Direct cardiac actions of erythropoietin (EPO): effects on cardiac contractility, BNP secretion and ischaemia/reperfusion injury

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ABSTRACT

EPO (erythropoietin) has recently been shown to have protective actions upon the myocardium; however, the direct effects of EPO upon cardiac contractile and secretory functions are unknown and the signalling mechanisms are not well defined. In the present study, we provide the first evidence of direct cardiac contractile actions of EPO. In isolated perfused Sprague–Dawley rat hearts, a 30 min infusion of EPO significantly increased contractility in a dose-dependent fashion (maximal change 18 ± 2 % with 1 unit/ml EPO; \( P < 0.005 \) compared with vehicle). Perfusion with ET-1 (endothelin-1) increased transiently during EPO infusion, and the ETA/ETB antagonist bosentan abolished the inotropic response to EPO. BNP (B-type natriuretic peptide) secretion (28 ± 8%; \( P < 0.05 \)) and nuclear transcription factor GATA-4 DNA-binding activity (51%; \( P < 0.05 \)) were both significantly increased by EPO and blocked by bosentan. In a model of global ischaemic injury, delivery of 1 unit/ml EPO during reperfusion significantly attenuated creatine kinase release (28 ± 12%; \( P < 0.05 \)) and significantly improved contractile recovery (\( P < 0.001 \)), independent of ETA blockade. Apoptotic indices [assessed by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) /cleaved caspase-3-positive cells] were significantly decreased (\( P < 0.01 \)) by 1 unit/ml EPO during reperfusion alone, coincident with significantly increased phosphorylation of myocardial JAK2 (Janus kinase 2) and STAT3 (signal transducer and activator of transcription 3). Thus EPO directly enhances cardiac contractility and BNP secretion and alleviates ischemia/reperfusion injury via ET-1-dependent and -independent mechanisms respectively.

INTRODUCTION

EPO (erythropoietin), a glycoprotein growth factor produced by the kidneys, is an important regulator of red blood cell maturation and is widely used to treat anaemia associated with end-stage renal disease [1]. The classical function of EPO resides in its increased synthesis and secretion in response to hypoxia, promoting red blood...
Recent research suggests that EPO may have other effects augmenting oxygen delivery, such as increasing atrial contractility in post-hypoxic mice [2] and protecting the cardiac myocardium from ischaemic injury in vivo [3–5] and in vitro [6,7]. The presence of the EPO receptor in the heart has been localized to endothelial cells, vascular smooth muscle cells, cardiac fibroblasts and cardiomyocytes [1,3,6]. Thus the complete EPO system appears to be present in the heart and mediates its cardioprotective actions [8].

Clinically, EPO has been studied in treatment of chronic heart failure patients with anaemia [9–11]. In these studies, EPO treatment significantly improved cardiac function and exercise capacity. Improved oxygen delivery due to elevated haemoglobin levels was suggested to be the main factor explaining the benefits, but the potential involvement of direct cardiac effects was not reported. Previously, EPO treatment in renal patients has been associated with changes in levels of known paracrine regulators of cardiac function, such as ET-1 (endothelin-1) [12,13], prostaglandins [12], catecholamines [14] and agents of the renin–angiotensin system [15]. Taken together, all these findings suggest that EPO may have important direct cardiac effects independent of its effects on red blood cell mass.

Accordingly, using an in vitro isolated heart model, we sought to determine the direct effects of EPO on cardiac contractile and secretory function and the potential cardiac signalling pathways and factors underlying these effects, and the effect of EPO on ischaemia/reperfusion injury in isolated hearts when administered during reperfusion alone or when administered before ischaemia.

**MATERIALS AND METHODS**

**Experimental model**

Male Sprague–Dawley rats (n = 144) weighing 250–350 g were anaesthetized by sodium pentobarbitone (50 mg/kg of body weight, intraperitoneally) and killed by decapitation. The isolated Langendorff perfused rat heart set up was prepared as described previously [16,17]. LVEDP (LV (left ventricular) end-diastolic pressure), DP (developed pressure) and the maximal and minimal derivatives of the LV pressure (+dP/dt max and −dP/dt min respectively) were measured with a liquid-filled balloon in the left ventricle. Perfusion pressure was monitored with a side-arm cannula above the aortic root. A constant flow rate of 12 mL/min was maintained with a peristaltic pump (MP-2; Gilson Minipuls).

The Animal Ethics Committee of the Christchurch School of Medicine, University of Otago approved the study protocol. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

**Ischaemia/reperfusion protocol**

The preparations for ischaemia/reperfusion experiments were paced with a stimulator (Digitimer) using a bipolar electrode placed on the right atrium (15 V, 1 ms, 300 beats/min). The temperature in the moisturized chamber where the heart was positioned was monitored to remain between 35–37 ◦C throughout experiments. In this set of experiments, we compared the cardioprotective effect of 1 unit/ml EPO under two different strategies: (i) a preconditioning effect prior to 35 min of global ischemia, and (ii) a direct ‘real time’ effect given at the initiation of 60 min of reperfusion. The treatments were given for 30 min either prior to ischaemia or starting at the time of reperfusion. During reperfusion, 35 min after reinitiating the coronary flow, LVEDP was temporarily set to 5 mmHg by adjusting the intraventricular balloon volume to obtain contractile parameters with comparable end-diastolic pressure.

**The nuclear factor GATA-4 DNA binding**

For GATA-4 DNA-binding activity experiments, isolated hearts were treated with a 30 min infusion of vehicle or EPO (1 unit/ml) alone or in combination with bosentan (1 µmol/l). Extraction of nuclear proteins and gel mobility-shift assays/super-shift assays on LV free wall samples were performed as described previously [18]. Double-stranded oligonucleotide corresponding to GATA-binding region (−68/−97) of the rat BNP (B-type natriuretic peptide) promoter was used for analysis of GATA DNA-binding activity and an oligonucleotide described previously for measurement of Oct-1 (octamer-1) DNA-binding activity [18].

**Drugs**

Recombinant human EPO (0.3–3 units/ml; Research Diagnostics), bosentan (1 µmol/l; Actelion), the AT1 [AngII (angiotensin II) type 1] receptor blocker CV-11974 (10 nmol/l; kindly provided by Dr Hidefumi, Takeda Pharmaceutical Company, Osaka, Japan) and indomethacin (1 µmol/l; Biomol) were infused into the hearts through a side-arm cannula placed above the aortic root. All doses of drugs were based on results from preliminary studies and previous reports [16–19].

**Measurement of cAMP, BNP, CK (creatine kinase) and ET-1**

cAMP levels were measured as described previously [19], with a commercially available RIA kit (Amersham Biosciences), according to the manufacturer’s protocols. Timed collections of the perfusate were obtained for measurement of BNP, ET-1 and CK. SepPak extraction of 10 and 30 ml of perfusate for BNP and ET-1...
assays respectively, was performed. BNP and ET-1 RIAs were performed as described previously [16]. CK activity assay was performed by Canterbury Health Labs, Christchurch, New Zealand, according to manufacturer’s protocols (Abbott).

**TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining and immunohistochemical detection of cleaved caspase 3**

DNA fragmentation was detected from formalin-fixed sections of LV free wall by using a kit (Chemicon International), according to the manufacturer’s protocol. From each heart, a cross-section at the mid-ventricle level was used for staining, and all the TUNEL-positive cells were counted. Sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole) to determine the total number of cells.

Caspase 3 is one of the terminal effectors of the apoptotic cascade. It exists in cells as an inactive 32 kDa protein, and in apoptotic cells it is cleaved to 20/17 kDa active form. An immunohistochemical detection for detection of cleaved caspase 3 was used. Briefly, formalin-fixed sections were deparaffinized, rehydrated and incubated in 1% (v/v) H2O2 for 30 min to quench endogenous peroxidase. Following antigen retrieval with heat, the sections were incubated overnight at 4 °C with a polyclonal rabbit antibody recognizing the cleaved form of human caspase 3 (Cell Signaling Technology). Primary antibody binding was detected with peroxidase-labelled polymer conjugated with goat anti-(rabbit IgG) (DAKO), with diaminobenzidine solution (DAKO) used as the substrate. The tissues were lightly counterstained with haematoxylin. PBS replaced the primary antibody as a negative control for these experiments. The mean number of caspase-3-positive cells per seven randomly selected 40× objective fields was counted in each sample.

**Isolation of mitochondrial and cytosolic proteins**

Cardiac LV free walls were homogenized in homogenization buffer [250 mmol/l sucrose, 10 mmol/l Tris/HCl (pH 7.4), 1 mmol/l EDTA, protease inhibitors and phosphatase inhibitors]. The lysate was centrifuged for 5 min at 1000 g to pellet the unbroken cells and nuclei. The supernatant was centrifuged further for 20 min at 13 000 g to pellet the unsoluble material. The supernatant was centrifuged further for 60 min at 100 000 g to separate the cytosolic fraction (the supernatant).

**Western blotting**

Western blotting was performed as described previously [18]. Antibodies against phospho-JAK2 (Janus kinase 2), phospho-STAT3 (signal transducer and activator of transcription 3) and phospho-eIF2α (eukaryotic initiation factor 2α) were from Cell Signaling Technology. The Omi/HtrA2 antibody was from Biovision, the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was from R&D Systems, and the vinculin antibody was from Sigma. The GATA-4 antibody was from Santa Cruz Biotechnology.

**Statistical analysis**

Results are presented as means ± S.E.M. Multiple-group comparisons were made by one-way or repeated-measures ANOVA as appropriate, followed by the post-hoc test for least significant differences. For the comparison between two groups, Student’s t test was used. Significance was assumed at P < 0.05. All the Statistical analyses were performed with SPSS (version 11.5).

**RESULTS**

**EPO dose-dependently increases cardiac contractility**

Administration of EPO at concentrations of 1 and 3 units/ml resulted in significantly increased contractile strength in isolated rat hearts (Figure 1). On average, the maximal change in DP in response to 1 unit/ml EPO was 18 ± 2% (P < 0.005 compared with vehicle; Figure 1B), with concomitant significant changes in +dP/dt max and −dP/dt min (P < 0.05 compared with vehicle; Figure 1C). The absolute change in DP averaged 10 mmHg (62 ± 7 mmHg at baseline compared with 72 ± 6 mmHg with 1 unit/ml EPO). Administration of EPO at 0.3 unit/ml had no effect on cardiac contractility.

**ET-1 is a paracrine mediator of EPO-induced cardiac inotropism**

The positive inotropic effect of EPO (1 unit/ml) was blocked by the mixed ET A/ET B antagonist bosentan (1 µmol/l; Table 1, and Figures 2A and 2B). Bosentan alone did not have a significant effect on any of the parameters (results not shown). The AT1 receptor antagonist CV-11974 (10 nmol/l) and the COX (cyclooxygenase) inhibitor indomethacin (1 µmol/l) did not significantly modify the EPO-induced increase in contractility (Figure 2). To confirm further the up-regulation of ET-1 in response to EPO, changes in perfusate ET-1 concentrations during EPO infusion were analysed by
Figure 1  Time course and dose responsiveness of inotropic response to EPO
(A) Time course of positive inotropism of EPO at a dose of 1 unit/ml. (B) Maximal increase of DP in response to EPO (E) at doses of 0.3, 1 and 3 units/ml. (C) Maximal response in $+\Delta P/\Delta t_{\text{max}}$ and $-\Delta P/\Delta t_{\text{min}}$ to different doses of EPO. Baseline for $+\Delta P/\Delta t_{\text{max}}$ was 1340 ± 180, 1540 ± 400, 1370 ± 130 and 1120 ± 80 mmHg/s and for $-\Delta P/\Delta t_{\text{min}}$ was -970 ± 150, -1140 ± 310, -1000 ± 110 and -810 ± 90 mmHg/s for vehicle, 0.3 unit/ml EPO, 1 unit/ml EPO and 3 units/ml EPO respectively. *P < 0.05 and †P < 0.005 compared with vehicle; ‡P < 0.05 compared with 0.3 unit/ml EPO. For the number of experiments in each group, see Table 1.

Table 1  Haemodynamic parameters of isolated hearts
PP, perfusion pressure at baseline; RPP, rate–pressure product (DP × heart rate); $\Delta\%$, change in RPP from baseline. *P < 0.05, †P < 0.01 and ‡P < 0.001 compared with vehicle; §P < 0.05 compared with 1 unit/ml EPO.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>DP (mmHg)</th>
<th>PP (mmHg)</th>
<th>RPP (mmHg · beats/min)</th>
<th>Baseline</th>
<th>30 min</th>
<th>$\Delta%$</th>
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<tr>
<td>Vehicle</td>
<td>12</td>
<td>62 ± 7</td>
<td>53 ± 2</td>
<td>16 050 ± 1760</td>
<td>15 620 ± 1710</td>
<td>-2.5 ± 1.2</td>
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<td>EPO</td>
<td>7</td>
<td>60 ± 4</td>
<td>51 ± 5</td>
<td>15 070 ± 1200</td>
<td>14 970 ± 1050</td>
<td>-0.8 ± 1.5</td>
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<td>0.3 unit/ml</td>
<td>5</td>
<td>61 ± 6</td>
<td>50 ± 4</td>
<td>15 890 ± 1810</td>
<td>16 800 ± 1670</td>
<td>7.7 ± 3.1†</td>
<td></td>
</tr>
<tr>
<td>1 unit/ml</td>
<td>9</td>
<td>53 ± 4</td>
<td>48 ± 5</td>
<td>15 070 ± 2200</td>
<td>16 690 ± 2690</td>
<td>9.6 ± 1.9‡</td>
<td></td>
</tr>
<tr>
<td>3 units/ml</td>
<td>3</td>
<td>53 ± 4</td>
<td>48 ± 5</td>
<td>15 070 ± 2200</td>
<td>16 690 ± 2690</td>
<td>9.6 ± 1.9‡</td>
<td></td>
</tr>
<tr>
<td>EPO (1 unit/ml)+ Bosentan (1 $\mu$mol/l)</td>
<td>7</td>
<td>63 ± 4</td>
<td>59 ± 5</td>
<td>16 240 ± 1670</td>
<td>15 460 ± 1390</td>
<td>-1.2 ± 1.8§</td>
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</tr>
<tr>
<td>CV-11974 (10 nmol/l)</td>
<td>5</td>
<td>58 ± 7</td>
<td>52 ± 6</td>
<td>17 260 ± 2480</td>
<td>18 480 ± 2710</td>
<td>7.1 ± 3.1†</td>
<td></td>
</tr>
<tr>
<td>Indomethacin (1 $\mu$mol/l)</td>
<td>3</td>
<td>56 ± 3</td>
<td>66 ± 18</td>
<td>16 140 ± 1230</td>
<td>17 690 ± 1590</td>
<td>8.8 ± 2.8§</td>
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</table>

specific RIA ($n = 6$ for EPO-treated hearts and $n = 7$ for vehicle-treated hearts). Perfusate ET-1 concentrations were significantly higher in EPO-perfused hearts compared with controls (38 ± 13 compared with 6 ± 6 % respectively; $P < 0.05$; Figure 2C). Baseline levels of ET-1 in the perfusate were 69 ± 8 and 66 ± 3 fmol/l in EPO and vehicle infusions respectively [$P = \text{NS}$ (not significant)].

To test for the possible role of cAMP involvement in the inotropic effect of EPO, LV tissue cAMP concentrations were measured after 5 min treatment with
1 unit/ml EPO. No significant difference was observed between vehicle- and EPO (1 unit/ml)-treated hearts (433±92 compared with 489±97 fmol/g of wet weight respectively; n=5 for both).

Effect of EPO on other haemodynamic variables
Infusion of EPO alone or in the presence of different antagonists did not affect LVEDP, perfusion pressure or contractile rate (in beats/min) in spontaneously beating hearts (results not shown). The significant inotropic effect of EPO was confirmed in paced rat heart preparations (300 beats/min, n=12 for EPO and n=9 vehicle), where 1 unit/ml EPO induced a sustained 9.2±1.4% increase in DP compared with a transient 5.4±1.5% increase with vehicle (P<0.02). Baseline DP in these experiments was 60±7 and 54±9 mmHg in vehicle- and EPO-treated hearts respectively.

EPO enhances cardiac BNP secretion
Infusion of 1 unit/ml EPO induced a significant increase (28±8%; P<0.05) in perfusate BNP concentrations (Figure 2D) compared with control infusions (baseline BNP concentrations were 990±100 and 860±100 fmol/l in vehicle- and EPO-treated hearts respectively; P=NS).
Up-regulation of GATA-4 DNA-binding by EPO

GATA DNA binding was increased by 51 ± 14% after 30 min of EPO infusion (P < 0.05) in the left ventricles of isolated rat hearts. This was determined as the GATA-4 isoform by a supershift assay using a GATA-4-specific antibody (Figure 3). When bosentan was administered together with EPO, this GATA-4-specific DNA-binding response was abolished (Figure 3).

EPO treatment during reperfusion attenuates cardiac ischaemia/reperfusion injury

EPO preconditioning for 30 min resulted in 82 ± 2% recovery of DP at 35 min of reperfusion (P < 0.01 compared with vehicle; Figure 4A), attenuation of CK release (55 ± 15%; P < 0.01 compared with vehicle; Figure 4D) and significant improvements in reperfusion LVEDP (P < 0.01 compared with vehicle; Figures 4B and 4C). EPO delivered at reperfusion alone also resulted in significant improvements in reperfusion DP (P < 0.01; Figure 4A), reperfusion LVEDP (P < 0.01; Figures 4B and 4C) and CK release (−28 ± 12%; P < 0.05) compared with vehicle-treated hearts. There were no significant differences in cardiac improvements when the two strategies were compared with one another. To test whether improved contractile recovery associated with EPO treatment was due to the ET-1-mediated inotropic effect, ischaemia/reperfusion experiments with combined infusion of EPO and the ET-1 antagonist bosentan were performed. At the end of the reperfusion period, there was no significant difference in the recovery of DP between hearts treated with either EPO alone or EPO + bosentan for 30 min at reperfusion (Table 2 and Figure 5A), but LVEDP in the hearts treated with EPO alone during reperfusion was significantly lower than that in hearts receiving EPO + bosentan (P < 0.05; Figure 5B). The recovery of DP when LVEDP was set to 5 mmHg (35 min after reperfusion) was 73 ± 4% compared with 63 ± 5% of baseline (P = NS when EPO was compared with EPO + bosentan; Figure 5C). EPO alone and in combination with bosentan were equally effective in decreasing CK release during reperfusion (Figure 5D).

EPO attenuates apoptosis and increases JAK2/STAT3 phosphorylation during ischaemia/reperfusion

EPO (1 unit/ml) administered before or after ischaemia attenuated the number of cells that stained positive for cleaved caspase 3 by 66 ± 16 and 64 ± 7% respectively (P < 0.01; Figure 6A) and the percentage of TUNEL-positive nuclei (0.20 ± 0.04 and 0.21 ± 0.03%; P < 0.01; Figure 6B) compared with vehicle infusion. In the search of the mechanisms underlying this protective action of EPO, we identified in EPO-treated hearts elevated levels of phospho-JAK2 and phospho-STAT3 compared with vehicle-treated hearts (Figure 7A). In contrast, although the phosphorylation states of the p38, ERK (extracellular-signal-regulated kinase) and JNK (c-Jun N-terminal kinase) were increased following ischaemia/reperfusion, administration of EPO had no

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**Figure 3** EPO induces GATA-4 DNA-binding activity

(A) GATA DNA binding in response to vehicle, or EPO (1 unit/ml) alone or in combination with bosentan (1 µmol/l) (Bos). 32P-Labelled double-stranded oligonucleotide corresponding to −68/−97 of rat BNP promoter was used as a GATA-factor-binding probe. The specificity of the effect of EPO on GATA factor DNA-binding activity was confirmed by measuring Oct-1 DNA-binding activity in parallel with GATA binding, the same nuclear extracts were incubated with a 32P-labelled Oct-1 probe prior to electrophoretic mobility-shift assay. *P < 0.05 compared with vehicle; †P < 0.05 compared with EPO alone (n = 3–4 in each group). (B) Supershift using a specific antibody to GATA-4. The supershift reaction was performed by incubating reaction mixtures with 1 µg of antibodies specific for GATA-4, followed by the addition of 32P-labelled double-stranded oligonucleotide corresponding to −68/−97 of rat BNP promoter.
Figure 4  EPO administered before or after 35 min of global ischaemia attenuates cardiac dysfunction and damage

Hearts were treated with vehicle, or 1 unit/ml EPO 30 min before (EPO pretreatment) or after (EPO reperfusion) ischaemia. (A) Recovery of DP during reperfusion. (B) LVEDP during reperfusion. (C) The recovery of DP at 35 min of reperfusion when the LVEDP was temporarily set to 5 mmHg. (D) Cumulative CK release during reperfusion. *P < 0.05 and †P < 0.01 compared with vehicle. For number of experiments in each group, see Table 2.

Table 2  Haemodynamic parameters in ischaemia/reperfusion experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>DP (mmHg)</th>
<th>PP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>At the end of ischaemia</th>
<th>At 60 min of reperfusion</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>DLVEDP (mmHg)</td>
<td>DP Δ%</td>
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<td>Vehicle</td>
<td>15</td>
<td>72 ± 5</td>
<td>50 ± 3</td>
<td>3.8 ± 0.5</td>
<td>25 ± 4</td>
<td>−28 ± 3</td>
</tr>
<tr>
<td>EPO pretreatment</td>
<td>7</td>
<td>69 ± 8</td>
<td>60 ± 5</td>
<td>4.3 ± 2.1</td>
<td>24 ± 4</td>
<td>−6 ± 5†</td>
</tr>
<tr>
<td>EPO during reperfusion</td>
<td>12</td>
<td>69 ± 5</td>
<td>52 ± 5</td>
<td>3.9 ± 0.5</td>
<td>23 ± 4</td>
<td>−17 ± 3*</td>
</tr>
<tr>
<td>Bosentan during reperfusion</td>
<td>5</td>
<td>81 ± 5</td>
<td>48 ± 2</td>
<td>3.8 ± 0.3</td>
<td>30 ± 3</td>
<td>−14 ± 4*</td>
</tr>
<tr>
<td>EPO + bosentan during reperfusion</td>
<td>7</td>
<td>82 ± 3</td>
<td>52 ± 2</td>
<td>4.0 ± 1.3</td>
<td>28 ± 2</td>
<td>−15 ± 5*</td>
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</tbody>
</table>

effect on this, and the phosphorylation of Akt was not affected by either ischaemia/reperfusion or treatment with EPO (results not shown). Furthermore, no increase in cytoplasmic Omi/HtrA2, a pro-apoptotic mitochondrial serine protease involved in cell death, was seen in response to 35 min of global ischaemia and 60 min of reperfusion (Figure 7B). An increase in response to ischaemia/reperfusion, but no treatment effect with EPO, was seen in the phosphorylation of eIF2α, a specific marker of ER (endoplasmic reticulum) stress (Figure 7B).

DISCUSSION

The present study provides the first evidence of direct cardioregulatory effects of EPO. Taken together, our results show that in isolated rat hearts: (i) EPO induces
a sustained positive inotropic effect that is mediated by local ET-1, (ii) EPO increases cardiac secretion of BNP, (iii) EPO induces the up-regulation of potentially cardioprotective GATA-4 DNA binding, and (iv) EPO is able to protect the heart against ischaemia/reperfusion injury and apoptosis via the JAK/STAT pathway when administered during reperfusion alone.

EPO has been reported to be the mediator of hypoxia-induced improvement in atrial contractility in mice [2]. Our results extend this finding to show that EPO exerts a small, but sustained and significant, positive inotropic effect on mammalian ventricular myocardium in an isolated perfused heart setting. The inotropic response to EPO in the present study was observed at pharmacologically and pathophysiologically relevant doses (1 unit/ml), which are achieved by single subcutaneous doses in healthy individuals [20]. Although plasma EPO levels in normal health range from 1 to 30 milli-units/ml, levels exceeding 10 units/ml have been documented from patients with severe anaemia [21]. The inotropic effect of EPO we report in the present study has not been described previously, presumably because of different experimental settings and the relatively small magnitude of the effect compared with some other inotropic peptides [17,19]. The in vivo significance of the inotropic effect of EPO remains to be determined, but an ET-1-mediated mechanism of EPO-induced bioactivity has been documented for ANP (atrial natriuretic peptide) secretion [22] and arterial vasoconstriction [12].

Aside from alterations in cardiac load, the cardiac secretion of ANP and BNP is regulated by humoral factors, including ET-1, AngII and the catecholamines [23,24]. In combination with previous studies [22], our results suggest that EPO is another potential humoral factor regulating ANP/BNP secretion. Thus secretion of ANP in an isolated rat atria preparation was increased acutely by EPO and inhibited by ETA/ETB receptor antagonism with bosentan [22], and a single study has reported a positive correlation between plasma EPO and BNP in haemodialysis patients [25]. Furthermore, plasma EPO concentrations are elevated in patients with chronic heart failure and decrease with ACE (angiotensin-converting enzyme)-inhibitor treatment [26]. It is unknown how EPO affects chronic changes in natriuretic peptide secretion in humans, but a recent study has documented that repeated administration of EPO with oral iron can

Figure 5  Effect of bosentan on the cardioprotective effect of EPO
Hearts were treated with vehicle, EPO (1 unit/ml), bosentan or EPO + bosentan. (A) Recovery of DP during reperfusion. (B) LVEDP during reperfusion. (C) The recovery of DP at 35 min of reperfusion when LVEDP was temporarily set to 5 mmHg. (D) Cumulative CK release during reperfusion. ∗P < 0.05 and †P < 0.005 compared with vehicle; ‡P < 0.05 when EPO was compared with EPO + bosentan. For number of experiments in each group, see Table 2.
Both acute inotropism and elevated BNP release are rarely seen properties with a cardioprotective agent. The inotropism we observed with EPO is relatively weak compared with isoprenaline or other known inotropic agents [19]. Interestingly, the inotropic effect we observed in the present study was mediated by ET-1, whereas the cardioprotective effect was not, as the ET-1 receptor antagonist bosentan had no significant effect on recovery from ischaemia/reperfusion. We suspect that this may relate to the timing of sample collection and length of EPO administration, as our tissue samples were collected after 60 min of reperfusion, only half of which received EPO treatment. In contrast, previous studies have used earlier times of tissue collection, immediately after EPO administration [6,28–30]. We also suspect that the dose of EPO used in our present study may have influenced this, as previous studies have used doses much higher...
Figure 7  Effect of ischaemia/reperfusion and EPO treatment in ischaemia/reperfusion on JAK2 and STAT3 phosphorylation
Samples were taken from control hearts, hearts following ischaemia/reperfusion (I/R) and hearts following EPO treatment during ischaemia/reperfusion (I/R + EPO). Representative Westerns blots showing the effects of different treatments on JAK2/STAT3 phosphorylation (P-JAK2 and P-STAT3 respectively), cytoplasmic Omi/HtrA2 and phospho-eIF2α (P-EIF2alpha). GAPDH and vinculin were used as loading controls. * P < 0.05 I/R + EPO compared with I/R; † P < 0.05 compared with control. For the number of experiments in each group, see Table 2.

(10 units/ml; [29,30]) or much lower (0.16 unit/ml; [28]) than in the present study. In this regard, the GATA-4 transcription factor is an important regulator of cardiac gene expression and it has been implicated in the up-regulation of BNP gene expression in myocardium in response to increased load [31,32]. Although ET-1 is a known regulator of GATA-4 DNA binding, and both ET-1 and GATA-4 have been implicated as mediators of hypertrophic response, previous findings suggest that GATA-4 may also protect cardiomyocytes from drug-induced apoptosis [32,33]. ET-1 has also been shown to promote cell survival under certain conditions [34]; however, our present results indicate that ET-1, although mediating the inotropic effect of EPO, is not involved in the cardioprotection. Intriguingly, recent results using genetically engineered mice with cardiac-specific deletion of GATA-4 indicate that GATA-4 not only mediates the hypertrophic response, but is also important for cardiomyocyte survival and, therefore, for cardiac function [35]. Clearly, further studies are needed to clarify the role of GATA-4 in ischaemia/reperfusion injury and the role of GATA-4 in the cardioprotective effect of EPO.

Our present findings indicate that 1 unit/ml EPO given at the time of cardiac reperfusion results in significant increases in phospho-JAK2 and phospho-STAT3 compared with vehicle treatment. Previous reports have suggested a crucial role for the PI3K (phosphoinositide 3-kinase)/Akt pathway in EPO-induced cardioprotection [28,30]; thus our findings add to these by suggesting that JAK2/STAT3 may be an important upstream regulator of PI3K/Akt activity in this setting. Interestingly, Wang et al. [36] showed that the JAK/STAT pathway potentiates GATA-4 activity through protein–protein interactions with STATs. Previously, an increase in phospho-Akt and phospho-ERK has been reported in response to EPO [5], but no difference was observed in ischaemia/reperfusion-treated hearts in our present study. This difference compared with earlier results may relate to the experimental models used or to the fact that in our present study the phosphorylation of these factors was studied in hearts that had been exposed to 30 min of EPO treatment during reperfusion and then buffer reperfusion for a further 30 min. In contrast, previous reports [6,28–30,37] have taken samples immediately after cessation of EPO treatment, which may partially account for the lack of effect upon phospho-PI3K/Akt in our model.

In conclusion, we provide the first evidence for direct haemodynamic cardiac effects of EPO when administered at low pharmacological doses to the isolated heart. This action depends upon ET-1 signalling, as EPO-induced increases in cardiac contractility and BNP secretion were blocked by bosentan administration. EPO administered during coronary reperfusion protects the heart through a mechanism involving the inhibition of cellular damage and apoptosis and an improvement in contractile recovery. In contrast with its contractile and endocrine actions, we provide the first evidence that
EPO-mediated cardioprotection, when given in 'real time' at reperfusion after ischaemia, may involve signalling through the EPO-receptor/JAK/STAT pathway and the nuclear transcription factor GATA-4. These findings demonstrate novel important cardioregulatory actions of EPO and provide a solid platform for future investigations of its potential cardioprotective efficacy.

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