Molecular Basis of Cardioprotection by Erythropoietin

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Abstract: Erythropoietin (EPO), a glycoprotein essential for red blood cell production acts on several non-erythropoietic tissues. The EPO receptor (EPOR) is expressed in a variety of cell types including neurons, endothelial cells, and cardiomyocytes. Recently, a number of reports have indicated that EPO preserves heart function in models of cardiac ischemia-reperfusion (I/R) injury. A diverse range of cellular/physiological processes is modulated by EPO and are thought to play a role in the preservation of heart function. *In vivo*, reductions in infarct size, apoptosis, oxidative stress, and inflammation have been reported. More recently, increases in angiogenesis and reductions in arrhythmias have been implicated in the cardioprotective effects of EPO. *In vitro*, EPO reduces apoptosis, oxidative stress, and inflammation. These cardioprotective effects appear to be mediated by a receptor interaction that is distinct from that responsible for EPO's erythropoietic effects. Downstream of receptor interactions, the activation of phosphatidylinositol-3 kinase (PI3-kinase) and Akt appear to mediate many of EPO's cardioprotective effects. However, there is emerging evidence for Akt-independent mechanisms of cardioprotection including the inhibition of glycogen synthase kinase 3β, as well as the activation of potassium channels, protein kinase C, and protein kinases such as ERK1/2. This review focuses on the effects of EPO in the heart and the molecular mechanisms by which EPO achieves its cardioprotective effects.

Keywords: Erythropoietin, cardioprotection, myocardial infarction, ischemia and reperfusion, apoptosis, nitric oxide, signal transduction, angiogenesis.

INTRODUCTION

Erythropoietin (EPO) is a 30 kilo-Dalton cytokine that is produced primarily in the peritubular interstitial cells of the kidney [1-3]. Though the term erythropoietin (for erythropoiesis-stimulating hormone) was not introduced until 1948 [4], the concept of hormonal regulation of hematopoiesis was first proposed by Paul Carnot in 1906 [5]. Subsequent studies outlined EPO's ability to facilitate the differentiation and development of red blood cells [6-8], and observed that EPO achieves this through the prevention of erythropoietic progenitor cell apoptosis [9-11]. It is now well known that EPO increases red blood cell mass mainly by altering the balance between erythropoiesis and apoptosis. The role of EPO in the regulation of erythropoiesis is, in fact, critical for survival as deletion of the EPO or EPOR genes results in death around embryonic day 13 due to severe anemia [12-14]. Because of its significant effects on red blood cell production, recombinant human erythropoietin (rhEPO) has been approved by the US Food and Drug Administration for the treatment of anemia [15, 16].

In the late 1990's however, the expression of EPO as well as its receptor (EPOR) were discovered in a number of non-erythropoietic tissues including the brain [17] and the endothelium [18]. In fact, following MI the heart is capable of producing EPO [19], though EPO production in the native heart may not be significant [20]. This has led to the investigation of non-erythropoietic effects of EPO in a variety of organs including the brain, kidney, liver, and heart. It is now becoming increasingly clear that EPO plays a protective role in the body outside of its erythropoietic effects. Indeed, protective effects of EPO have been described in models of cerebral ischemia [21-23], retinal degeneration [24-26], Alzheimer's disease [27], renal injury [28-31], Parkinson's disease [32], hepatic ischemia [33] and myocardial ischemia [20, 34, 35]. Recently, an increasing number of studies have focused on the protective role of EPO in models of cardiovascular disease both *in vitro* and *in vivo*. The cardioprotective effects are achieved primarily through the inhibition of myocardial apoptosis, the reduction in inflammation, and the induction of angiogenesis. A wide array of mechanisms has been proposed to mediate the cardioprotective effects of EPO depending on the time point, and model of disease. This article summarizes the various cardioprotective effects that have been established for EPO and attempts to outline the molecular mechanisms by which EPO achieves these effects.

ERYTHROPOIETIN RECEPTOR

EPO's erythropoietic effects are achieved through a 66-78 kDa membrane-spanning protein that is a member of the cytokine superfamily of receptors [36]. Upon binding to its receptor, EPO exerts its intracellular effects through tyrosine phosphorylation of a number of proteins [36]. However the EPOR does not possess endogenous tyrosine kinase activity [37, 38]. Instead, upon EPO binding, the preformed dimeric receptor undergoes a conformational change which in turn allows for the activation of Janus kinase 2 (JAK2) which is constitutively associated with the EPOR close to the transmembrane portion of the receptor [39]. JAK2 then phosphorylates signal transducer and activator of transcription factors (STATs), as well as 8 tyrosine residues on the cytoplasmic domain of the EPOR which act as docking sites for proteins containing Src homology 2 (SH2) domains [36]. After binding, these SH2-domain containing proteins are tyrosine phosphorylated and subsequently activated. The activation of phosphatidylinositol-3 (PI3) kinase [40], extracellular signal related kinases (ERK1/2) [41] and phospholipase C [42] is commonly seen following EPOR activation in this manner. A summary of the common signaling pathways activated by EPOR signaling is shown in Fig. (**1**).

Interestingly, the beta chain of the interleukin-3 and granulocyte-macrophage colony-stimulating factor receptors (also known as CD131) can form a heterodimer with the erythropoietin receptor [43, 44]. This heterodimer has no effect on erythropoiesis since bone marrow cells from mice lacking the beta chain exhibit normal erythropoietic response to EPO [45]. However recent work by Brines and colleagues suggests a EPOR/CD131 heterodimer may be responsible for the tissue protective effects of EPO [46]. In mice lacking the beta chain, EPO has no tissue protective effects in models of spinal cord injury or staurosporine-induced cardiomyocyte apoptosis [46]. Because of this heterologous receptor composition, it is possible to separate the tissue protective effects of EPO from the erythropoietic effects pharmacologically. Indeed several derivatives of EPO have been developed that have no erythropoietic effects but are protective in models of stroke and myocardial infarction (MI) [47-49]. The precise signaling pathways regulated by the EPOR/CD131 heterodimer have not been described, although several kinases have been implicated in the cardioprotective effects of EPO.

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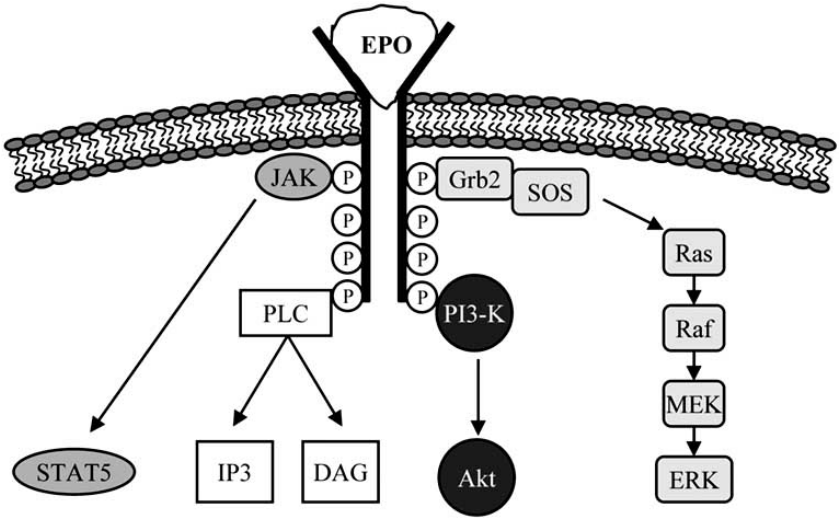

Fig. (1). Activation of intracellular signaling pathways by erythropoietin (EPO). EPO binds to its receptor (EPOR) and triggers activation of Janus Kinase 2 (JAK2). JAK2, in turn, phosphorylates tyrosine residues on the EPOR as well as signal transducer and activator of transcription factors (STATs). After EPOR phosphorylation, phosphatidylinositol-3 kinase (PI3-K), phospholipase C (PLC), and growth factor receptor-bound protein 2 (Grb2) bind to the receptor and become activated resulting in the stimulation of multiple intracellular signaling processes. PI3-K activates its downstream effector protein kinase B (Akt). PLC catalyzes the hydrolysis of phosphatidylinositol bisphosphate (PIP2) into two second-messenger proteins; inositol trisphosphate (IP3) and diacylglycerol (DAG). Finally Grb2 binds the Ras-guanine exchange factor son of sevenless (SOS) which, in turn activates Ras, which activates the classic mitogen activated protein kinase (MAPK) pathway involving Raf, MAPK/extracellular signaling related kinase (ERK) kinase, and ERK. Ultimately EPO treatment results in changes in gene transcription in response to activation of these intracellular signaling pathways.

ANGIOGENESIS

Ischemic heart disease is characterized by a reduction in blood supply to the myocardium. It has been suggested that angiogenesis, the formation of new capillaries from pre-existing vessels may be beneficial in heart disease through the restoration of perfusion of the heart [50]. Angiogenesis has been shown to improve heart function in dilated cardiomyopathy [51], pressure-overload induced hypertrophy [52, 53], and following MI [51, 54-57]. Because of this, a number of clinical studies have investigated the effectiveness of pro-angiogenic factors in ischemic heart disease [58-61]. Interestingly, endothelial cells express EPOR and are known to proliferate in response to EPO treatment [18, 62] and deletion of the EPO or EPOR is associated with angiogenic defects during development [63].

In the late 1990's several studies provided evidence that EPO plays an important role in controlling endothelial cell proliferation and angiogenesis [18, 62, 64-66]. One study by Carlini and colleagues, using rat aortic rings in a basement membrane matrix, demonstrated that EPO increases vessel growth in culture [64]. The increased vessel growth was associated with an increase in supernatant endothelin-1 levels. Furthermore, incubation with anti-endothelin-1 antibody blocked EPO-mediated angiogenic effects suggesting that EPO regulates angiogenesis through endothelin-1 release [64]. Endothelin-1 has previously been shown to increase expression of the pro-angiogenic factor vascular endothelial growth factor (VEGF) *via* protein kinase C (PKC) [67]. A second study by Carlini *et al*. demonstrated that EPO can inhibit endothelial cell apoptosis *in vitro* and suggested that this may be important in the angiogenic effects of EPO [65]. Ribatti and colleagues [66] analyzed the effects of EPO on cell proliferation in a human EA.hy926 cultured endothelial cell line, and vessel formation in the chick embryo chorioallantoic membrane. They found that EPO increases endothelial cell proliferation and vessel formation, which was coupled with JAK-2 phosphorylation and matrix metalloproteinase-2 (MMP-2) production. However, though JAK-2 [68] and MMP-2 [69] have been implicated in the promotion of angiogenesis in other models, the roles of JAK-2 activation and MMP-2 production in the angiogenic effects of EPO were not confirmed through the use of specific inhibitors. Furthermore, in contrast to the work by Carlini and co-workers, EPO's angiogenic effects were not mimicked by endothelin-1 treatment. Thus, the role of endothelin-1 as a mediator of EPO's angiogenic effects is not clear.

Until recently however, the angiogenic effects of EPO have not been investigated in the heart. Jaquet *et al.* addressed this question *in vitro* using endothelial cells derived from human myocardial tissue [70]. They found that EPO increased capillary growth, in cultured human myocardial tissue, to a level similar to that of VEGF. Interestingly, one study in mice has provided evidence that EPOR activation may regulate VEGF expression during peripheral ischemia [71]. The work of Jaquet and coleagues is supported by recent *in vivo* studies using rats subjected to myocardial infarction, which demonstrate that EPO stimulates angiogenesis and improves heart function in the ischemic heart [72, 73]. Nishiya *et al*. showed that over 4 weeks, EPO significantly improved ventricular function while increasing angiogenesis in the peri-infarct myocardium [73]. Van der Meer and colleagues demonstrated that over 9 weeks, the EPO analogue darbepoietin alfa increased capillary density and improved heart function [72]. Though these studies found clear evidence for the stimulation of angiogenesis, the molecular mechanisms by which darbepoietin achieved this were not investigated.

A number of recent studies looking at endothelial progenitor cells (EPCs) may provide insight into the mechanisms by which EPO achieves these angiogenic effects [74-78]. Hamed *et al*. investigated the effect of EPO treatment on EPCs derived from rats subjected to doxorubicin-induced cardiomyopathy [78]. EPO treatment increased the number of circulating EPCs and *in vitro* EPC vessel formation in doxorubicin-treated animals. These effects were associated with decreased mortality in these animals. Heeschen *et al*. examined the effect of EPO on EPC proliferation and mobilization in mice and in patients with coronary heart disease [76]. They found that EPO significantly increased EPC number as well as neovascularization in mice. Additionally, in patients with and without coronary heart disease, serum EPO levels correlated with EPC number [76]. This observation is supported by the work of Bahlmann and colleagues who found that EPO increased the number of circulating EPCs in healthy subjects and patients with renal anemia

[77]. Moreover, EPO activated the PI3-kinase/Akt pathway in EPCs *in vitro*. Recently, George *et al*. used cultured EPCs from healthy patients and demonstrated that EPO administration promoted EPC proliferation through the activation of PI3-kinase, an effect which could be blocked with PI3-kinase inhibitors [74]. The Akt dependence of EPO's angiogenic effects is supported by the work of Zhang and colleagues who examined the effectiveness of bone marrow stromal cell transplantation in promoting angiogenesis in a rat model of MI [79, 80]. They found that EPO enhances the angiogenic potency of transplanted stromal cells and that this enhancement was dependent upon a PI3-kinase and Akt pathway [79].

EPO not only increases EPC mobilization from the bone marrow, but also promotes EPC incorporation into the newly generated blood vessels. In this regard, Urao *et al*. demonstrated that EPO increases incorporation of EPC into the endothelium, in a model of acute-aortic-wire injury [81]. Similarly, Westenbrink and colleagues showed that EPO promotes neovascularization in a chronic model of heart failure post myocardial infarction in mice [75]. EPO-induced neovascularization is associated with increased mobilization, myocardial homing, and vascular incorporation of EPC, which comprise 30% of the newly formed vessels, resulting in improvement in cardiac repair. Thus, EPO promotes neovascularization in the ischemic myocardium by a number of mechanisms including EPC proliferation, mobilization, homing and incorporation into the endothelium.

Of note, anemia does not appear to impair angiogenesis. In fact, there is evidence to suggest an increase in angiogenesis in the heart during anemia [82, 83]. Rakusan *et al.* examined capillary density in anemic rats and reported increases in the average number of capillaries/cardiomyocyte [82]. Furthermore, a low oxygen environment may serve as a stimulus for angiogenesis (reviewed in [84]). Thus, the pro-angiogenic effects of EPO are likely independent of any erythropoietic effects. In fact, given that circulating EPO levels are higher in anemic patients than non-anemic patients [85], it may be possible that the increases in angiogenesis during anemia are actually mediated through EPO.

Thus, there is considerable evidence to suggest that EPO can promote angiogenesis, independent of its erythropoietic effects. The exact molecular mechanisms are unclear, and may or may not involve the stimulation of endothelin-1 release, JAK-2 activation or MMP-2. However, multiple laboratories have implicated PI3-kinase and Akt signaling in the promotion of angiogenesis following EPO treatment. The hypothesized mechanisms by which EPO promotes angiogenesis are summarized in Fig. (**2**).

INFLAMMATION

Cardiac inflammation is associated with poor prognosis during heart failure [86, 87]. Several inflammatory processes contribute to cardiac depression and dysfunction including the expression of inflammatory cytokines, transendothelial migration of polymorphonuclear leukocytes (PMN), and the expression of cyclooxygenase-2 (COX-2). Expression of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) directly influence contractility, fibrosis and cell death [87-91]. PMN, through the release of myeloperoxidase (MPO) can cause ventricular dilation and impaired cardiac function [92]. Additionally, COX-2 is up-regulated in models of heart failure and contributes to impaired ventricular function [93]. Thus, inflammation is a significant contributing factor to cardiac dysfunction during heart failure. Recently, several studies have identified a role for EPO in the reduction of inflammation in models of I/R or MI [94-98].

Our group reported one of the earliest descriptions of the anti-inflammatory effects of EPO in the heart using a murine model of I/R injury and observed EPO-mediated reductions in oxidative stress and PMN transendothelial migration [95]. Using pharmacological inhibitors, as well as genetically-altered mice we were able to demonstrate that these effects are dependent on the phosphorylation of PI3-kinase, the activation of the nuclear transcription factor AP-1, and the up-regulation of endothelial nitric oxide synthase (eNOS). Our study demonstrated that EPO administration protects the heart from an acute inflammatory response during myocardial I/R [95].

Two independent studies have subsequently found similar effects of EPO. Li *et al.* utilized a chronic murine model of heart failure post-MI and demonstrated that EPO treatment inhibited myocardial expression of several inflammatory cytokines including IL-6, TNF-α, and IL-1β [94]. *In vitro* experiments demonstrated

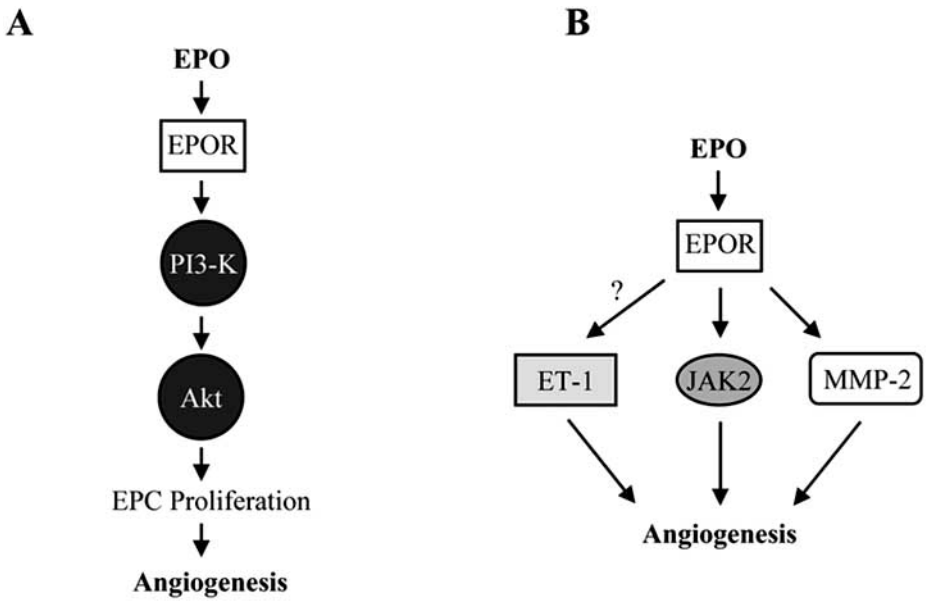

Fig. (2). Regulation of myocardial angiogenesis by EPO. As yet, it is unclear as to whether EPO mediates these effects through the homodimeric EPOR or the heterodimeric EPOR/CD131 receptor. A: EPO activates PI3-K and Akt which increase endothelial cell progenitor proliferation and thereby angiogenesis. B: EPO may also increase angiogenesis *via* up-regulation of endothelin-1 (ET-1), although this result has been questioned [66]. Additionally, JAK-2 and MMP-2 activation are associated with EPO-mediated increases in angiogenesis and may be involved in mediating these effects.

that the major source of cytokine production was from the cardiac fibroblasts themselves. Moreover, the use of specific pharmacological inhibitors suggested that these EPO-mediated reductions in cytokine expression were mediated by STAT signaling. In addition, inhibition of oxidative DNA damage by EPO treatment in cardiomyocytes was PI3-kinase dependent [94]. A study by Liu *et al.* used a rodent model of I/R and found that EPO reduces PMN infiltration, and the expression of the pro-inflammatory cytokines TNF-α and IL-6, while increasing expression of the anti-inflammatory cytokine IL-10 [97]. The involvement of PI3-kinase or STAT-mediated signaling in these effects was not investigated. However, in contrast to our work, the authors actually observed reductions in the activation of transcription factors AP-1 and NF-kappa B following EPO treatment. It is possible that the earlier time point used in our studies (4 hours vs. 24-27 hours) to measure AP-1 binding is responsible for this discrepancy.

COX-2 is an important inflammatory mediator. Recent studies have investigated the effects of EPO on COX-2 expression in cardiomyocytes. In a doxorubicin-induced cardiomyopathy mouse model, Li *et al.* showed that EPO protected hearts from doxorubicin-induced ventricular dilatation and dysfunction [99]. These protective effects were associated with inhibition of myocardial expression of COX-2 during development of cardiomyopathy over 2 weeks [99]. In contrast to the above studies, the authors found that EPO did not significantly alter expression of the inflammatory cytokines TNF-α or transforming growth factor beta (TGF-β). Doxorubicin did not cause a significant induction of these cytokines during the time points examined, and thus these cytokines were not likely contributing to the cardiac dysfunction in their model of cardiomyopathy [99]. Nevertheless EPO was able to reduce inflammatory cell infiltration and COX-2 expression. The mechanism by which EPO achieved these effects was not directly investigated, although EPO was shown to increase ERK1/2, but not Akt or Stat5 activation in this model. In contrast to these studies, work by Liu *et al.* in rodents subjected to I/R found that EPO administration actually increased COX-2 expression as well as levels of its products, prostaglandins E_2 and $F_{2\alpha}$ [96]. The increases in COX-2 expression and its end products were associated with reductions in infarct size, though the authors did not investigate the mechanism by which this was achieved. In contrast to the studies by Li *et al.*, which measured EPO's effects over a two-week period, Liu and colleagues observed the increases in COX2 expression within 24 hours of EPO administration. Thus the effects of EPO on COX-2 expression may vary depending on the timing of EPO administration and possibly on the experimental models used.

While there is a need for further investigation into the anti-inflammatory effects of EPO, particularly with regard to its role in regulating COX-2 expression, there is already considerable evidence to suggest an anti-inflammatory role for EPO. Multiple, independent studies have described EPO-mediated reductions in inflammation using models of I/R, MI and cardiomyopathy. These effects include reductions in expression of inflammatory cytokines and oxidative damage. A summary of the signal transduction mechanisms by which EPO inhibits inflammation is outlined in Fig. (**3**).

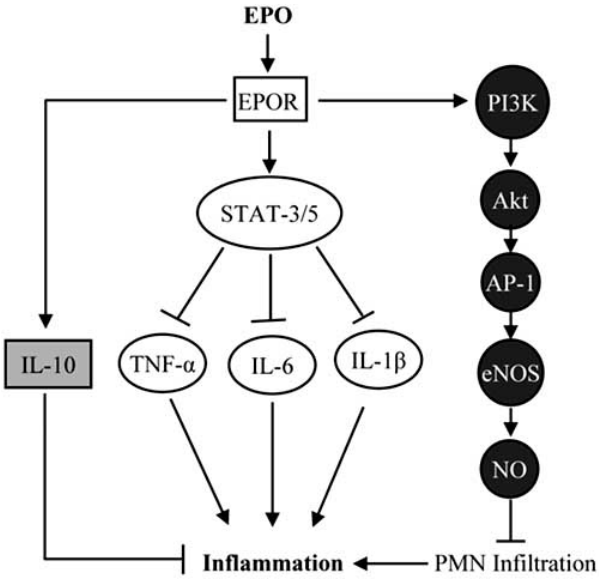

Fig. (3). Inhibition of myocardial inflammation by EPO. EPO activates PI3-K and Akt which increase endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) production *via* activator-protein 1 (AP-1). As yet, it is unclear as to whether EPO mediates these effects through the homodimeric EPOR or the heterodimeric EPOR/CD131 receptor. Downstream of Akt, eNOS-derived NO production inhibits polymorphonuclear leukocyte infiltration and reduces inflammation. EPO also increases expression of the anti-inflammatory cytokine interleukin 10 (IL-10) and also activates STAT 3 and STAT 5, which reduces pro-inflammatory cytokine expression.

CARDIAC HYPERTROPHY

Although the hypertrophic growth of the heart is a necessary component of heart development, the pathological hypertrophy of cardiomyocytes accompanies many forms of heart disease, including ischemic heart disease, hypertension, heart failure, and valvular disease [100]. Cardiomyocyte hypertrophy is considered an independent risk factor for cardiovascular mortality [101-104] and the reversal of hypertrophy is associated with a decreased risk of mortality and improved heart function [105-107]. Indeed, reductions in left ventricular hypertrophy are thought to be an important component of the beneficial effects of angiotensin converting enzyme (ACE) inhibitors [108-111]. However, despite the well-established cardioprotective effects of EPO in myocardial I/R and post-MI, the effect of EPO on hypertrophy remains a subject of debate in the literature.

Briest and colleagues analyzed cardiomyocyte size in transgenic mice that overexpress EPO [112, 113]. They demonstrated that at 7 months of age, ventricular weight was significantly higher in transgenic mice, and that this was accompanied by increased expression of the hypertrophic markers atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). A second study from the same laboratory found that EPO overexpressing mice exhibit significantly more hypertrophic growth in response to norepinephrine than their WT counterparts [113]. The plasma EPO levels in these mice were approximately ten-fold higher than their wild-type counterparts and these transgenic mice were also more likely to die of diastolic dysfunction than their WT counterparts [113, 114]. However, these mice also exhibited significant erythrocytosis with hematocrit levels of approximately 0.80. Increases of the hematocrit to these levels induce ventricular hypertrophy through increased blood viscosity and vascular resistance [115-117]. Thus, it is possible that the hypertrophic effects observed in the EPO overexpressing mice are due to the erythropoietic effects of EPO. Whether EPO exerts a direct hypertrophic effect on the heart in these studies is not clear.

In contrast to these studies, several laboratories have reported EPO-mediated reductions in ventricular hypertrophy. Li *et al.* examined the effect of EPO on ventricular hypertrophy in a murine model of MI [94]. They found that EPO administration following MI reduced heart weight in mice and this effect was accompanied by activation of STAT-3, STAT-5 and Akt in the myocardium. However, EPO treatment did not directly alter cardiomyocyte size in this model. Van der Meer *et al.* examined the effect of EPO on cardiac hypertrophy in a rat model of MI [72]. They found that EPO

administration significantly reduced plasma ANP levels and myocardial beta-myosin heavy chain expression. However, EPO did not significantly reduce ventricular weight and myocyte cross sectional size [72]. It should be noted that in both of these studies there was an elevation in hematocrit associated with EPO treatment. It is conceivable that supraphysiological hematocrit levels could have a direct impact on hypertrophy. A study by Asaumi and colleagues examined the effects of EPO, independent of erythropoietic effects, on hypertrophy of cardiomyocytes in a murine model of transverse aortic constriction (TAC) [118]. In this study, mice deficient in EPOR in non-erythropoietic tissues exhibited elevated ventricular weight and elevated expression of the hypertrophic marker ANP with increased mortality compared to their WT littermates. Importantly, these mice had normal hematocrit levels compared with their WT counterparts. The increases in hypertrophic markers were associated with an impairment of STAT 3 and p38 phosphorylation, and VEGF protein expression. However, once again, the cross sectional area of cardiomyocytes in these EPOR deficient mice was unchanged following TAC [118]. This raises the question as to whether or not endogenous EPO had a significant effect on hypertrophic growth of cardiomyocytes in any of these models.

On the other hand, the ability of EPO to reduce hypertrophy during anemia is relatively clear. For several decades it has been known that chronic anemia can result in cardiac hypertrophy [119-121]. In fact, it has been suggested that a decrease in hemoglobin of even 1g/dl, increases the risk of left ventricular hypertrophy [122]. Multiple studies in humans and mice have demonstrated that EPO administration during anemia reduces ventricular hypertrophy and improves heart function [123-129]. Importantly, cessation of EPO therapy is associated with a recurrence of ventricular hypertrophy [130]. Furthermore, correction of anemia by EPO and iron supplementation in patients with heart failure improves heart function and leads to a reduction in diuretic use and hospitalizations [131, 132]. However, ventricular hypertrophy was not studied in these heart failure patients. Thus, it's not clear that improvement in cardiac function is due to a decrease in cardiac hypertrophy.

In summary, it is clear that EPO is able to reduce ventricular hypertrophy in anemic patients through the correction of anemia. However, it is not clear whether or not EPO is able to reduce hypertrophy in heart failure patients and in animal models of heart failure independent of its erythropoietic effects. EPO does appear able to reduce hypertrophic markers and heart weight following MI but does not appear to reduce cardiomyocyte size at this time. Further studies are warranted in order to evaluate the effect of non-erythropoietic EPO on cardiac hypertrophy in heart failure patients with anemia, and in relevant animal models of these diseases.

ARRHYTHMIA

Arrhythmia is an irregularity in the electrical activity and rhythmic beating of the heart and a major cause of sudden cardiac death. It is estimated that following MI, approximately 70% of all patients will experience an arrhythmia [133, 134]. Furthermore the prevention of arrhythmias has been reported to reduce mortality and sudden death (reviewed in [135]). There is emerging evidence that EPO may prevent arrhythmia in the ischemic heart [136].

A study by Hirata *et al.* in an *in vivo* canine model of myocardial I/R provided the first, and as yet only, evidence of an anti-arrhythmic effect of EPO [136]. In this study, EPO was administered just prior to reperfusion and significantly reduced the incidence of ventricular fibrillation. The authors demonstrated that EPO mediates the reduction in fibrillation through a PI3-kinase dependent signaling pathway, since selective inhibition of this kinase blocked the antiarrhythmic effect. The role of Akt and other downstream mediators of PI3-kinase were not investigated. However, it is also important to consider that in this study the reduction in ventricular fibrillation was accompanied by a reduction in infarct size in the heart. Thus, it is possible that the anti-arrhythmic effects of EPO may simply have been due to infarct size reduction.

While the study by Hirata *et al.* is the only direct evidence of an anti-arrhythmic effect of EPO, there is evidence that EPO may stimulate signal transduction pathways that are important in the regulation of cardiac rhythm. Protein kinase C epsilon (PKC-ε) is important in I/R-induced arrhythmia since stimulation of PKC-ε reduces I/R-induced arrhythmia, and inhibition of PKC-ε exacerbates it [137]. Since EPO activates PKC-ε [138, 139], it is possible that EPO may reduce cardiac arrhythmia through activation of PKC. As yet however, this mechanism has not been investigated in any animal models and remains speculative.

There is emerging evidence to suggest that EPO may be able to inhibit ventricular arrhythmia during ischemia. However, further study is needed to confirm the role of PI3-kinase and other downstream mediators in the anti-arrhythmic effects of EPO. Moreover it is important to determine if EPO has any anti-arrhythmic effects independent of its other cardioprotective effects.

APOPTOSIS

The role of EPO in the prevention of cardiomyocyte apoptosis is a relatively well-studied cardioprotective effect of EPO. Apoptosis refers to programmed cell-death and differs from necrosis in that it is a regulated process initiated by the cell itself while necrosis is a direct result of severe tissue injury. Apoptotic cell death is of greater interest to the medical community because it may be more amenable to pharmacological intervention than necrotic cell death [140]. Inhibition of apoptotic cell death during heart failure may improve the clinical outcome. For example, excessive stimulation of the β_1 adrenergic receptor (β_1AR), such as that seen during heart failure, is known to induce myocardial apoptosis [141]. Beneficial effects of β receptor blockade in patients with heart failure may be in part due to an inhibition of β_1AR-induced apoptosis [142]. Additionally, a recent study by Chandrashekhar *et al.* demonstrated that inhibition of apoptosis improves cardiac function of rats after myocardial infarction [143]. Several studies have demonstrated a role for EPO in reducing cardiomyocyte apoptosis in models of I/R [34], cardiomyopathy [99], and MI [144].

Calvillo and colleagues were amongst the first to report anti-apoptotic effects for EPO in the heart [34]. They used *in vitro* and *in vivo* models of I/R in rats and found that EPO administration significantly reduced cardiomyocyte apoptosis following *in vitro* hypoxia and *in vivo* myocardial I/R. This reduction in myocyte death was accompanied by an improvement in heart function. This study was supported by the work of Cai and colleagues [20] who examined the effect of EPO on isolated mouse hearts subjected to global ischemia. In this model, EPO treatment prior to ischemia reduced apoptosis and improved heart function. Though the mechanisms by which EPO achieves these effects were not investigated in these two studies, both authors suggested a role for Akt in mediating this reduction in apoptosis. Akt is known to protect cardiomyocytes from apoptosis through the inhibition of pro-apoptotic proteins such as Bad, caspase-9, and pro-apoptotic transcription factors such as forkhead receptor ligand 1 (FKRL1) [145-147]. The suggestion that Akt is involved in the anti-apoptotic effects of EPO was quickly supported by a study by Tramontano *et al.* in rats [148]. As before, EPO significantly reduced cardiomyocyte apoptosis following hypoxia *in vitro* and cardiac ischemia *in vivo*. Using an Akt kinase activity assay, the authors demonstrated that EPO administration increased Akt activity. Furthermore, pharmacological inhibition of PI3-kinase blocked increased Akt activity as well as its anti-apoptotic effect [148]. The importance of Akt in the anti-apoptotic effects of EPO has since been supported and corroborated by data from several laboratories [35, 136, 144, 149, 150]. However, in many of these studies the role of Akt was confirmed using a pharmacological inhibitor of PI3-kinase, rather than direct inhibi-

tion of Akt. Our lab provided the first direct evidence of Akt involvement when we demonstrated a loss of EPO's anti-apoptotic effects following treatment with an Akt dominant-negative adenovirus [149]. A recent study by Fu and Arcasoy used a pharmacological inhibitor of Akt in neonatal rat cardiomyocytes and also found that direct inhibition of Akt blocks the anti-apoptotic effects of EPO [151].

Despite the large number of studies implicating Akt in the anti-apoptotic effects of EPO, very little research has been done into the downstream mediators. We recently demonstrated a role for eNOS-derived NO production in the anti-apoptotic effects of EPO [149]. eNOS-derived NO production reduces apoptosis by reducing oxidative stress [152], increasing expression of the anti-apoptotic protein Bcl-2 [153], and inhibiting the activities of the pro-apoptotic proteins caspase-3 and -8 through S-nitrosylation [154, 155]. Using genetically altered mice we demonstrated that EPO-mediated reductions in apoptosis require activation of eNOS during myocardial I/R [149]. Furthermore, we demonstrated *in vitro*, using pharmacological inhibitors of PI3-kinase and a dominant negative adenovirus to Akt, that EPO increases eNOS-derived NO production through a PI3-kinase and Akt-dependent pathway. Thus, we have provided evidence of EPO-mediated NO production from eNOS in cardiomyocytes, while others have also reported EPO-mediated increases of eNOS expression in endothelial cells [114, 156] and EPO-mediated increases in eNOS phosphorylation in the heart [150]. Additionally, there is evidence that glycogen synthase kinase (GSK)-3β inhibition *via* phosphorylation is involved in the anti-apoptotic effects of EPO downstream of Akt [157, 158]. Inhibition of GSK-3β is known to reduce apoptosis in cardiomyocytes by reducing mitochondrial permeability [157], increasing the expression of the anti-apoptotic protein Bcl-2 and inhibiting the expression of the pro-apoptotic protein Bax [159]. Fu and Arcasoy demonstrated a role for GSK-3β in the anti-apoptotic effects of EPO in cultured rat cardiomyocytes [151]. In this study, EPO inhibited doxorubicin-induced apoptosis and this reduction was coupled with activation of Akt and phosphorylation of GSK-3β to its inactive form [151]. Moreover, inhibition of PI3-kinase or Akt blocked EPO-mediated phosphorylation of GSK-3β. Pharmacological inhibition of GSK-3β blocked doxorubicin-induced apoptosis in a manner similar to EPO. A recent study by Nishihara *et al.* also found that EPO administration increased phosphorylation of GSK-3β [160]. These observations were further supported in a study by Kim *et al.* in mouse cardiomyocytes which showed that EPO-mediated increases in GSK-3β inactivation were PI3-kinase-dependent [158]. Interestingly, a study in breast cancer cell lines found that GSK-3β inhibition can increase eNOS expression [161]. Thus it is possible that GSK-3β inactivation may mediate the increases in eNOS observed in our study. As yet, it is unclear as to whether the Akt-dependent effects on eNOS and GSK-3β represent distinct effects or if they comprise components of the same signaling pathway.

Thus, there is compelling evidence to suggest that EPO protects cardiomyocytes from apoptosis through a PI3-kinase, Akt-dependent mechanism. Several independent laboratories have implicated this pathway in multiple animal models. Additionally, there is emerging evidence that eNOS and GSK-3β may be important downstream mediators of Akt signaling following EPO administration. A summary of proposed intracellular signaling events leading to the reduction in apoptosis is seen in Fig. (**4**).

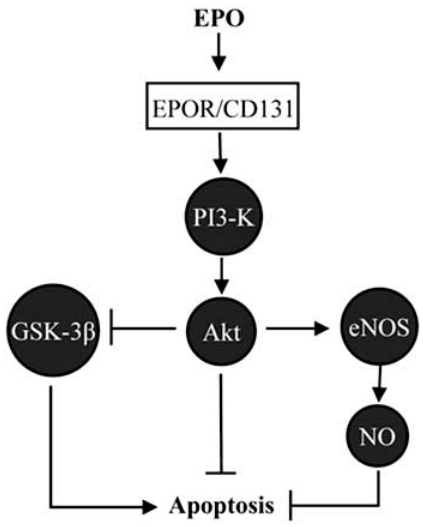

Fig. (4). EPO reduces cardiomyocyte apoptosis. EPO activates a heterodimeric receptor consisting of EPOR and the common beta subunit of the interleukin-3 and granulocyte-macrophage colony-stimulating factor receptors (CD131), then PI3-K and Akt. Akt reduces cardiomyocyte apoptosis directly, and by increasing eNOS-derived NO production as well as the inhibition of glycogen synthase kinase 3 β (GSK-3β).

MYOCARDIAL INFARCTION

Perhaps the best studied of EPO's cardioprotective effects is its infarct-size limiting effects. During myocardial infarction, tissue necrosis is caused by a loss of blood supply to the myocardium. The resultant infarct region is eventually replaced by fibroblasts and collagen as a result of inflammation and the post-MI repair process. The development of an infarct is a complex process involving apoptotic and necrotic cell death [162], neutrophil infiltration and inflammation [163, 164], changes in the extracellular matrix [165] and numerous other factors. An infarct can lead to sudden death through cardiac rupture [166], arrhythmias [167], or cardiac remodeling and heart failure [168]. Moreover, the reduction of infarct size is associated with a reduction of mortality in the ischemic heart [169]. Several laboratories have reported that EPO reduces infarct size in mice [149], rats [73, 150, 160], dogs [136], and rabbits [144].

In 2003, two independent laboratories examined the effects of EPO on infarct size following coronary artery ligation [34, 144]. Calvillo *et al.* used a model of I/R in rats and showed that while EPO, administered prior to ischemia, significantly improved heart function and reduced myocyte cell death, there was no effect on infarct size [34]. In contrast to this, Parsa and colleagues found that EPO administered prior to coronary artery ligation significantly reduced infarct size and improved heart function in rabbits following MI [144]. The reason that Calvillo *et al*. did not observe an effect on infarct size may be due to the relatively small infarct generated in this study. This group reported approximately 15-20% of the LV infarcted following myocardial ischemia, whereas other investigators have generated infarcts of anywhere from 30%-60% of the LV [139, 144, 149]. Indeed subsequent studies by the same laboratory using a more severe model of MI have found reductions in infarct size following treatment with carbamylated EPO (a derivative of EPO that has had all lysine residues transformed to homocitrulline and consequently does not bind to the EPOR homodimer) [170]. While one additional study has called into question the ability of EPO to reduce infarct size following MI [171], the infarct size-limiting effect of EPO has since been supported by a large number of independent investigations [48, 72, 98, 136, 149, 150, 172]. Interestingly, Parsa *et al.* showed *in vivo* activation of Akt kinase and ERK 1/2 following EPO treatment, though the involvement of these two kinases in the infarct-size limiting effects of EPO was not examined [144].

Hanlon and colleagues examined the effects of EPO on infarct size in isolated, Langendorff-perfused rat hearts subjected to global ischemia and provided insight into the signaling pathways required for EPO-mediated infarct size reduction [139]. They demonstrated

that EPO administration in the perfusate prior to ischemia significantly reduced infarct size. Furthermore, using pharmacological inhibitors, they identified critical, time-dependent roles for PI3-kinase/Akt signaling and PKC epsilon (PKC-ε). Administration of the PI3-kinase inhibitor LY294002 post-ischemia significantly reduced EPO-mediated increases in Akt activation as well as EPO-mediated decreases in infarct size. Similarly, administration of the PKC inhibitor chelerythrine pre-ischemia significantly inhibited EPO-mediated translocation of PKC-ε to the membrane and EPO-mediated decreases in infarct size. Interestingly, although EPO-mediated increases in ERK1/2 activation were seen, inhibition of ERK1/2 signaling did not significantly alter the infarct size-limiting effects of EPO. These observations are supported by the work of Nishihara *et al*. in rats subjected to myocardial I/R [160]. In this study, EPO-mediated reductions in infarct size were blocked by pharmacological inhibition of PI3-kinase or PKC administered prior to ischemia. The role of ERK1/2 and the time-dependence of PKC and PI3-kinase activation were not investigated. Taken together, these data suggest that EPO reduces infarction through a PKC-dependent pathway during ischemia, and an Akt-dependent pathway post-ischemia.

Recently, studies by Miki *et al.* suggested that ERK1/2 signaling may indeed be important in the infarct size-limiting effects of EPO following MI [172, 173]. Using a rat model of MI and pharmacological inhibitors, they examined the involvement of Akt and ERK1/2 signaling in the infarct-size limiting effects of EPO. They showed that 4 weeks post-MI, EPO was no longer able to increase Akt activation, but maintained the ability to increase ERK1/2 activation, and its infarct size limiting effect [173]. Furthermore, under these conditions, pharmacological inhibition of ERK1/2 blocked the infarct size-limiting effects of EPO. Thus, the mechanisms by which EPO reduces infarct size may differ depending on experimental models used.

As was the case in EPO's anti-apoptotic effects, activation of eNOS and inhibition of GSK-3β have been implicated downstream of Akt. Nishihara *et al*. in their study using rats subjected to myocardial I/R found that EPO inhibited GSK-3β through PI3-kinase dependent and independent signaling [160]. Furthermore, this inhibition of GSK-3β correlated well with EPO-mediated reductions in infarct size. Additionally, we demonstrated that EPO-mediated reductions in infarct size following myocardial I/R are significantly diminished in eNOS-deficient mice [149]. As is the case with the roles of GSK-3β and eNOS in the anti-apoptotic effects of EPO, it is unclear whether these two effects are linked. Thus, further study is needed to determine the precise involvement of these two molecules in the infarct size-limiting effects of EPO.

Finally, Xu *et al.* also examined the effects of EPO on infarct size in rats subjected to myocardial I/R [174]. The authors showed that EPO significantly reduced the size of infarction when administered 24 hours prior to coronary artery ligation. Additionally, they observed that this reduction in infarct size was associated with an increase in heat shock protein (HSP) 70 expression and decreased NF-kappa B expression. However, the authors did not use any pharmacological or genetic inhibition to demonstrate a direct role for HSP 70 or NF-kappa B in the reduction of infarction and they did not determine if the effects of EPO on HSP 70 or NF-kappa B are dependent upon ERK1/2 or PI3-kinase signaling. To date, this is the only study to report that EPO increases HSP70 expression in cardiomyocytes, though EPO-mediated increases in HSP70 have been reported in the kidney [175]. Thus, the specific role of HSP70 in mediating the cardioprotective effects of EPO remains to be determined.

Although two studies have found no effect of EPO on infarct size [34, 171], several independent laboratories have reported that EPO reduces the size of infarction in the myocardium during I/R or MI in multiple animal models [72, 97, 98, 136, 149, 150]. There is sufficient evidence to suggest that this effect is mediated by a PI3-kinase, Akt-dependent pathway; however, further investigation is needed to clarify the involvement of ERK1/2 signaling in the chronically damaged heart and the role of PKCε during the

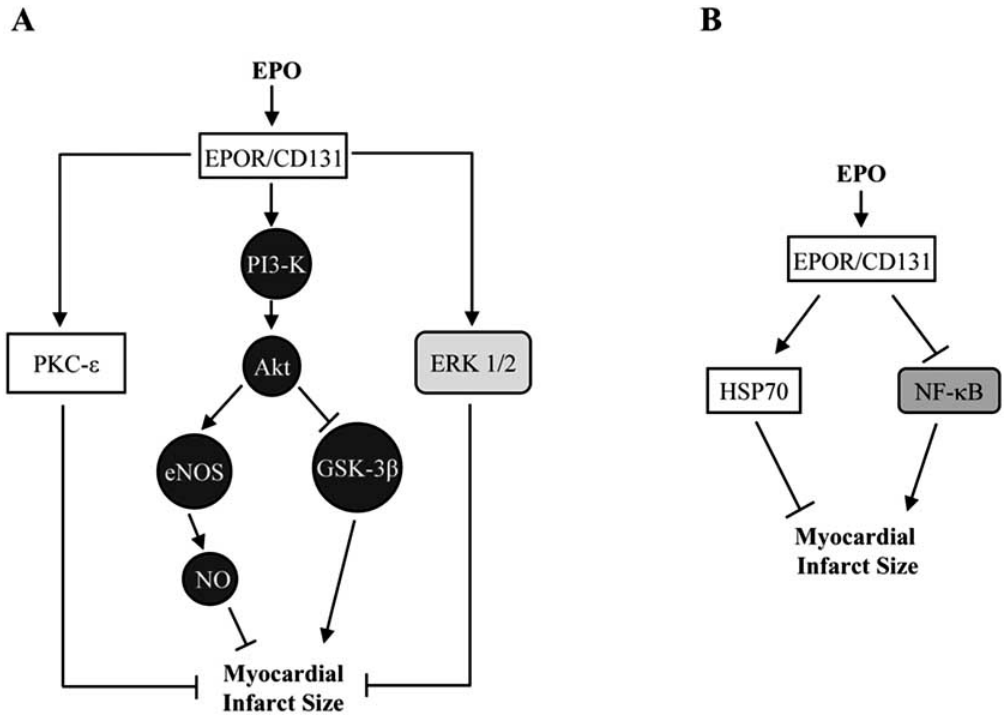

Fig. (5). Mechanisms of EPO-mediated reductions in myocardial infarct size. **A:** EPO activates a EPOR/CD131 heterodimeric receptor, then PI3-K and Akt. Akt increases eNOS-derived NO production and inhibits GSK-3β activation thereby reducing infarct size. Additionally, EPO activates ERK and protein kinase C epsilon (PKC-ε), which, in turn mediate a reduction in infarct size. **B:** EPO-mediated reductions in infarct size are associated with activation of heat shock protein 70 (HSP70) and inhibition of nuclear factor kappa B (NF-κB).

ischemic period. Moreover, while there is evidence to suggest the involvement of GSK-3β and eNOS downstream of Akt signaling [95, 149, 151, 158, 160], further investigation into the role of other downstream mediators, such as HSP70, is warranted. Fig. (**5**) illustrates the signaling pathways involved in the reduction of infarct size following EPO treatment.

OTHER STUDIES

Two studies by Shi and Rafiee in rabbits analyzed the role of EPO administration in I/R injury using isolated hearts [138, 176]. They showed that EPO treatment 15 minutes prior to global ischemia improved cardiac function as measured by left ventricular developed pressure. Using pharmacological inhibitors they demonstrated that the improvement in heart function was dependent upon p38 MAPK, JAK/STAT, PI3-kinase, ERK1/2, PKC, and ATP-dependent potassium channel activities [138, 176]. While the authors demonstrated clear involvement of these intracellular signaling processes in the improvement in heart function following EPO treatment, they did not delve into the mediating mechanisms by which EPO achieves this improvement in heart function. It is not clear, for example, whether there are alterations in apoptosis or inflammation as a result of the alterations in intracellular signaling.

The extracellular matrix plays an important role in the myocardium in response to pathological stress [177]. In fact, alterations in the composition of the extracellular matrix (ECM) occur in several cardiac diseases and may lead to diastolic dysfunction [178, 179]. There is now emerging evidence that EPO prevents ECM disruption in I/R injury [180]. Using isolated rat hearts Chan *et al.* demonstrated that EPO treatment prevents I/R-induced alterations in matrix metalloproteinases (MMP) 2 and 9, and tissue inhibitor of metalloproteinases (TIMP) 4 [180]. Furthermore attenuation of these alterations correlated with decreased collagen degradation and improved heart function. It remains unclear to what degree the observed improvement in heart function is dependent upon the maintenance of ECM composition and the contributions of the aforementioned mechanisms by which EPO confers cardioprotection.

EPO DOSING

It should be noted that the doses of recombinant human EPO administered in the majority of the animal studies cited are generally in the range of 1000-5000 U/kg, which is over 10 times higher than those currently used clinically in anemic patients with chronic renal failure [181]. A summary of the animal studies investigating the cardioprotective effects of EPO, and the doses used in these studies can be found in Table (**1**). Doses in this range are capable of increasing the hematocrit of rodents by 20-30% [34]. Given the high doses used, it is not possible to rule out the possibility that the cardioprotective effects of EPO are not, under some circumstances, achieved through an increase in oxygen delivery by red blood cells to the myocardium rather than through direct EPOR/CD131-receptor-mediated signaling. Furthermore, rapid or supranormal increases of the hematocrit in the clinical setting are associated with adverse effects such as hypertension and thromboembolism [182], which are important factors to consider in the chronic use of EPO in heart disease.

Of particular concern are the results of a recent meta-analysis which examined EPO treatment in anemic cancer patients [183]. The analysis found that treatment with EPO, or darbepoeitin increased the risk of thrombo-embolic events in these patients. Moreover, EPO may actually promote tumor growth through the promotion of angiogenesis [184]. Thus caution must be used with EPO when given to patients at risk of thrombo-embolic events and to those with cancer.

The question of whether extremely high doses of EPO are necessary to confer tissue protection was addressed in two recent studies [78, 136]. Hamed *et al.* used relatively low EPO doses (20 U/kg and 200 U/kg) in a rodent model of doxorubicin-induced cardiomyopathy and found that EPO improves heart function [78]. Interestingly, treatment with EPO at both doses significantly improved survival in these rats. A study by Hirata *et al.* compared low and high dose EPO and examined their effects on infarct size and arrhythmia in dogs [136]. They found that relatively high doses (1000 U/kg) of EPO reduced infarct size and the incidence of ventricular fibrillation following I/R in the canine heart. More clinically relevant, lower doses (100 U/kg) of EPO still reduced infarct size, though not as dramatically as the higher doses, and the incidence of ventricular fibrillation was not significantly different. Thus at clinically relevant doses EPO may provide some cardioprotection, but the full benefit of EPO-mediated cardioprotection may only be achieved through the use of high-dose EPO administration. This may be explained by the fact that the erythropoietic effects are mediated by the homodimeric EPOR while the tissue-protective effects are mediated by a separate heterodimeric receptor consisting of EPOR/CD131. It has been suggested that the affinity of EPO for the heterodimeric EPOR is much lower than that for the homodimeric EPOR [39, 46]. Thus, higher doses of EPO may be needed to achieve cardiac protection.

Finally, the optimal timing of delivery of EPO has not been determined with certainty. Several studies involving EPO-mediated cardioprotection have administered EPO prior to the ischemic insult. Pre-treatment with EPO may have some clinical benefit in high risk patients, or in the preservation of a heart during transplantation. However in patients with acute MI, treatment occurs typically at the time of reperfusion during angioplasty. There is growing evidence that EPO can still provide protection to the heart when administered at the time of reperfusion [136, 150]. Lipsic *et al.* used a rodent model of I/R and compared the effects of EPO administration prior to ischemia, at the start of ischemia, and after the onset of reperfusion [185]. They found that EPO administered after the onset of reperfusion provided similar reductions in infarct size and apoptosis as compared to EPO administered prior to ischemia. Moreover, a recent *ex vivo* study using human atrial appendages in patients undergoing coronary artery bypass surgery demonstrated that EPO administered right at reoxygenation reduced apoptosis and improved the force of contraction [186]. Thus, EPO may still be protective when administered at the time of reperfusion. However it is unclear whether reductions in inflammation are achieved when EPO is administered at reperfusion. Thus, whether or not the full cardioprotective benefit of EPO can be achieved when merely given at reperfusion is unclear.

DERIVATIVES OF ERYTHROPOIETIN

An alternative approach to the use of EPO for tissue protection is the use of derivatives of EPO that do not have erythropoietic effects. Modification of the structure of EPO can result in derivatives that have a very short half-life, or fail to bind to the homodimeric EPOR and trigger erythropoiesis [47, 49]. Asialoerythropoietin (AS-EPO) is a derivative of EPO that binds to the EPO receptor normally and provides tissue protection, but has a very short biological half-life and thus does not increase hematocrit [49]. Additionally, carbamylated EPO (C-EPO), another derivative of EPO does not appear to bind to the classic homodimeric EPOR, but can provide tissue protection *via* binding to the heterodimer EPOR/CD131 receptor [46]. Despite the lack of erythropoietic effect, several studies have now demonstrated that these derivatives of EPO maintain some of their tissue protective effects and it has been suggested that ultimately these derivatives will prove more useful as therapeutic agents than native EPO [187]. However, these derivatives have only been shown to reduce apoptosis and infarct size [48]. The ability of these derivatives to promote myocardial angiogenesis, for example, is not known. Thus it is unclear if these derivatives will exhibit the same range of cardioprotective effects as

Table 1. Dosing Protocols for EPO Treatment in Animal Models of I/R, MI, and Cardiomyopathy

Model	Dose Used	Outcome	Reference
Rat MI	· 5000 U/kg post MI (s.c., daily for 4 days) · 1000 U/kg post MI (s.c., 3 times per week for 4 weeks)	· Increased capillary density · Reduced infarct size · Increased heart function (EF%, LVEDP) · Reduced LV/BW ratio	[73]
Rat I/R	· 5000 U/kg 24 hours prior to I/R (i.p.)	· Reduced infarct size · Decreased neutrophil infiltration · Decreased pro-inflammatory cytokine expression (TNF-α, IL-6) · Increased anti-inflammatory cytokine expression (IL-10)	[97]
Rat I/R	· 5000 U/kg 24 hours prior to I/R (i.p.)	· Reduced infarct size · Increased COX-2 expression · Increased myocardial prostaglandin E_2 and $F_{1\alpha}$	[96]
Rat I/R	· 5000 U/kg 24 hours as well as 30 min prior to ischemia (i.p.) and daily for 7 days · 5000 U/kg at reperfusion (i.p.) and daily for 7 days	· Increased hematocrit · Increased myocyte survival	[34]
Rat I/R	· 5000 U/kg 15 min prior to I/R (i.v.)	· Reduced infarct size	[160]
Rat I/R	· 3000 U/kg 24 hours prior to I/R (i.p.)	· Reduced infarct size	[174]
Rat MI	· 40 μg/kg Darbepoietin alfa (approximately equivalent to 8000 U/kg of EPO), i.p. immediately following ligation, or immediately after ligation and once every 3 weeks for 9 weeks, or once every 3 weeks starting 3 weeks after ligation	· Reduced infarct size · Increased hematocrit · Increased heart function (LVEDP, LV d*P*/dt, LV developed pressure) · Decreased plasma ANP levels · Increased capillary density	[72]
Rat MI	· 5000 U/kg 24 hours prior to surgery (s.c. daily for 5 days)	· Increased hematocrit · No change in infarct size · Indeterminate effects on heart function · No change in EF · EF/infarct size ratio increased · Systolic volume/infarct size ratio increased	[171]
Rat Doxorubicin-induced cardiomyopathy	· 2500 U/kg at the start of doxorubicin administration (i.p. administered 6 times over 2 weeks)	· No change in hematocrit · Decreased apoptosis · Reduced fibrosis · Increased heart function (LVEDP, LV d*P*/dt, EF%)	[194]
Rat Doxorubicin-induced cardiomyopathy	· 20 U/kg beginning 1 week prior to doxorubicin administration (s.c. 3 times weekly for 3 weeks) · 200 U/kg beginning 1 week prior to doxorubicin administration (s.c. 3 times weekly for 3 weeks)	· Increased survival · Improved heart function (FS%)	[78]
Mouse I/R	· 5000 U/kg 24 hours prior to I/R (i.p.)	· Decreased neutrophil infiltration	[95]
Mouse I/R	· 2500 U/kg 24 hours as well as 30 min prior to ischemia (i.v.)	· Reduced infarct size · Decreased apoptosis	[149]
Mouse MI	· 1500 U/kg beginning 6 weeks after MI (s.c., twice a week for 4 weeks)	· Decreased pro-inflammatory cytokine expression (IL-1β, IL-6, TNF-α, TGF-β) · Increased hematocrit · Increased heart function (EF%, LVEDP, LV d*P*/dt) · Reduced heart/body weight ratio · Reduced myocardial fibrosis	[94]
Mouse Doxorubicin-induced cardiomyopathy	· 5000 U/kg at the start of doxorubicin administration (i.p., every 5 days for 2 weeks)	· Decreased COX-2 expression · Increased heart function (LVEDP, FS%, LV d*P*/dt) · Reduced myocardial fibrosis	[99]
Canine I/R	· 100 U/kg or 1000 U/kg 10 min prior to reperfusion (i.v.)	· Reduced infarct size · Reduced ventricular fibrillation · Decreased apoptosis	[136]

Abbreviations: EF, Ejection fraction; LVEDP, Left ventricular end diastolic pressure; LV dP/dt, First derivatives of left ventricular pressures.

EPO. Moreover, while EPO has been used clinically for more than 2 decades, there is no information regarding the safety (e.g. immunogenicity) and efficacy of these non-erythropoietic EPO derivatives in the clinical setting.

CLINICAL EPO TREATMENT IN HEART DISEASE

Clinically, recombinant human EPO has been in use for more than 2 decades to treat patients with anemia as a result of inadequate EPO production and/or decreased red blood cell production

from the bone marrow [183]. The use of EPO in this manner has proven to be effective and beneficial [183, 188]. Additionally, a recent study by Namiuchi *et al.* suggested that high serum EPO level correlates with smaller infarct size in patients with acute MI [189]. Given the growing evidence of cardioprotective effects of EPO in animal models, there is ostensible therapeutic value for EPO in patients with MI or chronic heart failure. Despite the aforementioned concerns regarding the timing and size of doses, evidence is emerging that the observations in animal models may indeed translate into clinical practice.

Lipsic *et al.* examined the safety and tolerability of the EPO analogue darbepoietin alfa (with an elimination half-life of about 1 week) in patients with acute MI [190]. They found the use of a single bolus of darbepoietin to be both safe, and well tolerated in patients. Additionally, Mancini *et al.* studied the effects of EPO on exercise capacity in patients with chronic heart failure and anemia [191]. In this study the authors also found that EPO was well tolerated by all patients over 3 months. Moreover EPO treatment enhanced exercise capacity in patients with chronic heart failure.

Anemia is frequently seen during heart failure with approximately 50% of all patients suffering from congestive heart failure also exhibiting anemia [132, 192]. The use of EPO in anemic heart failure patients has been examined. In a perspective randomized controlled study, Silverberg *et al.* examined the effects of subcutaneous EPO administration in combination with iron supplementation on cardiac function in severe heart failure patients with mild anemia [193]. In the treatment group (n=16), Hb was raised to above 12.5 g/dL. Following 8.2 months treatment, cardiac function and New York Heart Association functional class were significantly improved with less hospitalization and less use of diuretics compared to the control group. The study demonstrated that correction of anemia in patients with severe heart failure by EPO improves clinical outcome.

Given the preliminary data demonstrating successful use of EPO in the patient population, the large body of experimental evidence from animal models, and the longstanding history of use in the treatment of anemia, there is support for the initiation of larger clinical trials examining the use of EPO in the treatment of acute myocardial infarction and heart failure in the near future.

CONCLUSIONS

There is abundant evidence from several animal models to support the notion that EPO is capable of acting as a cardioprotective agent in a variety of disease models. EPO has beneficial effects through reduction of inflammation, inhibition of apoptosis, reduction in infarct size, the promotion of angiogenesis, remodeling, and the reduction in ventricular arrhythmias.

Though our understanding of the molecular signals that regulate these cardioprotective effects is still developing, the importance of PI3-kinase and Akt signaling in cardioprotection by EPO is becoming increasingly clear. Indeed, PI3-kinase and Akt signaling have been implicated in the cardioprotective effects of EPO regardless of the beneficial effect being examined. Additionally, ERK1/2 signaling, STAT signaling, and PKCε have also been implicated in mediating some of the cardioprotective effects of EPO. Emerging evidence that suggests roles for eNOS, and GSK-3β as downstream mediators of EPO's cardioprotective effects have advanced our understanding of the mechanisms by which EPO achieves its effects while the effects on COX-2, endothelin-1, and MMPs require further investigation.

While the cardioprotective effects of EPO are well established in animal models, clinical studies in patients with MI and heart failure are still limited. Though legitimate concerns regarding potential thromboembolism and other side effects exist such as disease progression in cancer patients undergoing EPO treatment, single doses of darbepoietin in patients with acute MI appear to be safe, and short term EPO treatment in patients with heart failure has shown promising beneficial effects. Furthermore, novel EPO analogues that do not have erythropoietic activity but retain tissue protective properties have been developed and tested in animal models. Ultimately, large-scale clinical trials are required to determine the efficacy and safety of EPO, and its analogues in patients with heart disease.

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