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ROUTINE ARTICLE

Chronic Erythropoietin Treatment Limits Infarct-size in the Myocardium in Vitro

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Summary. **It has been well established that erythropoietin (EPO) can limit myocardial ischemia/reperfusion injury in a variety of acute settings. However, despite EPO being used chronically to treat anemia the infarct limiting effects of long term treatment (chronic) have never been fully investigated. In this study we examined the effects of a 3 week treatment of EPO (5,000 IU/Kg) in male Sprague Dawley rats in limiting myocardial infarction after 35 min ischemia and 2 h reperfusion in an *in vitro* isolated heart perfusion model. Treating the animals 'once a week' failed to limit infarct size significantly compared to a saline control (54.1% ± 3.5 v 52.3% ± 4.4), whereas a '3 times a week' regime succeeded in significantly reducing infarct size (36.2% ± 3.2 v 52.3% ± 4.4, $p < 0.05$). To demonstrate that the effect was not due to improved oxygen supply caused by a raised hematocrit level, we also administered EPO 24 h prior to ischemia/reperfusion. This treatment again reduced infarct size compared to a saline control (39.9% ± 4.4 v 58.4% ± 5.0, $p < 0.05$). To examine the mechanism of protection we used the PI3K inhibitor wortmannin and the nitric oxide synthase inhibitor L-NAME to try to abrogate EPO mediated protection. Where wortmannin failed to block the effects of EPO (31.7% ± 6.0 v 36.2% ± 3.2), L-NAME did abrogate protection (51.6% ± 5.6 v 36.2% ± 3.2, $p < 0.05$). We demonstrate that chronic EPO treatment limits infarct size and that it does so in a nitric oxide dependent manner.**

Key Words. **ischemia, reperfusion, infarction, kinases**

Introduction

Erythropoietin (EPO) has long been used as a chronic treatment to correct anemia, whether as a result of kidney failure, cancer or some other pathology. Originally EPO had only been considered to increase the hematocrit content of blood and so any beneficial pleiotropic effect was attributed to an improved oxygen supply to tissues [1].

In recent years, however, it has become increasingly evident that EPO has beneficial effects that are independent of its hemopoietic properties. For example, EPO has demonstrated limitation of injury induced by ischemia/reperfusion in the heart and brain using models where blood was absent [2–4]. It has also been shown that derivatives of EPO that have no hemopoietic effect could also provide protection in the setting of ischemia/reperfusion [5,6].

More recently, we and others have demonstrated that EPO has an ability to limit infarction as a result of reperfusion-induced injury [7–9] in a manner that requires activation of the RISK pathway [9,10]. However, there is no evidence to suggest whether this protection occurs following chronic administration of EPO or whether this RISK pathway is maintained during such chronic treatment.

As EPO is routinely administered chronically to correct anemia providing improved outcome for patients [1], we undertook studies to determine whether the benefits could at least partially be due to a direct effect of EPO on the myocardium and to try and ascertain by which signalling pathways any protection is mediated.

Materials and Methods

Animals

All animals were obtained from Charles River (UK) and were treated in accordance with the United Kingdom Animal (Scientific Procedures) Act of 1986. The investigation conforms to the Guide for the Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85).

Materials

Wortmannin, was dissolved in DMSO and diluted into buffer such that the vehicle constituted less than 0.02% of the total volume. L-NAME was dissolved in water.

EPO was provided by Roche (Welwyn Garden City, UK).

Isolated heart preparation

Male Sprague-Dawley rats (300–400 g) were anaesthetised with sodium pentobarbital (50 mg/Kg ip). Heparin (300 IU) was administered concomitantly.

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Hearts were rapidly excised and placed in ice-cold buffer and mounted on a constant pressure (80 mm Hg) Langendorff-perfusion apparatus. They were perfused retrogradely through the aorta with modified Krebs-Henseleit buffer containing (in mM): NaCl 118, $NaHCO_3$ 25. KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.7 and glucose 12 and aerated with carbogen to maintain a pH of 7.3–7.5. Temperature was continuously monitored using a thermo-probe inserted into the pulmonary artery and maintained at 37°C ± 1°C. A latex balloon was introduced into the left ventricle through the left atrial appendage and inflated to give an end-diastolic pressure of 5–10 mm Hg. Regional ischaemia was achieved by tightening a silk suture around the main branch of the left coronary artery and confirmed by a substantial drop in both left ventricular developed pressure and coronary flow rate. Hearts underwent 10 min stabilisation, 35 min. regional ischaemia and 2 h reperfusion.

Infarct size determination

At the end of the protocol, the risk zone was determined by re-occluding the silk suture around the left coronary artery and injecting a 5% solution of Evans blue dye (2 ml) to mark the risk zone as unstained. Hearts were then frozen and subsequently cut into 2 mm slices for determination of viability by triphenyltetrazolium chloride (TTC) staining in which viable tissue stains red. The slices were then measured for infarct to risk ratio (% I/R) by computerized planimetry (Summa Sketch II, Summa Graphics, Seymour, CT, USA) [9].

Chronic administration of EPO

All animals within the chronic study were treated for 3 weeks and dosed by sub-cutaneous injection (volume 200μL). EPO (5000 IU/kg) was either given once a week (1× Wk), or 3 times a week (3× Wk).

The control group had saline administered 3 times a week. Hearts from additional groups that had received the 3× Wk regime had either the PI3-Kinase inhibitor wortmannin (100nM) or the nitric oxide synthase inhibitor L-NAME (100 μM) perfused for 10 min. prior to ischemia on the isolated perfused preparation.

Acute administration of EPO

EPO was administered by sub-cutaneous injection 24 h prior to index ischemia.

A control group of a single dose of saline was administered by sub-cutaneous injection 24 h prior to index ischemia.

Hematocrit analysis

Hematocrits were determined using a KX-21 manufactured by Sysmex UK Ltd, Milton Keynes, UK.

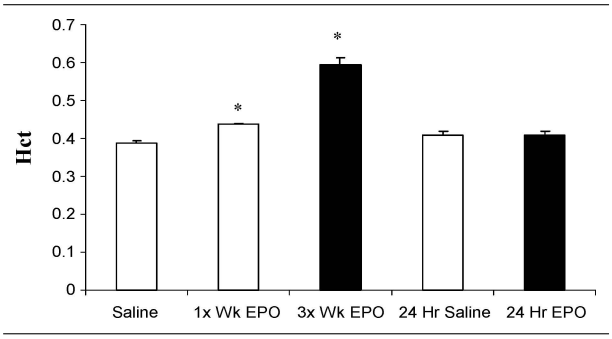

Fig. 1. *The hematocrit (Hct) levels after treatment with a 3 times a week dose of saline for 3 weeks, once a week dose of EPO (5000 IU/Kg) for 3 weeks (1× Wk EPO), 3 times a week dose of EPO for 3 weeks (3× Wk EPO), a single dose of saline 24 h before and a single dose of EPO 24 h before.* * *= $p < 0.05$ v saline control. ($n = 7$–12)*

Statistics

Data were statistically analyzed by one-way ANOVA using Statview 4.5 (Abacus Concepts Inc., Berkley, California, USA). A p-value <0.05 was considered to be statistically significant. Results are presented as means ± SEM.

Results

Effect of EPO on hematocrit

A 'once a week treatment' of EPO for 3 weeks significantly increased hematocrit compared to a saline-treated control (0.44 ± 0.006 v 0.39 ± 0.008, $p < 0.05$) (see Fig. 1). A '3 times a week' treatment of EPO increased hematocrit significantly compared to both the 'once a week' treatment and the saline control (0.60 ± 0.018 v 0.44 ± 0.006 v 0.39 ± 0.008, $p < 0.05$). A single treatment of EPO administered 24 h prior to the experiment produced no alteration in hematocrit compared to its saline control (0.41 ± 0.08 v 0.41 ± 0.08).

Haemodynamic effect of EPO

There was no significant difference in left ventricular developed pressure or coronary flow between the different groups.

The effect of chronic treatment on infarct size in vitro

A '3 times a Week' treatment of EPO successfully limited infarct size compared to its saline control (I/R 36.2% ± 3.2 v 52.3% ± 4.4, $p < 0.05$) (see Fig. 2). A 'once a week' treatment of EPO failed to elicit any protection against ischaemia/reperfusion compared to the saline control (54.1% ± 3.5 v 52.3% ± 4.4).

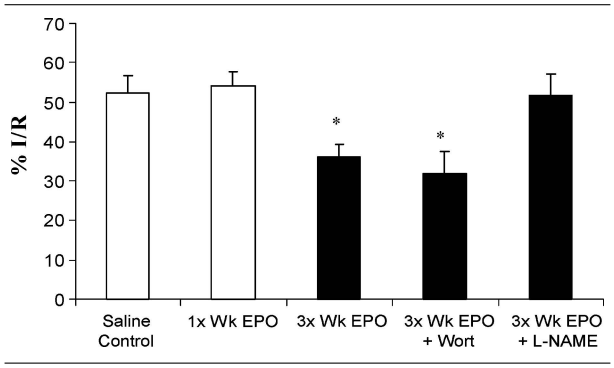

Fig. 2. *The effect upon infarct size after 35 min ischemia and 2 h reperfusion of treatment with a 3 times a week dose of saline for 3 weeks, once a week dose of EPO (5000 IU/Kg) for 3 weeks (1× Wk EPO), 3 times a week dose of EPO for 3 weeks (3× Wk EPO), 3 times a week EPO plus treatment with wortmannin (3× Wk EPO + Wort), and 3 times a week EPO treatment plus L-NAME (3× Wk EPO + L-NAME) ∗=$p < 0.05$ v saline control. ($n = 5–7$)*

The mechanism of action of chronic EPO treatment

Treatment of the 3 times a week EPO group with the PI3-Kinase inhibitor, wortmannin, prior to index ischemia did not block EPO's infarct limiting effect (I/R 31.7% ± 6.0 v 36.2% ± 3.2). However, the nitric oxide synthase inhibitor L-NAME did significantly blunt EPO's protective properties (I/R 51.6% ± 5.6 v 36.2% ± 3.2, $p < 0.05$).

The effect of acute administration of EPO

A single sub-cutaneous injection of EPO (5000 U/Kg) 24 h prior to ischemia reperfusion provided a significant reduction in infarct size compared to its saline control (I/R 39.9% ± 4.4 v 58.4% ± 5.0, $p < 0.05$) (see Fig. 3).

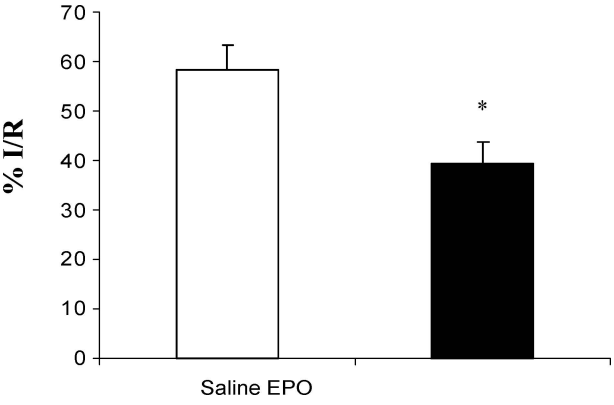

Fig. 3. *The effect upon infarct size after 35 min. ischemia and 2 h reperfusion of dose of saline and a single dose of EPO (5000 IU/Kg) 24 h prior to index ischemia. ∗ = $p < 0.05$ v saline control. ($n = 6–10$)*

Discussion

In this study we have demonstrated that a '3 times a week' treatment of EPO for 3 weeks can significantly attenuate myocardial infarct size as a result of a period of ischemia and reperfusion *in vitro*, whereas a 'once a week dose' is insufficient to elicit any protection (see Fig. 1).

We had previously reported that EPO managed to limit infarct size *in vivo* and *in vitro* when administered at the point of reperfusion after a period of ischemia [9]. This protection was attenuated by inhibitors of PI3-kinase LY294002 and wortmannin, as well as the ERK 1/2 inhibitor, U0126. This involvement of PI3-kinase in mediating the protection of EPO in a cardiac setting has been widely reported by other groups [11–13] at a variety of time points and settings. It is thus interesting that wortmannin should fail to abrogate the protection afforded by EPO in our chronic setting. One possibility to explain this finding is that the activation of PI3-kinase and Akt is a short-lived signal being regulated by phosphatases such as PP2A [14] and PHLPP [15]. The initial signal being sufficient to provide acute protection, but is damped down by phosphatases in the chronic setting resulting in a failure to abrogate chronic EPO mediated protection. To clarify a potential connection between PI3 kinase and NOS upregulation it would be important to measure NOS activity subsequent to PI3 kinase inhibition. It has been shown in the past, within the myocardium, that eNOS is downstream of PI3K-Akt in an insulin-induced signalling cascade and confers a limitation in infarct size in an *in vivo* model of ischemia/reperfusion [16]. It is, therefore, possible that PI3K conferred activation of eNOS prior to its signal being suppressed, and that the eNOS activation was sufficient to provide long-term protection.

It has been shown that extended treatment of endothelial cells with EPO caused a marked rise in nitric oxide synthase (NOS) levels [18]. Furthermore, it has also been demonstrated in rats, kept at high altitude, that they also raised hematocrit and iNOS. [19]. As NOS has been implicated in protection from ischemia/reperfusion using other agents [20,21], it is feasible to assume that NOS could be playing a mediatory role in this chronic setting. Interestingly, there has been one study examining the role of NOS, in EPO-mediated protection, that found it could not block EPO's protection using L-NAME [22]. However, it should be noted that this study examined the role of NOS in an acute pre-treatment context. Therefore, it was important to determine the role of NOS in the chronic setting. With the NOS specific blocker L-NAME totally abrogating the reduction of infarction seen, it suggests that NOS does indeed play a key mediating role in EPO's protection in the chronic setting. An 'acute' dose of EPO given 24 h prior to index ischemia caused a limitation of infarct size (see Fig. 2) compared to its saline control, despite the fact that

there was no increase in haematocrit (see Fig. 1). This data in conjunction with the use of the blood free perfusion apparatus suggests that the protection due to acute *in vivo* administration of EPO is not due to any increase in oxygen supply to the myocardium from the blood.

Recent research has demonstrated further mechanistic possibilities in EPO-mediated protection. This includes the involvement of PKC in EPO-mediated protection [17]. Similarly, Xu et al. demonstrated that administration of EPO, 24 h prior to index ischaemia, elevated the HSP70 and diminished the expression of NFκB, both phenomena have been previously associated with improved protection in ischemia reperfusion [23].

An additional possibility is that the chronic effect of EPO is mediated through its recently discovered effect on the common beta-receptor [24].

In conclusion, our findings suggest that improved outcome of chronic heart failure patients undergoing chronic treatment with EPO may well be at least partially due to a direct effect on the myocardium via a nitric oxide dependent pathway, rather than solely due to improved oxygen supply from the blood.

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