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Erythropoietin protects the heart from ventricular arrhythmia during ischemia and reperfusion via neuronal nitric oxide synthase

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Running Head: EPO inhibits ventricular arrhythmia via nNOS

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ABBREVIATIONS: EPO, Erythropoietin; I/R, ischemia-reperfusion; nNOS, neuronal nitric oxide synthase; ADV-dnAkt1, dominant negative adenovirus to Akt1; WT, wild-type; SR, sarcoplasmic reticulum; PVCs, premature ventricular contractions; VT, ventricular tachycardia; CsCl, cesium chloride; MI, myocardial infarction; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; ECG, electrocardiogram; TTC, triphenyltetrazolium chloride; PI3-kinase, phosphatidylinositol 3 kinase.

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ABSTRACT

Erythropoietin (EPO) is a potent cardioprotective agent in models of myocardial ischemia and reperfusion (I/R). Recently, it has been suggested that EPO may also reduce ventricular arrhythmia following I/R. The present study investigated the role of neuronal nitric oxide synthase (nNOS) in the antiarrhythmic effects of EPO. EPO treatment increased nNOS expression in isolated neonatal mouse ventricular myocytes. Co-treatment with the PI3-kinase inhibitor LY294002, 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. Pre-treatment with EPO 24 hours prior to 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 to this, 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 protects the heart from spontaneous and CsCl-induced ventricular arrhythmia during myocardial I/R via nNOS.

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Introduction

Erythropoietin (EPO) is a 30 kilodalton 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. Indeed, EPO has been shown to act in a number of non-hematopoietic 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-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). Recently, a report by Hirata *et al.* suggested that EPO may also reduce I/R-induced ventricular arrhythmia via protein kinase B (Akt) activation (Hirata et al., 2005).

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 (SR) (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). Indeed, 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; Burkard et al., 2007), and a decrease in SR- Ca^{2+} ATPase function (Burkard et al., 2007). A recent report by Gonzalez and colleagues found that

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deficiency in nNOS leads to spontaneous Ca^{2+} waves in isolated cardiomyocytes (Gonzalez et al., 2007). These spontaneous waves were associated with deficient nitrosylation of ryanodine receptors (RyR) and are thought to be pro-arrhythmogenic. Interestingly, inhibition of nitric oxide (NO) production has been shown to be pro-arrhythmogenic in isolated rat and rabbit hearts suggesting that nNOS-derived NO production may indeed play an important antiarrhythmic role in the heart (Pabla and Curtis, 1995; Pabla and Curtis, 1996).

While 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.

Methods

Animals. WT and $nNOS^{-/-}$ mice of C57BL/6 background were purchased from 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-hour light and dark cycles. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published

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by the US National Institutes of Health (NIH publication No. 85-23) 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 hours were minced in Hanks' balanced salt solution and cardiomyocytes were dispersed by 22.5 µg/ml liberase blendzyme IV at 37 ˚C for 30 minutes. Isolated cells were pre-plated on polystyrene plates for 60 minutes to allow for the removal of non-cardiomyocytes. Cardiomyocytes were then plated in M199 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 hours after which they were treated with recombinant human EPO (Eprex ®, Ortho Biotech, Canada) and/or the indicated reagents.

Real Time RT-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 M-MLV reverse transcriptase and random primers (Invitrogen, Burlington, ON). Real-time PCR was conducted using SYBR Green PCR Master Mix as per the manufacturer's instructions (Eurogentec, San Diego, CA). 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. The levels of nNOS were compared to that of 28S rRNA, and the relative expression of nNOS was obtained.

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Western Blot Analysis. Total nNOS protein levels in cardiomyocytes and heart tissues (non-ischemic region of the LV myocardium) were measured by western blot analysis. Briefly, 40 µg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% gels. Proteins were then transferred to nitrocellulose membranes and blots were probed with antibodies against nNOS (1:2000, Sigma) and β-actin (1:2000; Sigma, Mississauga, ON). Blots were then washed and probed with horseradish peroxidase-conjugated secondary antibodies (1:2000; BioRad, Mississauga, ON) and detection was performed using an enhanced chemiluminescent detection method and exposed to Kodak X-ray film 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 to 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 pfu/cell. Adenovirus-mediated gene transfer was implemented by adding a minimal volume of M199 with 2% fetal bovine serum containing Adv-dnAkt1. Viral constructs containing green fluorescence protein (GFP) 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.

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***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 hours prior to surgery, and then a second time 30 minutes prior to 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 hyper-inflated using positive end-expiratory pressures (3 cm H_2O), and the thorax was closed. The artery was occluded for 45 minutes to induce ischemia after which the suture was loosened to allow reperfusion for 45 minutes.

Monitoring of 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 (AD Instruments, Colorado Springs, CO).

Cesium Chloride Infusion. Following 6 hours of reperfusion, mice were re-anesthetized and the jugular vein was cannulated and cesium chloride (CsCl) was infused using a syringe pump (Sage Instruments). CsCl has previously been reported to induce VT in dogs (Jones et al., 2001). ECG

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was monitored using limb lead I with needle electrodes and recorded using PowerLab. The minimum dose of CsCl required to induce ventricular tachycardia (VT) was determined.

ECG Analysis. Ventricular arrhythmias were analyzed offline 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 ventricular tachycardia (VT) was defined as a run of three or more premature QRS complexes. The number of singlet and doublet PVCs, as well as the incidence of VT, were quantified.

Measurement of Infarct Size. Infarct size was measured as described previously (Burger et al., 2006). Briefly, following 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. 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 as a percentage of overall area using SigmaScan 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.

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Statistical Analysis. All data are expressed as mean ± SEM. Statistical significance was assessed using the Student *t*-test, ANOVA, or two-way ANOVA followed by the Bonferroni post-hoc test where appropriate using Prism 4.0 (GraphPad Software, La Jolla, CA). Differences were considered significant at the level of $P<0.05$.

Results

Erythropoietin treatment increases nNOS expression in cardiomyocytes. To determine if EPO treatment can increase nNOS expression levels in cardiomyocytes, cells were treated with 20 U/mL of 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 hours (Fig. 1A, $P<0.05$) and nNOS protein expression peaking at 16 hours (Fig. 1B, $P<0.05$).

Role of PI3-kinase/Akt in EPO-mediated increases in nNOS expression. To determine if 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 previously been shown 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; however EPO-mediated increases in nNOS protein expression were blocked in the presence of LY294002 or Adv-dnAkt1 (Fig. 2A and B).

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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 minutes of ischemia followed by 45 minutes of reperfusion. Pre-treatment 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 pre-treatment had no effect on I/R-induced PVCs or the incidence of VT in nNOS$^{-/-}$ mice (Fig. 3B and C). No changes were observed in heart rate amongst any treatment groups (Fig. 3D). Representative tracings are shown in Figure 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 pre-treatment on infarct size in WT and nNOS$^{-/-}$ mice. EPO pre-treatment using Protocol #1 significantly reduced infarct size to a similar extent in both WT and nNOS$^{-/-}$ mice following 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 following CsCl infusion. Clinically, extended pre-treatment such as that used earlier in this study may not be possible in patients with acute MI. To determine if EPO confers cardioprotection when administered after the onset of ischemia, we examined the effects of EPO (2500 U/kg, i.v., Protocol #2) administered

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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 following I/R in WT mice (Fig. 6B). The protective effects of EPO were not seen in sham-operated or ischemia-reperfused nNOS$^{-/-}$ mice (Fig. 6A 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.

The role of EPO in the protection of the heart from injury has been extensively studied 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). Indeed, 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 following 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 as they may not behave in the same way as the human

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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.* observed reductions in infarct size and an improvement in heart function following EPO treatment in rabbits with I/R (Parsa et al., 2004). 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 non-anemic 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 if EPO treatment had any effect on cardiac electrophysiology. The present study demonstrated that EPO pre-treatment 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.* who observed PI3-kinase-dependent reductions in ventricular fibrillation following myocardial I/R in dogs (Hirata et al., 2005).

Interestingly, we found that EPO-mediated reductions in spontaneous arrhythmia following pre-treatment, and EPO-mediated reductions in 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. Pre-treatment 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 12-48 hours

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after the onset of MI (Campbell et al., 1981; Antman et al., 2004). In our model, EPO significantly reduced CsCl-induced arrhythmia 6 hours after reperfusion. CsCl was chosen to induce arrhythmia because spontaneous arrhythmia was no longer present after 6 hours 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 following myocardial ischemia. Thus it is important to replicate the 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. While further studies on the anti-arrhythmic effects of EPO, when administered post-ischemically, 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 since EPO's effects on nNOS were blocked by the PI3-kinase inhibitor LY294002 or an adenoviral dominant negative construct

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targeted to Akt. Additionally, EPO-mediated reductions in arrhythmia appear to be mediated, at least in part, by nNOS since no antiarrhythmic effects were seen following 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 if EPO-mediated increases in nNOS were involved in EPO's infarct size-limiting effects, we measured infarct size in EPO-treated WT and nNOS$^{-/-}$ mice following I/R. As expected, EPO significantly reduced infarct size following 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 and colleagues which found that nNOS does not exert any effects on infarct size following I/R (Jones et al., 2000). Importantly however, 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.

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References

Antman EM, Anbe DT, Armstrong PW, Bates ER, Green LA, Hand M, Hochman JS, Krumholz HM, Kushner FG, Lamas GA, Mullany CJ, Ornato JP, Pearle DL, Sloan MA, Smith SC, Jr., Alpert JS, Anderson JL, Faxon DP, Fuster V, Gibbons RJ, Gregoratos G, Halperin JL, Hiratzka LF, Hunt SA and Jacobs AK (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 and Shimokawa H (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.

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JPET Fast Forward. Published on March 23, 2009 as DOI: 10.1124/jpet.109.150896
This article has not been copyedited and formatted. The final version may differ from this version.

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, Neyses L, Maier LS, Maier SK, Renne T, Schuh K and Ritter O (2007) Conditional neuronal nitric oxide synthase overexpression impairs myocardial contractility. *Circ Res* **100**:e32-44.

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 USA* **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 USA* **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.

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JPET Fast Forward. Published on March 23, 2009 as DOI: 10.1124/jpet.109.150896
This article has not been copyedited and formatted. The final version may differ from this version.

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**:H314-321.

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, Takashima S, Shinozaki Y, Mori H, Shiraga M, Kitakaze M and Hori M (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, Komamura K, Takashima S, Shinozaki Y, Mori H, Tomoike H, Hori M and Kitakaze M (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.

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JPET Fast Forward. Published on March 23, 2009 as DOI: 10.1124/jpet.109.150896
This article has not been copyedited and formatted. The final version may differ from this version.

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 and Parrillo JE (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.

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JPET Fast Forward. Published on March 23, 2009 as DOI: 10.1124/jpet.109.150896
This article has not been copyedited and formatted. The final version may differ from this version.

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, Hirano H, Sasaguri Y, Nakashima Y and Yanagihara N (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, Tada H, Sakuma M, Watanabe J, Ichihara T and Shirato K (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

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on March 23, 2009 as DOI: 10.1124/jpet.109.150896
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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, Harron DW and et al. (1988) The Lambeth Conventions: guidelines for the

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on March 23, 2009 as DOI: 10.1124/jpet.109.150896
This article has not been copyedited and formatted. The final version may differ from this version.

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 USA* **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.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on March 23, 2009 as DOI: 10.1124/jpet.109.150896
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Footnotes:

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Legends for Figures:

Fig. 1. Erythropoietin (EPO) increases nNOS expression in neonatal mouse ventricular cardiomyocytes. A: EPO (20 U/mL) increased nNOS mRNA levels as measured by real time RT-PCR in cardiomyocytes after 4 and 8 hours of treatment. B: EPO increased nNOS protein levels as measured by western blot in cultured cardiomyocytes after 8, 16, and 24 hours of treatment. Data are mean ± SEM from 4-6 independent experiments *$P<0.05$ vs. control (Ctrl).

Fig. 2. Inhibition of PI3-kinase/Akt-1 decreases erythropoietin (EPO)-mediated nNOS expression. Cultured cardiomyocytes were treated with 20 U/mL of EPO alone or in combination with 10 μM of the PI3-kinase inhibitor LY294002 (LY, A) or 100 pfu/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 are representative blots. Data are mean ± SEM from 3-4 independent experiments. *P<0.05 vs. control (Ctrl), †P<0.05 vs. EPO treatment alone.

Fig. 3. Effect of erythropoietin (EPO) on nNOS expression and ventricular arrhythmia *in vivo*. Wild-type (WT) mice were treated with EPO (2500 U/kg, IP) 24 hours as well as 30 minutes prior to analysis. A: EPO increased myocardial nNOS expression as measured by western blot analysis. Data are expressed as the ratio of nNOS/actin. Inserts are representative blots. *P<0.05 vs. control (Ctrl), n=4 mice per group. B-D: WT and $nNOS^{-/-}$ mice were treated with EPO (2500 U/kg, IP) or equivalent volume saline 24 hours as well as 30 minutes prior to surgery. Mice were then subjected to 45 minutes of myocardial ischemia followed by 45 minutes of reperfusion and cardiac electrical activity was monitored using a single limb lead I electrocardiogram (ECG). B:

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Total number of premature ventricular contractions (PVCs). C: Incidence of ventricular tachycardia (VT). D: Heart rate was not significantly different across all treatment groups. *P<0.05 vs. genotype saline treatment, †P<0.05 vs. WT saline treatment, n=8-15 mice per group.

Fig. 4. Representative limb lead I electrocardiogram (ECG) tracings of saline and erythropoietin (EPO) treated wild-type (WT) and nNOS$^{-/-}$ mice. Shown are representative premature ventricular contractions (PVCs, indicated by the arrows) following myocardial ischemia and reperfusion (I/R) in anesthetized and artificially ventilated mice.

Fig. 5. Erythropoietin (EPO) reduces infarct size in wild-type (WT) and nNOS$^{-/-}$ mice. Mice were pre-treated with saline or 2500 U/kg of EPO 24 hours, as well as 30 minutes prior to surgery. Myocardial ischemia was induced by occlusion of coronary artery for 45 minutes followed by 45 minutes of reperfusion. Infarct size was expressed as a percentage of area at risk. Inserts are representative sections of TTC stained hearts corresponding to each treatment group. Data are mean ± SEM from n=5-6 mice per group. *P<0.05 vs. genotype saline treatment.

Fig. 6. Effects of erythropoietin (EPO) treatment on VT threshold dose of Cesium chloride (CsCl) in WT and nNOS$^{-/-}$ mice. WT and nNOS$^{-/-}$ mice were subjected to sham surgery (A) or 45 minutes ischemia followed by 6 hours of reperfusion (B). EPO (2500U/kg) was administered immediately after ischemia. Following reperfusion, mice were exposed to increasing concentrations of CsCl and the minimum dose required to induce ventricular tachycardia (VT) was determined. Data are mean ± SEM from n=4-5 mice per group. *P<0.05 vs. WT saline treatment, †P<0.05 vs WT EPO treatment. C. Representative limb lead I ECG tracings

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demonstrating VT threshold dose of CsCl in WT and nNOS$^{-/-}$ mice post I/R with and without EPO treatment. Arrows indicate VT.

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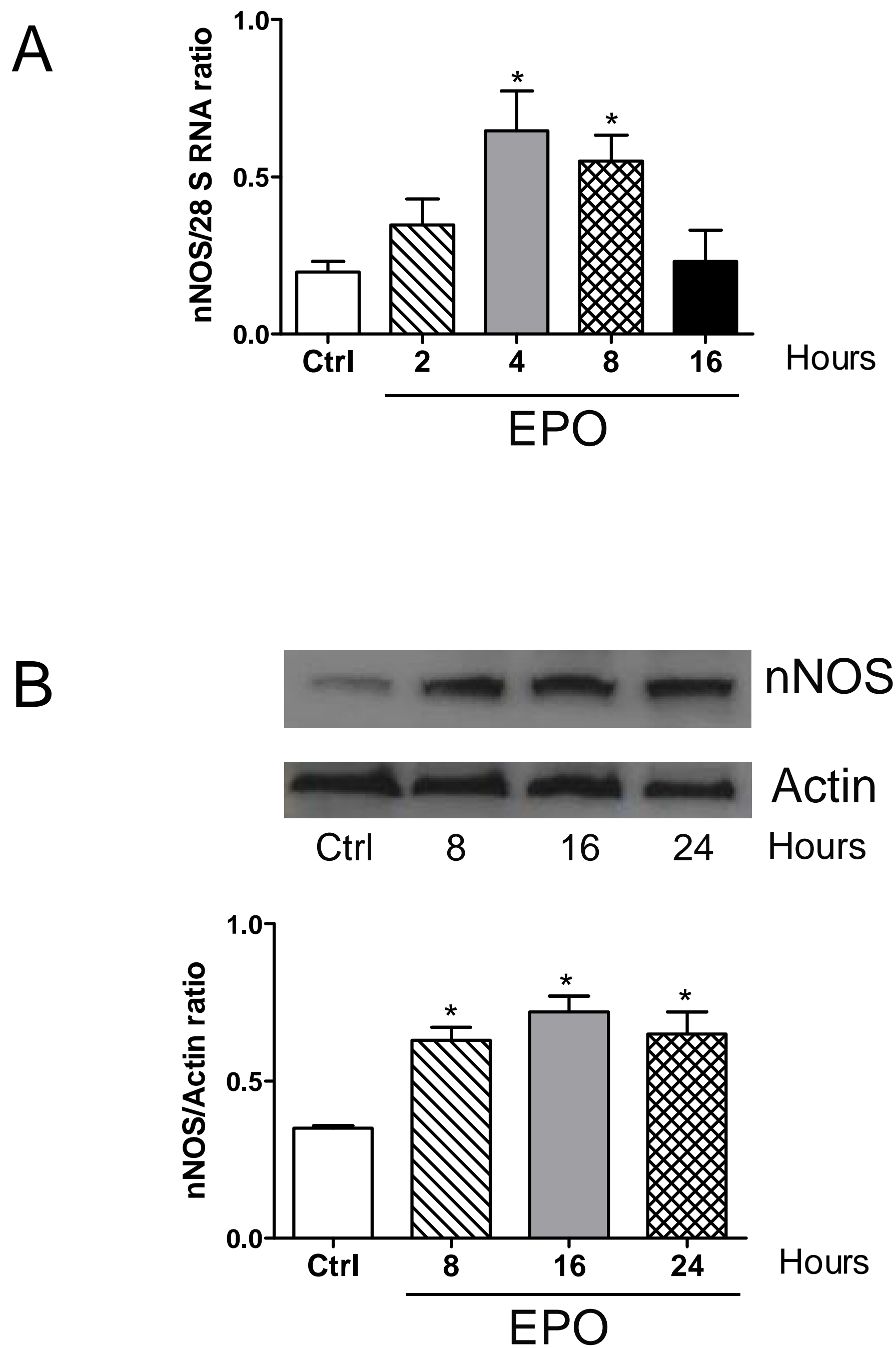

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Fig. 1

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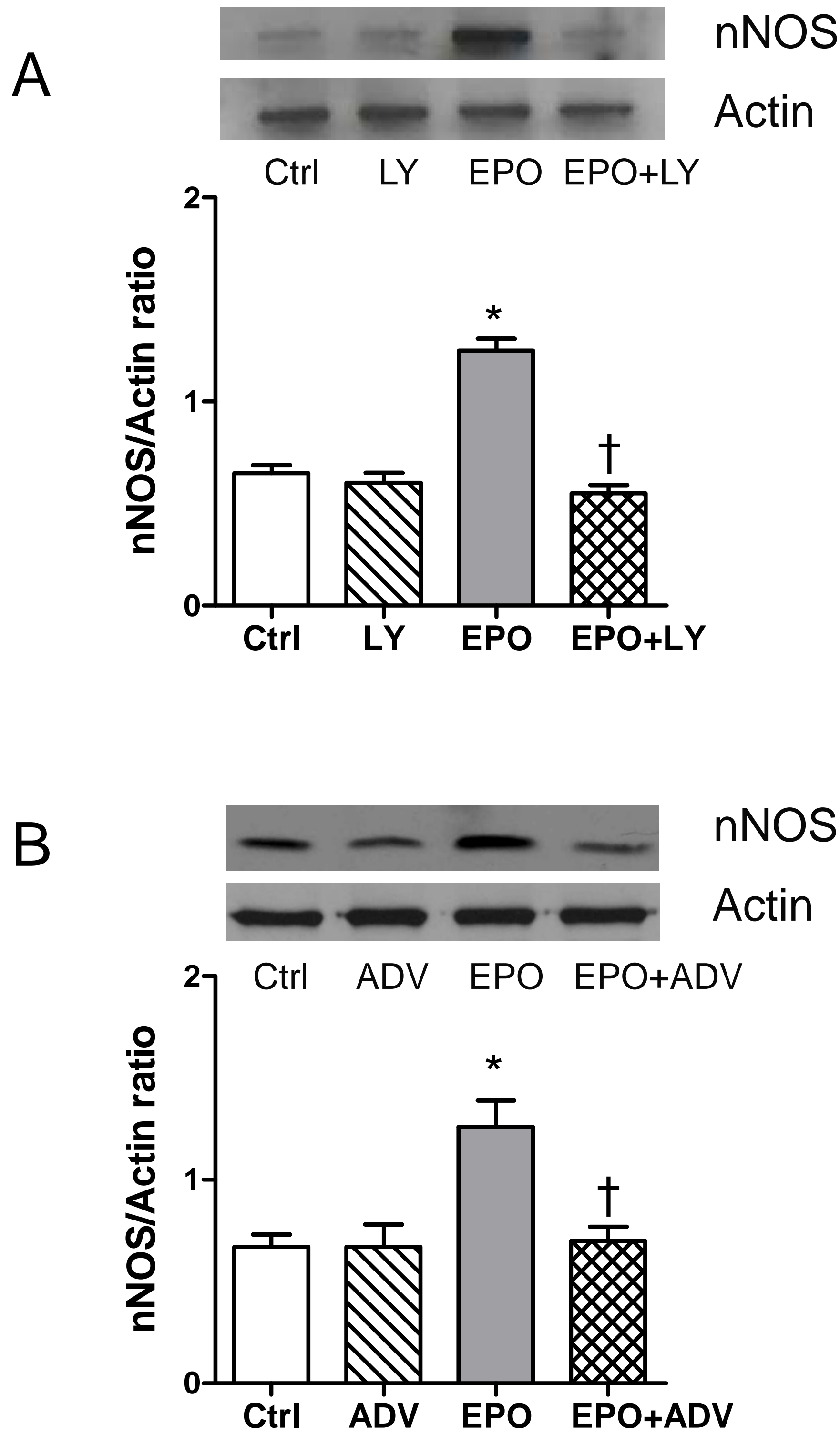

Fig. 2

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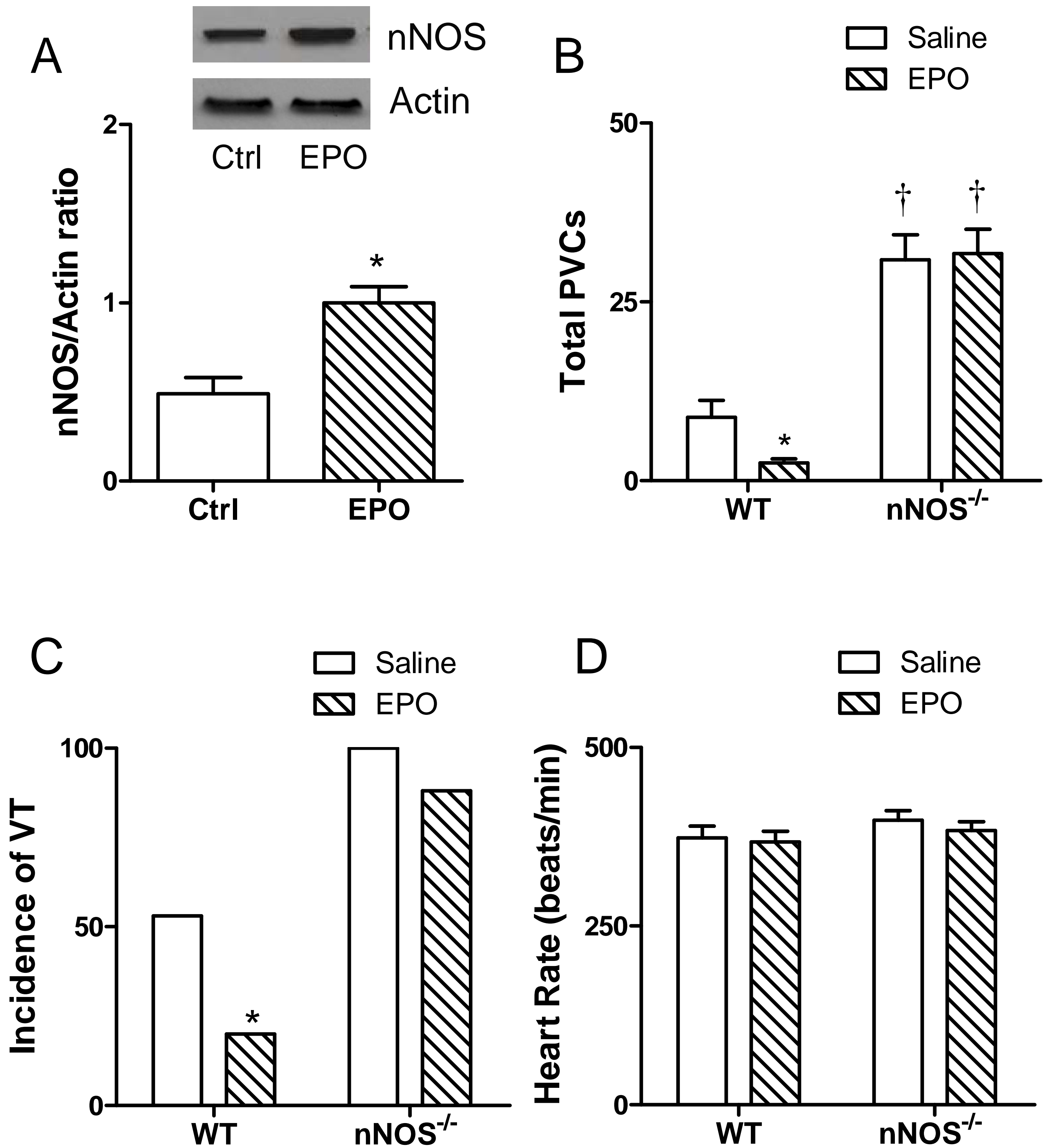

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Fig. 3

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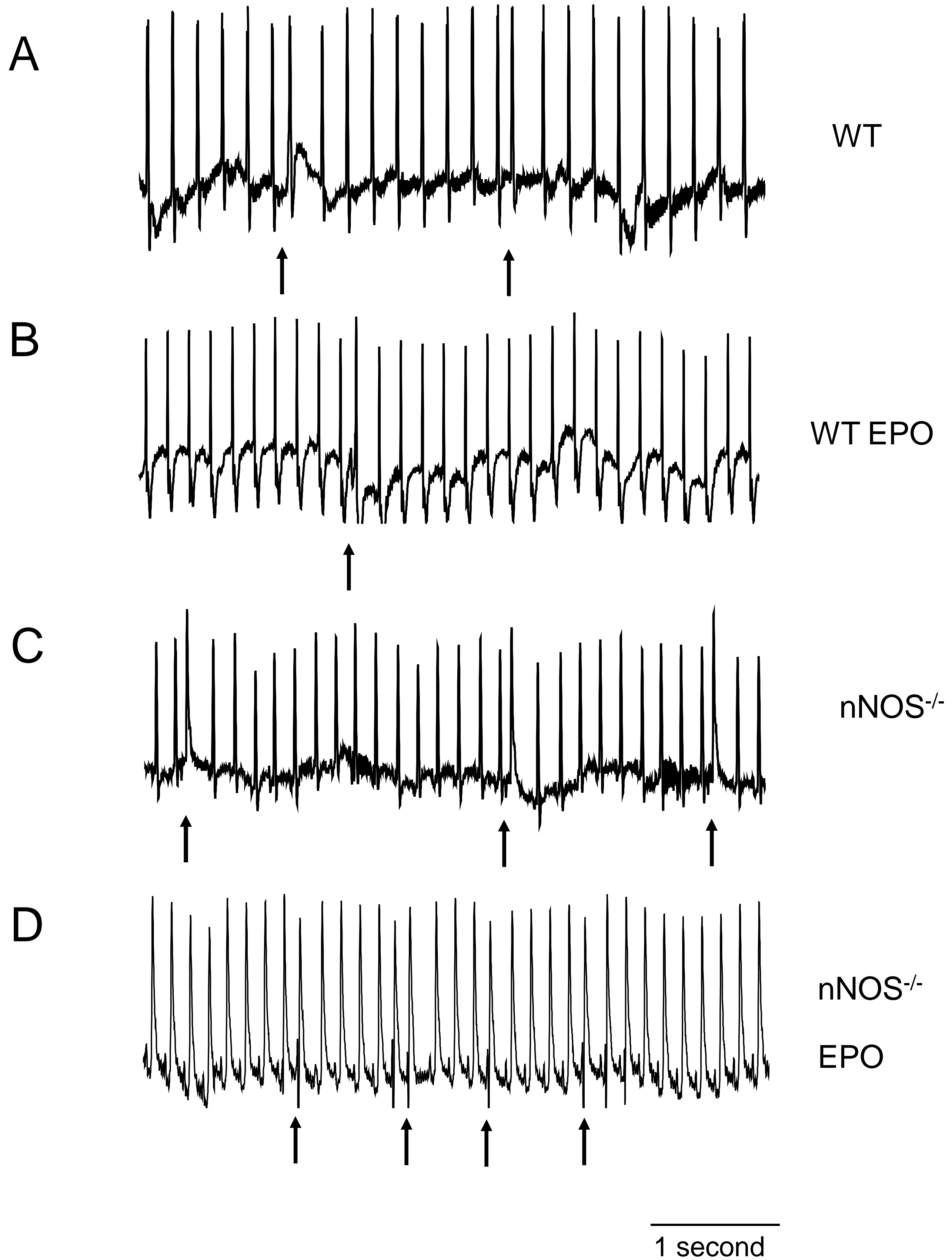

Fig. 4

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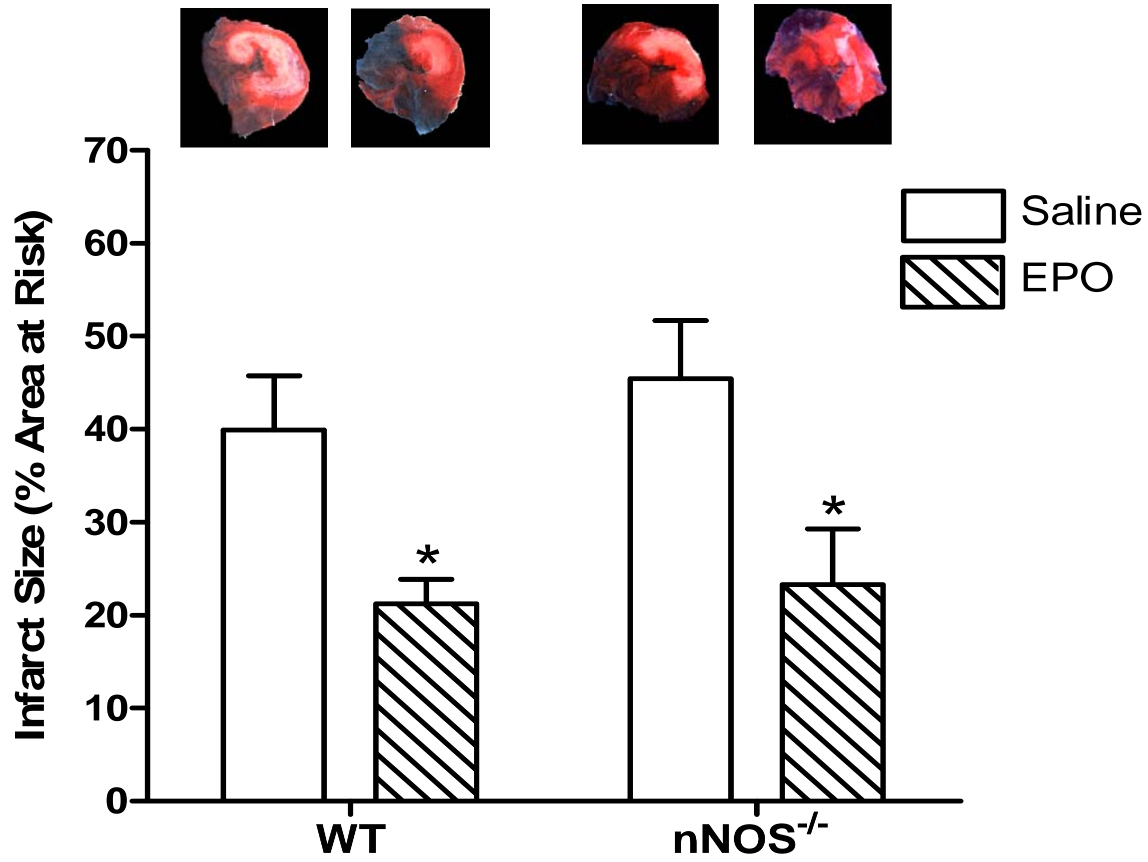

Fig. 5

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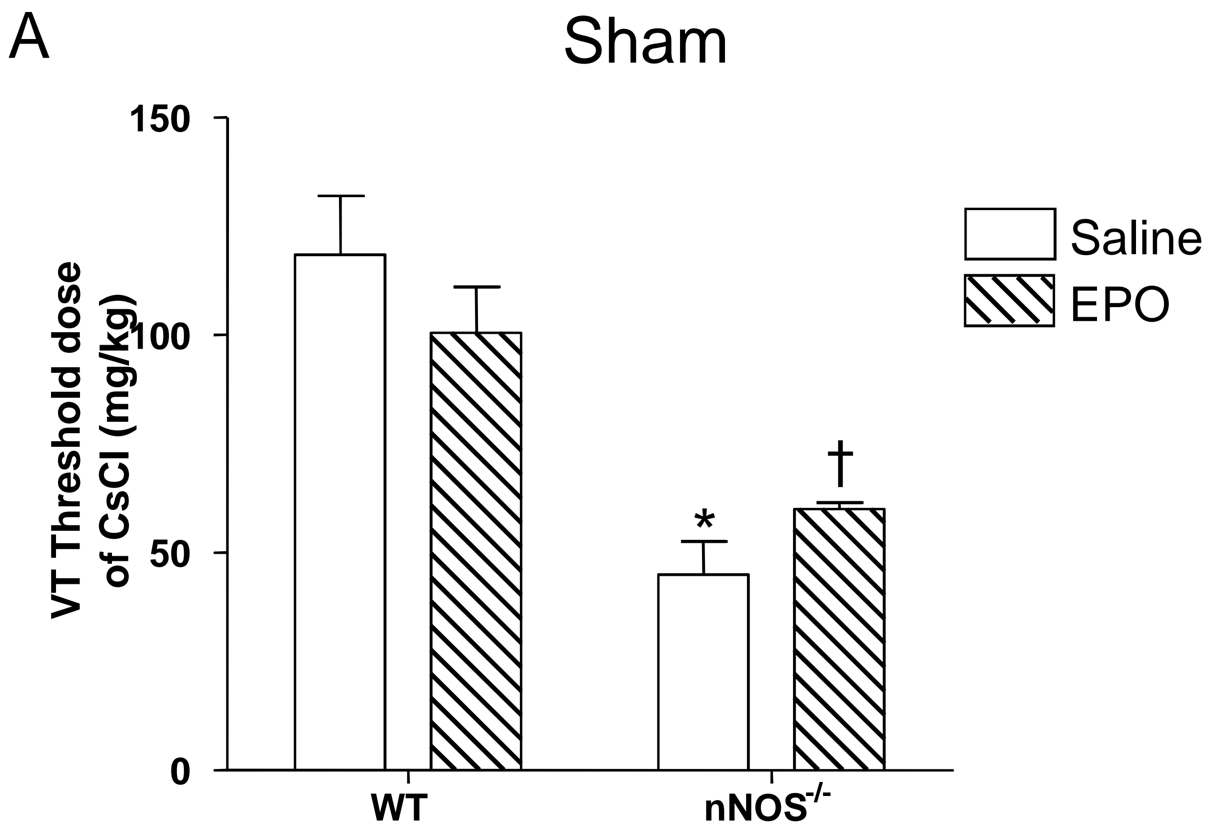

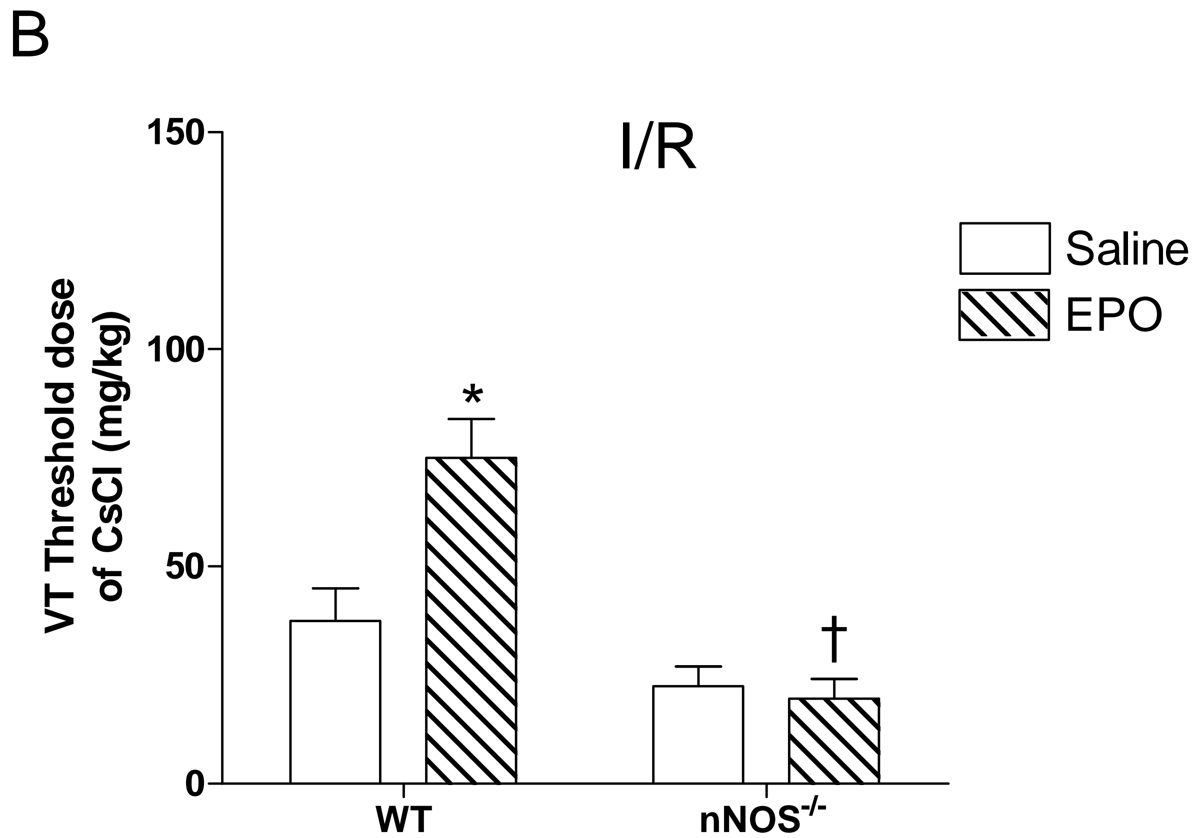

Fig. 6A and B

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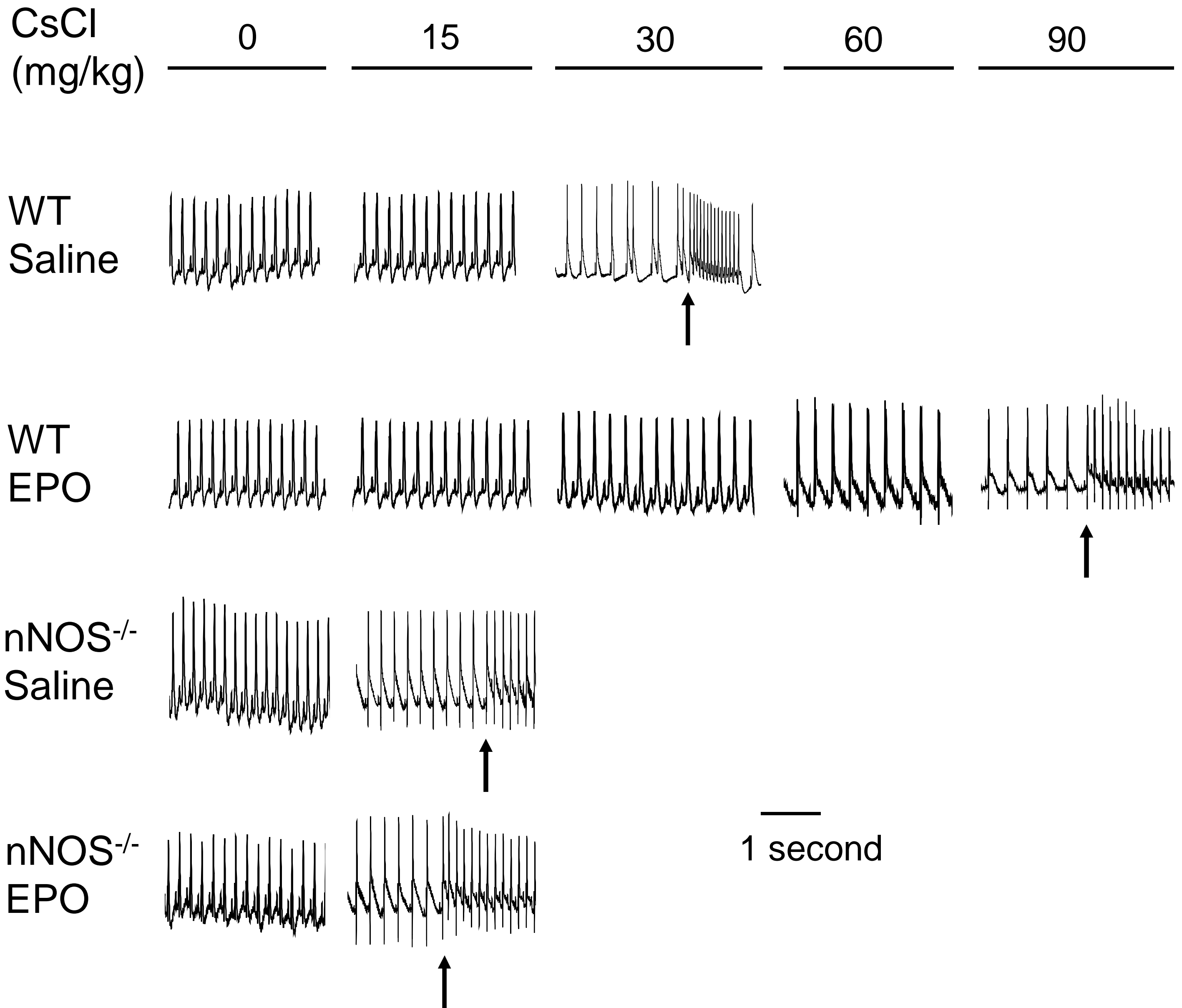

Fig. 6C

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