A Small Nonerythropoietic Helix B Surface Peptide Based upon Erythropoietin Structure Is Cardioprotective against Ischemic Myocardial Damage

Ismayil Ahmet,[1] Hyun-Jin Tae,[1] Magdalena Juhaszova,[1] Daniel R Riordon,[1] Kenneth R Boheler,[1] Steven J Sollott,[1] Michael Brines,[2] Anthony Cerami,[2] Edward G Lakatta,[1] and Mark I Talan[1*]

[1]Laboratory of Cardiovascular Sciences, National Institute on Aging, NIH, Baltimore, Maryland, United States of America; and
[2]Araim Pharmaceuticals, Ossining, New York, United States of America

Strong cardioprotective properties of erythropoietin (EPO) reported over the last 10 years have been difficult to translate to clinical applications for ischemic cardioprotection owing to undesirable parallel activation of erythropoiesis and thrombogenesis. A pyroglutamate helix B surface peptide (pHBP), recently engineered to include only a part of the EPO molecule that does not bind to EPO receptor and thus, is not erythropoietic, retains tissue protective properties of EPO. Here we compared the ability of pHBP and EPO to protect cardiac myocytes from oxidative stress *in vitro* and cardiac tissue from ischemic damage *in vivo*. HBP, similar to EPO, increased the reactive oxygen species (ROS) threshold for induction of the mitochondrial permeability transition by 40%. In an experimental model of myocardial infarction induced by permanent ligation of a coronary artery in rats, a single bolus injection of 60 µg/kg of pHBP immediately after coronary ligation, similar to EPO, reduced apoptosis in the myocardial area at risk, examined 24 h later, by 80% and inflammation by 34%. Myocardial infarction (MI) measured 24 h after coronary ligation was similarly reduced by 50% in both pHBP- and EPO-treated rats. Two wks after surgery, left ventricular remodeling (ventricular dilation) and functional decline (fall in ejection fraction) assessed by echocardiography were significantly and similarly attenuated in pHBP- and EPO-treated rats, and MI size was reduced by 25%. The effect was retained during the 6-wk follow-up. A single bolus injection of pHBP immediately after coronary ligation was effective in reduction of MI size in a dose as low as 1 µg/kg, but was ineffective at a 60 µg/kg dose if administered 24 h after MI induction. We conclude that pHBP is equally cardioprotective with EPO and deserves further consideration as a safer alternative to rhEPO in the search for therapeutic options to reduce myocardial damage following blockade of the coronary circulation.

INTRODUCTION

Erythropoietin (EPO), a well-known hematopoietic cytokine (1), is used widely clinically for the treatment of anemia (2). Recently EPO has emerged as a potent tissue protective cytokine (3,4). Its cardioprotective properties were demonstrated in a number of *in vitro* and *in vivo* experimental models (5,6). Specifically, in cardiomyocytes, human recombinant EPO was shown to increase tolerance to ischemic damage and to increase the mitochondrial permeability threshold to oxidative stress (7); in rodent experimental models of myocardial infarction (MI) induced either by a permanent ligation of coronary artery or in ischemia-reperfusion models, administration of rhEPO at the time of MI induction reduced the extent of early myocardial damage and, thus, the rate of development of the subsequent dilated cardiomyopathy (5,6).

Translation of significant advances demonstrated in preclinical animal experiments into clinical practice, however, has been hindered by an understandable caution regarding the classic properties of EPO to stimulate the production of red blood cells and to enhance the risk of thrombosis, an additional cardiovascular risk for already vulnerable patients. Even a single dose of rhEPO can be associated with undesirable platelet elevation (8). These, paradoxically "negative" effects of EPO prompted a search to develop a molecule that retains the tissue-protective properties of EPO, but does not stimulate erythropoiesis. Recently, it was established that the tertiary structure of erythropoietin consists of three helices that interact with the erythropoietic receptor, while a fourth region within helix B is believed to interact with a separate receptor to promote tissue protection (9,10,11). A small peptide (molecular weight 1,257), helix B surface peptide

(pHBP), was developed and shown to be neuroprotective *in vitro* and in several models of experimental ischemic stroke, peripheral nerve trauma and wound healing (11). Even repeated injections of this peptide in the rat and rabbit model did not result in elevated red blood cell, hematocrit or platelet count (11). pHBP also was shown to be comparable to rhEPO in the reduction of apoptosis induced in cardiomyocytes by TNF-α, and within the myocardium of dilated cardiomyopathic hamster (12).

Here we report the results of an extensive evaluation of the cardioprotective properties of pHBP. At the single cardiomyocytes level, we compared the effectiveness of rhEPO and pHBP to increase the ROS threshold for induction of the mitochondrial permeability transition. In a rat model of MI, we also compared the effectiveness of systemic administration of pHBP as a single dose immediately after coronary ligation with a single dose of rhEPO: 1) to reduce apoptosis and inflammation in the area of the myocardium at risk (AAR) and MI size 24 hours after induction of MI, 2) and to attenuate the ensuing left ventricular remodeling and MI size at the end of 8 weeks of observation.

MATERIALS AND METHODS

Materials

Pyroglutamate helix B surface peptide (pHBP), ARA290, was obtained from Araim Pharmaceuticals (Ossining, NY, USA). rhEPO (Procrit, Amgen Inc., Thousand Oaks, CA, USA) was purchased from Henry Schein Inc. (Denver, PA, USA).

In Vitro Protocols

Left ventricular myocyte isolation for mitochondrial permeability transition experiments. Single ventricular myocytes were isolated by a previously described method, with minor modifications (13). Briefly, 2- to 4-month-old Sprague-Dawley rats were anesthetized with sodium pentobarbital, and the hearts were rapidly excised and perfused with 40 mL of

Ca^{2+}-free bicarbonate buffer gassed with 95% O_2 to 5% CO_2 at 37°C. The buffer had the following composition: 116.4 mmol/L NaCl, 5.4 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L NaH2PO$_4$, 5.6 mmol/L glucose, and 26.2 mmol/L NaHCO$_3$, pH 7.4. The hearts were continuously perfused with bicarbonate buffer containing 0.1% collagenase type B, 0.04 mg/mL protease XVI, and 0.1% bovine serum albumin type V for 4 min, and 50 μmol/L Ca^{2+} was added. After 10 min of perfusion, the left ventricle was minced and incubated for 10 min at 37°C in bicarbonate buffer containing 100 μmol/L Ca^{2+}. Myocytes then were resuspended in HEPES buffer with gradually increasing Ca^{2+} concentration up to 1 mmol/L and kept at room temperature until use. The HEPES buffer had the following composition: 137 mmol/L NaCl, 4.9 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L NaH_2PO_4, 15 mmol/L glucose, 20 mmol/L HEPES, and 1.0 mmol/L $CaCl_2$ (adjusted pH to 7.4). The isolated cardiac myocyte viability was typically 70% to 80%.

Confocal microscopy and determination of MPT-ROS threshold. Myocytes were loaded with 125 nmol/L TMRM for at least 1 h at room temperature and imaged with an LSM-510 inverted confocal microscope (Carl Zeiss Inc., Jena, Germany). Line scan images at 2 Hz were recorded from mitochondria arrayed along individual myofibrils with excitation at 568 nm and collecting emission at > 560 nm, using a Zeiss Plan-Apochromat 63×/1.4 numerical aperture oil immersion objective, and the confocal pinhole was set to obtain spatial resolutions of 0.4 μm in the horizontal plane and 1 μm in the axial dimension. Images were processed by MetaMorph software (Universal Imaging, Downingtown, PA, USA). Experiments were conducted as described previously (7), using a method to quantify the ROS susceptibility for the induction of MPT in individual mitochondria within cardiac myocytes (14). Briefly, isolated cardiac myocytes were exposed *in vitro* to conditions that mimic oxidative stress by repetitive laser scan-

ning of a row of mitochondria in a myocyte loaded with tetramethylrhodamine methyl ester. This results in incremental, additive exposure of only the laser-exposed area to the photodynamic production of ROS and consequent MPT induction. The occurrence of MPT is clearly identified by the immediate dissipation of mitochondrial membrane potential, ΔΨ. The ROS threshold for MPT induction (tMPT) was measured as the average time necessary to induce MPT in a row consisting of ~25 mitochondria. Experiments were carried out at 23°C. In parallel experiments, cells were exposed for 20 min to equal concentrations of rhEPO or pHBP prior to tMPT measurements.) Wortmannin (50 μmol/L) was also applied to block the effect of pHBP.

In Vivo Experiments

Animals and experimental design. We housed and studied 168 male Sprague-Dawley rats, 4 months of age, in conformance with the *Guide for the Care and Use of Laboratory Animals* (Washington, DC: National Academy Press: 1996), with Institutional Animal Care and Use Committee approval.

Experiment I: proof of concept. Rats were anesthetized by isoflurane (2% in oxygen). After opening the left side of the chest, the left anterior descending coronary artery was ligated by 7–0 surgical suture at the level of left atrial apex to induce myocardial infarction (MI), as described previously (15). Immediately after coronary ligation (< 5 min), all animals received a single intraperitoneal (i.p.) injection of either pHBP (60 μg/kg; n = 18), or rhEPO (3000 U/kg; n = 16), or normal saline (1 mL/kg; n = 17). Twenty four h after surgery, survivors were anesthetized by isoflurane, chests were opened bilaterally, and 2 mL of 5% Evans blue was injected rapidly into the left ventricle (LV) via apex, while the aorta was tightly closed by forceps. The hearts were removed, rapidly rinsed in phosphate-buffered cold saline, and atriums and great vessels were dissected from the ventricles. The ventricle was cut into four pieces along the short axis. One piece was

snap frozen in liquid nitrogen for histo-logical analysis. The remaining three pieces were kept in triphenyltetrazolium chloride (TTC) at 37°C for 30 min. TTC sections were photographed, digitized and analyzed offline. Serial, 7 μm thick cryostat sections were prepared and used for hematoxyline and eosin and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, respectively. The MI size from TTC staining sections was assessed, as described previously (Ahmet *et al.*, 2005). Myocardial apoptosis was assessed in area at risk (AAR) as described previously (15). Briefly, in each section, the number of cardiomyocytes and the number of TUNEL-positive cardiomyocyte nuclei were counted and totaled in 10 randomly selected fields of the AAR at ×400 amplification. Only nuclei clearly located inside cardiomyocytes were counted. Inflammation in the AAR was assessed by counting the neutrophils and macrophages in 10 randomly selected fields at × 200 amplification in H&E stained sections.

Experiment IA: signaling. A total of 25 rats were subjected to a surgical induction of MI and treated either with rhEPO or HBP, or with saline as described above. Rats that survived surgery were euthanized at 3 h and 24 h after MI (three rats/time point/group). The ischemic myocardium and normal myocardium (septum), identified by tissue color changes, were dissected and frozen in liquid N_2. Myocardial tissue samples were subjected to a Western blot analysis for phosphorylated signal transducer and activator of transcription 3 (STAT3) and mitogen-activated protein (MAP) kinase assessments.

Experiment II: dose response of HBP. Experiment II was done simultaneously with Experiment I. All rats were subjected to the same surgical procedure as described above. In addition to two groups of rats treated with 60 μg/kg of pHBP (n = 18) or saline (n = 17), three more dose/group of pHBP were tested: 10 μg/kg (n = 7); 1 μg/kg (n = 11); and 0.1μg/kg (n = 6). As described in Experiment I, survivors were euthanized 24 h

later, and MI size was measured using Evans blue and TTC straining.

Experiment III: remote effect of treatment. Rats were subjected to a coronary ligation or sham operation (n = 10), as described above. Immediately after coronary ligation, rats were given an i.p. injection of one of the following: (1) rhEPO (3000 U/kg; n = 12); (2) pHBP (60μg/kg; n = 13); or (3) saline (1mL/kg; n = 25). An additional group of rats was treated with pHBP (60 μg/kg; n = 8) 24 h after surgery. Baseline (prior to surgery) echocardiography (Echo) was conducted in sham-operated animals only. A follow up Echo was conducted in all animals at 2, 4, 6 and 8 wks after surgery. At the end of an 8-wk period, all animals were euthanized and hearts were harvested for histological evaluation.

Echocardiography

Cardiac function was assessed by echocardiography (HP Sonos 5500, Hewlett Packard, Palo Alto, CA, USA) with a 12MHz probe under light general anesthesia with isoflurane (2% in oxygen) as described previously (15). Briefly, 2D images of LV were obtained at parasternal long axis, short axis and four chamber views. LV end-diastolic volume (EDV) and LV end-systolic volume (ESV) were calculated by Modified Simpson's Rule. LV ejection fraction (EF%) was derived as EF = (EDV-ESV)/EDV × 100. All measurements were made by one observer who was blinded to treatment modalities. All measurements were averaged over three to five consecutive cardiac cycles. The reproducibility and variability of measurements did not exceed 5%.

Protein Analysis

Frozen heart tissue was ground into a fine powder in liquid nitrogen using a mortar and pestle and then lysed for 1 h rocking at 4°C in 7 volumes (wt/v) of RIPA Lysis buffer (150 mmol/L NaCl, 2 mmol/L EDTA, 1.0% IGEPAL, 0.5% Deoxycholate, 0.1% SDS, 50 mmol/L Tris, pH 8.0, 1 mmol/L PMSF, 1 mmol/L Na_3VO_4, 1 mmol/L NaF and 1:100 dilution of a protease inhibitor cocktail

[Sigma P8340, Sigma-Aldrich Corp., St. Louis, MO, USA]). Samples were triturated up to 50× through a 25G needle and syringe on ice, reincubated rocking at 4°C for another hour, and spun at 10,000*g* at 4°C to remove nonlysed tissue and cellular debris. The supernatant was transferred to a new tube and proteins quantified using the BioRad, D.C. protein assay. 40 μg of protein were denatured for 5 min at 95°C in 1 × LDS Sample Buffer (Novex, Invitrogen, Carlsbad, CA, USA) containing 1 mmol/L DTT, separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were probed with the following primary antibodies from Cell Signaling Technology Inc. (Danvers, MA, USA): mouse Stat3, mouse P-Stat3 (S727), rabbit p44/42 MAPK, and rabbit P-p44/42 MAPK (T202/Y204). Blots were re-probed with HRP-conjugated goat antimouse IgG (H+L) or goat antirabbit IgG (H+L) (Invitrogen), and horseradish peroxidase detected with Pierce Super-Signa West Pico or West Dura ECL substrate kits (Thermo Fisher Scientific Inc., Rockford, IL, USA) and chemiluminescence captured on Phenix Blue X-ray film (Kodak Corp., Rochester, NY, USA). Films were imaged using a Kodak Gel Logic 2200 Imaging System and bands analyzed using Kodak MI SE software (Kodak Corp.).

Statistical Analyses

Statistical significance of differences in Echo indices among groups with regard to changes of these indices over time was determined using analysis of variance (ANOVA) for repeated measurements. A *post hoc* comparison among groups was conducted at specific time points. In the multigroup experiments at a single time point measurement, a one-way ANOVA with Bonferroni *post hoc* correction was used. Statistical significance was assumed at $P < 0.05$.

RESULTS

In Vitro Experiments

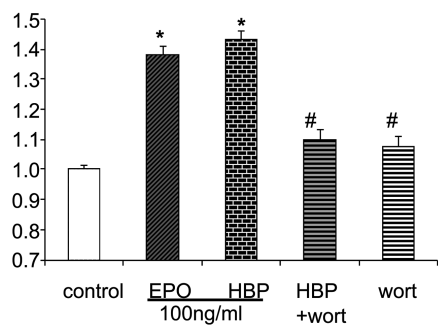**Assessment of MPT-ROS threshold.** Figure 1 presents the cardioprotective

Figure 1. Effects of rhEPO and pHBP on the MPT-ROS threshold (tMPT) in rat cardiac myocytes. Wort, Wortmannin; *$P < 0.05$ versus control; #$P < 0.05$ versus rhEPO and pHBP (Bonferroni correction).

effects of rhEPO and pHBP in isolated cardiac myocytes as indexed by the ROS threshold for MPT induction (tMPT). rhEPO exposure resulted in increased MPT-ROS threshold by ~40% above the untreated control. The effect of pHBP exposure was equally effective and completely blocked by 50 μmol/L of Wortmannin.

In Vivo Experiment

Mortality. Perioperative (<24 h after surgery) mortality ranged from 25% to 40% and was not affected by treatment or dose of drug. In the chronic arm of experiments there was no mortality after the first 24 h. The final number of animals in experimental groups is indicated in the corresponding Figure legends.

Experiment I. Results of histological assessment of the hearts 24 h after coronary ligation are presented in Figures 2–4. Size of AAR, averaged at 47% to 51%, was not affected by treatment and did not differ between EPO and pHBP treatment groups (Figure 2). In the untreated group, the MI size was 54% of AAR and 28% of LV respectively. The MI size in EPO- or HBP-treated groups was approximately half of the MI size in untreated hearts, and did not differ between treatment modalities.

Figure 3 illustrates examples of TUNEL staining and average data for TUNEL positive cardiomyocytes within the AAR. The number of TUNEL-positive car-
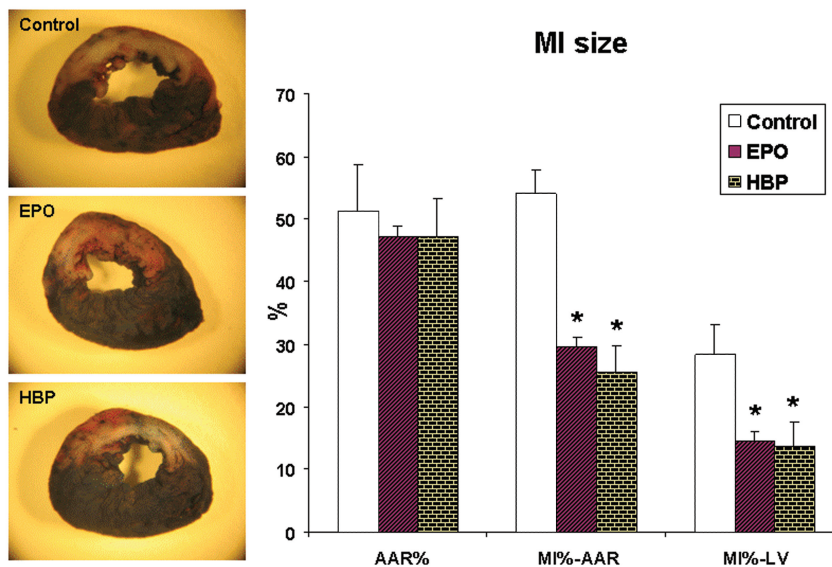

Figure 2. Effect of pHBP on MI size 24 h after its induction. Left, photographs of representative examples of untreated heart and hearts treated with rhEPO and pHBP after incubation in TTC. Right, average MI size expressed as % of area at risk (AAR) or LV. The number of animals per group: Control, n = 8; rhEPO, n = 8; HBP, n = 9. *$P < 0.05$ versus control (Bonferroni correction).
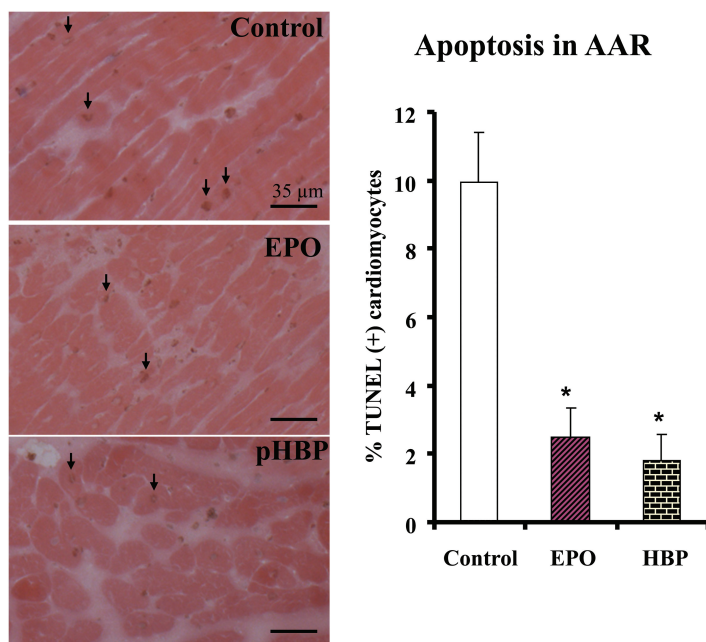

Figure 3. TUNEL positive cardiomyocytes in the AAR of myocardium 24 h after coronary ligation and treatment with rhEPO or HBP. Left, representative examples of TUNEL staining in the AAR of myocardium. Right, percent of TUNEL positive cardiomyocytes in the AAR. The number of animals per group: Control, n = 8; rhEPO, n = 8; HBP, n = 9. $P < 0.05$ versus control (Bonferroni correction).

diomyocytes in the AAR was reduced in both treatment groups by approximately 80%. There were no differences between the effects of EPO or pHBP treatment.

The effect of EPO and pHBP treatments on inflammation is presented in Figure 4. The number of inflammatory cells (neutrophils and macrophages) within the AAR was reduced by 30% and by 34% in EPO and pHBP treated groups respectively. The effect did not differ between treatment groups.

Experiment IA. Results of Western blot analyses of antiapoptotic signaling pathways are illustrated in Figure 5. Results of STAT3 and P44/P42 expression were measured in infarcted area of myocardium 3 h and 24 h after coronary ligation, normalized for nonischemic tissue, and expressed as a ratio of phosphorylated to a total protein. Compared to untreated rats (control) both signaling pathways were activated in infarcted myocardium, however response to rhEPO was observed only at 24 h, while response to pHBP appeared at 3 h after coronary ligation and reduced to normal at 24 h.

Experiment II. Results of the experiment testing the effects of different doses of pHBP treatment on MI size measured histologically 24 h after coronary ligation are presented in Figure 6. The average AAR measured at 24 h after surgery was similar among five groups (four treated and one untreated) and ranged between 47% and 58%. A single injection of 0.1 μg/kg of pHBP did not affect the MI size measured 24 h following coronary ligation, which was similar to MI size in saline-treated group. However, three other treatment doses (1, 10, and 60 μg/kg) resulted in significant reduction of MI size compared with MI size in untreated animals ($P < 0.001$). Differences between effective doses were not statistically significant.

Experiment III. The indices of LV end-diastolic, end-systolic volumes, and EF measured at serial Echos during 8 wks following coronary ligation in untreated group, three treatment groups, and sham operated rats are presented in Figure 7 (A, B, and C). Treatment groups consisted of
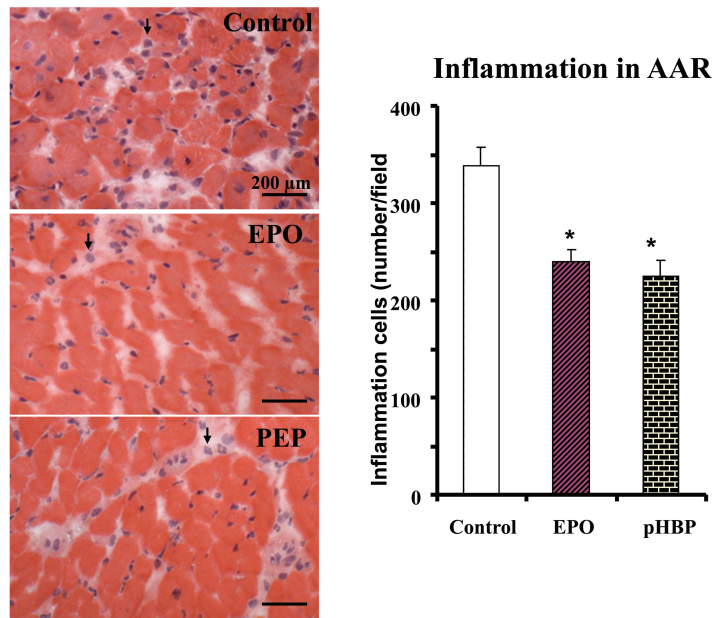

Figure 4. Inflammation in the AAR of myocardium 24 h after coronary ligation and treatment with rhEPO or HBP. Left, representative examples of H&E stained section of myocardium. Right, combined number of neutrophils and microphages in ten randomly selected fields of vision. The number of animals per group: Control, n = 8; rhEPO, n = 8; HBP, n = 9. *$P < 0.05$ versus control (Bonferroni correction).
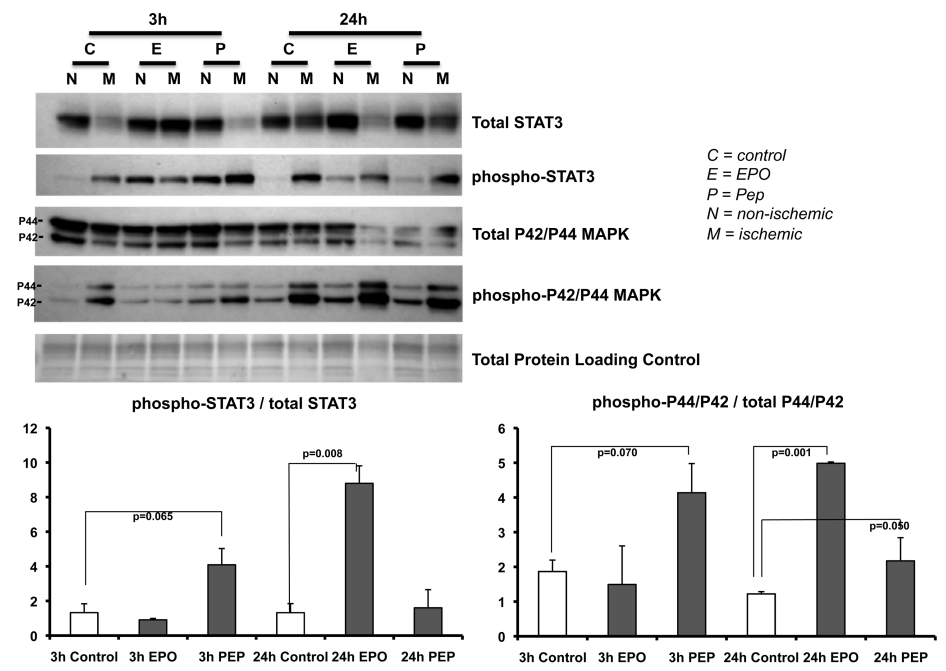

Figure 5. Antiapoptotic signaling pathways in the AAR of myocardium 3 h and 24 h after coronary ligation in untreated rats and rats treated with pHBP or rhEPO. Expression of STAT3 and P44/P22 in the AAR was normalized for nonischemic tissue and presented as a ratio of phosphorylated and total protein.
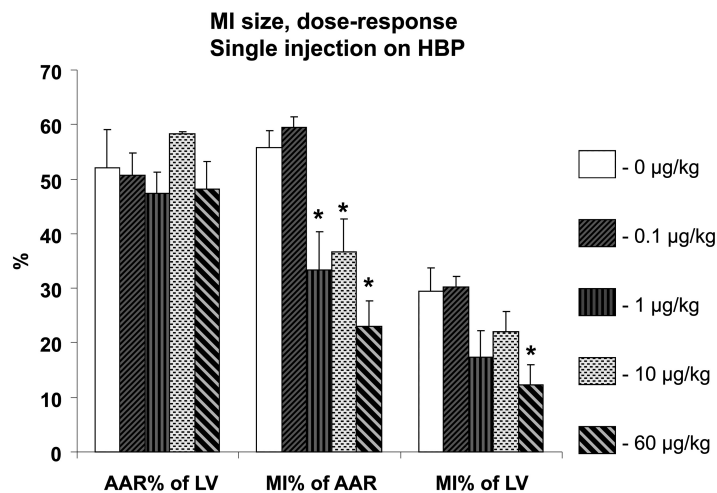
Figure 6. MI size expressed as percent of AAR or LV 24 h after coronary ligation followed by different doses of pHBP treatment. The number of animals per group: Control, n = 13; pHBP 60 µg/kg, n = 11; pHBP 10 µg/kg, n = 5; pHBP 1 µg/kg, n = 8; pHBP 0.1 µg/kg, n = 5. *$P < 0.05$ versus control (Bonferroni correction).

animals that received a single injection of rhEPO (1) or pHBP (2) immediately after coronary ligation and animals that received a single injection of pHBP 24 h after coronary ligation (3). In the untreated MI group, 2 wks after surgery, the EDV and ESV became 55% and 148% higher, respectively, than in sham operated, while EF was 49% lower ($P < 0.05$). The severity of LV dilatation and functional decline expressed in the untreated group at the first 2 wks after surgery was maintained during the following 6 wks, and all Echo indices remained significantly different from those in sham operated group. The Echo indices in the rhEPO and pHBP groups also were significantly different from sham group, but remodeling was less severe than in untreated animals. ESV in rhEPO and pHBP groups was smaller than in nontreated (nT) group ($P < 0.05$) at 2 wks and EF was higher than in nT at all time points ($P < 0.05$). All values for the group treated with pHBP 24 h after surgery were similar to those in the untreated group. The results of Echo measurements of MI size are presented in Figure 7D. The MI size in the untreated group was 28% of LV at 2 wks after surgery, and gradually expanded to 35% of LV during the next 6 wks. The MI size in the delayed treatment group was

similar to that in untreated animals. Both rhEPO and HBP-treated groups, however, showed significant improvement in their MI sizes (approximately 25% lower than in untreated group at all time points, $P < 0.05$). The beneficial effect on MI size was similar for pHBP and EPO groups.

DISCUSSION

This study demonstrates that helix B surface peptide, a small molecule designed and synthesized to represent the amino acids in space of the B helix of erythropoietin that are responsible for tissue-protective properties (11) entirely replicates the cardioprotective properties of the parent erythropoietin. A single systemic application of pHBP reduced the size of MI induced in the rat experimental model by permanent ligation of a coronary artery to the same extent as that of rhEPO (16). Reduction of MI was trans-
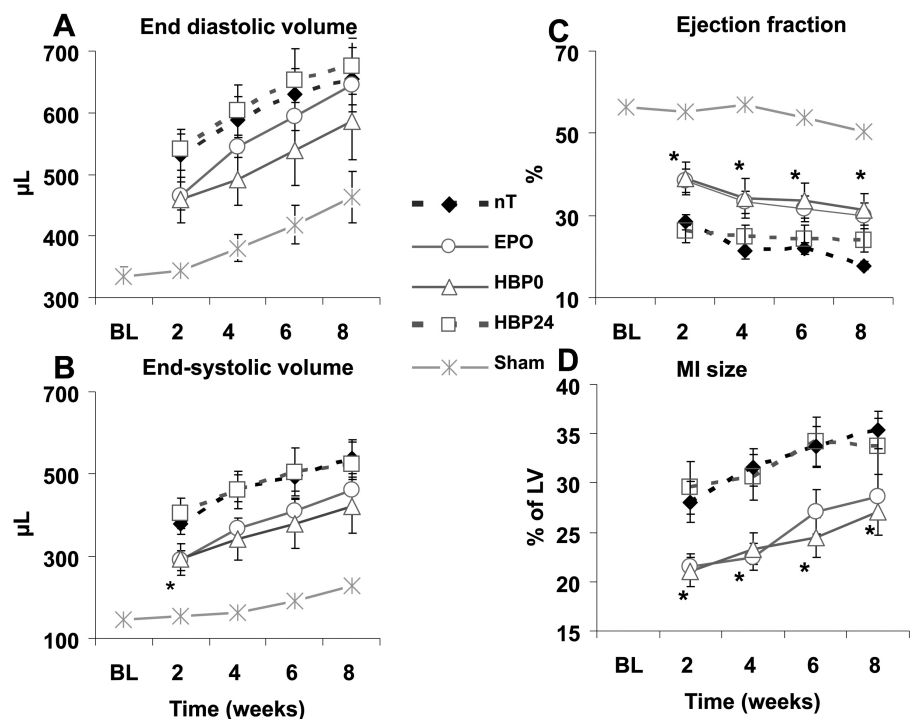

Figure 7. LV remodeling and MI expansion assessed by serial Echo during wk 8 of observation after induction of MI in untreated rats (n = 15) and in rats treated immediately after coronary ligation with a single injection of either rhEPO (n = 9), or pHBP (n = 10), or a single injection of pHBP 24 h after coronary ligation (n = 8). Data for sham operated rats (n = 10) presented for comparison. *$P < 0.05$ versus control at specific time points (Bonferroni correction).

lated into attenuated LV dilatation and loss of function and reduced the MI expansion during the week following MI induction (16). Similar to the effect of rhEPO, reduction of MI size by pHBP treatment was associated with a significant reduction of necrosis, apoptosis and inflammation measured 24 hours after MI induction in the area at risk (16). While the main experiment reported in this study was conducted with the i.p. dose of pHBP 60 μg/kg, a similar effect was observed with a dose as small as 1 μg/kg. The different doses of pHBP that reduced the size of resulting MI were injected during the first 5 minutes after coronary ligation. The pHBP was completely ineffective even in the highest dose when injected 24 hours after coronary ligation. In this regard the pHBP also was similar with rhEPO (17).

Multiple mechanisms are implicated in cardioprotective properties of erythropoietin (18, 19). Activation of signaling cascades of cardioprotection, especially the Pl3K/Akt pathway, is responsible for reduction of apoptosis and inflammation. Increased angiogenesis and possible mobilization of progenitor cells also has been reported. Experiments involving mitochondrial permeability threshold of single cardiomyocytes conducted in this study demonstrated that application of pHBP (as well as with a use of rhEPO [7] or nonerythropoietic modification of rhEPO, carbamylated EPO [20]), increased the mitochondrial permeability threshold to ROS, a final common pathway for antiapoptotic signaling. The blockade of the effect of pHBP on mitochondrial permeability by Wortmannin indicated that, as well as with the effects of rhEPO and CEPO, the Pl3K signaling was predominantly involved.

Results of Western blot analyses at 3 hours and 24 hours after coronary ligation indicated that both rhEPO and pHBP participate in activation of antiapoptotic pathways. Interestingly, while greater pathway activation associated with rhEPO was observed 24 hours after treatment, application of pHBP resulted in the greater activation of antiapoptotic pathways 3 hours after application and the effect subsided at 24 hours. These differ-

ences in time delay are apparently associated with differences in the plasma half life of rhEPO and pHBP, about 8 hours for the former and 2 minutes for the latter (11).

In summary, pHBP fully recapitulates the cardioprotective effects of rhEPO in an experimental model of MI. Since pHBP cannot activate erythropoiesis, it deserves further consideration as a safer alternative to rhEPO in the search of therapeutic options to reduce myocardial damage following interruption of coronary circulation. No negative effects of pHBP in the dose range similar to this study were observed during the phase I safety human volunteer trial (unpublished data of Araim Pharmaceuticals). Cardioprotection without erythropoiesis, combined with a very small size of the peptide and very short plasma half life (~2 min after intravenous (i.v.) injection) (11), makes pHBP an attractive prospect for therapeutic scenarios that require repeated application, such as chronic heart failure.

ACKNOWLEDGMENTS

DISCLOSURES

M Brines and A Cerami work for Araim Pharmaceuticals, which owns the intellectual property of the peptide.

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