Molecular Medicine

Delayed Administration of Pyroglutamate Helix B Surface Peptide (pHBSP), a Novel Nonerythropoietic Analog of Erythropoietin, Attenuates Acute Kidney Injury

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In preclinical studies, erythropoietin (EPO) reduces ischemia-reperfusion–associated tissue injury (for example, stroke, myocardial infarction, acute kidney injury, hemorrhagic shock and liver ischemia). It has been proposed that the erythropoietic effects of EPO are mediated by the classic EPO receptor homodimer, whereas the tissue-protective effects are mediated by a heterocomplex between the EPO receptor monomer and the β-common receptor (termed "tissue-protective receptor"). Here, we investigate the effects of a novel, selective-ligand of the tissue-protective receptor (pyroglutamate helix B surface peptide (pHBSP)) in a rodent model of acute kidney injury/dysfunction. Administration of pHBSP (10 μg/kg intraperitoneally (i.p.) 6 h into reperfusion) or EPO (1,000 IU/kg i.p. 4 h into reperfusion) to rats subjected to 30 min ischemia and 48 h reperfusion resulted in significant attenuation of renal and tubular dysfunction. Both pHBSP and EPO enhanced the phosphorylation of Akt (activation) and glycogen synthase kinase 3β (inhibition) in the rat kidney after ischemia-reperfusion, resulting in prevention of the activation of nuclear factor-κB (reduction in nuclear translocation of p65). Interestingly, the phosphorylation of endothelial nitric oxide synthase was enhanced by EPO and, to a much lesser extent, by pHBSP, suggesting that the signaling pathways activated by EPO and pHBSP may not be identical.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2012.00093

INTRODUCTION

Acute kidney injury (AKI) and chronic transplant nephropathy are defined by a progressive functional deterioration of the kidney that is associated with interstitial fibrosis, tubular atrophy and glomerulosclerosis leading to late renal allograft failure. Ischemia/reperfusion injury (IRI) is an associated risk factor for both AKI and chronic transplant nephropathy. *In vivo* models of renal IRI have extensively demonstrated the evolution of AKI and the consequent long-term chronic lesions that mimic those demonstrated in chronic transplant nephropathy (1). Two separate long-term retrospective outcome studies of patients surviving AKI on discharge, with a median follow-up of 7.2 and 8 years, revealed significant deterioration in renal function that progressively leads to end-stage renal disease in 19 and 37% of patients, respectively (2,3). During AKI, the S3 segment of the proximal tubule and the outer medullary thick ascending limb of the nephron are subject to the most severe injury, resulting in cell death. The proximal tubule absorbs two-thirds of all NaCl in the glomerular filtrate, and the thick ascending limb is responsible for Na^+ reabsorption through several sodium transporters, which allows the loop of Henle to generate a high osmolarity to concentrate urine. Therefore, fractional excretion of sodium together with glomerular filtration rate is used to monitor tubular as well as glomerular function. Deterioration in both these markers is indicative of AKI and has been shown to change during the evolution of AKI (4). During the recovery phase of AKI, viable nephrons undergo

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Submitted February 28, 2012; Accepted for publication March 7, 2012; Epub (www.molmed.org) ahead of print March 8, 2012.

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hyperfiltration and hypertrophy that is linked to the synthesis of cytokines and growth factors to cause a systemic inflammatory response syndrome and extracellular matrix accumulation due to the induction of transforming growth factor-β (a key fibrogenic cytokine). Therefore, therapeutic strategies that can ameliorate AKI early may prevent the later onset of end-stage renal disease. Thus, patients have to be treated during the early phases of the evolution of AKI.

Erythropoietin (EPO) is beneficial in preclinical models of IRI including myocardial infarction (5), hind-limb ischemia (6), liver ischemia (7), AKI (8,9) and hemorrhagic shock (10), when given before injury or at the onset of reperfusion. Leist *et al.* (11) suggested that EPO mediates tissue-protection through a receptor (tissue-protective receptor) that is pharmacologically distinct from the classic EPO receptor that is known to mediate erythropoiesis (11). The tissue-protective receptor exhibits a lower affinity for EPO, forms distinct molecular species in cross-linking experiments (12) and is a heteromer composed of the EPO receptor and CD131 (the β-common receptor [βcR]) (13). The βcR also forms receptor complexes with the α receptor subunits specific for granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3 and IL-5 and hence has been termed the "common" receptor (14). We have recently discovered that (a) the helix B of EPO has tissue-protective properties that are similar to properties of EPO, and (b) a peptide constructed to mimic the external, aqueous surface of EPO without primary sequence similarity (pyroglutamate helix B surface peptide [pHBSP]) mimics the tissue-protective effects of EPO in a mouse model of renal IRI when given as multiple administrations in the course reperfusion (15).

The βcR plays an integrative role in the EPO signaling-mediated activation of endothelial nitric oxide synthase (eNOS) (16). eNOS is believed to be responsible for the maintenance of physiological renal hemodynamics and function (17). During the early phase of reperfusion injury, that is, the first 6 h, eNOS activation is significantly attenuated, whereas the inducible isoform of NOS (iNOS) is significantly upregulated (18). We have previously shown that renal IRI is effectively attenuated by genetic deletion or pharmacological inhibition of iNOS (19). During the later stages of reperfusion, that is, by 24 h, eNOS activation is restored back up to basal levels, whereas iNOS expression continues to increase (18). Surprisingly, ischemic preconditioning (18) or treatment with nitrite (20) in models of renal IRI prevent the expression of iNOS and the decline in eNOS activity caused by ischemia followed by 6 h of reperfusion. This finding suggests that strategies that enhance eNOS (and prevent iNOS expression) may protect the kidney against IRI.

Therefore, we set out to investigate whether a single, delayed administration of either pHBSP or EPO (during the early reperfusion period) reduces the tissue injury and dysfunction caused by renal IRI. Having discovered that the delayed administration of EPO or pHBSP (4 or 6 h after onset of reperfusion, respectively) reduces glomerular and tubular dysfunction, we investigated (a) the effects of the above interventions on disease-relevant markers (including plasma clusterin and osteopontin) and (b) signaling pathways known to play key roles in tissue injury and/or inflammation (including the phosphorylation of Akt on Ser^{473}, phosphorylation of glycogen synthase kinase [GSK]-3β on Ser^{9}, phosphorylation of eNOS on Ser^{1177}, activation of p38 mitogen-activated protein kinase (MAPK) and activation of nuclear factor [NF]-κB [measured as nuclear translocation of p65]).

MATERIALS AND METHODS

The animal protocols followed in this study were approved by the local Animal Use and Care Committee in accordance with the derivatives of both the Home Office *Guidance on the operation of the Animals (Scientific Procedures) Act 1986* (21) and the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (22).

Surgical Procedure and Quantification of Organ Injury/Dysfunction

This study was carried out on 42 male Wistar rats (Charles River, Margate, UK) receiving a standard diet and water *ad libitum*. Animals were anesthetized using a ketamine (150 mg/kg) and xylazine (15 mg/kg) mixture (1.5 mL/kg intraperitoneally [i.p.]). The hair was shaved and the skin was cleaned with 70% alcohol (v/v). The animals were then placed on a homoeothermic blanket set at 37°C. Animals received 0.1 mg/kg subcutaneous buprenorphine (0.1 mL/kg) before commencing surgery. A midline laparotomy was then performed. The renal pedicles (consisting of the renal artery, vein and nerve) were isolated and clamped using nontraumatic microvascular clamps at time 0. After 30 min of bilateral renal ischemia, the clamps were removed to allow reperfusion for 48 h. For reperfusion, the renal clamps were removed and the kidneys were observed for a further 5 min to ensure reflow, after which 8 mL/kg saline at 37°C was injected into the abdomen and all incisions were sutured in two layers (Ethicon Prolene 4-0). Animals were then allowed to recover on the homothermic blanket and placed into cages upon recovery. At 24 h of reperfusion, rats were placed into metabolic cages for the collection of urine. At sacrifice (after 48 h of reperfusion), blood was taken by cardiac puncture into nonheparinized syringes and immediately decanted into 1.3 mL serum gel tubes (Sarstedt, Ltd., Leicester, UK). The blood was centrifuged at 9,900*g* for 3 min to separate serum. All biochemical markers in serum and urine were measured in a blinded fashion by a commercial veterinary testing laboratory (IDEXX, West Sussex, UK).

Experimental Design

Rats were randomly allocated into the following groups: (a) Sham (n = 6); (b) IRI (n = 11); (c) IRI + pHBSP (pHBSP, 10 μg/kg i.p., administered 6 h after the onset of reperfusion, n = 11); and (d) IRI + EPO (EPO, 1,000 IU/kg i.p., adminis-

tered 4 h after the onset of reperfusion, n = 14). The volume of phosphate-buffered saline (vehicle) administered was equal to the volume of pHBSP or EPO administered (1 mL/kg). Sham-operated rats underwent identical surgical procedures but without IRI. It was previously shown that after an intraperitoneal administration of 1,000 IU/kg EPO, peak plasma concentrations of EPO were achieved 2 h later (23). As the peak plasma concentration of pHBSP is achieved at the time of administration, that is, 6 h into reperfusion (15), EPO was administered at 4 h into reperfusion to achieve a peak plasma concentration at 6 h into reperfusion.

Measurement of Serum Clusterin and Osteopontin

Analysis for the kidney injury markers of clusterin and osteopontin was carried out using the Novagen® Widescreen® Rat Kidney Toxicity Panel 2 on the Luminex® xMAP® System per the manufacturer's protocol.

Western Blot Analysis

Western blots were carried out as previously described (24). Three separate experiments of Western blot analysis were performed for each marker, and tissues were done separately for each Western blot experiment. Briefly, rat kidney samples were homogenized and centrifuged at 4,000*g* for 5 min at 4°C. Supernatants were removed and centrifuged at 15,000*g* at 4°C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer. The suspensions were centrifuged at 15,000*g* for 20 min at 4°C. The resulting supernatants containing nuclear proteins were carefully removed, and protein content was determined using a bicinchoninic acid protein assay following the manufacturer's directions. Proteins were separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldenedifluoride membrane, which was then incubated with a primary antibody (rabbit anti-total GSK-3β, dilution 1:200; goat anti-phospho GSK-3β Ser^{9} dilution 1:200; rabbit anti-total Akt dilution 1:1,000; mouse anti-phospho Akt Ser^{473} dilution 1:1,000; rabbit anti-total eNOS dilution 1:200; goat anti-phospho eNOS Ser^{1177} dilution 1:200; rabbit anti-NF-κB p65 dilution 1:400; mouse anti-phospho p38, dilution 1:1,000; rabbit anti-total p38 dilution 1:1,000). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1:10,000) and developed using the ECL detection system. The immunoreactive bands were visualized by autoradiography. The membranes were stripped and incubated with β-actin monoclonal antibody (dilution 1:5,000) and subsequently with an anti-mouse antibody (dilution 1:10,000) to assess gel-loading homogeneity. Densitometric analysis of the bands was performed using Gel Pro® Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA), and optical density analysis was expressed as the fold-increase versus the sham group. In the sham group, the immunoreactive bands of the gel were respectively measured and normalized against the first immunoreactive band (standard sham sample), and the results of all the bands belonging to the same group were expressed as mean ± standard error of the mean (SEM). The membranes were stripped and incubated with β-actin monoclonal antibody and subsequently with an anti-mouse antibody to assess gel-loading homogeneity. Relative band intensity was assessed and normalized against parallel β-actin expression. Each group was then adjusted against corresponding Sham data to establish relative protein expression when compared with Sham animals.

Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich (Poole, Dorset, UK). All stock solutions were prepared using nonpyrogenic saline (0.9 % [w/v] NaCl; Baxter Healthcare, Thetford, Norfolk, UK). Ringer's Lactate was purchased from Baxter Healthcare. Antibodies for Western blot analysis were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). pHBSP was supplied by Araim Pharmaceuticals (Ossining, NY, USA). EPO was supplied by the Barts and The London Pharmacy.

Statistical Analysis

All values described in the text and figures are expressed as mean ± SEM for n observations. Each data point represents biochemical measurements obtained from up to 14 separate animals. Statistical analysis was carried out using GraphPad Prism 5.0d (GraphPad Software, San Diego, CA, USA). Data without repeated measurements was assessed by one-way analysis of variance (ANOVA) followed by Dunnett post hoc test. Data with repeated measurements were assessed by two-way ANOVA followed by a Bonferroni post hoc test. A P value of <0.05 was considered significant.

RESULTS

Effect of pHBSP and EPO on Renal, Glomerular and Tubular Dysfunction

When compared with sham-operated rats, renal ischemia (30 min) and reperfusion (48 h) caused significant increases in serum creatinine, serum urea, creatinine clearance and fractional excretion of sodium (Figure 1). Rats were administered 10 μg/kg pHBSP at 6 h into reperfusion after ischemia. Administration of pHBSP to rats subjected to IRI caused an attenuation in serum creatinine, serum urea, creatinine clearance and fractional excretion of sodium when compared with rats subjected to IRI only (see Figure 1).

pHBSP has a half-life of ~2 min in the rodent (15) and, hence, it was surprising to see an effect after such a late administration of 6 h in this model. Therefore, we wanted to determine if EPO would have a similar beneficial effect if given late into reperfusion. EPO reaches peak plasma concentrations 2 h after an intraperitoneal administration (23); therefore, rats were administered 1,000 IU/kg EPO at 4 h into reperfusion after ische-

mia. Administration of EPO to rats subjected to IRI caused an attenuation in serum creatinine, serum urea and fractional excretion of sodium, with only a small insignificant increase in creatinine clearance when compared with rats subjected to IRI only (see Figure 1).

Effect of pHBSP and EPO on Plasma Biomarkers of Renal Injury

When compared with sham-operated rats, renal ischemia (30 min) and reperfusion (48 h) also caused significant increases in plasma clusterin and osteopontin (Figure 2). pHBSP significantly attenuated the rise in clusterin and osteopontin (see Figure 2) when compared with rats subjected to IRI only. In contrast, EPO did not attenuate the rise in osteopontin (see Figure 2), but did attenuate the rise in clusterin (see Figure 2) when compared with rats subjected to IRI only.

Effect of pHBSP and EPO on the Phosphorylation of Akt, GSK-3β, eNOS and p38 in the Kidneys of Rats That Underwent Renal IRI

To gain a better insight into the potential mechanism(s) underlying the observed beneficial effects of pHBSP and EPO, we investigated the effects of this peptide on cell signaling pathways known to confer tissue protection or to inhibit inflammation in the kidney. When compared with sham-operated rats, the kidneys of rats subjected to IRI that had been treated with vehicle showed small (nonsignificant) decreases in the phosphorylation of Akt on Ser^{473} and GSK-3β on Ser^{9} (Figures 3A, B). Treatment of IRI rats with pHBSP or EPO resulted in a substantial increase in the phosphorylation of Akt and GSK-3β to levels that were significantly higher than levels measured in sham-operated rats (see Figures 3A, B). The degree of phosphorylation of eNOS on Ser^{1177} was similar in sham-operated rats and IRI rats treated with vehicle, indicating that renal IRI did not affect eNOS phosphorylation (Figure 3C). Treatment of IRI rats with pHBSP resulted in a significant (~1-fold) increase in the phosphorylation of eNOS when compared with IRI rats treated with vehicle alone (see Figure 3C). Interestingly, treatment of IRI rats with EPO also resulted in a significant, but larger (approximately three-fold) increase in the phosphorylation of eNOS when compared with IRI rats treated with vehicle alone (see Figure 3C). When compared with sham-operated rats, IRI rats treated with vehicle exhibited significant increases in the phosphorylation of p38 in the kidney (Figures 3D). Treatment of IRI rats with pHBSP or EPO significantly attenuated the degree of p38 phosphorylation in the kidney (see Figure 3D) when compared with rats subjected to IRI only.

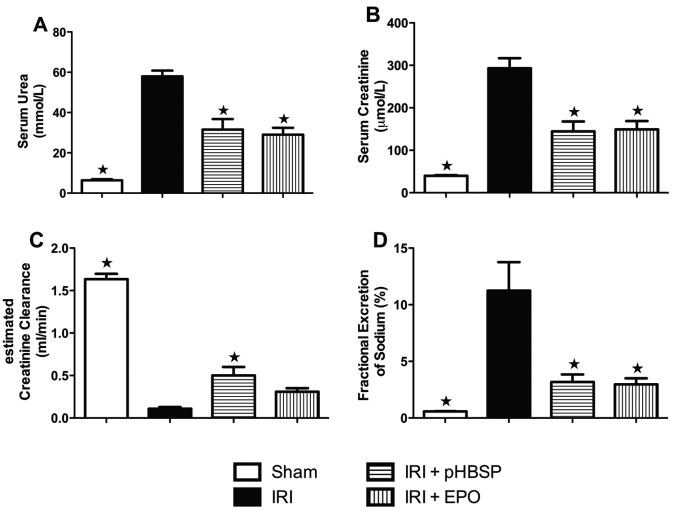

Figure 1. Renal and tubular dysfunction in rats treated with pHBSP or EPO after 30 min ischemia and 48 h reperfusion. Serum urea (A), serum creatinine (B), estimated creatinine clearance (C) and fractional excretion of sodium (D) were measured after sham operation (Sham, n = 6) or renal ischemia-reperfusion injury (IRI, n = 11; IRI + pHBSP, n = 11 (10 μg/kg pHBSP administered 6 h into reperfusion)); and IRI + EPO, n = 14 (1,000 IU/kg EPO administered 4 h into reperfusion)). Data are expressed as means ± SEM for n number of observations. $P < 0.05$ versus IRI.

Effect of pHBSP and EPO on the Nuclear Translocation of the p65 NF-κB Subunit in the Kidneys of Rats That Underwent Renal IRI

When compared with sham-operated rats, the kidneys of IRI rats treated with vehicle exhibited significant increases in the nuclear translocation of the p65 NF-κB subunit (Figure 4), indicating the activation of NF-κB. Treatment of IRI rats with pHBSP or EPO resulted in a significant reduction in nuclear translocation of p65 and, hence, the activation of NF-κB in the kidney (see Figure 4).

DISCUSSION

Several molecular interaction studies have identified the regions of EPO that interact with the EPO receptor, which include portions of helices A and C, as well as helix D and the loop connecting helices A-B (25–28). Modification of the amino acids of EPO within portions of helices A and C or in the loop connecting helices A-B abolishes the ability of EPO to bind to its receptor, thus making these modified EPOs nonerythropoietic *in vivo* and *in vitro*. However, the most interesting aspect of these EPO analogs is that many are still able to demonstrate tissue-protective properties (11). Chemical modification of lysine residues or amino acid substitutions made within helices A and C or in the loop connecting helices A-B of EPO do not affect tissue protection, suggesting that other regions of EPO may contain the recognition site(s) for the βcR (see Introduction). The interaction of the hydrophobic content of the

four α-helices of EPO constrain the molecule into a compact, relatively rigid, globular structure and, hence, give EPO a well-defined tertiary structure in aqueous media. When EPO is bound to the EPO receptor, helix B and parts of the loops connecting helices A-B and C-D face the aqueous medium, away from the homodimer binding sites (Protein Data Bank [PDB] ID code 1EER). These regions do not contain lysine and, therefore, are not modified by carbamylation of EPO, a procedure that produces a tissue-protective compound that is unable to bind to the classic EPO receptor (11). Subsequent studies have since identified that the novel tissue-protective peptide, pHBSP, can confer protection in a number of disease models (29–37). The interesting aspect of pHBSP is that it has many of the pleiotropic effects of EPO. In particular, pHBSP has been shown to accelerate wound healing of punch biopsy wounds in the rat (15).

Results from previous experiments have shown that a peptide fragment of EPO comprising the amino acid sequence corresponding to helix B exhibited tissue-protective effects similar to EPO and its nonerythropoietic derivatives in a variety of *in vitro* and *in vivo* models (15). In the same study, we demonstrated a dose-dependent renoprotective effect of pHBSP when administered at 1 min, 6 h and 12 h after the onset of reperfusion after 30 min of renal ischemia (15). The degree of protection observed was similar to that of EPO previously reported in a murine model of 30 min renal ischemia and 24 h reperfusion (9). We demonstrate here that a single administration of pHBSP at 6 h after the onset of reperfusion protects the kidney from IRI and dysfunction. We found that this delayed administration of pHBSP attenuated the renal, glomerular and tubular dysfunction and injury associated with 30 min of ischemia and 48 h of reperfusion. In rodents, maximal plasma levels of EPO are achieved at 2 h after a single intraperitoneal injection (23). We show here that a single intraperitoneal injection of a moderate dose of EPO (1,000 IU/kg) also caused a pronounced reduction in renal IRI and dysfunction, which was similar to the one afforded by pHBSP. Thus, it appears that the delayed administration (as late as 6 h into the reperfusion period) of either EPO or its nonerythropoietic analog pHBSP dramatically improves the functional recovery after IRI in the rat.

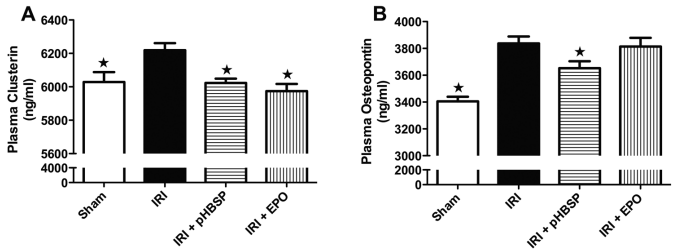

Figure 2. Plasma biomarkers of renal injury in rats treated with pHBSP or EPO following 30 min ischemia and 48 h reperfusion. Clusterin (A) and osteopontin (B) levels were measured after sham operation (Sham, n = 6) or renal ischemia-reperfusion injury (IRI, n = 10; IRI + pHBSP, n = 6 (10 μg/kg pHBSP administered 6 h into reperfusion); and IRI + EPO, n = 8 (1,000 IU/kg EPO administered 4 h into reperfusion)). Data are expressed as means ± SEM for n number of observations. $P < 0.05$ versus IRI.

In recent years, clusterin and osteopontin were used as biomarkers of kidney injury (38). Clusterin is an ambiguous protein with evidence supporting a transport/chaperone role not too dissimilar to that of heat shock proteins (39). Although clusterin is ubiquitously expressed, an increased expression of clusterin is associated with injury to the proximal tubular epithelium in human renal diseases (40). In our rodent study, we observed a significant rise in plasma clusterin at 48 h after the onset of reperfusion. This rise in clusterin was abolished when rats subjected to renal IRI were treated with either pHBSP or EPO.

Osteopontin, which is found in numerous organs including the bone, nervous system and kidney, predominantly functions to modulate nitric oxide (NO), regulate calcium, maintain bone and the extracellular matrix, and maintain migration/adhesion of macrophages and lymphocytes (41). Kidney mRNA expression of osteopontin is localized to the long loop of Henle, distal convoluted tubule, proximal tubule and glomerular epithelium, with increased gene expression occurring after injury (42). There is some evidence that suggests that osteopontin is proinflammatory (43). In our study, the rise in plasma osteopontin caused by IR was attenuated by pHBSP but not EPO. This result may suggest that pHBSP is able to target tissue repair in areas of the kidney not possible by EPO, such as the distal tubular and glomerular epithelium, which may explain the difference in effect with respect to glomerular function.

There is some evidence that the beneficial effects of pHBSP and/or EPO are secondary to the activation of the survival kinase Akt (44,45). Akt is a member of the phosphoinositide 3-kinase signal transduction enzyme family, which regulates cellular activation, inflammatory responses, chemotaxis and apoptosis (46). When phosphorylated by its upstream regulator, phosphoinositide-dependent kinase, Akt modulates cell survival and growth (46). We report here that renal IRI at 48 h of reperfusion results in a small reduction in the phosphorylation of Akt in the kidney. A reduction in the activation of this survival pathway, if biologically significant, will make organs more susceptive to injury and inflammation (47,48). Most notably, pHBSP and EPO (when given 6 h into reperfusion) significantly enhanced Akt phosphorylation (at 48 h) to levels that were significantly higher than levels seen in sham-operated rats. The enhanced activation of this important survival pathway should make

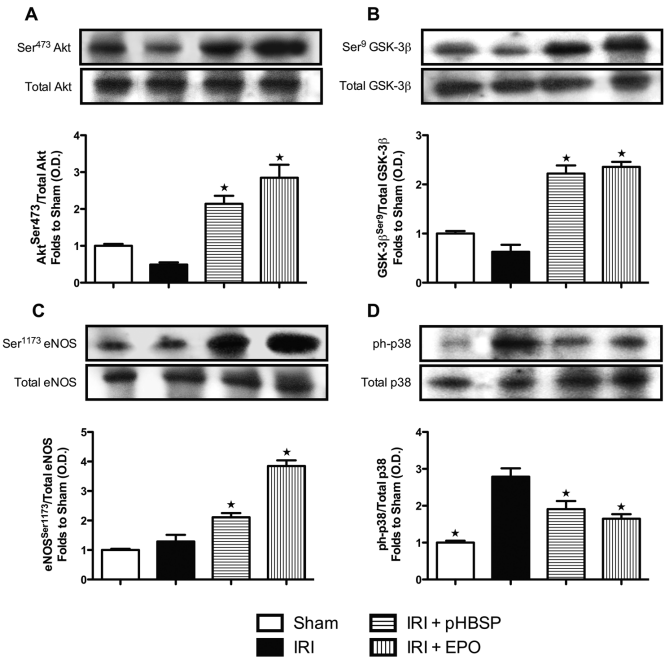

Figure 3. Effect of pHBSP and EPO on the phosphorylation of Ser^{473} on Akt in the kidney (A), Ser^{9} on GSK-3β in the kidney (B), Ser^{1177} on eNOS in the kidney (C), p38 in the kidney (D) after sham operation (Sham, n = 3) or renal ischemia-reperfusion injury (IRI, n = 3; IRI + pHBSP, n = 3 (10 μg/kg pHBSP administered 6 h into reperfusion); and IRI + EPO, n = 3 (1,000 IU/kg EPO administered 4 h into reperfusion). Data are expressed as means ± SEM for n number of observations. $P < 0.05$ versus IRI. O.D., optical density.

the kidney of animals treated with pHBSP or EPO more resistant to injury. Interestingly, both EPO and pHBSP also enhance the phosphorylation of Akt in cardiomyocytes subjected to tumor necrosis factor (TNF)-α (29).

GSK-3β is a serine-threonine kinase that was originally recognized as a kinase that phosphorylates glycogen synthase. In contrast to most other kinases, GSK-3β is active in a resting cell state; however, it is inactivated by phosphorylation of Ser^{9}. GSK-3β is regulated by multiple signaling pathways including the Akt pathway, which inhibits this kinase by causing Ser^{9} phosphorylation (49,50). Consistent with the small decline in the phosphorylation/activation of Akt reported here (at 48 h of reperfusion), IRI also caused a small decline in the phosphorylation of GSK-3β on Ser^{9}. This result may indicate an excessive activation of GSK-3β, which in turn drives both inflammation (51) and tissue injury (52).

Most notably, pHBSP and EPO (when given 6 h into reperfusion) significantly enhanced Ser^{9} phosphorylation on GSK-3β (at 48 h) to levels that were significantly higher than those seen in sham-operated rats. This enhanced phosphorylation of Ser^{9} will result in the inhibition of the activity of GSK-3β, which should make the tissues more resistant against inflammation and injury. It should be noted that other molecules that increase in Ser^{9} phosphorylation resulting in inhibition of GSK-3β exert potent anti-inflammatory (51,53) and antiischemic effects in a number of organs including the kidney (52,54,55). Interestingly, inhibition of GSK-3β also mediates the cardioprotective effects of EPO (52).

Downstream of GSK-3β, several studies have now reported an association between GSK-3β and NF-κB activity *in vitro* (56,57) and *in vivo* (51,58). NF-κB is a transcriptional factor that plays an important role in regulating the transcription of a number of genes (for example, *iNOS*, cyclooxygenase-2, *IL-1*, *TNF* and *IL-6*), especially those involved in producing mediators involved in local and systemic inflammation, such as cytokines, chemokines, cell adhesion molecules, apoptotic factors and other mediators (59). Treatment of TNF-α–stimulated hepatocytes with a specific GSK-3β inhibitor resulted in a decrease of the NF-κB–dependent gene transcription (60). This study also indicated four potential phosphorylation sites for GSK-3β on the NF-κB subunit p65. Most notably, pretreatment with a number of chemically distinct inhibitors of GSK-3β attenuates organ injury and dysfunction in a rodent model of polymicrobial septic shock (51,53) and renal IRI (55). This protective effect was associated with inhibition of the activation of NF-κB and NF-κB–dependent proinflammatory genes, along with a reduced phosphorylation of Ser^{536} on the NF-κB p65 subunit. In addition, GSK-3β may also inhibit the activation of NF-κB by phosphorylating and degrading IκBα, which is required to prevent NF-κB translocation (57). We report here that IRI at 48 h of reperfusion results in a significant increase in the activation of NF-κB (measured here as nuclear translocation of p65), which was attenuated when pHBSP or EPO was given 6 h into reperfusion. All of the above findings support the view that pHBSP and EPO restore the activation of Akt, resulting in inhibition of GSK-3β (after phosphorylation on Ser^{9}) and inhibition of the activation of NF-κB.

In addition to inhibiting the activation of GSK-3β, activation of Akt results in the phosphorylation of eNOS on Ser^{1177}, which in turn causes activation of eNOS resulting in an enhanced formation of NO in the microcirculation. In our study, at 48 h of reperfusion, IRI did not affect eNOS phosphorylation on Ser^{1177} in the kidney. Delayed administration of pHBSP or EPO during reperfusion, however, caused an increase in eNOS phosphorylation and, hence, activity (to levels that were significantly higher than levels

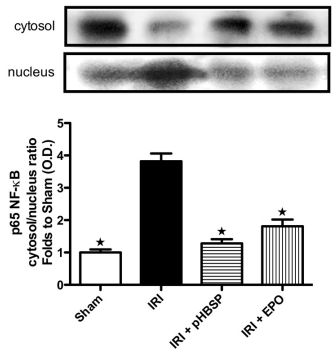

Figure 4. Effect of pHBSP and EPO on NF-κB activation in the kidney after sham operation (Sham, n = 3) or renal ischemia-reperfusion injury (IRI, n = 3; IRI + pHBSP, n = 3 (10 μg/kg pHBSP administered 6 h into reperfusion); and IRI + EPO, n = 3 (1,000 IU/kg EPO administered 4 h into reperfusion)). Data are expressed as means ± SEM for n number of observations. $P < 0.05$ versus IRI. O.D., optical density.

seen in sham-operated rats). The observed increase in eNOS phosphorylation/activity was greatest after the administration of EPO. In conditions associated with IRI, activation of eNOS is beneficial, since the enhanced formation of NO causes local vasodilation, inhibits adhesion of platelets and neutrophils and regulates angiogenesis (61). There is good evidence that agents that release NO or enhance the formation of endogenous NO attenuate organ injury/dysfunction in AKI (62,63). Agents that also inhibit the formation of NO from inducible NOS (iNOS) also attenuate the multiple organ failure associated with AKI (64). Inhibition of eNOS activity also attenuates the cardioprotective effects of EPO, suggesting that activation of eNOS importantly contributes to the protection of the heart by EPO (65). Recently, Su *et al.* (16) demonstrated that the βcR, when activated by EPO, causes phosphorylation of Akt and in turn increases the interaction between βcR and eNOS. This increase in interaction results in the enhanced activation of eNOS and the production of NO. Thus, activation of eNOS (possibly secondary to activation of Akt) may contribute to the beneficial effects of EPO reported here.

Activation of p38 MAPK occurs in response to ischemia in many organs and promotes cellular stress responses such as proliferation, differentiation and production of proinflammatory cytokines (66,67). IRI of the kidney results in the activation of p38 MAPK, whereas inhibition of the activity of this p38 MAPK in renal IRI attenuates dysfunction and injury (68). We report here that IRI results in a significant activation of p38 MAPK (when measured at 48 h after onset of reperfusion) in the kidney, which was attenuated by both pHBSP and EPO when given 6 h into reperfusion. Our data are consistent with the hypothesis that prevention of the activation of p38 MAPK contributes to the observed beneficial effects of pHBSP and EPO.

It should be duly noted that the kidney is not the only organ to demonstrate early recovery from injury. The brain has also been shown to have a similar therapeutic temporal window of up to 6 h after injury. It has been hypothesized that the ability of EPO to induce late protection in the brain may be due to a modulation of apoptosis, necrosis or immune-mediated injury (69). A similar scenario may also exist for both the kidney and pHBSP, as demonstrated by the data presented here.

CONCLUSION

This report states for the first time that a delayed, single intraperitoneal administration of EPO and particularly the nonhematopoietic EPO analog pHBSP attenuates the renal dysfunction (glomerular and tubular) and injury (clusterin) caused by ischemia (30 min) and reperfusion (48 h) in the rat. To gain a better insight into the signaling pathways involved in the tissue-protective and/or antiinflammatory effects of pHBSP, we investigated the effects of pHBSP on signaling pathways known to play a role in tissue injury/survival and/or inflammation. Because this peptide is based on helix B of EPO, we carried out these mechanistic studies in kidney samples from animals also treated with EPO, in the hope to find

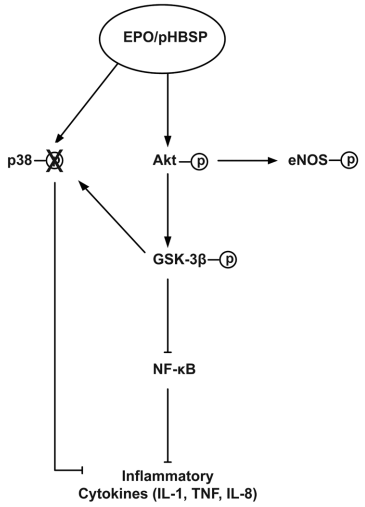

Figure 5. Antiinflammatory mechanisms of tissue-protective molecules. The antiinflammatory mechanism of EPOR-βcR activation is associated with (a) the activation of the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway, which leads to the inhibition of the activation of GSK-3β, which subsequently suppresses NF-κB activity leading to reduced expression of the NF-κB–driven gene transcription of proinflammatory mediators; (b) in parallel inhibition of the MAPK-p38 signaling pathway, which subsequently suppresses the production of proinflammatory mediators; and (c) the activation of the PI3K-Akt signaling pathway, which leads to increased activation of eNOS, resulting in an enhanced formation of NO.

common signaling events that contribute to the observed beneficial effects of pHBSP *in vivo*. In the kidney, ischemia-reperfusion injury resulted in a small reduction in the activation of the survival kinase Akt (measured as reduction in the phosphorylation on Ser^{473}). Treatment of rats with pHBSP and EPO restored the phosphorylation and, hence, activation of Akt, which in turn resulted in inhibition of GSK-3β (secondary to phosphorylation on Ser^{9}) and inhibition of the activation of NF-κB. There is now good evidence that therapeutic strategies that enhance the activation of Akt and reduce the activation of GSK-3β enhance the resistance of organs to noxious stimuli (including ischemia) and reduce inflamma-

tion via inhibition of NF-κB (57). Activation of Akt by EPO (and to a lesser extent pHBSP) also resulted in activation of eNOS (measured as phosphorylation on Ser^{1177}), which in turn results in an increase in eNOS activity and an enhanced formation of NO in the microcirculation. In addition, pHBSP attenuated the ischemia-reperfusion–induced activation of p38 MAPK, which is known to contribute to the development of organ injury/inflammation in ischemia-reperfusion injury (70,71) (Figure 5). We propose that all of the above signaling events initiated by pHBSP and/or EPO contribute to the beneficial effects of these two molecules in AKI. Because the beneficial effects of EPO in patients with acute kidney injury, or even chronic kidney disease, are limited by side effects due to excessive erythropoiesis, we speculate that nonhematopoietic analogs of EPO such as pHBSP may be useful to mimic the tissue-protective effects of EPO without causing the well-documented side effects.

ACKNOWLEDGMENTS

NSA Patel was supported by a Kidney Research UK Post-Doctoral Fellowship (PDF4/2009). This work was supported in part by the William Harvey Research Foundation and forms part of the research themes contributing to the translational research portfolio of Barts and the London Cardiovascular Biomedical Research Unit, which is supported and funded by the National Institute of Health Research.

NSA Patel, M Brines, A Cerami and C Thiemermann were involved in the conception, hypotheses delineation and design of the study. NSA Patel, HL Kerr-Peterson, M Collino, M Rogazzo, R Fantozzi, E G Wood, FL Johnson, MM Yaqoob, and C Thiemermann were involved in the acquisition of the data or the analysis and interpretation of such information. NSA Patel, M Brines, MM Yaqoob, A Cerami, and C Thiemermann were involved in writing the article or in substantial involvement in its revision before submission.

DISCLOSURE

M Brines and A Cerami are officers of Araim Pharmaceuticals and currently hold stock in the company.

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