOS(−/−) mice after I/R ($P < 0.05$, Fig. 5), suggesting that nNOS is not involved in EPO-mediated reductions in infarct size.

EPO Treatment at Ischemia Reduces VT Threshold after CsCl Infusion. Clinically, extended pretreatment such as that used earlier in this study, may not be possible in patients with acute MI. To determine whether EPO confers cardioprotection when administered after the onset of ischemia, we examined the effects of EPO (2500 U/kg i.v., protocol 2) administered immediately after ischemia. Six hours after reperfusion, animals were exposed to increasing concentrations of CsCl, and the threshold concentration of CsCl to induce VT was determined. EPO treatment did not significantly reduce the VT threshold dose in sham-operated animals (Fig. 6A). However, EPO treatment significantly increased the threshold dose of CsCl to induce VT after I/R in WT mice (Fig. 6B). The protective effects of EPO were not seen in sham-operated or ischemia-reperfused nNOS(−/−) mice (Fig. 6, A and B). Representative tracings of WT and nNOS(−/−) mice subjected to I/R are seen in Fig. 6C.

Discussion

The principal finding of this study is that treatment with EPO increases nNOS expression in cardiomyocytes and in the myocardium. These increases in nNOS expression are mediated via PI3-kinase and Akt and appear to be involved in the antiarrhythmic effects of EPO. Therefore, we confirmed an antiarrhythmic effect for EPO and demonstrate for the first time, the requirement of nNOS in EPO-mediated reductions in ventricular arrhythmia during myocardial I/R in mice.

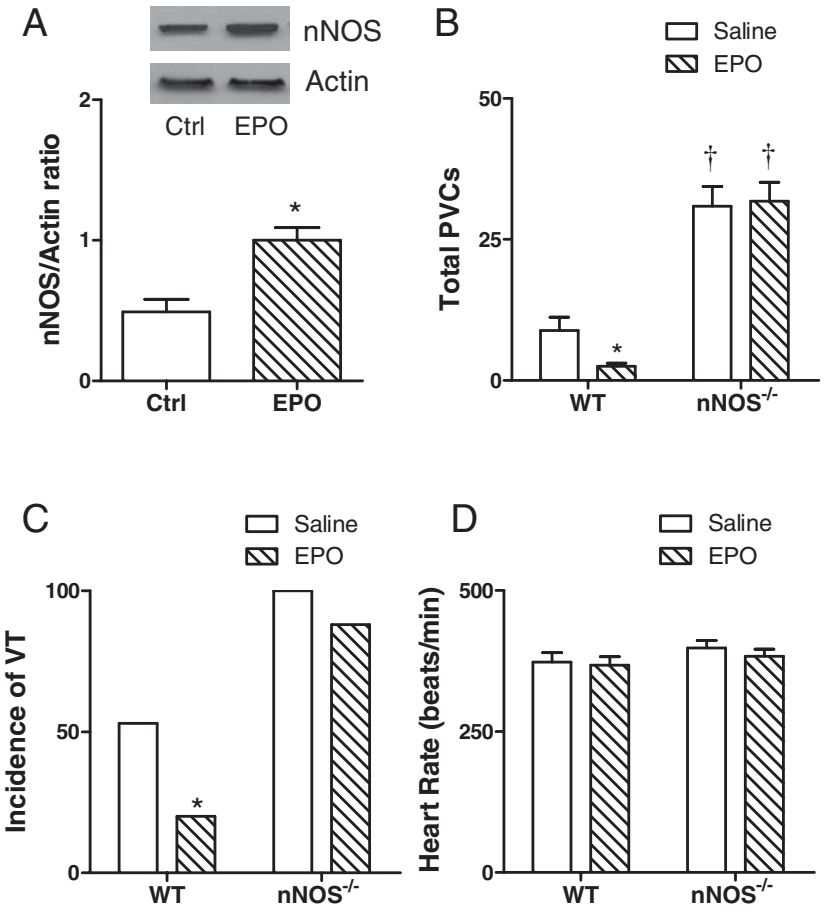

Fig. 3. Effect of EPO on nNOS expression and ventricular arrhythmia in vivo. WT mice were treated with EPO (2500 U/kg i.v.) 24 h and 30 min before analysis. A, EPO increased myocardial nNOS expression as measured by Western blot analysis. Data are expressed as the ratio of nNOS/actin. Inserts, representative blots. *, $P < 0.05$ versus control (Ctrl), $n = 4$ mice per group. B to D, WT and nNOS(−/−) mice were treated with EPO (2500 U/kg i.v.) or equivalent volume saline 24 h and 30 min before surgery. Mice were then subjected to 45 min of myocardial ischemia followed by 45 min of reperfusion, and cardiac electrical activity was monitored using a single limb lead I ECG. B, total number of PVCs. C, incidence of VT. D, heart rate was not significantly different across all treatment groups. *, $P < 0.05$ versus genotype saline treatment; †, $P < 0.05$ versus WT saline treatment, $n =$ 8 to 15 mice per group.

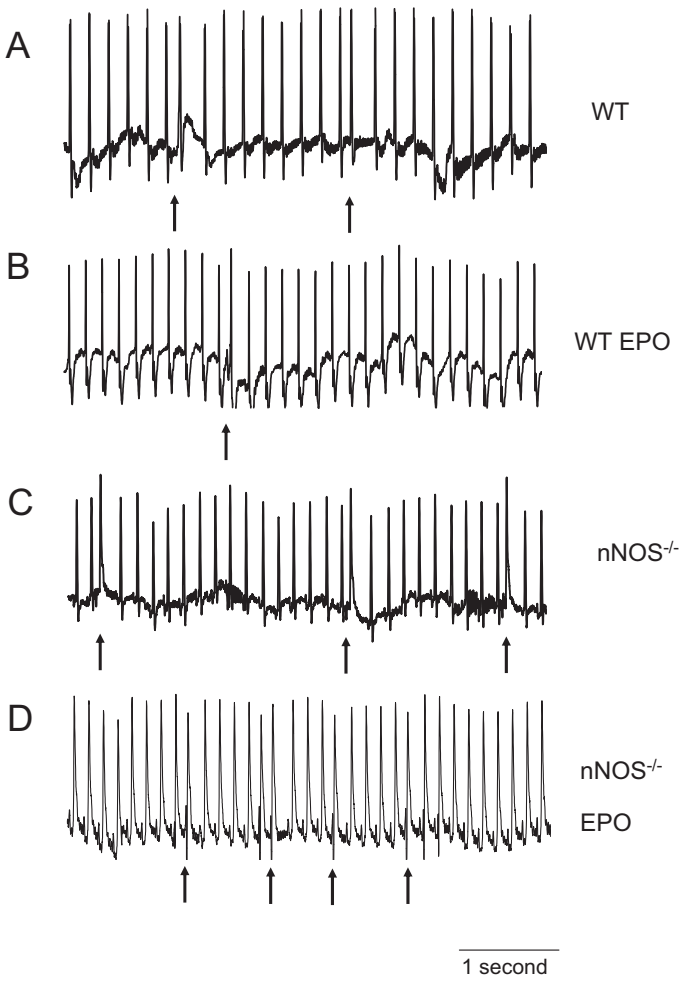

Fig. 4. Representative limb lead I ECG tracings after myocardial I/R in anesthetized and artificially ventilated mice. A, saline-treated WT. B, EPO-treated WT. C, saline-treated nNOS(−/−) mice. D, EPO-treated nNOS(−/−) mice. PVCs are indicated by the arrows.

The role of EPO in the protection of the heart from injury has been studied extensively, and it is becoming increasingly clear that EPO provides significant protection from cell death and cardiac dysfunction (Joyeux-Faure et al., 2005; Burger et al., 2009). EPO may even decrease mortality in rodent models of cardiomyopathy and pressure overload (Hamed et al., 2006; Asaumi et al., 2007). In this study, we found that EPO reduced infarct size and arrhythmia after I/R in mice. Small animal models are of significant benefit because of the ease of tissue collection and the finite control of select experimental conditions. However, small animal models have inherent limitations because they may not behave in the same way as the human population. For example, the mouse heart is approximately 1/2000th the size of the adult human heart and is more resistant to VF than the human heart (Verheule et al., 2006). Nevertheless, the protective effects of EPO during I/R have also been demonstrated in several large animal models. Parsa et al. (2004) observed reductions in infarct size and an improvement in heart function after EPO treatment in rabbits with I/R. Similar effects have been found in dogs (Hirata et al., 2006) and pigs (Krishnagopalan et al., 2002), although I/R in sheep did not have reductions in infarct size (Olea et al., 2006). In humans, high serum levels of EPO are associated with smaller infarct sizes in patients with acute myocardial infarction (Namiuchi et al., 2005), and EPO administration improves heart function in anemic patients with heart failure (Mancini et al., 2003). However, the protective effects of EPO in nonanemic patients with a coronary occlusion have yet to be confirmed, although clinical studies are ongoing (Belonje et al., 2008). Until recently, it was not known whether EPO treatment had any effect on cardiac electrophysiology. The present study demonstrated that EPO pretreatment significantly reduced ventricular arrhythmia in a mouse model of myocardial I/R. This observation is in agreement with the initial observations of Hirata et al. (2005), who observed PI3-kinase-dependent reductions in ventricular fibrillation after myocardial I/R in dogs.

In the present study, we showed that EPO decreased spontaneous arrhythmia after pretreatment and inhibited CsCl-induced arrhythmia when administered after ischemia. The observation that EPO treatment still provides protection when administered after the onset of ischemia is of significant therapeutic relevance. Pretreatment with EPO may be of some benefit in high-risk individuals; however, in patients suffering from coronary artery occlusions, therapeutic intervention usually begins after the onset of symptoms. In the clinical setting in patients, episodes of ventricular arrhythmia are present up to 12 to 48 h after the onset of MI (Campbell et al., 1981; Antman et al., 2004). In our model, EPO significantly reduced CsCl-induced arrhythmia 6 h after reperfusion. CsCl was chosen to induce arrhythmia because spontaneous arrhythmia was no longer present after 6 h of reperfusion in our mice. There is evidence of a role for the L-type Ca^{2+} channel in I/R-induced ventricular arrhythmia (Zaugg, 2004) and in CsCl-induced ventricular arrhythmia as a result of early afterdepolarizations (Marban et al., 1986). However, it should be noted that the precise mechanisms by which CsCl and I/R induce ventricular arrhythmia may differ, and CsCl administration may not model what is clinically seen with delayed ventricular arrhythmia after myocardial ischemia. Thus, it is important to replicate the

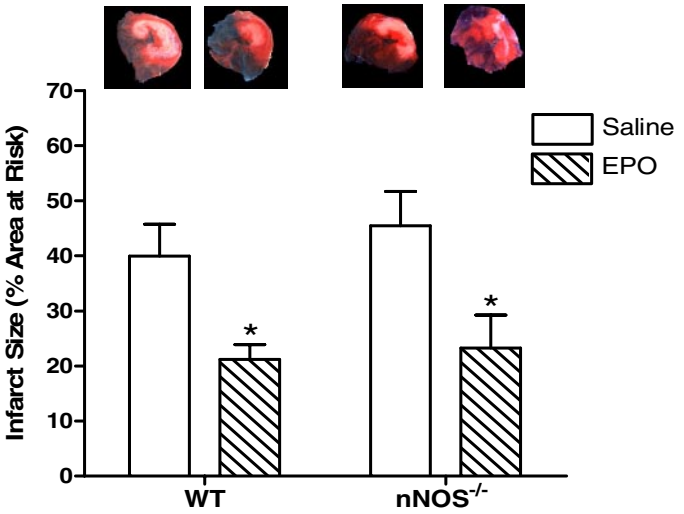

Fig. 5. EPO reduces infarct size in WT and nNOS(−/−) mice. Mice were pretreated with saline or 2500 U/kg EPO (i.v.) for 24 h and 30 min before surgery. Myocardial ischemia was induced by occlusion of coronary artery for 45 min followed by 45 min of reperfusion. Infarct size was expressed as a percentage of area at risk. Inserts, representative sections of TTC-stained hearts corresponding to each treatment group. Data are mean ± S.E.M. from $n = 5$ to 6 mice per group. *, $P < 0.05$ versus genotype saline treatment.

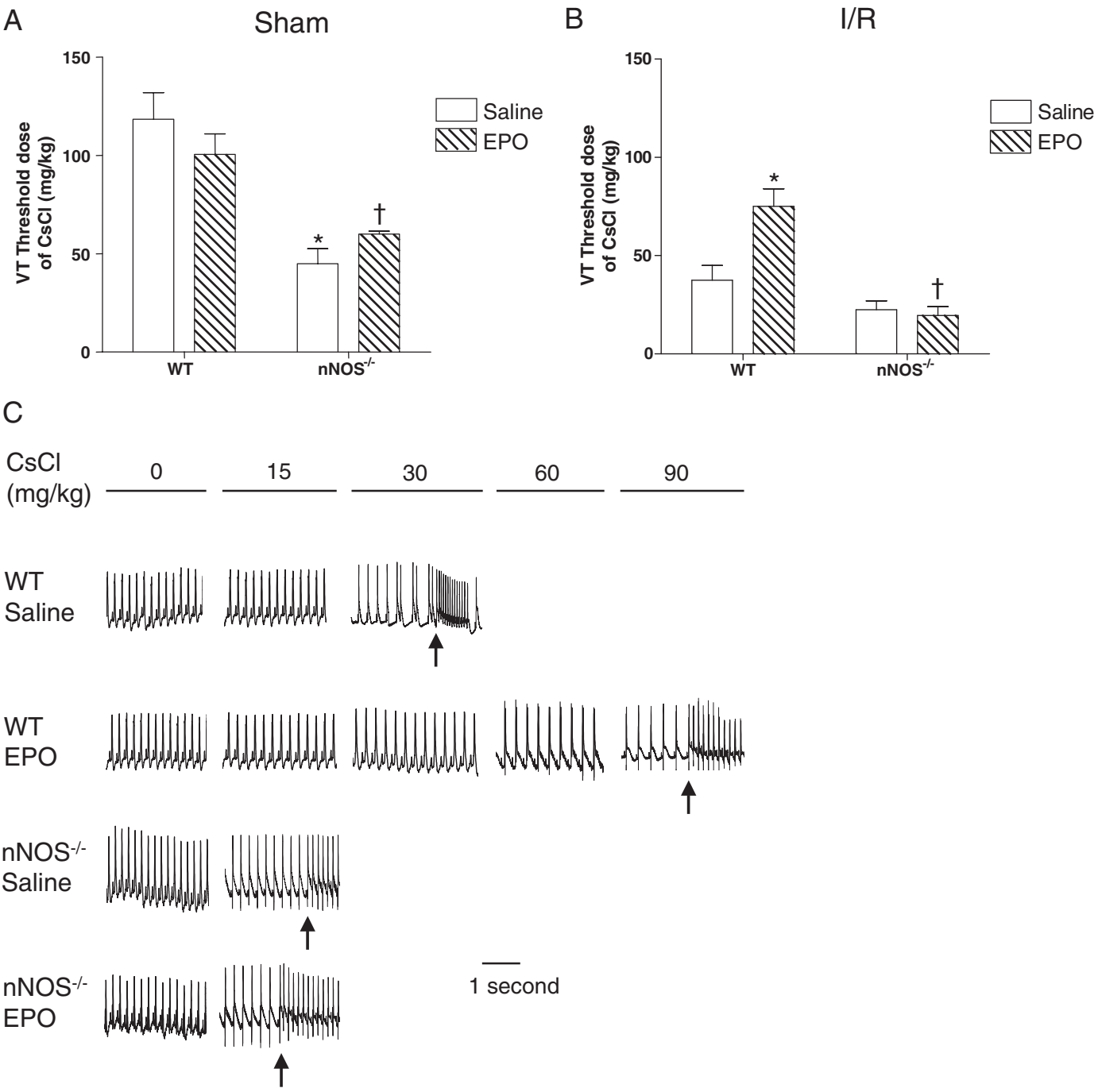

Fig. 6. Effects of EPO treatment on VT threshold dose of CsCl in WT and nNOS(−/−) mice. WT and nNOS(−/−) mice were subjected to sham surgery (A) or 45 min of ischemia followed by 6 h of reperfusion (B). EPO (2500 U/kg i.v.) was administered immediately after ischemia. After reperfusion, mice were exposed to increasing concentrations of CsCl, and the minimum dose required to induce VT was determined. Data are mean ± S.E.M. from n = 4 to 5 mice per group. *, $P < 0.05$ versus WT saline treatment; †, $P < 0.05$ versus WT EPO treatment. C, representative limb lead I ECG tracings demonstrating VT threshold dose of CsCl in WT and nNOS(−/−) mice post-I/R with and without EPO treatment. Arrows, VT.

effects of EPO and nNOS on ventricular arrhythmia in a large animal model, such as the canine coronary occlusion, and in the patient population using selective nNOS inhibitors. Nevertheless, these results suggest that EPO-mediated increases in nNOS protect mice from spontaneous ventricular arrhythmia and CsCl-induced ventricular arrhythmia during I/R. Although further studies on the antiarrhythmic effects of EPO, when administered postischemically, are merited, the present study suggests that EPO may be of therapeutic benefit to the patient population post-MI.

nNOS deletion is associated with arrhythmogenic spontaneous Ca^{2+} waves in isolated myocytes (Gonzalez et al., 2007). However, to date, the role of nNOS in cardiac electrophysiology in vivo has not been clarified. Our study provides the first in vivo evidence of increased arrhythmia in nNOS(−/−) mice. Furthermore, we tested the hypothesis that EPO protects the heart from I/R-induced ventricular arrhythmia via up-regulation of nNOS. In support of this, we demonstrated that EPO treatment increases nNOS expression in vitro and in vivo. The increase in nNOS expression

appears to be mediated by Akt because the effects of EPO on nNOS were blocked by the PI3-kinase inhibitor LY294002 or an adenoviral dominant-negative construct targeted to Akt. In addition, EPO-mediated reductions in arrhythmia appear to be mediated, at least in part, by nNOS because no antiarrhythmic effects were seen after EPO treatment in nNOS(−/−) mice. Thus, our study provides strong evidence of a role for Akt-dependent increases in nNOS expression in the antiarrhythmic effects of EPO.

Recently, we reported eNOS-dependent reductions in infarct size in a mouse model of I/R (Burger et al., 2006). To determine whether EPO-mediated increases in nNOS were involved in the infarct size-limiting effects of EPO, we measured infarct size in EPO-treated WT and nNOS(−/−) mice after I/R. As expected, EPO significantly reduced infarct size after I/R in WT mice. However, the infarct size-limiting effects of EPO were also seen in nNOS(−/−) mice, suggesting that nNOS is not involved in EPO's infarct size-limiting effects. This result is not entirely surprising given a previous report by Jones et al. (2000), which found that nNOS does not exert any effects on infarct size after I/R. However, it is important that the observation that nNOS mediates the antiarrhythmic, but not infarct size-limiting effects of EPO, rules out the possibility of EPO's antiarrhythmic effects being completely attributable to infarct size reduction in our model.

In summary, the present study demonstrated that EPO treatment increases nNOS expression in cardiomyocytes. Moreover, we established an important role for nNOS in mediating the antiarrhythmic effects of EPO during myocardial I/R in mice. Therefore, this study broadens our understanding of the mechanisms by which EPO exerts its antiarrhythmic effects in myocardial I/R.

References

Antman EM, Anbe DT, Armstrong PW, Bates ER, Green LA, Hand M, Hochman JS, Krumholz HM, Kushner FG, Lamas GA, et al. (2004) ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Revise the 1999 Guidelines for the Management of patients with acute myocardial infarction). *J Am Coll Cardiol* **44:**E1–E211.

Asaumi Y, Kagaya Y, Takeda M, Yamaguchi N, Tada H, Ito K, Ohta J, Shiroto T, Shirato K, Minegishi N, et al. (2007) Protective role of endogenous erythropoietin system in nonhematopoietic cells against pressure overload-induced left ventricular dysfunction in mice. *Circulation* **115:**2022–2032.

Belonje AM, Voors AA, van Gilst WH, Anker SD, Slart RH, Tio RA, Zijlstra F, and van Veldhuisen DJ (2008) Effects of erythropoietin after an acute myocardial infarction: rationale and study design of a prospective, randomized, clinical trial (HEBE III). *Am Heart J* **155:**817–822.

Bernaudin M, Marti HH, Roussel S, Divoux D, Nouvelot A, MacKenzie ET, and Petit E (1999) A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *J Cereb Blood Flow Metab* **19:**643–651.

Burger D, Lei M, Geoghegan-Morphet N, Lu X, Xenocostas A, and Feng Q (2006) Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase. *Cardiovasc Res* **72:**51–59.

Burger D, Xenocostas A, and Feng Q (2009) Molecular basis of cardioprotection by erythropoietin. *Curr Mol Pharmacol* **2:**56–69.

Burkard N, Rokita AG, Kaufmann SG, Hallhuber M, Wu R, Hu K, Hofmann U, Bonz A, Frantz S, Cartwright EJ, et al. (2007) Conditional neuronal nitric oxide synthase overexpression impairs myocardial contractility. *Circ Res* **100:**e32–e44.

Calvillo L, Latini R, Kajstura J, Leri A, Anversa P, Ghezzi P, Salio M, Cerami A, and Brines M (2003) Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. *Proc Natl Acad Sci U S A* **100:**4802–4806.

Campbell RW, Murray A, and Julian DG (1981) Ventricular arrhythmias in first 12 hours of acute myocardial infarction: natural history study. *Br Heart J* **46:**351–357.

Fisher JW (2003) Erythropoietin: physiology and pharmacology update. *Exp Biol Med (Maywood)* **228:**1–14.

Gonzalez DR, Beigi F, Treuer AV, and Hare JM (2007) Deficient ryanodine receptor S-nitrosylation increases sarcoplasmic reticulum calcium leak and arrhythmogenesis in cardiomyocytes. *Proc Natl Acad Sci U S A* **104:**20612–20617.

Hamed S, Barshack I, Luboshits G, Wexler D, Deutsch V, Keren G, and George J (2006) Erythropoietin improves myocardial performance in doxorubicin-induced cardiomyopathy. *Eur Heart J* **27:**1876–1883.

Hammoud L, Xiang F, Lu X, Brunner F, Leco K, and Feng Q (2007) Endothelial nitric oxide synthase promotes neonatal cardiomyocyte proliferation by inhibiting tissue inhibitor of metalloproteinase-3 expression. *Cardiovasc Res* **75:**359–368.

Han G, Ma H, Chintala R, Miyake K, Fulton DJ, Barman SA, and White RE (2007) Nongenomic, endothelium-independent effects of estrogen on human coronary smooth muscle are mediated by type I (neuronal) NOS and PI3-kinase-Akt signaling. *Am J Physiol Heart Circ Physiol* **293:**H314–H321.

Hanlon PR, Fu P, Wright GL, Steenbergen C, Arcasoy MO, and Murphy E (2005) Mechanisms of erythropoietin-mediated cardioprotection during ischemia-reperfusion injury: role of protein kinase C and phosphatidylinositol 3-kinase signaling. *FASEB J* **19:**1323–1325.

Heaton DA, Lei M, Li D, Golding S, Dawson TA, Mohan RM, and Paterson DJ (2006) Remodeling of the cardiac pacemaker L-type calcium current and its beta-adrenergic responsiveness in hypertension after neuronal NO synthase gene transfer. *Hypertension* **48:**443–452.

Hirata A, Minamino T, Asanuma H, Fujita M, Wakeno M, Myoishi M, Tsukamoto O, Okada K, Koyama H, Komamura K, et al. (2006) Erythropoietin enhances neovascularization of ischemic myocardium and improves left ventricular dysfunction after myocardial infarction in dogs. *J Am Coll Cardiol* **48:**176–184.

Hirata A, Minamino T, Asanuma H, Sanada S, Fujita M, Tsukamoto O, Wakeno M, Myoishi M, Okada K, Koyama H, et al. (2005) Erythropoietin just before reperfusion reduces both lethal arrhythmias and infarct size via the phosphatidylinositol-3 kinase-dependent pathway in canine hearts. *Cardiovasc Drugs Ther* **19:**33–40.

Jones DL, Petrie JP, and Li HG (2001) Spontaneous, electrically, and cesium chloride induced arrhythmia and afterdepolarizations in the rapidly paced dog heart. *Pacing Clin Electrophysiol* **24:**474–485.

Jones SP, Girod WG, Huang PL, and Lefer DJ (2000) Myocardial reperfusion injury in neuronal nitric oxide synthase deficient mice. *Coron Artery Dis* **11:**593–597.

Joyeux-Faure M, Godin-Ribuot D, and Ribuot C (2005) Erythropoietin and myocardial protection: what's new? *Fundam Clin Pharmacol* **19:**439–446.

Katavetin P, Inagi R, Miyata T, Shao J, Sassa R, Adler S, Eto N, Kato H, Fujita T, and Nangaku M (2007) Erythropoietin induces heme oxygenase-1 expression and attenuates oxidative stress. *Biochem Biophys Res Commun* **359:**928–934.

Kim KH, Oudit GY, and Backx PH (2008) Erythropoietin protects against doxorubicin-induced cardiomyopathy via a phosphatidylinositol 3-kinase-dependent pathway. *J Pharmacol Exp Ther* **324:**160–169.

Krishnagopalan S, Kumar A, Parrillo JE, and Kumar A (2002) Myocardial dysfunction in the patient with sepsis. *Curr Opin Crit Care* **8:**376–388.

Lee ST, Chu K, Sinn DI, Jung KH, Kim EH, Kim SJ, Kim JM, Ko SY, Kim M, and Roh JK (2006) Erythropoietin reduces perihematomal inflammation and cell death with eNOS and STAT3 activations in experimental intracerebral hemorrhage. *J Neurochem* **96:**1728–1739.

Li Y, Takemura G, Okada H, Miyata S, Maruyama R, Li L, Higuchi M, Minatoguchi S, Fujiwara T, and Fujiwara H (2006) Reduction of inflammatory cytokine expression and oxidative damage by erythropoietin in chronic heart failure. *Cardiovasc Res* **71:**684–694.

Liu X, Shen J, Jin Y, Duan M, and Xu J (2006) Recombinant human erythropoietin (rhEPO) preconditioning on nuclear factor-kappa B (NF-kB) activation & proinflammatory cytokines induced by myocardial ischaemia-reperfusion. *Indian J Med Res* **124:**343–354.

Mancini DM, Katz SD, Lang CC, LaManca J, Hudaihed A, and Androne AS (2003) Effect of erythropoietin on exercise capacity in patients with moderate to severe chronic heart failure. *Circulation* **107:**294–299.

Marban E, Robinson SW, and Wier WG (1986) Mechanisms of arrhythmogenic delayed and early afterdepolarizations in ferret ventricular muscle. *J Clin Invest* **78:**1185–1192.

Nakata S, Tsutsui M, Shimokawa H, Yamashita T, Tanimoto A, Tasaki H, Ozumi K, Sabanai K, Morishita T, Suda O, et al. (2007) Statin treatment upregulates vascular neuronal nitric oxide synthase through Akt/NF-kappaB pathway. *Arterioscler Thromb Vasc Biol* **27:**92–98.

Namiuchi S, Kagaya Y, Ohta J, Shiba N, Sugi M, Oikawa M, Kunii H, Yamao H, Komatsu N, Yui M, et al. (2005) High serum erythropoietin level is associated with smaller infarct size in patients with acute myocardial infarction who undergo successful primary percutaneous coronary intervention. *J Am Coll Cardiol* **45:**1406–1412.

Nishiya D, Omura T, Shimada K, Matsumoto R, Kusuyama T, Enomoto S, Iwao H, Takeuchi K, Yoshikawa J, and Yoshiyama M (2006) Effects of erythropoietin on cardiac remodeling after myocardial infarction. *J Pharmacol Sci* **101:**31–39.

Olea FD, Vera Janavel G, De Lorenzi A, Cuniberti L, Yannarelli G, Cabeza Meckert P, Cearras M, Laguens R, and Crottogini A (2006) High-dose erythropoietin has no long-term protective effects in sheep with reperfused myocardial infarction. *J Cardiovasc Pharmacol* **47:**736–741.

Pabla R and Curtis MJ (1995) Effects of NO modulation on cardiac arrhythmias in the rat isolated heart. *Circ Res* **77:**984–992.

Pabla R and Curtis MJ (1996) Endogenous protection against reperfusion-induced ventricular fibrillation: role of neuronal versus non-neuronal sources of nitric oxide and species dependence in the rat versus rabbit isolated heart. *J Mol Cell Cardiol* **28:**2097–2110.

Parsa CJ, Kim J, Riel RU, Pascal LS, Thompson RB, Petrofski JA, Matsumoto A, Stamler JS, and Koch WJ (2004) Cardioprotective effects of erythropoietin in the reperfused ischemic heart: a potential role for cardiac fibroblasts. *J Biol Chem* **279:**20655–20662.

Rui T, Feng Q, Lei M, Peng T, Zhang J, Xu M, Abel ED, Xenocostas A, and Kvietys PR (2005) Erythropoietin prevents the acute myocardial inflammatory response induced by ischemia/reperfusion via induction of AP-1. *Cardiovasc Res* **65:**719–727.

Sears CE, Bryant SM, Ashley EA, Lygate CA, Rakovic S, Wallis HL, Neubauer S, Terrar DA, and Casadei B (2003) Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. *Circ Res* **92:**e52–59.

Song W, Lu X, and Feng Q (2000) Tumor necrosis factor-alpha induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes. *Cardiovasc Res* **45:**595–602.

Verheule S, Sato T, and Olgin JE (2006) Cardiac electrophysiology, in *A Handbook of Mouse Models of Cardiovascular Disease* (Xu Q ed) pp 349–362, John Wiley & Sons, Chichester, UK.

Walker MJ, Curtis MJ, Hearse DJ, Campbell RW, Janse MJ, Yellon DM, Cobbe SM, Coker SJ, Harness JB, and Harron DW (1988) The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion. *Cardiovasc Res* **22:**447–455.

Xu KY, Huso DL, Dawson TM, Bredt DS, and Becker LC (1999) Nitric oxide synthase in cardiac sarcoplasmic reticulum. *Proc Natl Acad Sci U S A* **96:**657–662.

Zaugg CE (2004) Current concepts on ventricular fibrillation: a vicious circle of cardiomyocyte calcium overload in the initiation, maintenance, and termination of ventricular fibrillation. *Indian Pacing Electrophysiol J* **4:**85–92.

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