A Nonerythropoietic Peptide that Mimics the 3D Structure of Erythropoietin Reduces Organ Injury/Dysfunction and Inflammation in Experimental Hemorrhagic Shock

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Recent studies have shown that erythropoietin, critical for the differentiation and survival of erythrocytes, has cytoprotective effects in a wide variety of tissues, including the kidney and lung. However, erythropoietin has been shown to have a serious side effect—an increase in thrombovascular effects. We investigated whether pyroglutamate helix B-surface peptide (pHBSP), a nonerythropoietic tissue-protective peptide mimicking the 3D structure of erythropoietin, protects against the organ injury/dysfunction and inflammation in rats subjected to severe hemorrhagic shock (HS). Mean arterial blood pressure was reduced to 35 ± 5 mmHg for 90 min followed by resuscitation with 20 mL/kg Ringer Lactate for 10 min and 50% of the shed blood for 50 min. Rats were euthanized 4 h after the onset of resuscitation. pHBSP was administered 30 min or 60 min into resuscitation. HS resulted in significant organ injury/dysfunction (renal, hepatic, pancreas, neuromuscular, lung) and inflammation (lung). In rats subjected to HS, pHBSP significantly attenuated (i) organ injury/dysfunction (renal, hepatic, pancreas, neuromuscular, lung) and inflammation (lung), (ii) increased the phosphorylation of Akt, glycogen synthase kinase-3β and endothelial nitric oxide synthase, (iii) attenuated the activation of nuclear factor (NF)-κB and (iv) attenuated the increase in p38 and extracellular signal-regulated kinase (ERK)1/2 phosphorylation. pHBSP protects against multiple organ injury/dysfunction and inflammation caused by severe hemorrhagic shock by a mechanism that may involve activation of Akt and endothelial nitric oxide synthase, and inhibition of glycogen synthase kinase-3β and NF-κB.

INTRODUCTION

The mortality associated with closed femur fractures or gunshot wounds to the abdomen prior to World War I was 50% and 90%, respectively. However, even with the introduction of the advanced trauma life support protocol it remains the leading cause of mortality and morbidity in the United States and is the number one killer of Americans under the age of 34 (1). Severe hemorrhage accounts for nearly 40% of all trauma deaths and is the leading cause of preventable trauma death (2). If exsanguination after severe trauma is not the primary cause of death, then prolonged shock is known to increase the incidence of multiple organ failure (MOF) and late mortality (3). There have been many recent improvements in the care of trauma hemorrhage including high-ratio transfusion strategies, hypotensive resuscitation, hemostatic dressings, tourniquets and novel resuscitation fluids (4). Novel resuscitation fluids designed to reduce the inflammatory response and to improve patient outcome after trauma are at the forefront of research in this field.

For 20 years, erythropoietin (EPO) has been used widely for the treatment of anemia associated with chronic kidney disease and cancer chemotherapy (5). EPO binds and activates a preformed receptor homodimer (EPO-R) to regulate the survival, proliferation, and differentiation of erythroid progenitor cells. There is now good evidence that the effects of EPO go beyond erythropoiesis: EPO exerts potent antiapoptotic, antioxidant and angiogenic effects in animal models

of ischemia-reperfusion injury (brain, kidney and liver) and local or systemic inflammation (6–8). The therapeutic use of high doses of EPO is, however, associated with side effects, including hypertension (9) and thrombosis (10) which limit its clinical use.

In a recent prospective, randomized, placebo-controlled trial that enrolled 1,460 critically ill patients, Corwin and colleagues reported that EPO (30,000 IU per week for 3 weeks) reduced 28-d mortality in a subset of trauma patients ($n =$ 402 treated with EPO; $n =$ 391 treated with vehicle) without affecting the number of red blood cell transfusions. The survival advantage afforded by EPO therapy in trauma patients, however, was associated with a significant increase in thrombovascular events (11). It has been proposed that the beneficial effects of EPO are secondary to the binding of EPO to a receptor complex consisting of EPO-R and the β-common receptor (CD131) subunit (12), while the above side effects are due to activation of the "classic" EPO-R homodimer. Thus, molecules that activate the proposed tissue-protective receptor without activation of EPO-R may confer tissue protection without causing significant side effects.

The proposed tissue-protective receptor exhibits a lower affinity for EPO and forms distinct molecular species in crosslinking experiments (13). CD131 also forms receptor complexes with the α receptor subunits specific for GM-CSF, IL-3 and IL-5 and has been termed the "common" receptor (14). We recently have shown that (i) the helix B of EPO has tissue-protective properties representative of the full molecule and (ii) a peptide constructed to mimic the external, aqueous surface of EPO without primary sequence similarity (pyroglutamate helix B surface peptide–pHBSP) mimics, and possibly accounts for, the protective effects of EPO in rodent models of ischemic stroke and renal ischemia reperfusion injury (IRI) (15).

This study investigates whether pHBSP attenuates organ injury/dysfunction and inflammation in rats

subjected to severe hemorrhagic shock (HS). Having documented that severe hemorrhage and resuscitation (HR) in the rat results in renal dysfunction, hepatic injury, pancreatic injury, neuromuscular injury and lung inflammation, we have investigated the effect of pHBSP when given either 30 min or 60 min after the onset of resuscitation in these animals. Subsequently, we have investigated the mechanism(s) underlying the observed beneficial effects of pHBSP in HS by investigating a number of signaling pathways which play a role in tissue injury and inflammation, including the phosphorylation of Akt on Ser[473], phosphorylation of glycogen synthase kinase-3β (GSK-3β) on Ser[9], phosphorylation of endothelial nitric oxide synthase (eNOS) on Ser[1177], activation of NF-κB (measured as nuclear translocation of p65), activation of p38 MAPK and activation of extracellular signal-regulated kinase (ERK)1/2.

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 Animals (Scientific Procedures) Act 1986 published by Her Majesty's Stationary Office and the *Guide for the Care and Use of Laboratory Animals* of the National Research Council.

Surgical Procedure and Quantification of Organ Injury/Dysfunction

This study was carried out on 54 male Wistar rats (Charles River Ltd, Margate, UK) weighing 230 to 400*g* receiving a standard diet and water *ad libitum*. Rats were anesthetized with sodium thiopentone, and anesthesia was maintained by supplementary injections (~10 mg/kg intravenously [i.v.]) of sodium thiopentone for the duration of the experiment. Blood was withdrawn via a cannula inserted in the right carotid artery to achieve a fall in mean arterial pressure (MAP) to 35 ± 5 mmHg within 10 min. From this point onwards, MAP was

maintained at 35 ± 5 mmHg for a period of 90 min, either by further withdrawal of blood during the compensation phase or by administration of Ringer Lactate i.v. during the decompensation phase. The average volume of blood withdrawn during hemorrhage was 10.13 ± 0.25 ml (n = 40, across all hemorrhaged groups). At 90 min after initiation of hemorrhage, resuscitation was performed with 20 mL/kg Ringer Lactate over a period of 10 min, and then half the shed blood mixed with 100 IU/mL heparinized saline over a period of 50 min. At the end of 1 h resuscitation, an i.v. infusion of Ringer Lactate (1.5 mL/kg/h) was started as fluid replacement and maintained throughout the experiment for a further 3 h, at which point 1.2-mL blood samples were collected via the carotid artery into S/1.3 tubes containing serum gel (Sarstedt, Numbrecht, Germany), after which the heart was removed to terminate the experiment. The samples were centrifuged (6000*g* for 3 min) to separate serum from which creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lipase and creatine kinase were measured within 24 h (IDEXX Laboratories Ltd., West Yorkshire, UK). Additionally, lung and kidney samples were taken and stored at –80°C for further analysis.

Experimental Design

Rats were allocated randomly into the following groups: (i) Sham (n = 10); (ii) Sham + pHBSP 60 min after resuscitation (n = 4); (iii) HS + saline (n = 10); (iv) HS + pHBSP 0 min after resuscitation (pHBSP 1 μg/kg, i.v. administered 0 min after the onset of resuscitation, $n = 10$); (v) HS + pHBSP 30 min after resuscitation (pHBSP 1 μg/kg, i.v. administered 30 min after the onset of resuscitation, $n = 10$); (vi) HS + pHBSP 60 min after resuscitation (pHBSP 1 μg/kg, i.v. administered 60 min after the onset of resuscitation, $n = 10$) and (vii) HS + pHBSP 90 min after resuscitation (pHBSP 1 μg/kg, i.v. administered 90 min after the onset of resuscitation, $n = 10$). The volume of saline (vehicle) administered was equal to the volume of pHBSP administered

(1 mL/kg). Sham-operated rats underwent identical surgical procedures but without hemorrhage or resuscitation.

Histology

At the end of the experiment, lung samples were excised and fixed in 10% (w/v) formalin, buffered with phosphate buffered saline (PBS, 0.01 M, pH 7.4) for 1 wk. Samples then were dehydrated using graded ethanol, embedded in paraffin wax and cut into sections using a Leica rotary microtome (thickness, 5 μm). Sections were deparaffinized with xylene, stained with Gills hematoxylin and washed. Sections then were subsequently counterstained with 1% eosin, dehydrated with ethanol and cleared with Neo-Clear (Darmstadt , Germany) before mounting using HistoMount (Atlanta, GA, USA). Sections were analyzed using a Leica DM2000 upright microscope (Wetzlar, Germany). To determine lung injury, the sections were examined blind using a grading scale (0: none, 1: rare, 2: mild, 3: moderate and 4: severe) as described by Akinci OI *et al.* (16) with minor modifications. Features examined were inflammatory cell infiltration, pulmonary congestion and thickening of the alveolar septa. A total of ten fields were evaluated randomly for each sample. The score for each group was the average score for all samples in the group.

Western Blot Analysis

Western blots were carried out as described previously (17). Three separate experiments of Western blot analysis were performed for each marker, and tissues were done separately for each Western blot experiment. Briefly, rat liver and kidney samples were homogenized and centrifuged at 4,000g for 5 min at 4°C. Supernatants were removed and centrifuged at 15,000g at 4°C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer. The suspensions were centrifuged at 15,000g for 20 min at 4°C. The resulting supernatants containing nuclear proteins were removed carefully, and protein content was determined using a
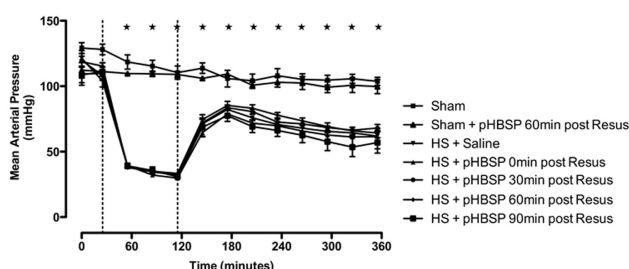

Figure 1. Effect of pHBSP on mean arterial pressure; mean arterial pressure was monitored throughout HR in rats subjected to sham operation (Sham, $n = 10$; Sham + pHBSP 60 min post after resuscitation (1 μg/kg 60 min after the onset of sham resuscitation), $n = 4$) or HS (HS + saline, $n = 10$; HS + pHBSP 0 min after resuscitation (1 μg/kg 0 min after the onset of resuscitation), $n = 5$; HS + pHBSP 30 min after resuscitation (1 μg/kg 30 min after the onset of resuscitation), $n = 10$; HS + pHBSP 60 min after resuscitation (1 μg/kg 60 min after the onset of resuscitation), $n = 10$; HS + pHBSP 90 min after resuscitation (1 μg/kg 90 min after the onset of resuscitation), $n = 5$). Data represent mean ± SEM for n observations, *$P < 0.05$ sham versus HS + saline.

bicinchoninic acid (BCA) protein assay following the manufacturer's directions. Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldenedifluoride (PVDF) membrane, which then was incubated with a primary antibody (rabbit anti-ICAM-1, rabbit anti–total GSK-3β, dilution 1:200; goat anti-pGSK-3β Ser[9] dilution 1:200; rabbit anti–total Akt dilution 1:1,000; mouse anti-pAkt Ser[473] dilution 1:1,000; rabbit anti–total eNOS dilution 1:200; goat anti-peNOS Ser[1177] dilution 1:200; rabbit anti-NF-κB p65 dilution 1:400; rabbit anti–total ERK1/2 dilution 1:2,000; mouse anti–phospho ERK1/2 dilution 1:2,000; mouse anti–phospho p38, dilution 1:1,000; rabbit anti–total p38 dilution 1:1,000). Blots then were 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 antimouse 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 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 ± SEM. This provides SEM for the sham group where a value of one is relative to the first immunoreactive band. The membranes were stripped and incubated with β-actin monoclonal antibody and, subsequently, with an antimouse 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 Company Ltd. (Poole, Dorset, UK). All stock solutions were prepared using nonpyrogenic saline (0.9% [w/v] NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK). Ringer Lactate was purchased from Baxter Healthcare Ltd. Antibodies for Western blot analysis were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg,
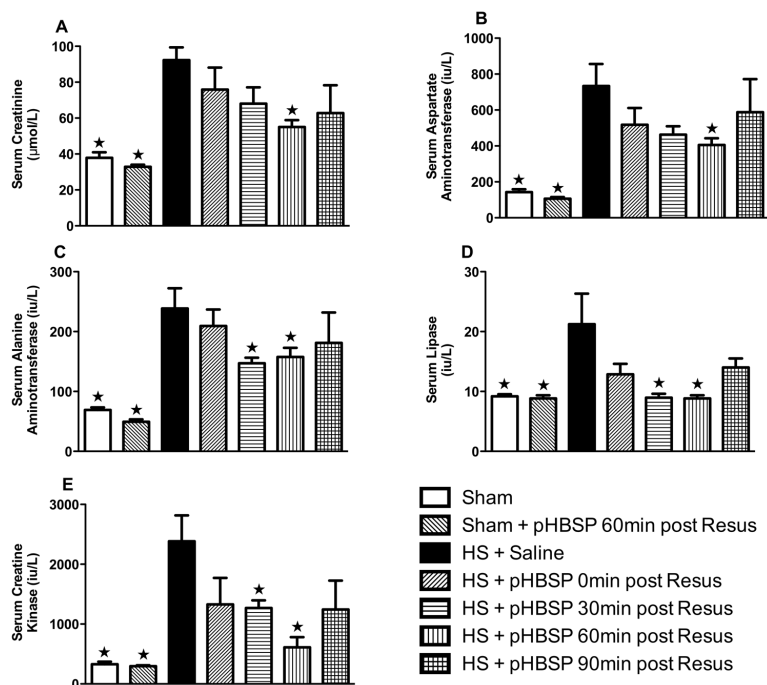
Figure 2. Effect of pHBSP on renal function (A); serum creatinine levels, liver injury (B,C); serum aspartate aminotransferase and alanine aminotransferase levels, pancreatic injury (D); serum lipase levels, and neuromuscular injury (E); serum creatine kinase levels were measured after sham operation (Sham, $n = 10$; Sham + pHBSP 60 min after resuscitation (1 μg/kg 60 min after the onset of sham resuscitation), $n = 4$) or HS (HS + saline, $n = 10$; HS + pHBSP 0 min after resuscitation (1 μg/kg 0 min after the onset of resuscitation), $n = 5$; HS + pHBSP 30 min after resuscitation (1 μg/kg 30 min after the onset of resuscitation), $n = 10$; HS + pHBSP 60 min after resuscitation (1 μg/kg 60 min after the onset of resuscitation), $n = 10$; HS + pHBSP 90 min after resuscitation (1 μg/kg 90 min after the onset of resuscitation), $n = 5$). Data represent mean ± SEM for n observations, *$P < 0.05$ HS + saline.

Germany). pHBSP was supplied by Araim Pharmaceuticals Inc. (Ossining, NY, USA).

Statistical Analysis

All values described in the text and figures are expressed as mean ± standard error of the mean (SEM) for n observations. Each data point represents biochemical measurements obtained from up to 10 separate animals. For histological scoring, each data point represents analysis of kidneys taken from four individual animals. Statistical analysis was carried out using GraphPad Prism 5.0d (GraphPad Software, San Diego, California, USA). Data without repeated measurements was assessed by one-way ANOVA followed by the Dunnett *post hoc* test. Data with repeated measurements was assessed by two-way ANOVA followed by the Bonferroni *post hoc* test. A P value of less than 0.05 was considered to be significant.

RESULTS

Effect of pHBSP on the Circulatory Failure Caused by Hemorrhagic Shock

When compared with sham-operated rats, HS rats treated with vehicle demonstrated a significant reduction in MAP during the resuscitation period (Figure 1). The administration of pHBSP at either 0, 30, 60 or 90 min after the onset of resuscitation failed to attenuate the decline in MAP caused by hemorrhage during the resuscitation phase (see Figure 1).

Effect of pHBSP on the Multiple Organ Failure Caused by Hemorrhagic Shock

When compared with sham-operated rats, HS rats treated with vehicle developed significant increases in serum creatinine (Figure 2A), AST (Figure 2B), ALT (Figure 2C), lipase (Figure 2D) and creatine kinase (Figure 2E) indicating the development of renal dysfunction, liver injury, pancreatic injury and skeletal-muscle injury. Treatment of HS rats with pHBSP (60 min into resuscitation) significantly attenuated the rises in serum creatinine (see Figure 2A), AST (see Figure 2B), ALT (see Figure 2C) and creatine kinase (see Figure 2E), and abolished the rise in serum lipase (see Figure 2D). However, treatment of HS rats with pHBSP (0 or 90 min into resuscitation) failed to significantly attenuate the rises in serum creatinine, AST, ALT, lipase and creatine kinase (Figure 2). Thus, treatment of HS rats with pHBSP as late as 60 min into resuscitation attenuated the multiple organ injury/dysfunction caused by HS.

Hemorrhage frequently is associated with acute lung injury and acute respiratory distress syndrome (ARDS) (18). When compared with sham-operated rats (Figures 3A, 4A), the lung histology of HS rats treated with vehicle confirmed inflammatory cell infiltration ($P < 0.05$, Figures 3B, D), alveolar septal thickening (Figures 3B, E) and pulmonary congestion (Figures 4B, D), suggesting development of lung injury/inflammation. Treatment of HS rats with pHBSP (60 min into resuscitation) significantly attenuated the degree of inflammatory cell infiltration (Figures 3C, D) and alveolar septal thickening (Figures 3C, E) and reduced pulmonary congestion (Figures 4C, D). To confirm this further, when compared with sham-operated rats (Figure 3F), HS rats treated with vehicle demonstrated a significant increase in the expression of lung ICAM-1 protein (see Figure 3F). Treatment of HS rats with pHBSP (60 min into resuscitation) significantly attenuated the increase in the expression of ICAM-1 caused by HS (see Figure 3F).

Figure legend:
☐ Sham
■ HS + Saline
▥ HS + pHBSP 60min post Resus
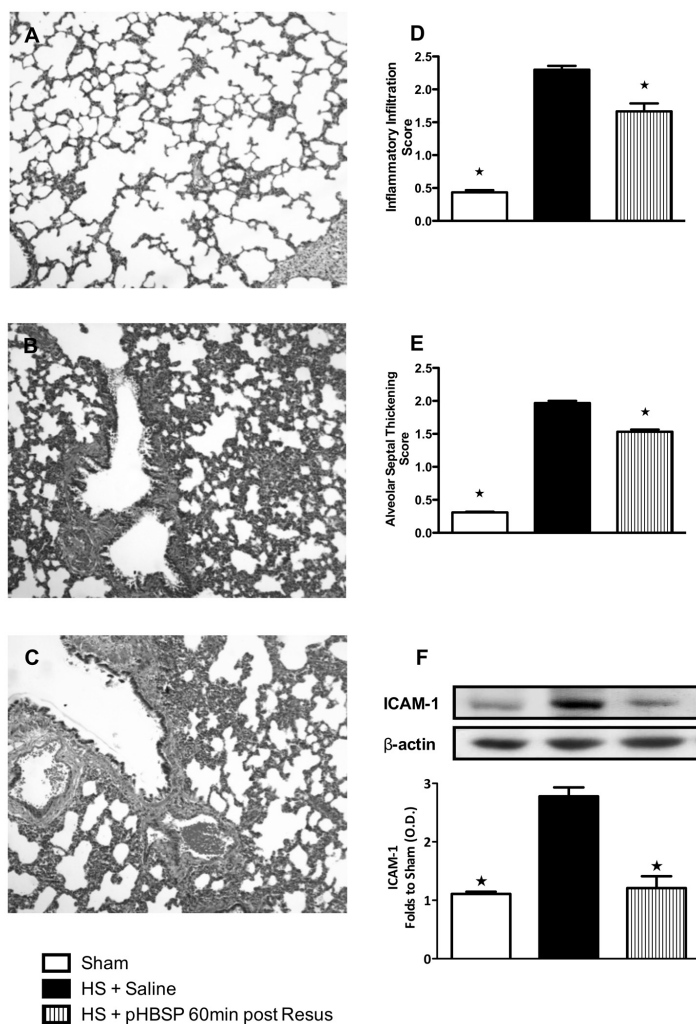
Figure 3. Effect of pHBSP on lung injury and inflammation. Lung histological sections taken from a sham-operated rat with inflammatory cell infiltration grade 1 and no observable alveolar septa thickening (A); a rat subjected to HS with inflammatory cell infiltration grade 3 and widespread alveolar septa thickening (B); and a rat subjected to HS and administered 1 μg/kg pHBSP 60 min after the onset of resuscitation with inflammatory cell infiltration grade 2 and focal alveolar septa thickening (C). Hematoxylin and eosin, magnification × 100, Figures are representative of at least three experiments performed on different days. Effect of pHBSP on lung inflammatory cell infiltration (D), alveolar septal thickening (E) and ICAM-1 expression, corrected for the corresponding β-actin content and normalized using the related sham band (F), after sham operation (Sham, $n = 3$) or HS (HS + saline, $n = 3$; HS + pHBSP 60 min after resuscitation (1 μg/kg 60 min after the onset of resuscitation), $n = 3$). Data represent mean ± SEM for n observations, *$P < 0.05$ HS + saline.

Effect of pHBSP on the Phosphorylation of Akt, GSK-3β and eNOS in the Livers and Kidneys of Rats that Underwent Hemorrhage and Resuscitation

To gain a better insight into the potential mechanism(s) underlying the observed beneficial effects of pHBSP, we investigated the effects of this peptide on cell signaling pathways known to confer tissue protection or to inhibit inflammation in liver and kidney. When compared with sham-operated rats, HS rats treated with vehicle developed significant decreases in the phosphorylation of Akt on Ser[473] and GSK-3β on Ser[9] in liver and kidney (Figures 5A–D). Treatment of HS rats with pHBSP attenuated (30 or 60 min into resuscitation) the decline in the phosphorylation of Akt and GSK-3β caused by HR in both liver and kidney (see Figures 5A–D). The degree of the phosphorylation of eNOS on Ser[1177] was similar in sham-operated rats and HS rats treated with vehicle indicating that HR alone is not sufficient to affect eNOS phosphorylation (Figures 5E, F). Treatment of HS rats with pHBSP (60 min in kidney and 30 and 60 min into resuscitation in the liver) resulted in a significant increase in the phosphorylation of eNOS when compared with HS rats treated with vehicle alone (see Figures 5E, F).

Effect of pHBSP on the Nuclear Translocation of the p65 NF-κB Subunit in the Livers and Kidneys of Rats that Underwent Hemorrhage and Resuscitation

When compared with sham-operated rats, HS rats treated with vehicle developed significant increases in the nuclear translocation of the p65 NF-κB subunit in both liver and kidney indicating the activation of NF-κB (Figure 6). Treatment of HS rats with pHBSP (60 min into resuscitation) resulted in a significant reduction in nuclear translocation of p65 and, hence, the activation of NF-κB in both liver and kidney (see Figure 6).

Effect of pHBSP on the Phosphorylation of p38 in the Livers and Kidneys of Rats that Underwent Hemorrhage and Resuscitation

When compared with sham-operated rats, HS rats treated with vehicle exhibited significant increases in the phosphorylation of p38 in liver and kidney (Figures 7A, B). Treatment of HS rats with pHBSP did not affect p38 phosphorylation in the kidney (see Figures 7A, B). Treatment of HS rats with pHBSP (30 or 60 min into resuscitation) did, however, attenuate the degree of p38 phosphorylation in the liver significantly (see Figures 7A, B).
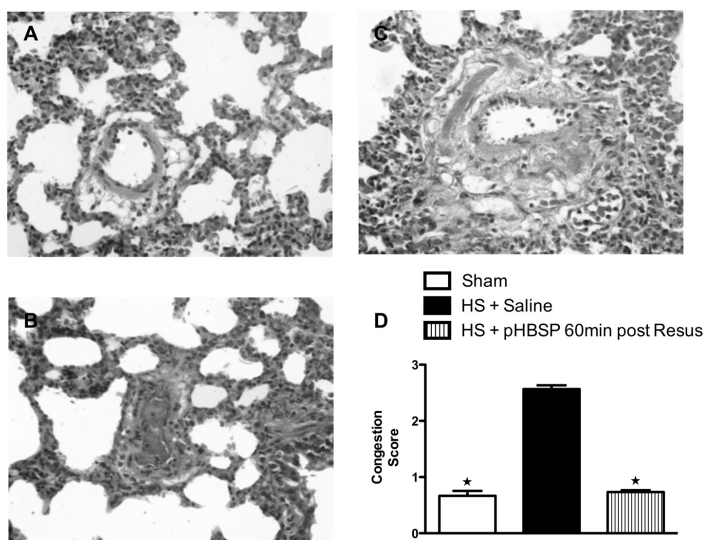
Figure 4. Effect of pHBSP on pulmonary congestion. Lung histological sections taken from a sham-operated rat with congestion grade 0 (A); a rat subjected to HS with congestion grade 3 (B); and a rat subjected to HS and administered 1 μg/kg pHBSP 60 min after the onset of resuscitation with congestion grade 0 (C). Hematoxylin and eosin, magnification 400×, figures are representative of at least three experiments performed on different days. Effect of pHBSP on pulmonary congestion (D) after sham operation (Sham, $n = 3$) or HS (HS + saline, $n = 3$; HS + pHBSP 60 min after resuscitation (1 μg/kg 60 min after the onset of resuscitation), $n = 3$). Data represent mean ± SEM for n observations, *$P < 0.05$ HS + saline.

Effect of pHBSP on the Phosphorylation of ERK1/2 in the Livers and Kidneys of Rats that Underwent Hemorrhage and Resuscitation

When compared with sham-operated rats, HS rats treated with vehicle developed significant increases in the phosphorylation of ERK1/2 in the liver and kidney (Figures 7C, D). Treatment of HS rats with pHBSP (30 or 60 min into resuscitation) attenuated the increase in ERK phosphorylation caused by hemorrhage and resuscitation in both organs (see Figures 7C, D).

DISCUSSION

There is now substantial evidence that EPO protects organs and tissues against a number of noxious stimuli including ischemia (6). In 2004, we discovered that the administration of EPO (300 IU/kg) on resuscitation reduces the organ injury/dysfunction associated with severe trauma hemorrhage by antiapoptotic and antiinflammatory mechanisms

(19). Although EPO also improved survival in critically ill patients with trauma, this came at the expense of a significant increase in thrombotic events (11). The recent discovery of a nonerythropoietic, tissue-protective peptide mimicking the 3D structure of EPO (pHBSP) allowed us to test the hypothesis that the tissue-protective effects of EPO can be obtained in trauma hemorrhage in the absence of significant side effects. We report here that a single injection of pHBSP given intravenously to rats after the onset of resuscitation attenuated the renal dysfunction, liver injury, pancreatic injury and neuromuscular injury caused by severe HR in the anesthetized rat. There is evidence that EPO attenuates the degree of lung inflammation and injury caused by hyperoxic injury (20), ischemia-reperfusion (21) and acute necrotizing pancreatitis (22). We report here that pHBSP attenuated the degree of inflammatory cell infiltration, alveolar septal thickening and congestion

in the lungs of rats subjected to HR. Thus, similar to EPO, pHBSP exerts tissue-protective and antiinflammatory effects *in vivo*.

What, then, is the mechanism(s) by which pHBSP exerts these beneficial effects? Clearly, pHBSP affected neither the metabolic acidosis nor the hemodynamic abnormalities caused by HR. There is some evidence that the beneficial effects of EPO are secondary to the activation of the survival kinase Akt (23,24). Akt is a member of the phosphoinositide 3-kinase signal transduction enzyme family, which regulates cellular activation, inflammatory responses, chemotaxis and apoptosis (25). When phosphorylated by its upstream regulator, phosphoinositide-dependent kinase, Akt modulates cell survival and growth (25). We report here that HR results in a significant reduction in the phosphorylation of Akt in both the liver and kidney. A reduction in the activation of this important survival pathway will make organs more susceptive to injury and inflammation (26,27). Most notably, pHBSP restored the degree of Akt phosphorylation to the level seen in sham-operated animals even when the EPO-mimetic was given as late as 60 min into resuscitation. Both EPO and pHBSP also enhance the phosphorylation of Akt in cardiomyocytes subjected to TNF-α (28).

GSK-3β is a serine-threonine kinase that originally was 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 inactivates it by causing Ser[9] phosphorylation (29,30). Consistent with decline in the phosphorylation/activation of Akt reported here, HR also caused a significant decline in the phosphorylation of GSK-3β on Ser[9]. This indicates an excessive activation of GSK-3β which would drive both inflammation (31) and tissue-injury (32). Similar to the above-reported effects on Akt phosphorylation, pHBSP restored the degree of Ser[9] phosphorylation on GSK-3β
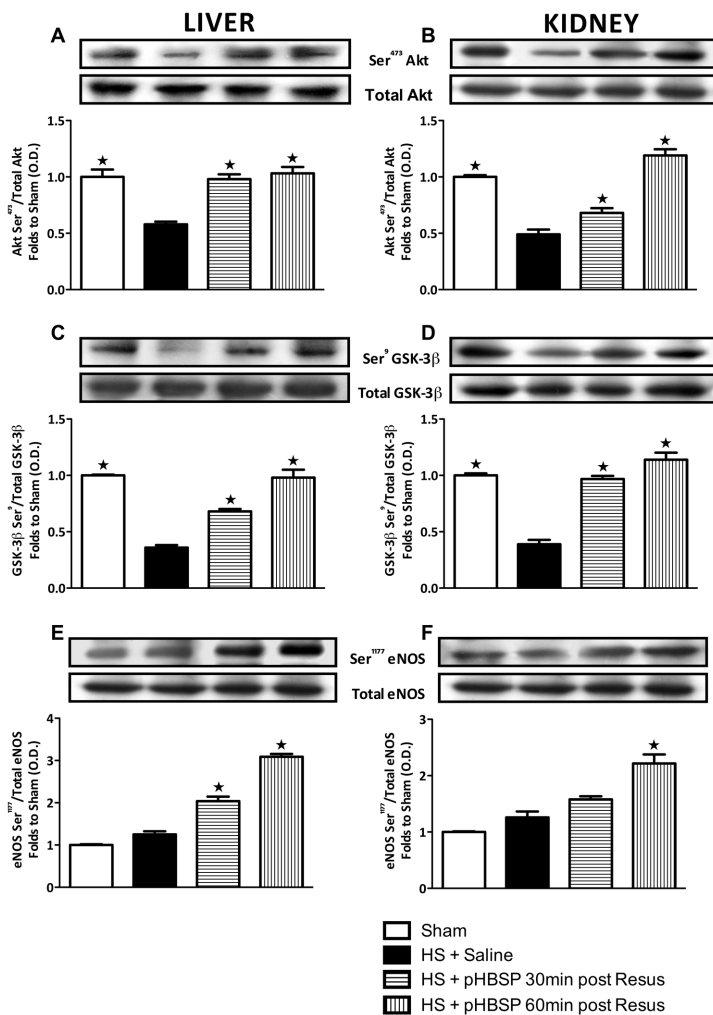
Figure 5. Effect of pHBSP on the phosphorylation of Ser[473] on Akt in the liver (A) and kidney (B), Ser[9] on GSK-3β in the liver (C) and kidney (D), and Ser[1177] on eNOS in the liver (E) and kidney (F) after sham operation (Sham, $n = 4$) or HS (HS + saline, $n = 4$; HS + pHBSP 30 min after resuscitation (1 μg/kg 30 min after the onset of resuscitation), $n = 4$; HS + pHBSP 60 min after resuscitation (1 μg/kg 60 min after the onset of resuscitation), $n = 4$). Data represent mean ± SEM for n observations, *$P < 0.05$ HS + saline.
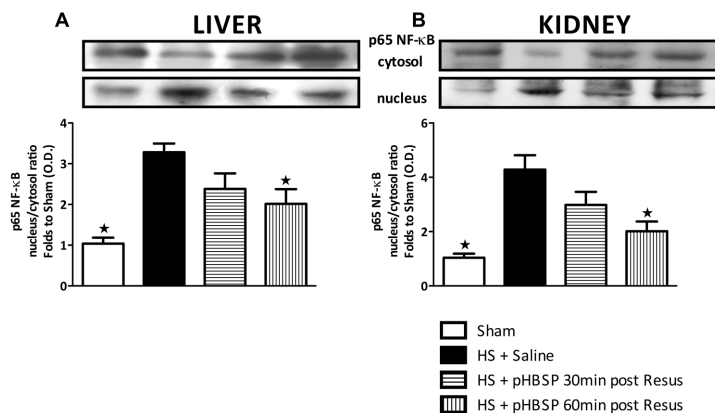
sites for GSK-3β on the NF-κB subunit p65. Most notably, pretreatment with a number of chemically distinct inhibitors of GSK-3β attenuates organ injury and dysfunction caused by hemorrhage and resuscitation and endotoxemia (31,33). This protective effect was associated with inhibition of the activation 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 also may inhibit the activation of NF-κB by phosphorylating and degrading IκBα, which is required to prevent NF-κB translocation (37). We report here that HR results in a significant increase in the activation of NF-κB (measured here as nuclear translocation of p65) which was attenuated when pHBSP was given 60 min into resuscitation. All of the above findings support the view that pHBSP restores 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 nitric oxide (NO). In our study, HR did not affect eNOS phosphorylation on Ser[1177]. Administration at 60 min into resuscitation of pHBSP, however, caused a pronounced increase in eNOS phosphorylation and, hence, activity. In conditions associated with ischemia-reperfusion injury, activation of eNOS is beneficial as the enhanced formation of NO causes local vasodilation, inhibits adhesion of platelets and neutrophils and regulates angiogenesis (41). There is good evidence that agents which release NO or enhance the formation of endogenous NO attenuate organ injury/dysfunction in HS (42). Agents that also inhibit the formation of NO from inducible NOS (iNOS) also attenuate the multiple organ failure associated with HS (43). Inhibition of eNOS activity also attenuates the cardioprotective effects of EPO (44). Recently, Su *et al*. (45) have demonstrated that CD131, when activated by EPO, is able to activate Akt

to the levels seen in sham-operated animals even when the EPO-mimetic was given as late as 60 min into resuscitation. An increase in Ser[9] phosphorylation results in inhibition of this kinase and inhibitors of GSK-3β exert potent antiinflammatory (31,33) and antiischemic effects in a number of organs (32,34,35). Interestingly, inhibition of GSK-3β also mediates the cardioprotective effects of EPO (32).

Downstream of GSK-3β, several studies have now reported an association between GSK-3β and NF-κB activity *in vitro*

(36,37) and *in vivo* (31,38). NF-κB is a transcriptional factor that plays an important role in regulating the transcription of a number of genes, especially those involved in producing mediators involved in local and systemic inflammation, such as cytokines, chemokines, cell adhesion molecules, apoptotic factors and other mediators (39). Treatment of TNF-α stimulated hepatocytes with a specific GSK-3β inhibitor resulted in a decrease of the NF-κB–dependent gene transcription (40). This study also indicated four potential phosphorylation

Figure 6. Effect of pHBSP on NF-κB activation in the liver (A) and kidney (B) after sham operation (Sham, *n* = 4) or HS (HS + saline, *n* = 4; HS + pHBSP 30 min after resuscitation (1 µg/kg 30 min after the onset of resuscitation), *n* = 4; HS + pHBSP 60 min after resuscitation (1 µg/kg 60 min after the onset of resuscitation), *n* = 4). Data represent mean ± SEM for *n* observations, *$P < 0.05$ HS + saline.
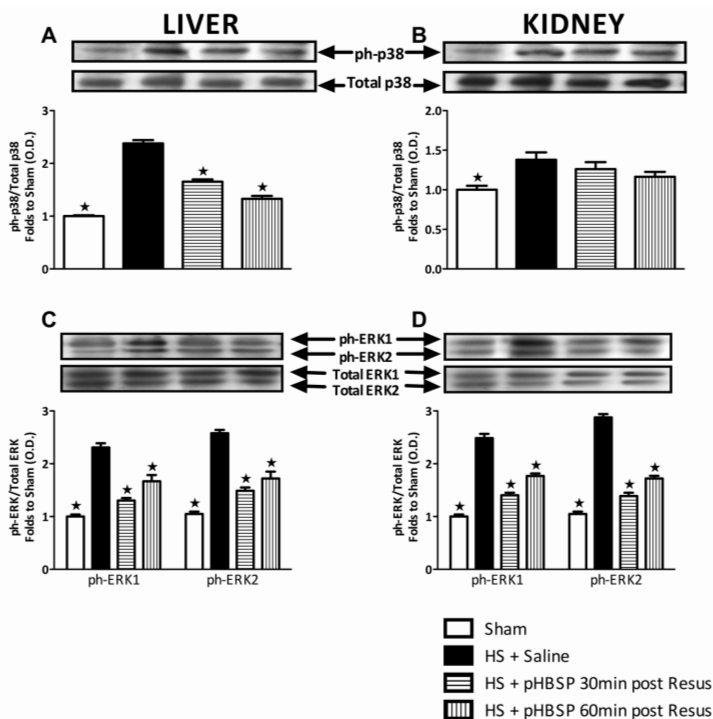
Figure 7. Effect of pHBSP on the phosphorylation of p38 in the liver (A) and kidney (B), and ERK-1/2 in the liver (C) and kidney (D) after sham operation (Sham, *n* = 4) or HS (HS + saline, *n* = 4; HS + pHBSP 30 min after resuscitation (1 µg/kg 30 min after the onset of resuscitation), *n* = 4; HS + pHBSP 60 min after resuscitation (1 µg/kg 60 min after the onset of resuscitation), *n* = 4). Data represent mean ± SEM for *n* observations, *$P < 0.05$ HS + saline.

and, in turn, increases the interaction between CD131 and eNOS. This increase in interaction results in the activation of eNOS and production of NO (45). Thus,

activation of eNOS (possibly secondary to activation of Akt) may contribute to the beneficial effects of pHBSP reported here.

Activation of p38 MAPK promotes cellular stress responses such as proliferation, differentiation and production of proinflammatory cytokines and occurs in response to ischemia and hemorrhage in a number of organs (46,47). HR results in the activation of p38 MAPK (in male animals), while inhibition of the activity of this p38 MAPK in hemorrhagic shock attenuates renal, cardiac and lung injury (48–50). We report here that HR results in a moderate activation of p38 MAPK (even when measured at 4 h after onset of resuscitation) in the kidney and a more marked activation in the liver, the latter of which was attenuated by pHBSP when given either at 30 or 60 min into resuscitation. Our data are consistent with the hypothesis that prevention of the activation of p38 MAPK contributes to the observed beneficial effects of pHBSP, at least, in the liver. It is well documented that activation of p38 peaks 1 h after hemorrhage in the kidney and thereafter gradually declines. Thus, it is possible that a more marked activation of p38 MAPK occurs early after hemorrhage which may have been attenuated by pHBSP.

Like ischemia, severe HR results in the activation of ERK1/2 and drugs that prevent the activation of ERK1/2 in HS exert beneficial effects (51). We report here that the activation of both ERK1/2 caused by HR is attenuated by pHBSP. Like trauma hemorrhage, traumatic brain injury results in a significant activation of ERK1/2 which drives brain edema. Interestingly, EPO attenuates brain edema in this model by preventing the activation of ERK1/2 (52). Similarly, the beneficial effects of EPO in a rat model of spinal cord injury have been attributed to the ability of EPO to reduce the phosphorylation of ERK (53).

In conclusion, we have discovered that the nonhematopoietic EPO-analogue pHBSP attenuates the multiple organ injury and dysfunction when given during resuscitation of rats subjected to severe hemorrhage. It should be noted that pHBSP was still effective when given as late as 60 min after the onset of resuscita-

tion. Surprisingly, some of the effects (on outcome or signaling) observed with pHBSP were more pronounced when the peptide was given at 60 min, rather than 30 min, into the resuscitation period. We have reported previously that the efficacy of pHBSP increased when given as late as 6 hours after the onset of reperfusion of the previously ischemic kidney (15). We do not fully understand this phenomenon, but it is possible that the receptor targeted by pHBSP (and EPO) is upregulated in response to injury, leading to a more pronounced response after binding of the ligand. In addition, pharmacokinetic studies have confirmed that pHBSP has a very short plasma half life of ~2 min in the rat (15) suggesting that an agent present within the circulation for only a short time after i.v. dosing elicits protective effects equivalent to EPO or CEPO with plasma half lives of 4–6 hours. Whatever the mechanism, this finding is likely to be of therapeutic relevance, as it allows a late intervention in HS and other conditions associated with ischemia-reperfusion. This could be of great importance when aiming to extend the "golden hour" after a major insult.

The acute model used here necessitates the use of heparin to prevent the formation of clots in the catheters used to extract the blood as well as the hemorrhaged blood. It has been known for some time that heparin does have potential therapeutic applications beyond anticoagulation (54) and these should be considered when evaluating data from this model. However, the model used here demonstrates significant multiple organ failure (MOF) compared with animals subjected to sham operation, suggesting that the dose of heparin used here to prevent coagulation does not produce any observable beneficial effects.

To gain a better insight into the signaling pathways involved in the tissue-protective and/or antiinflammatory effects of pHBSP, we have investigated the effects of pHBSP on signaling pathways known to play a role in tissue injury/survival and/or inflammation. As the time-course in the activation of these signaling cascades may vary between tissues, we have carried out these mechanistic studies in both liver and kidney, two organs that were markedly protected by pHBSP, in the hope to find common signaling events that contribute to the observed beneficial effects of pHBSP *in vivo*. In both liver and kidney, HR resulted in a significant inactivation of the survival kinase Akt (measured as reduction in the phosphorylation on Ser^{473}). Treatment of rats with pHBSP 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 very good evidence that therapeutic strategies which enhance the activation of Akt and reduce the activation of GSK-3β enhance the resistance of organs to noxious stimuli (including ischemia) and reduce inflammation via inhibition of NF-κB (37). Activation of Akt by pHBSP also resulted in activation of eNOS (measured as phosphorylation on Ser^{1177}) which should result in an enhanced formation of NO in the microcirculation. In addition, pHBSP attenuated the HR-induced activation of p38 MAPK and ERK1/2, both of which are known to contribute to the development of organ injury/inflammation in HS (49,51). We propose that all of the above signaling events initiated by pHBSP contribute to the beneficial effects of this nonhematopoietic EPO analogue in HS. As the beneficial effects of EPO in patients with trauma are limited by side effects owing to excessive erythropoiesis, we speculate that nonhematopoietic analogues of EPO such as pHBSP may be useful to mimic the tissue-protective effects of EPO without causing the well documented side effects.

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DISCLOSURE

M Brines was an employee of Araim Pharmaceuticals until July 1, 2009 and currently holds stocks/shares in the company. A Cerami is the CEO of Araim Pharmaceuticals and currently holds stocks/shares in the company.

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