

# Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin

Michael Brines<sup>\*†‡</sup>, Nimesh S. A. Patel<sup>§</sup>, Pia Villa<sup>¶</sup>, Courtenay Brines<sup>\*</sup>, Tiziana Mennini<sup>¶</sup>, Massimiliano De Paola<sup>¶</sup>, Zubeyde Erbayraktar<sup>\*\*</sup>, Serhat Erbayraktar<sup>\*\*</sup>, Bruno Sepodes<sup>††</sup>, Christoph Thiemermann<sup>§</sup>, Pietro Ghezzi<sup>¶</sup>, Michael Yamin<sup>\*</sup>, Carla C. Hand<sup>†</sup>, Qiao-wen Xie<sup>\*†</sup>, Thomas Coleman<sup>\*†‡‡</sup>, and Anthony Cerami<sup>\*†‡</sup>

<sup>\*</sup>Warren Pharmaceuticals, Ossining, NY 10562; <sup>†</sup>The Kenneth S. Warren Institute, Ossining, NY 10562; <sup>§</sup>Centre for Translational Medicine and Therapeutics, William Harvey Research Institute, Barts and London School of Medicine and Dentistry, Queen Mary, University of London, London EC1M 6BQ, England; <sup>¶</sup>Mario Negri Institute for Pharmacological Research, 20-20156 Milan, Italy; <sup>\*\*</sup>Dokuz Eylül University, Izmir 35340, Turkey; <sup>††</sup>Faculty of Pharmacy, University of Lisbon, 1600 Lisbon, Portugal; and <sup>¶</sup>National Research Council, Institute of Neuroscience, 20129 Milan, Italy

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Erythropoietin (EPO), a member of the type 1 cytokine superfamily, plays a critical hormonal role regulating erythrocyte production as well as a paracrine/autocrine role in which locally produced EPO protects a wide variety of tissues from diverse injuries. Significantly, these functions are mediated by distinct receptors: hematopoiesis via the EPO receptor homodimer and tissue protection via a heterocomplex composed of the EPO receptor and CD131, the  $\beta$  common receptor. In the present work, we have delimited tissue-protective domains within EPO to short peptide sequences. We demonstrate that helix B (amino acid residues 58–82) of EPO, which faces the aqueous medium when EPO is bound to the receptor homodimer, is both neuroprotective *in vitro* and tissue protective *in vivo* in a variety of models, including ischemic stroke, diabetes-induced retinal edema, and peripheral nerve trauma. Remarkably, an 11-aa peptide composed of adjacent amino acids forming the aqueous face of helix B is also tissue protective, as confirmed by its therapeutic benefit in models of ischemic stroke and renal ischemia–reperfusion. Further, this peptide simulating the aqueous surface of helix B also exhibits EPO's trophic effects by accelerating wound healing and augmenting cognitive function in rodents. As anticipated, neither helix B nor the 11-aa peptide is erythropoietic *in vitro* or *in vivo*. Thus, the tissue-protective activities of EPO are mimicked by small, nonerythropoietic peptides that simulate a portion of EPO's three-dimensional structure.

cognition | cytoprotection | excitotoxicity | ischemia–reperfusion injury | wound healing

In its hormonal role, the cytokine erythropoietin (EPO) is released by the kidney into the circulation in response to hypoxia and binds to a preformed receptor homodimer (EPOR)<sub>2</sub> present on the cell membrane of erythrocytic progenitors. Subsequently, a molecular cascade begins with the phosphorylation of Janus tyrosine kinase 2 and ultimately results in inhibition of programmed cell death, fostering the survival and maturation of erythroid precursors to erythrocytes [reviewed by Fisher (1)]. However, over the last 15 years, it has been discovered that EPO is also synthesized locally by many tissues, especially in response to metabolic stress. This pool of EPO acts as a multifunctional protective molecule [reviewed by Brines and Cerami (2)]. In this paracrine/autocrine role, EPO inhibits apoptosis in a wide variety of cell types and activates multiple mechanisms to protect stressed tissues, e.g., reducing inflammation and local edema. EPO also plays crucial roles during development (3). Therefore, it is not surprising that in the adult organism, EPO mediates multiple trophic effects, leading to accelerated healing and tissue regeneration. Finally, EPO has been shown to enhance cognition in normal (4) as well as diseased (5) human subjects.

The molecular interaction of EPO with the erythropoietic receptor (EPOR)<sub>2</sub> has been studied intensively, such that the regions of EPO that interact with (EPOR)<sub>2</sub> have been identified

(Fig. 1A). These include portions of helices A and C (site 2), as well as helix D and the loop connecting helices A and B (site 1) (6–9). Chemical or mutational modifications of amino acid residues within these two regions of EPO abolish its binding to (EPOR)<sub>2</sub> and, therefore, these modified EPOs are not erythropoietic *in vivo* or *in vitro*. Remarkably, a number of these modified EPOs retain potent tissue-protective properties (10). Clearly, sites 1 and 2, which are essential for erythropoiesis, are not required for tissue protection.

These observations suggest that an additional receptor for EPO mediates tissue protection. This receptor is pharmacologically distinct from that of erythropoiesis, because it exhibits a lower affinity for EPO and forms distinct molecular species in cross-linking experiments (11). In prior studies, we have provided evidence that the receptor that promotes tissue protection is a heteromer composed of EPOR and CD131, the  $\beta$  common receptor ( $\beta$ cR) (12). CD131 also forms receptor complexes with the  $\alpha$  receptor subunits specific for GM-CSF, IL-3, and IL-5 and has been termed the “common” receptor [reviewed by Murphy and Young (13)].

Results from experiments showing that chemical modification of lysine residues or amino acid substitutions made within sites 1 and/or 2 do not affect tissue protection suggest that other regions of EPO contain the recognition site for the tissue-protective receptor. Notably, in aqueous media, EPO's tertiary structure is relatively well defined because of the interaction of the hydrophobic content of its four  $\alpha$ -helices, constraining the molecule into a compact, relatively rigid, globular structure. When EPO is bound to the hematopoietic receptor (14), helix B and parts of the AB and CD loops face the aqueous medium, away from the homodimer binding sites [Protein Data Bank (PDB) ID code 1EER; Fig. 1]. These regions do not contain lysine and therefore are not modified by carbamylation of EPO, a procedure that produces a selectively tissue-protective compound (10). In view of these observations, we hypothesized that tissue protection, as distinct from erythropoiesis, depends on a region within helix B and/or loop AB within the EPO molecule.

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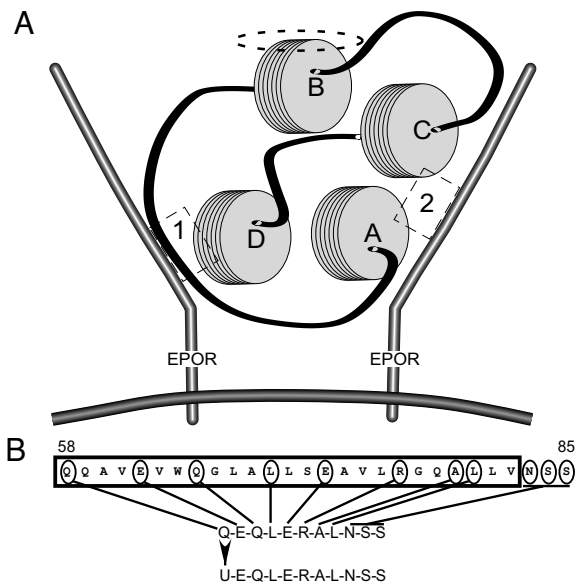
Conflict of interest statement: M.B., C.B., M.Y., Q.-w.X., T.C., and A.C. were employees of Warren Pharmaceuticals when this work was performed. Warren Pharmaceuticals is developing erythropoietin analogues and tissue-protective compounds for potential clinical uses.

<sup>†</sup>To whom correspondence may be addressed: mbrines@warrenpharma.com or acerami@warrenpharma.com.

<sup>††</sup>Present address: Feinstein Institute for Medical Research, Manhasset, NY 11030.

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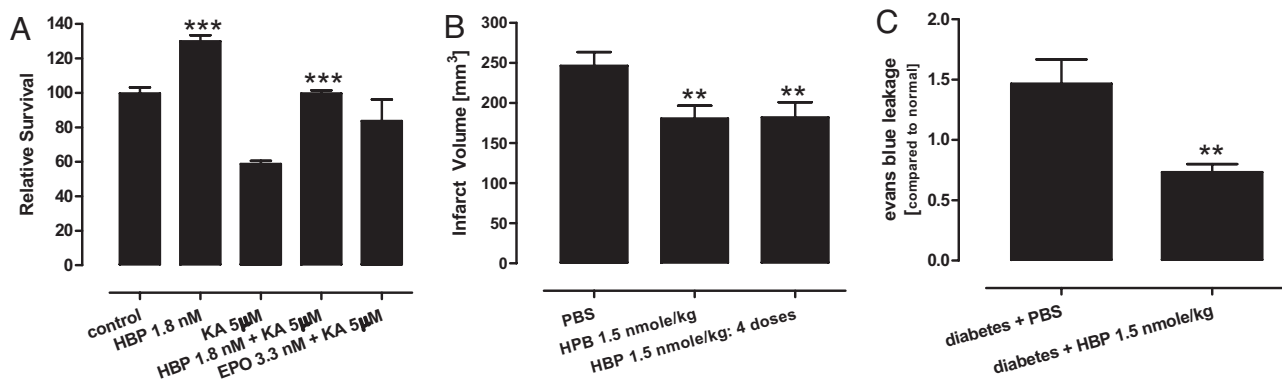
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**Fig. 1.** Structure of EPO indicating tissue protective domains and sequences. (A) Schematized drawing of EPO bound to the hematopoietic receptor dimer (EPOR)<sub>2</sub>, shown inserted into the plasma membrane. Helices A–D associate via hydrophobic interactions to form a compact, globular structure. Sites 1 and 2 (indicated by dashed boxes) within the topography of the EPO molecule bind with high affinity to each EPOR monomer. The aqueous face of helix B faces away from the interior of the receptor, as indicated by the dashed ellipse. (B) Helix B peptide (HBP) was synthesized as the linear sequence of helix B (amino acids 58–82; boxed region; single-letter code). Circled residues show those amino acid residues on the aqueous face of the 4-3  $\alpha$ -helix B. Leucine in position 80 is also on the aqueous face. A linear peptide comprising only these residues was synthesized as HBSP. Residues 83–85 are relatively rigidly oriented because of the associated helices. Glutamine in the N-terminal position can spontaneously undergo cyclization into pyroglutamate (U), forming pH-BSP (bottom peptide).

## Results and Discussion

Based on data available from PDB ID code 1EER, we began the study by synthesizing helix B peptide (HBP; residues 58–82). As expected, this molecule, like carbamylated EPO [CEPO (10)], was not erythropoietic in the UT-7 EPO cell assay or *in vivo* (see supporting information (SI) *Materials and Methods* and Fig. S1).



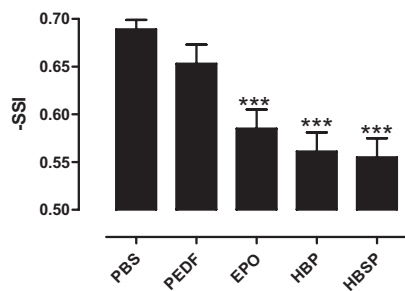
**Fig. 2.** Helix B peptide (HBP) is tissue protective *in vitro* and *in vivo*. (A) HBP protects against kainic acid (KA)-induced motoneuron excitotoxic death *in vitro*. Mixed anterior horn cultures obtained from the ventral horn of the spinal cord of 14-day rat embryos were treated on the sixth day *in vitro* by incubation for 48 h with kainic acid (5  $\mu$ M) alone or in cotreatment with EPO or HBP. Data are means and SEM; \*\*\*,  $P < 0.001$  compared with kainic acid alone. (B) HBP is neuroprotective in a stroke model. A single dose of HBP administered i.v. immediately after a 1-h arterial occlusion significantly reduces infarct volume determined after 24 h. Additional doses of HBP do not improve protection. Data are means and SEM; \*\*,  $P < 0.01$  compared with saline. (C) HBP ( $n = 16$ ) prevents the development of retinal edema in a rat model of diabetes (PBS;  $n = 14$ ). Results of three experiments are shown. Evans blue extravasation in normal retinas ( $n = 12$ ) was  $6 \pm 0.2$  ng/mg of dry retina. Data are means and SEM; \*\*,  $P < 0.01$ .

However, HBP possessed potent neuroprotective activity comparable to EPO (Fig. 2A) and to CEPO (15) in a rat motoneuron model *in vitro*. In this model, the neurotoxic effects of the glutamate receptor agonist kainic acid were blocked by either HBP (1.8 nM) or EPO (3.3 nM). Because HBP is small and does not contain features designed to resist proteolysis or decrease clearance, its plasma half-life is presumably very short. It was therefore of great interest to determine whether HBP exhibited protective properties *in vivo*.

HBP was protective in a rat model of middle cerebral artery occlusion that has previously shown large protective effects for EPO (16), asialo-EPO (17), and CEPO (10) (Fig. 2B). In this experiment, HBP administered as a single i.v. dose [1.5 nmol/kg of body weight (bw)] reduced infarct volume as determined by tetrazolium salt staining 24 h after reperfusion. HBP was also associated with an improved behavioral outcome (foot faults of the saline group  $24.6 \pm 2$  versus  $16.4 \pm 2$  in the single dose HBP group and  $14.5 \pm 0.9$  in the four-dose HBP group;  $P < 0.05$  between saline and treated groups). Notably, additional doses of HBP administered i.p. at 2-h intervals for three additional doses did not further improve the extent of neuroprotection.

Confirming that HBP was neuroprotective *in vitro* and *in vivo*, we then assessed whether HBP possessed other properties consistent with EPO's nonerythropoietic activities. For example, EPO reduces injury-related local edema in a number of tissues (18–21), including the retina (22). Specifically, in models of diabetic retinopathy, hyperglycemia produces endothelial injury, leading to vascular leakage and retinal edema. Further, it is notable that in a small, retrospective study of diabetic patients with macular edema, EPO treatment was associated with an increase in visual acuity and a decrease in retinal exudates (23). To determine whether HBP could inhibit diabetes-related retinal edema, rats were administered streptozotocin. After the confirmation of the diabetic state, HBP (1.5 nmol/kg of bw) or saline was administered i.p. 5 days each week. After 3 weeks of hyperglycemia, retinal leakage in the HBP group (as assessed by extravasation of Evans blue dye) was not different from animals without diabetes (Fig. 2C). In contrast, retinas from animals that received only saline exhibited significant edema.

The results of these experiments showed 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. Previous study has shown that peptides can



**Fig. 3.** HBSP is equipotent to HBP and EPO in a sciatic nerve injury model. PEDF, pigment epithelium-derived factor. Compounds were administered at a dose of 0.3 nmol/kg of bw i.v. immediately after a 1-min compression of the sciatic nerve at the level of the mid-thigh. Data are means and SEM plotted as the negative of the static sciatic index (SSI).  $n = 6-8$  for each group; \*\*\*,  $P < 0.001$  compared with PBS.

be synthesized to mimic the helical structure of a protein that interacts with its receptor to reproduce the biological activities of the full molecule (24). Upon further consideration, however, we reasoned that because helix B is amphipathic and of the 4-3  $\alpha$ -helix type, specific amino acid residues within the hydrophilic portion face the external, aqueous face (i.e., every fourth and third residue in the b and f position, respectively). Spatially (but not linearly) adjacent residues therein could constitute a recognition site for the tissue-protective receptor.

Data obtained from crystallographic studies of EPO bound to (EPOR)<sub>2</sub> show that the aqueous face of helix B consists of amino acids QEQLERAL (PDB ID code 1EER; Fig. 1B). Thus, a peptide derived from surface-simulation analysis of EPO should possess the biological activities of helix B. To test this hypothesis, a peptide was synthesized to include these surface amino acids as well as the three residues within the proximal portion of the BC loop that are relatively constrained by the rigid structure of the associated helices. The resulting 11-mer helix B surface peptide (HBSP: QEQLERALNSS), unrelated in primary sequence to EPO, was thus intended to mimic a particular feature of EPO's three-dimensional structure, notwithstanding the possible steric constraints of spatially but not linearly adjacent residues bonded directly together. We subsequently assessed whether this peptide was a nonerythropoietic, tissue-protective molecule.

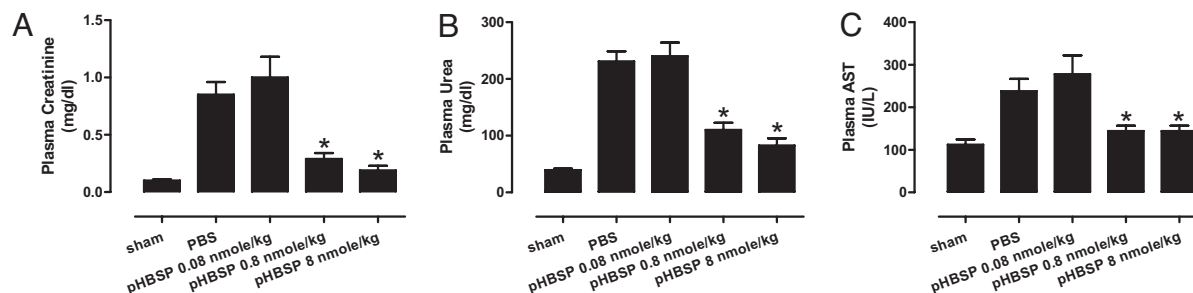
HBSP, which does not contain site 1 or 2, was, as predicted, not erythropoietic *in vitro* (UT7-EPO cells) or *in vivo* in the rat (data not shown). However, HBSP was highly active in reducing the degree of injury observed in a sciatic nerve crush injury model to a degree identical on a molar basis to EPO and chemical derivatives of EPO that are not erythropoietic (10, 17). In this model, the sciatic nerve was reversibly compressed by

using a ligature for a duration of 1 min and single doses of HBP or HBSP (0.3 nmol/kg of bw) administered i.v. immediately after removal of the constriction. The tissue-protective potency of these molecules was assessed by the static sciatic index as we have previously reported for asialo-EPO (17) and CEPO (10) and were found to be equivalent (Fig. 3). In contrast, an equimolar amount of a 20-mer fragment of pigment epithelium-derived factor (amino acids 102–121), derived from a biologically active region of this molecule (25), was inactive.

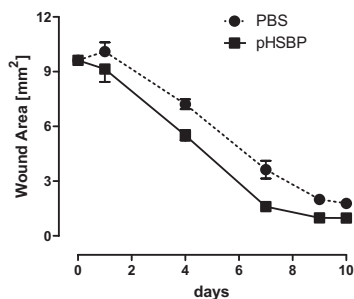
Thus, a peptide designed to mimic the external, aqueous face of helix B resembled EPO sufficiently to activate tissue-protective pathways. In the past, successful surface-simulation synthesis has been reported for antigenic determinants in proteins (26), as well as the surface of the  $\alpha$ -helix of HIV-1 virus (27). Although there have also been claims that peptides exhibiting enzymatic activity can be synthesized from surface simulation analysis of the catalytic site of an enzyme (28), these have not been substantiated (29). In retrospect, it seems very unlikely that a small peptide could effectively reproduce the complex three-dimensional structure required for enzymatic activity, because binding sites for proper orientation of the substrate to the catalytic site require a rigid, three-dimensional scaffold not structurally attainable by using a small peptide. In the case of receptor-mediated biological activity, however, binding and activation of a receptor can topologically be much simpler. Hence, a number of examples exist of small peptides that reproduce the biological activity of a larger protein. For example, the clinically useful parathyroid hormone fragment (1–34) possesses the same biological activity as the full 84-aa protein (30). Further, it should be noted that a 17-mer peptide derived from a portion of EPO's AB loop (residues 30–47) has been reported to possess neurotrophic activity (31). It is currently unclear whether this peptide interacts with the tissue-protective receptor subtype or mediates its biological effects by a different mechanism.

With respect to the primary structure of HBSP, however, it is well known that N-terminal glutamine residues can undergo a spontaneous, irreversible cyclization (particularly at room temperature under acidic conditions) into pyroglutamate (32). In confirmation of this fact, amino acid analysis of production batches of HBSP revealed that  $\approx 90\%$  of the product possessed a free N-terminal glutamine, whereas the remainder was cyclized. Thus, HBSP was actually a mixture of two peptides. To determine whether pyroglutamate HBSP (pHBSP) was biologically active, it was synthesized *de novo* (Fig. 1B).

pHBSP (which was nonerythropoietic; see Figs. S2–S5) was evaluated in a rodent model of renal ischemia-reperfusion injury. Specifically, mice were randomized into five experimental groups and administered either vehicle or various amounts of pHBSP as an i.p. bolus at 1 min, 6 h, and again at 12 h after reperfusion. Twenty-four hours later, plasma creatinine and



**Fig. 4.** Pyroglutamate Helix B surface peptide improves renal ischemia-reperfusion injury. Plasma creatinine (A), urea (B), and aspartate aminotransferase (AST) (C) were measured from mice ( $n = 12$  each group) as biochemical markers of renal dysfunction and injury subsequent to sham-operation or renal ischemia-reperfusion injury (bilateral renal pedicle occlusion for 30 min). PBS or pHBSP (8.0 nmol/kg of bw) was administered i.p. 1 min, 6 h, and 12 h into reperfusion. Data represent mean and SEM; \*\*\*,  $P < 0.001$  versus PBS.



**Fig. 5.** Full-thickness punch biopsy wounds placed over the scapular region of a rat heal more rapidly after pHBSBP (24 nmol/kg of bw) administered daily via the s.c. route.  $n = 9$  animals each group. Curves differ at the  $P < 0.05$  level by repeated-measures analysis.

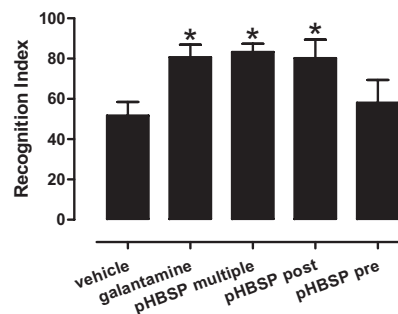
urea were obtained to estimate renal function and aspartate aminotransferase to assess injury. The results show a dose-dependent renoprotective effect, with the lowest dose administered (0.08 nmol/kg of bw) ineffective (Fig. 4). The degree of protection observed was similar to previous observations of EPO in this model (33).

Results of administering pHBSBP in a stroke model as a single i.v. dose (1.5 nmol/kg of bw) upon reperfusion after 1 h of occlusion, followed by three additional injections at 2-h intervals, demonstrated a significant reduction in infarct volume at 24 h [ $225 \pm 20 \text{ mm}^3$  for pHBSBP ( $n = 8$ ) compared with  $291 \pm 23 \text{ mm}^3$  for saline ( $n = 7$ );  $P < 0.05$ ] and an improvement in neurological function (saline group foot faults  $20.2 \pm 0.8$  versus  $11.2 \pm 1.1$  in the pHBSBP group;  $P < 0.001$ ). In contrast, a scrambled version of HBSP (LSEQARNQSEL;  $n = 6$ ) was biologically inactive ( $20.1 \pm 2.1$  foot faults;  $P < 0.05$  versus the pHBSBP group). This observation provides additional support that the surface structure of helix B is specific for tissue-protective activities of EPO.

As noted above, EPO has also been observed to mediate other biological activities in addition to purely tissue-protective effects (reviewed in refs. 2 and 34). Among these pleiotropic effects, EPO accelerates wound healing and modulates cognitive function. For example, EPO has been observed to promote incisional wound closure in rodent models by reducing ischemic and reperfusion injury, mobilizing endothelial progenitor cells, augmenting angiogenesis, and decreasing inflammation (35). To determine whether pHBSBP could also provide benefits in wound healing, we examined its effect in the healing of punch biopsy wounds.

In this experiment, 3.5-mm-diameter full-thickness skin wounds were placed at the corners of a 3-cm-wide square on the shaved and depilated scapular region of the rat. pHBSBP (24 nmol/kg of bw) or PBS was administered s.c. daily for 10 days. The area of open wound, measured in a blinded fashion from serial digital photographs, exhibited faster healing in animals that received peptide compared with saline controls (Fig. 5). Previous studies using systemically administered EPO have shown acceleration of wound healing in association with increased expression of inducible NO synthase (iNOS) and increased angiogenesis, vascular endothelial growth factor, and wound collagen content (reviewed in ref. 34).

Historically, one of the first nonerythropoietic activities noted for EPO was a strong neurotrophic effect (36). EPO and its nonerythropoietic tissue-protective derivatives have been shown to possess these properties *in vitro*, as well as *in vivo* for injured (37–39) and normal (40) animals. Recently, by using functional MRI, EPO has also been shown to enhance cognition in normal human volunteers (4, 41, 42). To determine whether pHBSBP modulates cognitive function, we used the novel object recognition paradigm in rats, in which memory recall for previously



**Fig. 6.** Effect of pHBSBP (24 nmol/kg of bw) and the positive control galantamine (3 mg/kg of bw) on novel object recognition memory test performed with adult Wistar rats. Data represent mean and SEM recognition index at the 24-h retention test.  $n = 8$  each group; \*,  $P < 0.05$  versus saline.

experienced objects is evaluated. Specifically, rats were exposed to novel test objects and then reexposed to them 24 h later. Animals having received galantamine (3 mg/kg i.p. 1 h before testing; a positive control) displayed enhanced memory retention of previously experienced objects (Fig. 6). Similarly, animals receiving pHBSBP (24 nmol/kg of bw i.p.) 3 h after the first exposure to the objects to be learned, or receiving twice daily doses 5 days before training and continued through the day immediately after training, showed enhanced memory for the objects. In contrast, animals that received pHBSBP 1 h before the first object exposure did not show enhancement. Because pHBSBP was effective only when administered after training, this molecule likely acts by intensifying the consolidation phase of memory acquisition.

In summary, using a variety of *in vitro* and *in vivo* models, we have shown that helix B of EPO has tissue-protective activities representative of the full molecule. Further, a peptide constructed to mimic the external, aqueous surface of EPO without primary sequence similarity recapitulates EPO's tissue-protective, neurotrophic, and reparative properties. Peptide doses that exhibited tissue protection were similar on a molar basis to those observed for EPO and are higher than those required for EPO-mediated erythropoiesis. For example, in the renal ischemia model, 0.08 nmol/kg of bw (equivalent to  $\approx 300$  units/kg of bw of EPO) was ineffective, whereas a 10-fold higher dose elicited strong tissue protection.

Finally, pharmacokinetic studies confirm that pHBSBP possesses a plasma half-life of  $\approx 2$  min in the rat and rabbit (see *SI Materials and Methods*, Figs. S6 and S7, and Tables S1 and S2). It is especially notable that, similar to asialo-EPO (17), 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 h. Tissue-protective peptides may therefore be of use as pharmacological reagents to delineate aspects of timing in tissue protection and trophic effects, in addition to potentially being of therapeutic benefit in a wide variety of clinical scenarios.

## Materials and Methods

The animal protocols followed in this study were approved by the respective Animal Use and Care Committees of each institution in accordance with the directives of the Guide for the Care and Use of Laboratory Animals of the National Research Council or the Home Office Guidance on the operation of animals (Scientific Procedures) Act 1986 published by Her Majesty's Stationery Office or in compliance with national (D.L. n. 116, G.U., suppl. 40, Feb. 18, 1992) and international laws and policies (EU Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987).

**Materials.** Peptides were obtained from commercial manufacturers. The UT-7 EPO hematopoietic assay (10, 17), motoneuron excitotoxicity study (15, 43),

middle cerebral artery occlusion model (44), and sciatic nerve compression injury (10) were performed as previously reported.

**Diabetic Retinal Edema.** Fasting male Sprague–Dawley rats weighing  $\approx 250$  g were administered streptozotocin (60 mg/kg of bw) i.p., and diabetes was confirmed by a fasting blood glucose of  $>250$  mg/dl 2 days later. Diabetic animals were administered HBP (1.5 nmol/kg of bw) or saline i.p. 5 days a week, while a third group of normal animals received saline. After 3 weeks, animals were anesthetized by using isoflurane, and Evans blue solution (45 mg/kg of bw as a 7.5 mg/ml solution) was injected via the tail vein. Three hours later, the animals were reanesthetized, a small blood sample was obtained to determine plasma concentration of Evans blue, and each rat was perfused with pH 7.4 citrate buffer at 120 mmHg for 2 min and, thereafter, both eyes were immediately removed. Under an operating microscope, the eyes were bisected along the equator and the retinas were removed. The retinas were desiccated at 60°C overnight in a vacuum, weighed, crushed in 120  $\mu$ l of formamide, and incubated at 70°C for 18 additional hours. The retinal formamide solution was filter-centrifuged at 15,000  $\times$  g for 30 min to remove retinal debris. Evans blue concentration was determined by a background-subtracted absorbance at wavelengths of 620 nm (maximum) and 740 nm (minimum). Fluid extravasation was calculated as Evans blue ( $\mu$ g)/retina dry weight (g). Data were analyzed by analysis of variance (ANOVA) followed by Dunnett's post hoc test comparison.

**Renal Ischemia–Reperfusion Model.** Sixty male C57/BL6 mice ( $\approx 25$  g; Charles River Laboratories) were anesthetized with ketamine (150 mg/kg) and xylazine (15 mg/kg) i.p. Each animal was placed on a homeothermic blanket set at 37°C, and after a mid-line laparotomy, the renal pedicles were clamped for 30 min by using nontraumatic microvascular clamps. pHBSP was administered at the indicated dose via i.p. injection at 1 min, 6 h, and 12 h after reperfusion. Twenty-four hours later, mice were reanesthetized and blood was obtained by cardiac puncture. Plasma urea and creatinine were used as indicators of renal dysfunction and aspartate aminotransferase was used as an indicator of renal injury. Data were analyzed by ANOVA followed by Dunnett's post hoc test comparison.

**Wound Healing.** Methods were adapted from the protocol of Padgett *et al.* (45). Male Sprague–Dawley rats ( $\approx 200$  g) were fasted from the evening before

the procedure. Under isoflurane anesthesia, a 5  $\times$  5 cm region of skin was shaved on the dorsum in the subscapular region and washed with povidone iodine solution. Four full-thickness wounds (3.5-mm diameter) were placed at the corners of a square of 3-cm sides by using a biopsy punch. The wound edge was then infiltrated with 1% lidocaine solution and lidocaine-saturated gel foam was attached with adhesive tape. Wound assessment was obtained by serial digital photographs that included a 3.5-mm diameter standard. Area was determined by using digital planimetry and the four measurements were averaged. Data were analyzed by using a repeated-measures analysis.

**Novel Object Recognition in Rats.** This model is based on the greater spontaneous exploration of a novel object, compared with a familiar object, shown by rodents (46). Male Wistar rats were assessed for cognitive ability in a test apparatus comprising an open-field arena placed in a sound-attenuated room under dim lighting.

After a 5-min habituation period, each rat was placed into the test arena in the presence of two identical plastic shapes, and the time spent actively exploring the objects during a 5-min test period (T1) was recorded. The rat was returned to its home cage between tests. After 24 h, each rat was again placed in the test arena for 5 min (T2) in the presence of one of the familiar objects and a novel object, and the time spent exploring each object was again recorded. A recognition index for each object, the ratio of the time spent exploring either the familiar object or the novel object over the total time spent exploring both objects (during retention session T2), was used to measure cognitive (memory) function.

Rats ( $n = 8$  each group) were treated with the test compounds before the test period (T1), after T1, or chronically for 5 days before T1, via the i.p. route. Groups consisted of those that received vehicle, galantamine (3 mg/kg of bw) administered 1 h before the first 5-min exposure to the two identical objects to be learned, pHBSP (24 nmol/kg of bw) administered 1 h before the first 5-min exposure to the two identical objects to be learned, pHBSP (24 nmol/kg of bw) administered 3 h after the first 5-min exposure to the two identical objects to be learned, or pHBSP (24 nmol/kg of bw) administered every 12 h for 5 days before training and then 12 and 24 h after training (the last dose was administered 1 h before the novel object exposure). Data were analyzed by ANOVA followed by Dunnett's post hoc test comparison.

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