**Objective**—Erythropoietin (EPO) may be protective for early stage diabetic retinopathy, although there are concerns that it could exacerbate retinal angiogenesis and thrombosis. A peptide based on the EPO helix-B domain (helix B-surface peptide [pHBSP]) is nonerythropoietic but retains tissue-protective properties, and this study evaluates its therapeutic potential in diabetic retinopathy.

**Research Design and Methods**—After 6 months of streptozotocin-induced diabetes, rats (n = 12) and age-matched nondiabetic controls (n = 12) were evenly split into pHBS and scrambled peptide groups and injected daily (10 μg/kg per day) for 1 month. The retina was investigated for glial dysfunction, microglial activation, and neuronal DNA damage. The vasculature was dual stained with lectin and collagen IV. Retinal cytokine expression was quantified using real-time RT-PCR. In parallel, oxygen-induced retinopathy (OIR) was used to evaluate the effects of pHBS on retinal ischemia and neovascularization (1–30 μg/kg pHBS or control peptide).

**Results**—pHBSP or scrambled peptide treatment did not alter hematocrit. In the diabetic retina, Müller glial expression of glial fibrillary acidic protein was increased when compared with nondiabetic controls, but pHBS significantly reduced this stress-related response (P < 0.001). CD11b+ microglia and proinflammatory cytokines were elevated in diabetic retina responses, and some of these responses were attenuated by pHBS (P < 0.01–0.001). pHBS significantly reduced diabetes-linked DNA damage as determined by 8-hydroxydeoxyguanosine and transferase-mediated dUTP nick-end labeling positivity and also prevented acellular capillary formation (P < 0.05). In OIR, pHBS had no effect on preretal neovascularization at any dose.

**Conclusions**—Treatment with an EPO-derived peptide after diabetes is fully established can significantly protect against neuroglial and vascular degenerative pathology without altering hematocrit or exacerbating neovascularization. These findings have therapeutic implications for disorders such as diabetic retinopathy.

Beyond its established hormonal role in maintaining erythrocyte mass, erythropoietin (EPO) also functions in a paracrine manner to protect tissues during ischemic, toxic, and traumatic insults (1). EPO is highly expressed in many organs (2), and its upregulation can prevent apoptosis and associated inflammation (3). Preclinical studies demonstrate that exogenous recombinant EPO can prevent ischemia-related damage in the brain (4) and heart (5).

Although used clinically to reverse anemia, recombinant EPO or erythropoiesis stimulating agent treatment of patients with stroke or myocardial infarction has shown variable efficacy (6–8). Some trials have also highlighted significant safety issues related to unwanted erythropoiesis and thrombosis (6). Furthermore, for cancer patients being treated for anemia, there has been considerable concern that EPO could activate the EPO receptor (EPO-R) on tumor cells and accelerate growth or metastasis (9). This, combined with the known proangiogenic effects of EPO, suggest that there are considerable limitations on using recombinant EPO for its tissue-protective effects, especially in at-risk patients such as those with cancer, thrombotic disorders, or diabetes (10).

As the most common complication of diabetes, retinopathy is the leading cause of blindness in the working population of many industrialized countries (11). The vascular-degenerative phase of diabetic retinopathy is also accompanied by neuroglial abnormalities and eventual depletion of ganglion cells (12). Retinal nonperfusion leads to increasing hypoxia (13), and this eventually drives breakdown of the blood–retinal barrier (BRB) and preretal neovascularization, which constitute the sight-threatening end points of diabetic retinopathy.

In degenerative retinopathies, exogenous EPO can inhibit neuronal apoptosis (14); however, during retinal ischemia, this cytokine may enhance pathological, preretal neovascularization (15). Blockade of EPO expression using interference RNA (16) or antagonism of EPO-R (17) can effectively prevent neovascularization, and this has raised concerns that using EPO in diabetic patients could serve to accelerate proliferative retinopathy. This assertion has been countered by recent studies that show some forms of erythropoiesis stimulating agent could prevent lesions associated with early stage diabetic retinopathy (18,19) and reverse retinal ischemia (20) in rodent models. While it is clear that EPO has the capacity to protect against pathology, its constitutive hormonal role and diversity of action on different cells currently limits its clinical potential.

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The molecular basis for the multiple roles of EPO is incompletely understood but may be linked to the differential binding affinities of its two main receptors (21). Classically, EPO binds to the homodimeric EPO-R, with high affinity on cells within the bone marrow, and controls erythropoiesis. In other tissues, a heterodimer of EPO-R and the β-common receptor can form similarly to receptors for other type I cytokines (21). This so-called tissue-protective receptor (TPR) is primarily expressed only during metabolic stress and tissue injury, and since it binds with lower affinity than to the EPO-R dimer, it responds only to local rather than circulating EPO (22). The nature of the TPR has been explored by recent studies that demonstrate a helix B-surface peptide (pHBSP) mimics the three-dimensional structure of EPO. This does not bind to the EPO-R dimer and is nonerythropoietic (23) but is tissue protective (24).

In the current study, we have sought to evaluate the potential of the TPR using a unique peptide analog in two models of diabetic retinopathy: one reproducing the early vascular and neuroglial degenerative stage and the other the ischemia-induced neovascular stage. In the clinical context, it is important to evaluate the most likely scenario in which patients can be treated; therefore, we devised an intervention strategy in which treatment was given after diabetic retinopathy was well established. We show that pHBSP is highly effective at preventing clinically relevant lesions of diabetic retinopathy without exacerbating neovascularization.

RESEARCH DESIGN AND METHODS

**Diabetic rat model.** All experiments conformed to U.K. Home Office regulations and were approved by Queen’s University Belfast Ethical Review Committee. Diabetes was induced in male SD rats (n = 12) at ~100 g body wt by a single intraperitoneal injection of streptozotocin (STZ: Sigma) (65 mg/kg in 0.1 mol/L citrate buffer, pH 4.6). A control group received citrate buffer alone (n = 12). At 1 week after STZ injection, hyperglycemia was confirmed using glucometric analysis of tail prick blood samples (Ascensia Breeze; Bayer, Cambridge, U.K.). Animals with blood glucose concentrations >11 mmol/L were considered to have diabetes and enrolled into the study. Blood glucose and weight were monitored monthly.

After 6 months of hyperglycemia, the diabetic rats and age-matched controls were randomly assigned to treatment with daily intraperitoneal injections for a further 28 days of either 10 μg/kg scrambled peptide (pSLEARNQSEL) or active peptide (pHBSP; p[His]-bridged 18 acid peptide of molecular weight 1,258 synthesized by standard F-moc solid phase peptide synthesis and purified by high-performance liquid chromatography and ion-exchange chromatography (23)). A total of 24 rats were divided into four groups. Groups 1 (n = 6) and 2 (n = 6) consisted of control, nondiabetic rats treated with either scrambled peptide or pHBSB. Groups 3 (n = 6) and 4 (n = 6) were diabetic rats that received scrambled peptide or pHBSP.

**Blood analysis.** At killing, whole blood from a cardiac puncture was taken for analysis. To determine the long-term diabetic state of the rats, HbA$_{1c}$ (glycated hemoglobin) was assessed (Glyko Affinity column; Helena Biosciences Europe, U.K.). For rats, hematocrit was quantified using the Sysmex SC9500 analyzer (Japan).

**Preparation of rat retina and immunofluorescence staining.** At killing, the eyes (one eye per rat; n = 6 per group) were immediately enucleated. One eye was fixed by immersing in freshly prepared 4% paraformaldehyde for 1 h at 4°C then washed in PBS. For immunofluorescence, the eye was dissected into two. The retina was removed from the eyecup for flat-mount staining. The other half of the eye was embedded in OCT and cryosections were cut (Alexa Fluor488 or Alexa Fluor568; Invitrogen, U.K.). Negative controls were conducted in neonatal C57BL6 wild-type mice (20), during which there is acute retinal ischemia in the central retina followed by a potent preretinal neovascular response between postnatal day (P) 15 and P21. A total of 34 mice were divided into six groups. Group 1 consisted of P12 controls (n = 6) and groups 2 to 6 were diabetic retina treated with either scrambled peptide or pHBSP. Groups 3 (n = 6) and 4 (n = 6) were diabetic rats that received scrambled peptide or pHBSP. Groups 5 (n = 6) and 6 (n = 6) were diabetic rats treated with either scrambled peptide or pHBSP. Groups 3 (n = 6) and 4 (n = 6) were diabetic rats that received scrambled peptide or pHBSP. Groups 5 (n = 6) and 6 (n = 6) were diabetic rats treated with either scrambled peptide or pHBSP.

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OIR pathology assessment. One eye from each animal (n = 6) had retinal RNA isolated and qPCR conducted as outlined above. The fellow eye (n = 6) was enucleated and immediately fixed in 4% paraformaldehyde (n = 6 per treatment group). Retinal flat mounts were stained with isoelectric B4 (Sigma) and the corresponding streptavidin Alexa Fluor 488 (Molecular Probes). Stained retinae were visualized and imaged using Nikon TE-2000 C1 confocal system. Avascular and preretinal neovascularization were quantified using Lucia Version 4.0 software as previously described (20).

Retinal hypoxia can be assessed using the bioreductive drug pimonidazole (hypoxprobe [HP], 60 mg/kg), which forms irreversible adducts with thiol groups on tissue proteins when pH < 10 mM Hg (26). Retinal flat mounts were incubated with an anti-HP rabbit polyclonal antibody (HP2-100kit; HIP Inc., MA) used at a dilution of 1:500 in PBS/T0.1% TX-100 and then a goat anti-rabbit antibody labeled with Alexa-594 (Molecular Probes).

qPCR was conducted as for rat retina, except that mouse sequence-specific primers for vascular endothelial growth factor (VEGF) and the housekeeping gene phosphophoryn PO (ABP368B) were used as previously described (27).

Statistical analysis. Data are presented as mean ± SEM. Statistical analyses were performed using Prism V4.02 (GraphPad Software, San Diego, CA). All datasets were tested to verify that they fulfilled requirements for a normal distribution. Two-way ANOVA was conducted to compare overall treatment differences and P < 0.05 was deemed significant. When a statistically significant difference was detected, post hoc multiple pairwise comparisons were performed using Tukey multiple comparison test.

RESULTS

Characteristics of diabetic animals. Analysis of body weight revealed a 50% reduction in diabetic rats compared with age-matched nondiabetic controls (Fig. 1A). HbA1c at killing showed a 2.5-fold increase in diabetic rats compared with control rats (P < 0.001) (Fig. 1B). pHBSP peptide did not alter these diabetes parameters, nor did it alter hematocrit counts (Fig. 1C). Diabetes induced a significant increase in the number of reticulocytes and pHBSP prevented this change (Fig. 1D).

We assessed the thickness of the retinal layers and found that the outer nuclear layer (ONL) was reduced in the diabetic retina with both the scrambled pHBSP and the pHBSP (P < 0.001). No significant difference was observed in the GCL, IPL, INL, and outer plexiform layer between control and diabetic retina groups (Supplementary Fig. 1).

pHBSP attenuates diabetes-related glial and neuronal dysfunction. In the nondiabetic retina, GFAP was localized to the astrocytes and a population of retinal Müller glia (Fig. 2A and B). This was typical of the GFAP staining pattern in aging rat retina (28). Diabetes induced a strong upregulation of this protein in both astrocytes and retinal Müller glia (P < 0.05). pHBSP peptide treatment of diabetic rats significantly prevented this glissosis response in Müller glia, as indicated by a reduction in the intensity of GFAP staining in the innermost retinal layers and the number of GFAP-positive fibers in the IPL (P < 0.05) (Fig. 2A and B).

As has been previously reported in rats of comparable diabetes duration (29), there was a significant increase in TUNEL positive cells in the retina (P < 0.001) (Fig. 3A and B). TUNEL positive cells, indicating DNA strand breaks, were apparent in the GCL, INL, and ONL. Treatment with pHBSP decreased TUNEL positivity in the GCL by 49% when compared with diabetic controls (P < 0.01) (Fig. 3B).

In terms of overall diabetes-related TUNEL positivity in the retina, pHBSP provided significant protection when compared with diabetic with scrambled peptide (P < 0.005) (Fig. 3C). Caspase-3 positive cells were observed in the INL mainly; however, there were no differences observed between the groups (Supplementary Fig. 2). And SOD1/Hg positive cells were found only in the diabetic retina on the border of the ONL both in those that received the scrambled peptide and to a lesser extent in those that received the active pHBSP (Supplementary Fig. 3).

pHBSP regulates microglial activation and cytokine expression in the diabetic retina. As depicted in retinal sections, diabetes was associated with an increase in CD11b positive microglia in the neuropile, especially within the IPL (P < 0.05) (Fig. 4A and B). There was also a significant shift in phenotype toward activated, amoeboid cells (Fig. 4C–F). pHBSP had no influence on overall microglia numbers, but this treatment significantly increased the proportion of cells with a dendritic phenotype and reduced amoeboid cells when compared with diabetic rats treated with the scrambled pHBSP (P < 0.05) (Fig. 4A and B). Lectin-stained microglia also showed that diabetes was associated with an increase in microglia compared with the controls in the inner plexus in the central (P < 0.01) and peripheral retina (P < 0.05) (Supplementary Fig. 4).

In keeping with the pattern observed with the resident immune cells within the retina, transcripts for the anti-inflammatory cytokine IL-10 were significantly decreased in the diabetic rat retina relative to age-matched controls (P < 0.001), and pHBSP treatment returned these expression levels to that seen in controls (Fig. 5A). By contrast, mRNAs for the proinflammatory cytokines TNF-α and IL-6 were significantly elevated in diabetic retina (P < 0.001), and pHBSP prevented this increase and restored expression to normal levels in both cases (Fig. 5B and C). STZ-induced diabetes caused a reduction in IL-1β levels when compared with control tissue treated with scrambled peptide (P < 0.001). Treatment with pHBSP caused a reduction in IL-1β levels both in nondiabetic tissue and diabetic tissue (P < 0.001) (Fig. 5D).

pHBSP protects retinal capillaries during diabetes. As diabetes progresses, the death of retinal pericytes and endothelium results in acellular capillary formation, a lesion that takes ~5–6 months to become obvious in diabetic rats (30). While various approaches have been used to visualize acellular capillaries, we used the fact that such naked basement membrane tubes remain positive for collagen IV and negative for isoelectric and can be quantified using confocal microscopy (Fig. 6A). The diabetic retina contained approximately fourfold increased numbers of acellular capillaries when compared with nondiabetic control retina (P < 0.05) (Fig. 6A and B). pHBSP treatment for 1 month, after 6 previous months of diabetes, significantly reduced acellular capillaries in the retina, and there were no differences between these treated patients with diabetes in comparison with nondiabetic control groups (Fig. 6B).

pHBSP does not exacerbate ischemia-induced neovascularization. As determined in the murine OIR model, pHBSP did not increase the percentage of reticulocytes in peripheral blood over an acute time frame (Supplementary Fig. 5). A dose of 1 μg/kg of pHBSP resulted in a decrease in the ischemic region of the retina. The higher doses of the peptide returned the ischemic region to normal (Fig. 7A). Consistent with the well-characterized OIR response, hyperoxia induced a temporal pattern of central retina vascular insufficiency upon return to room air at P12, which led to a reproducible preretinal neovascularization observable on flat mounts (Fig. 7B). Over a dose response range, pHBSP administered from P12 to P16 inclusive demonstrated no significant increase in ischemia-driven preretinal neovascularization (Fig. 7B–D).

In OIR, neovascularization is driven by ischemic hypoxia (akin to that observed in long-term diabetic retinopathy in patients), so the degree of hypoxia was evaluated. Retinal
HP deposition was reduced in the pHBSP-treated mice when compared with controls (Fig. 8A). The hypoxia-induced angiogenic factor VEGF was increased at the P17 time point as previously reported (27), and pHBSP decreased this expression in a dose-dependent manner (Fig. 8B).

DISCUSSION

Using two established preclinical models for the early and late stages of diabetic retinopathy, we show that a novel EPO-derived peptide has a significant protective effect. When administered for 1 month after 6 previous months of diabetes, pHBSP can prevent several important lesions of diabetic retinopathy. These include GFAP upregulation in the Müller glia, neuronal death, retinal inflammatory responses, and capillary degeneration. The current investigation builds on a previous study using a full-length pHBSP, which demonstrates prevention of BRB dysfunction during diabetes (22), and adds compelling evidence that elements of EPO-R signaling can be harnessed for treating diabetic retinopathy.

Many previous studies have used an array of therapeutic approaches and shown effective protection against various...
FIG. 2. pHBS prevents diabetes-related GFAP expression in Müller cells. Retinal sections were processed for GFAP immunoreactivity and assessed by confocal microscopy. Cntl, control; Sc, scrambled pHBS; and Db, diabetic. A: Bar chart shows the numbers of GFAP-positive fibers crossing the IPL and INL, which is significantly increased in diabetic rats receiving scrambled peptide when compared with nondiabetic controls (*P < 0.05). pHBS prevented the diabetes-related increase in GFAP, and there was no significant difference between this group and the non-diabetic groups. Data are mean ± SEM; n = 6 per group. B–E: Retina from nondiabetic and diabetic groups treated with pHBS or scrambled peptide exhibit GFAP immunoreactivity within astrocytes and a subpopulation of Müller cells. More extensive GFAP was observed in the Müller cells crossing the IPL and INL (depicted by dashed line) in the diabetic animals that received the scrambled peptide. This was reduced in the diabetic animals receiving the pHBS peptide. (A high-quality digital representation of this figure is available in the online issue.)
FIG. 3. pHBSP protects against DNA damage in diabetic retina. pHBSP treatment prevents diabetes-induced ganglion cell death. Cntl, control; Sc, scrambled pHBSP; and Db, diabetic. A: Cell count of terminal deoxynucleotidyl TUNEL positive cells in the GCL was assessed by counting the number of fluorescent TUNEL positive cells in the GCL divided by total number of cells present in the GCL. TUNEL of pHBSP-treated rats showed a significant increase in the percentage of cells with DNA strand breaks in the GCL of diabetic rats compared with control (**P < 0.001). Treatment with pHBSP decreased TUNEL positivity by 49% when compared with diabetic rat with scrambled pHBSP (++P < 0.01). Counts were assessed by image analysis in multiple sections. Images were taken at three separate points (three fields at 300 μm² each) on the central retina at ×40 magnification and presented as the average nuclei in the GCL. B: Cell count of TUNEL positive cells in the entire retina was assessed by counting the number of fluorescent TUNEL positive cells divided by total number of cells present. The diabetic retina treated with the scrambled peptide displayed TUNEL positive cells. This was reduced to control levels of ~10% with the pHBSP peptide (***P < 0.01). Data are mean ± SEM;
pathological end points during diabetic retinopathy. However, these treatments were commenced at establishment of experimental diabetes, and very few studies have evaluated efficacy once diabetes is well advanced. It is significant that the current investigation provides evidence that an intervention strategy, evoking EPO-mediated tissue protection in the diabetic retina, has significant benefits. Such a regimen has considerable clinical relevance since many diabetic patients have established retinopathy at diagnosis.

While EPO has established protective properties in a range of tissues, there are major drawbacks to its clinical use for tissue injury. A typical dose for treatment of anemia in patients is ~100 IU/kg, although to achieve tissue protection, much larger doses are needed (~500 IU/kg). While EPO can sometimes be delivered directly to a damaged tissue, this therapy can result in unwanted elevations in hematocrit with associated vascular thrombosis and hypertension (31). Although delivered systemically in both models, pHBSB induced no changes in reticulocytes or hematocrit in the mouse or rat, respectively, despite the latter being delivered over a 28 day period. Such delivery method appears valid since EPO crosses the BBB (14) and, therefore, it is likely that this 11aa peptide also crosses the retinal vascular endothelium. As demonstrated, this EPO analog appears to possess the tissue-protective advantages of EPO without the disadvantages.

Hyperglycemia is known to be associated with an increased erythrocyte turnover and therefore increased reticulocyte production, and in the current study, we observed increased reticulocytes in diabetic rats, a response that was attenuated by pHBSB treatment. Diabetes increases narrow production of reticulocytes to maintain hematocrit, and there is evidence that diabetes-induced oxidative damage may be more prominent in reticulocytes compared with other tissues as a result of their high content of iron and polyunsaturated fatty acids. Indeed, diabetic reticulocytes show increased lipid peroxidation and decreased levels of antioxidants when compared with nondiabetic counterparts. The assay we used did not assess viability of reticulocytes, so there may be a greater turnover in the diabetic rats. Certainly there was no increase in erythrocytes between groups. Tissue-protective measures, such as pHBSB, are active in attenuating these pathological processes, and although not determined in our experiments, presumably, this peptide could serve to reduce early erythrocyte senescence.

EPO is known to be strongly proangiogenic through typical VEGF-mediated pathways (20). While EPO-mediated angiogenesis can significantly improve wound repair (32), postinfarction myocardial vascular remodeling (33), and reperfusion of cerebral ischemia (34), such angiogenic responses in the context of retinopathy raise significant concerns. This is reinforced by reports that EPO levels are raised in the vitreous of patients with proliferative diabetic retinopathy (17). However, several experimental studies show that early administration of EPO can protect neurons, prevent vessel degeneration, and subsequently suppress the stimulus for hypoxia-induced neovascularization (15) by evoking beneficial intraretinal angiogenesis (20). Nevertheless, the disease phase at which EPO is introduced may alter the outcome, and EPO treatment—while the retina is experiencing hypoxia—may enhance pathological, preretinal neovascularization (15). Therefore, a major finding of the current study is that pHBSB not only prevents early stage pathology but also fails to evoke preretinal neovascularization and, thus, carries considerably less risk than recombinant EPO if used in diabetic patients.

Proinflammatory pathways contribute to neuroglial and microvascular lesions during diabetic retinopathy, as evidenced by the upregulation of proinflammatory cytokines from the Müller glia (35) and microglia (36), which show strong associative links to capillary degeneration (37). Microglia in particular are the resident immune cells of the retina, and their activation, combined with infiltration of circulating monocytes into the neuripile, could play an important regulatory role in diabetic retinopathy through cytokine expression and related cell responses (38). It is interesting that pHBSB reduced constitutive expression levels of some cytokines in retina from normal retina, for example, IL-1β. The reason for this is unclear but may be related to slightly elevated levels in older rats and the potential for pHBSB to interfere with proinflammatory signaling. In brain ischemia, microglial responses have been shown to be suppressed by EPO-related therapy (39), and there may be similar dampening of microglial activation, and associated proinflammatory cytokine expression is suppressed by pHBSB in diabetic retinopathy.

Protection against neuroglial apoptosis and associated inflammatory cascades is an established benefit of EPO treatment to damaged tissues such as ischemic brain. There are likely to be comparable benefits for the diabetic retina since progressive retinal neuronal and Müller cell dysfunction, DNA damage, and cell death have been previously demonstrated (40), although in rats this may take >4 months to become evident (25). In the current study, the TUNEL positive cells in the retina are likely to represent DNA damage in a range of neurons and glia but not necessarily apoptotic death. This argument is strengthened by the caspase-3 data in this study. The 8-OHdG staining observed in the ONL of the diabetic retina suggests that there is extensive DNA damage, which is also indicated by TUNEL. While EPO can inhibit neural cell apoptosis (14), it can also upregulate enzymes that scavenge oxygen radicals during brain ischemia (41) and protect against DNA damage (42). Wang et al. (18) have recently reported EPO-mediated protection against oxidative damage in the diabetic retina long before ischemia is present, and this may account for the observed reduction in DNA damage evoked by pHBSB treatment. In the current study, pHBSB (1 µg/kg) reduced retinal ischemia in the murine model, although the response was more apparent at the lower concentration. This may reflect commonly observed cytokine responses that are likely related to receptor dynamics and are often characterized by a U-shaped dose response curve (hormesis).

\[ n = 6 \text{ per group. Counts were assessed by image analysis in multiple sections. Images were taken at three separate points (three fields at 300 µm each) on the central retina at ×40 magnification and presented as the average nuclei in the GCL. pHBSB treatment prevents diabetes-induced ganglion cell death because there are more TUNEL positive cells in the diabetic with scrambled pHBSB–treated animals relative to the control and scrambled pHBSB in the GCL and in the entire retina. Retinal nuclei were also counterstained with PI. This is evident in the images showing TUNEL positivity in (C) control and Sc, (D) control and pHBSB, (E) diabetic and Sc, and (F) diabetic and pHBSB. (A high-quality digital representation of this figure is available in the online issue.)]
Suppression of DNA damage, apoptosis, inflammation, and oxidative stress-related pathways undoubtedly affect capillary degeneration during diabetic retinopathy (43). While pHBSF treatment could significantly modulate all these pathways, it is perhaps surprising that 1 month of treatment with pHBSF after 6 previous months of diabetes could have such a marked effect on reversal of acellular vessels. Death of retinal capillary component cells during...
diabetes is not necessarily linear, and the progressive nature of many pathogenic pathways indicates that endotheliopathy and pericyte death are the result of accumulative abnormalities in the early stages of diabetes. Indeed, acellular capillaries are not usually evident until at least 4–5 months of hyperglycemia (44). Although not evaluated in the current investigation, it should also be considered that activation of the EPO-Rs could mobilize vasoreparative endothelial progenitor cells (EPCs) into the circulating blood to target areas of hypoxia. The mechanism(s) by which EPCs mobilize and home to areas of ischemia are complex but involve stimulatory factors such as EPO (45) and VEGF (46). Diabetes causes EPC dysfunction, and this is associated with impaired vascular repair in diabetic retinopathy (47); it is possible that systemic delivery of pHBSP in diabetic rats could have enhanced EPC mobilization and thereby improved reparative function in a comparable manner to that described for EPO (48). This requires further study.

In summary, the current study indicates that the TPR pathway could play a key role in preventing early stage diabetic retinopathy and thereby progression to the sight-threatening late stages. The ability to use a therapeutic approach that harnesses all the tissue-protective and anti-inflammatory benefits of EPO without risking the potentially damaging collateral effects would be a major advance for neurovascular degenerative diseases such as diabetic retinopathy.
FIG. 6. pHBSF prevents retinal capillary degeneration during diabetes. The retinal vasculature was visualized in flat mounts using concomitant labeling of endothelium (isolectin) and basement membrane (collagen IV). Acellular capillaries can be visualized by continuance of collagen IV positivity but loss of endothelium. Cntl, control; Sc, scrambled pHBSF; and Db, diabetic. A: Graph showing the number of acellular capillaries in retina in the peripheral and central retina. Diabetic rats with scrambled pHBSF displayed more acellular capillaries than control rats, which received the scrambled pHBSF (*P < 0.05). The pHBSF peptide significantly reduced the number of the acellular capillaries to normal levels in the inner retina (P < 0.05). Data are mean ± SEM; n = 6 per group. B and C: Images showing acellular capillaries (arrows) in the diabetic animals that received the scrambled peptide. Acellular capillaries are observed when vessels are collagen IV positive (red) and negative for lectin (green). There are more acellular vessel profiles present in the diabetic retina that received the scramble peptide relative to the diabetic animals that received the pHBSF peptide. Sc’pHBSF, scrambled pHBSF. (A high-quality digital representation of this figure is available in the online issue.)
FIG. 7. pHBSP decreases ischemia at 1 μg/kg and exacerbates preretinal neovascularization in OIR. The murine retinal vasculature was assessed in flat mounts after OIR using isolectin labeling and confocal microscopy. A: Retina ischemia: pHBSP at 1 μg/kg decreases the ischemic region (** P < 0.001), while the higher doses of 10 and 30 μg/kg have no effect on ischemia. B: Retinal neovascularization: upon quantification of preretinal vessels, pHBSP had no significant effect on neovascularization at any of the doses tested (1, 10, and 30 μg/kg) when compared with the scrambled peptide or PBS control. Data are mean ± SEM; n = 6 per group. PBS, PBS control; Sc, scrambled pHBSP. C and D: Retinal flat mounts showing hyperfluorescent preretinal neovascularization in (C) PBS and (D) 1 μg/kg of pHBSP-treated OIR mice (arrow). (A high-quality color representation of this figure is available in the online issue.)
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C.M.M. designed the experiments, researched data, and wrote the manuscript. R.H. designed the experiments and researched data. L.M.C. researched data. T.A.G. was involved in the design of the experiments and contributed to the manuscript. M.B. designed the peptide for the study and was involved in the design of the experiments. A.C. designed the peptide for the study and contributed to the manuscript. A.W.S. led the project, obtained funding, designed the experiments, and wrote the manuscript.

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FIG. 8. pHBSP reduces retinal hypoxia and VEGF mRNA expression. A: VEGF mRNA levels were reduced with pHBSP at 1, 10, and 30 μg/kg relative to the scrambled pHBSP (n = 6; **p < 0.001). B–D: Retinal hypoxia was assessed by HP, which deposits an insoluble adduct in tissue at <10 mmHg. Retinal flat mounts at P17 show HP immunoreactivity in the ischemic regions (green), and this is reduced in area with 1 μg/kg when compared with the PBS and scrambled peptide control. The vasculature is visualized by isolectin (red). Shown is a representative retinal image for PBS, scrambled pHBSP, and 1 μg/kg of pHBSP. Data are mean ± SEM; n = 6 per group. PBS, PBS control; Sc, scrambled pHBSP. (A high-quality digital representation of this figure is available in the online issue.)
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