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Original Research

Heparin-binding epidermal growth factor–like growth factor improves erectile function in streptozotocin-induced diabetic mice

Fitri Rahma Fridayana, MD,[1,2], Jiyeon Ock, PhD[1], Fang-Yuan Liu, BA[1], Lashkari Niloofar, BA[1,2], Minh Nhat Vo, MD[1], Yan Huang, BA[1,2], Guo Nan Yin, PhD[1,*], Ji-Kan Ryu, MD, PhD[1,2,*]

[1]National Research Center for Sexual Medicine and Department of Urology, Inha University College of Medicine, Incheon 22332, Republic of Korea
[2]Program in Biomedical Science & Engineering, Inha University, Incheon 22332, Republic of Korea

*Corresponding authors: National Research Center for Sexual Medicine and Department of Urology, Inha University School of Medicine, 7-206, 3rd St, Shinheung-Dong, Jung-Gu, Incheon 22332, Republic of Korea. E-mail: 217052@inha.ac.kr and National Research Center for Sexual Medicine and Department of Urology, Program in Biomedical Science & Engineering, Inha University School of Medicine, 7-206, 3rd St, Shinheung-Dong, Jung-Gu, Incheon 22332, Republic of Korea. E-mail: rjk0929@inha.ac.kr

Abstract

Background: Heparin-binding epidermal growth factor–like growth factor (HB-EGF) serves as a pro-angiogenic factor; however, there is to our knowledge currently no reported research on the relationship between HB-EGF and diabetic erectile dysfunction (ED).
Aim: In this study we aimed to determine whether HB-EGF can improve the erectile function of streptozotocin-induced diabetic mice and to explore the related mechanisms.
Methods: Eight-week-old male C57BL/6 mice were used for diabetes induction. Diabetes mellitus (DM) was induced by low-dose injections of streptozotocin (50 mg/kg) for 5 consecutive days. Eight weeks after streptozotocin injections, DM was determined by measuring blood glucose and body weight. Diabetic mice were treated with two intracavernous administrations of phosphate-buffered saline (20 μL) or various doses of HB-EGF (days −3 and 0; 1, 5, and 10 μg in 20 μL of phosphate-buffered saline). The angiogenesis effect of HB-EGF was confirmed by tube formation and migration assays in mouse cavernous endothelial cells and mouse cavernous pericytes under high-glucose conditions. Erectile function was measured by electrical stimulation of the cavernous nerve, as well as histological examination and Western blot analysis for mechanism assessment.
Outcomes: In vitro angiogenesis, cell proliferation, in vivo intracavernous pressure, neurovascular regeneration, cavernous permeability, and survival signaling were the outcomes measured.
Results: Expression of HB-EGF was reduced under diabetic conditions. Exogenous HB-EGF induced angiogenesis in mouse cavernous endothelial cells and mouse cavernous pericytes under high-glucose conditions. Erectile function was decreased in the DM group, whereas administration of HB-EGF resulted in a significant improvement of erectile function (91% of the age-matched control group) in association with increased neurovascular content, including cavernous endothelial cells, pericytes, and neuronal cells. Histological and Western blot analyses revealed a significant increase in the permeability of the corpus cavernosum in DM mice, which was attenuated by HB-EGF treatment. The protein expression of phospho-Akt Ser473 and phosphorylated endothelial nitric oxide synthase Ser1177 increased after HB-EGF treatment.
Clinical Implications: The use of HB-EGF may be an effective strategy to treat ED associated with DM or other neurovascular diseases.
Strengths and Limitations: Similarly to other pro-angiogenic factors, HB-EGF has dual roles in vascular and neuronal development. Our study focused on broadly evaluating the role of HB-EGF in diabetic ED. In view of the properties of HB-EGF as an angiogenic factor, its dose concentration should be strictly controlled to avoid potential side effects.
Conclusion: In the diabetic ED mouse model in this study erectile function was improved by HB-EGF, which may provide new treatment strategies for patients with ED who do not respond to phosphodiesterase 5 Inhibitors.

Keywords: angiogenesis; diabetes; erectile dysfunction; HB-EGF; permeability.

Introduction

Diabetes mellitus (DM) is a multifactorial metabolic disorder, the main cause of which is an absolute or relative deficiency of insulin or insensitivity of peripheral tissues to insulin.[1] The incidence and prevalence of DM are increasing, posing a significant health problem due to associated complications such as macroangiopathy, microangiopathy, and neuropathy.[2] The prevalence of erectile dysfunction (ED) among diabetic patients is as high as 50%-75%.[3-5]

A study by Bae et al[6] has broadened our horizons through a systematic single-cell analysis of normal and diabetic mouse penile tissue. In particular, targeting ligand–receptor interactions between pericytes (fibroblast-like cells with extensive cytoplasmic processes that wrap around endothelial cells)[7] and other cell types involved in angiogenesis provides us with many promising therapeutic targets, such as vascular endothelial growth factor A (VEGFA) and VEGFB, placental growth factor, heparin-binding epidermal growth factor–like growth factor (HB-EGF), and fibroblast growth factor receptor 1 (FGFR1) and FGFR2. A member of the EGF ligand family, HB-EGF was originally synthesized as a membrane-bound growth factor known as proHB-EGF[8] and is a key molecular

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component in many normal physiological processes, including wound healing,[9] reproduction,[10] angiogenesis,[11] and adipogenesis.[12] Recently, Maachi et al demonstrated that increased levels of HB-EGF in the pancreatic duct enhance islet function by promoting beta cell production in various low-dose streptozotocin (STZ) diabetic rats.[13] However, this study only revealed the critical role of HB-EGF/EGF receptor signaling in glucose-induced rat pancreatic beta cell proliferation. The relationships and underlying mechanisms between HB-EGF and diabetic ED remain poorly understood.

Therefore, in the present study we aimed to determine whether exogenous HB-EGF protein could improve erectile function in STZ-induced type 1 diabetic mice. We assessed HB-EGF expression under normal and diabetic conditions in vivo (penile tissues) and in vitro (primary cultured mouse cavernous endothelial cells and primary cultured mouse cavernous pericytes). We also demonstrated that HB-EGF treatment induces endothelial cell angiogenesis, migration, and proliferation under high-glucose conditions. Exogenous administration of HB-EGF can reduce penile tissue permeability, promote vascular and neuronal cell content, and activate the AKT/ endothelial nitric oxide synthase (eNOS) signaling pathway, thereby improving erectile function in diabetic ED mice.

Materials and methods

Ethics statement and animal study design

Eight-week-old male C57BL/6 J mice (weight, 20-25 g; Orient Bio, Inc., Seongnam, Korea) were used with approval from the Institutional Animal Care and Use Committee of our university (approval number: 220222-813). The animals' health and behavior were observed daily, and they were fed disinfected commercial standard laboratory food and water ad libitum. The mice were maintained in a controlled environment with a mean (SD) room temperature of 23 C(2°C), relative humidity of 40%-60%, and a 12-hour light/dark cycle and were confirmed to be free from specific pathogens. In this study, a total of 110 adult male C57BL/6 J mice were used for diabetes mouse model preparation, intra-cavernous pressure (ICP) assessment, and related experiments (70 mice); primary culture of mouse cavernous endothelial cells (MCECs) and related experiments (20 mice); and primary culture of mouse cavernous pericytes (MCPs) and related experiments (20 mice). All animals received intramuscular injections of ketamine (100 mg/kg; Yuhan Corp., Seoul, Korea) and xylazine (5 mg/kg; Bayer Korea, Seoul, Korea) to induce anesthesia. The penis was exposed using a sterile technique. For euthanasia, a closed container was used, and 100% CO_2 gas was administered at a rate of 10%-30% of the container's volume per minute. Tissues were harvested only after confirming the cessation of heartbeat and respiration. Notably, no mice died during any of the experimental procedures, and all experiments were conducted in a blinded manner.

Animals and treatment

Induction of DM was performed with low-dose injections of streptozotocin (STZ, 50 mg/kg body weight in 0.1 M citrate buffer, Sigma-Aldrich, St. Louis, MO, United States) for 5 consecutive days in 8-week-old male C57BL/6 J mice as described previously.[14] Eight weeks after STZ injections, only mice with a tail vein blood glucose level higher than 300 mg/dL and significantly decreased body weights were considered to have DM. An Accu-Check blood glucose meter (Roche Diagnostics, Mannheim, Germany) was used to measure both fasting and postprandial blood glucose levels. To test the efficacy of HB-EGF, mice with STZ-induced type 1 DM received two intracavernous injections of phosphate-buffered saline (PBS; Gibco, Carlsbad, CA, United States) or recombinant mouse HB-EGF proteins (ProSpec, East Brunswick, NJ, United States; days −3 and 0; 1, 5, and 10 μg in 20 μL of PBS). A vascular clamp was applied to the base of the penis immediately before injection and was left in place for 30 minutes to restrict blood outflow and HB-EGF leakage into the systemic circulation.[15] After 2 weeks, the erectile function of the mice was assessed by electrical stimulation of the cavernous nerve. Penis tissue was then harvested for Western blot and histological examination studies. Mean systolic blood pressure (MSBP) was evaluated using a noninvasive tail-cuff system (Visitech System, Apex, NC, United States).

Cell culture and treatment

Primary MCECs were prepared and maintained as previously described.[16] Briefly, penis tissue was collected and placed into sterile vials containing Hank's balanced salt solution (Gibco, Carlsbad, CA, United States). The tissue underwent two washes in PBS (Gibco) before the glans, urethra, and dorsal neurovascular bundle were removed. Only the corpus cavernosum (CC) tissue was used for MCEC culture. Tissues were cultured in medium 199 (M199; Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (Gibco), 0.5 mg/mL heparin (Sigma-Aldrich, St. Louis, MO, United States), and 5 ng/mL VEGF (R&D Systems Inc., Minneapolis, MN, United States) in a 5% CO_2 atmosphere incubator at 37°C for 5 minutes. Once the cells reached confluence (after approximately 12-14 days of culture), sprouting cells were subcultured onto gelatin-coated dishes (0.2% gelatin, Sigma-Aldrich). Cells from passages two to four were used for all subsequent experiments. For all in vitro experiments using MCECs, we starved the cells overnight using M199 containing 2% FBS and 1% penicillin/streptomycin and used this medium during all experimental phases.

The primary culture of MCPs was established following the previously described protocol.[17] The CC tissue was cut into several 1-mm pieces, and these fragments settled by gravity into collagen I–coated 35-mm cell culture dishes (BD Biosciences, San Jose, CA, United States). After 30 minutes of incubation at 37°C with 300 μL of complement low-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 nM human pigment epithelium-derived factor (Sigma-Aldrich), an additional 900 μL of complement medium was added, and the samples were incubated at 37°C in a 5% CO_2 atmosphere. The medium was changed every 2 days. Once the cells were confluent and spread over the bottom of the dish (after approximately 12-14 days of culture), sprouting cells were subcultured onto dishes coated with 50 μL/mL collagen I (Advanced BioMatrix). Cells between passages two and three were used for experiments. For all in vitro experiments using MCPs, we starved cells overnight using DMEM containing 2% FBS and 1% penicillin/streptomycin and used this medium during all experimental phases.

To mimic diabetes-induced angiopathy conditions, cells were initially deprived of serum and other growth factors

overnight and then exposed to either normal glucose (NG; 5 mM, Sigma-Aldrich) or high glucose (HG; 30 mM, Sigma-Aldrich) conditions for a duration of 5 days at 37°C in a humidified atmosphere containing 5% CO_2.[18] To investigate the effect of HB-EGF, cells were treated with HB-EGF (0.1 μg/mL) for 72 hours under high-glucose conditions.

Tube formation assay

Tube formation assays were performed following the previously described protocol.[19] Approximately 100 μL of growth factor–reduced Matrigel (BD Biosciences) was placed into each well of a 48-well culture plate, which was maintained at 4°C. The plate was then incubated at 37°C for at least 10 to 15 minutes to allow the Matrigel to gel. Pretreated cells were seeded onto the Matrigel at a density of 1×10^5 cells per well in 200 μL of M199 containing 2% FBS. Tube formation was observed for 18 hours using a phase-contrast microscope (CKX41, Olympus, Tokyo, Japan), and the quantification of master junctions was performed by analyzing data from four independent experiments in a blinded manner with the aid of ImageJ software (National Institutes of Health 1.34, https://imagej.net/ij/nih-image/).

Cell migration assay

To achieve consistent scratches for a cell migration scratch assay, we seeded the pretreated cells in the SPLScar™Block system (SPL Life Sciences, Pocheon-si, Gyeonggi-do, Korea) with confluence over 95% on 60-mm culture dishes as described previously.[20] After 5 hours of seeding, the blocks were removed, and cells were incubated in M199 medium containing 2% FBS and thymidine (2 mM, Sigma-Aldrich) for 24 hours. A phase-contrast microscope (Olympus) was used to capture images, and cell migration was analyzed by determining the percentage of cells that moved into the frame line shown in the figures from four independent experiments in a blinded manner using ImageJ.

Measurement of erectile function

The measurement of erectile function was conducted as described previously.[14] In summary, mice were anesthetized through the administration of ketamine (100 mg/kg) and xylazine (5 mg/kg). A midline abdominal incision exposed the bladder and prostate. Bipolar platinum wire electrodes (BIOPAC Systems Inc., Goleta, CA, United States) were placed around the cavernous nerve for electrical stimulation. Electrical stimulation was applied to the nerve at 5 volts, with a frequency of 12 Hz and a pulse width of 1 millisecond, lasting for 1 minute. The maximal ICP during tumescence was recorded, and the total ICP was calculated as the area under the curve from the beginning of cavernous nerve stimulation to 20 seconds after the termination of the stimulus. Normalization of systemic blood pressure variations among individuals was achieved by calculating the ratio of maximal ICP or total ICP to MSBP.

Histological examinations

For immunofluorescence analysis, penis tissues were fixed in 4% paraformaldehyde at 4°C for 24 hours, and cell samples were fixed in 4% paraformaldehyde at 4°C for 15 minutes at room temperature, as described previously.[21] Frozen tissue sections (12 μm thick), MCECs, and MCPs were washed and then incubated overnight at 4°C with the following antibodies: primary antibody against HB-EGF (1:100, Santa Cruz Biotechnology Inc., Dallas, TX, United States), platelet/endothelial adhesion molecule 1 (PECAM-1; 1:100; Millipore, Temecula, CA, United States), NG2 (1:50; Millipore), phosphohistone H3 (1:50; Millipore), neuronal NOS (nNOS; 1:100; Santa Cruz Biotechnology Inc.), neurofilament (1:50; Sigma-Aldrich), occludin (1:100; Novus Biologicals, Littleton, CO, United States), claudin-5 (1:100; Invitrogen, Carlsbad, CA, United States), oxidized low-density lipoprotein (Ox-LDL: 1:100; Abcam, Cambridge, MA, United States), and phospho-eNOS (1:50; Invitrogen). After several washes with PBS (Gibco), sections were incubated for 2 hours at room temperature with species-appropriate tetramethyl rhodamine isothiocyanate– or fluorescein isothiocyanate–conjugated secondary antibodies (1:100; Zymed Laboratories, South San Francisco, CA, United States), and then mounted in a solution containing DAPI (Vector Laboratories, Inc.) for nuclei staining. The confocal microscope (K1-Fluo; Nanoscope Systems, Inc., Daejeon, Korea) was used to visualize the fluorescent signals. Quantitative histological analyses were performed using ImageJ. We normalized the final measured integrated density values using the area values obtained from ImageJ for each image. For each immunofluorescent staining experiment, at least four different samples were evaluated.

Western blot

Penis tissue, MCECs, and MCPs were lysed using RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitors (GenDEPOT, Katy, TX, United States) and phosphatase inhibitors (GenDEPOT). Equal quantities of protein (30 μg per lane) from whole-cell or tissue lysates were separated by SDS-PAGE on 8% to 12% gels and then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat dried milk for 1 hour at room temperature, then incubated with the following primary antibodies overnight at 4°C: HB-EGF (1:1000, Novus Biologicals), p-eNOS ^{Ser1177} (1:1000; Cell Signaling, Beverly, MA, United States), eNOS (1:1000; BD Biosciences, Mountain View, CA, United States), p-AKT^{Ser473} (1:1000, Cell Signaling), AKT (1:1000, Cell Signaling), and β-actin (1:5000, Cell Signaling). The intensity of the bands was determined using the densitometry feature of ImageJ.

Statistical analysis

Results are expressed as mean ± SEM of values from at least four independent experiments. Unpaired *t*-tests were used for the comparison of two groups, and one-way ANOVA (analysis of variance) followed by Tukey's post hoc test was used for comparisons of more than three groups. Probability value analysis was performed using GraphPad Prism version 8 (GraphPad Software, Inc., Boston, MA, United States), and *P*-values $<.05$ were considered statistically significant.

Results

Metabolic variables

Eight weeks after the completion of STZ injections, fasting and postprandial blood glucose concentrations were found to be significantly elevated in treated mice compared to control mice. Additionally, the body weight of DM mice (16-week-old male C57BL/6 J mice) was significantly reduced. No

Table 1. Physiologic and metabolic parameters: 2 weeks after treatment with HB-EGF.

	Control	STZ-induced diabetic mice				
		NT	PBS	HB-EGF, μg		
				1	5	10
Body weight (g)	34.4 ± 1.7	24.5 ± 0.9*	24.5 ± 1.2*	24.9 ± 1.0*	25.0 ± 0.5*	25.1 ± 1.5*
Fasting glucose (mg/dL)	100.2 ± 2.2	541.8 ± 13.9*	557.0 ± 14.4*	542.0 ± 15.7*	548.0 ± 13.4*	537.6 ± 23.4*
Postprandial glucose (mg/dL)	172.2 ± 13.5	589.8 ± 6.3*	578.8 ± 6.0*	593.6 ± 5.2*	581.6 ± 11.0*	583.4 ± 9.7*
MSBP (mm Hg)	106.8 ± 3.1	102.2 ± 2.8	102.4 ± 1.9	101.6 ± 3.4	100.2 ± 2.2	98.8 ± 0.8

Abbreviations: HB-EGF, heparin-binding epidermal growth factor–like growth factor; MSBP, mean systolic blood pressure; NT, not treated; STZ, streptozotocin. Values are the mean ± SEM for n = 5 animals per group. *$P < .05$ vs control group.

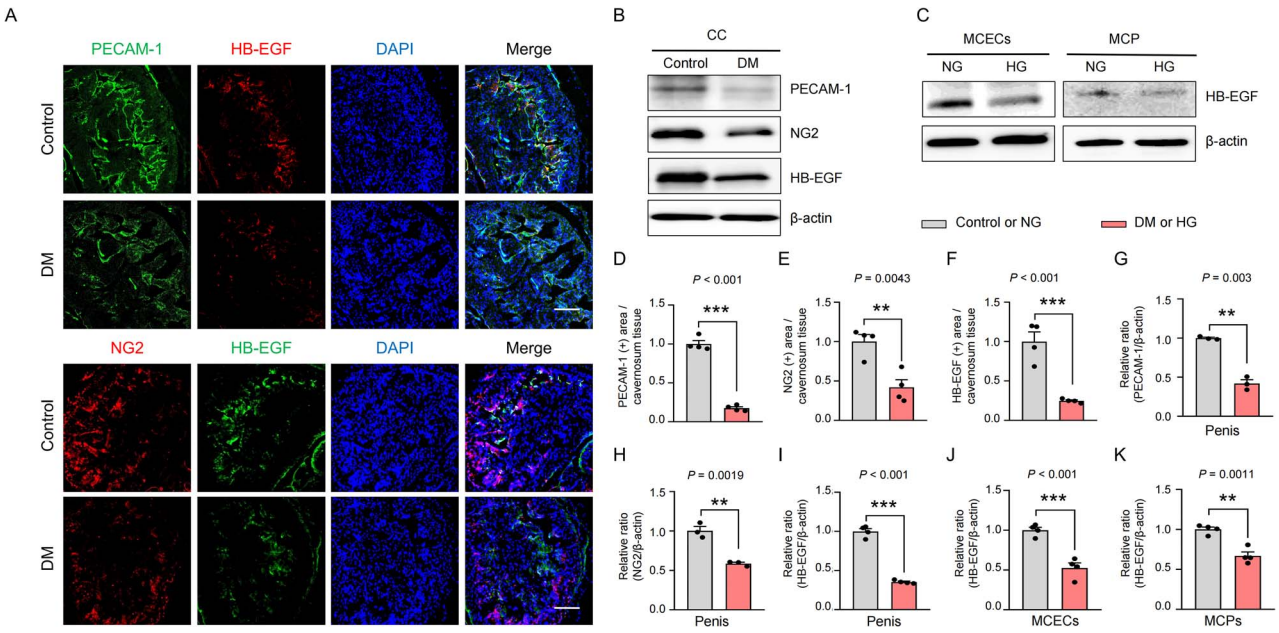

Figure 1. Expression of HB-EGF is decreased under diabetic conditions. (A) Double-immunofluorescence staining of PECAM-1/HB-EGF (top panel) and NG2/HB-EGF (bottom panel) in penile tissues of age-matched control and diabetic mice. Nuclei were stained with DAPI (blue). Scale bar, 100 μm. (B) Representative Western blots for PECAM-1, NG2, and HB-EGF in corpus cavernosum tissue from age-matched control and diabetic mice. (C) Representative Western blots for HB-EGF in MCECs, and MCPs exposed to NG and HG conditions. (D-F) Normalized areas of PECAM-1, NG2, and HB-EGF–immunopositive staining were quantified using ImageJ software (n = 4). (G-K) Normalized band intensity values for indicated targets were quantified using ImageJ software (n = 4). Results are presented as mean ± SEM. **$P < .01$; ***$P < .001$. The relative ratio of the control or NG group was arbitrarily set to 1. DAPI, 4′,6-diamidino-2-phenylindole; DM, diabetes mellitus; HB-EGF, heparin-binding epidermal growth factor–like growth factor; HG, high glucose; MCEC, mouse cavernous endothelial cell; MCP, mouse cavernous pericyte; NG, normal glucose; PECAM-1, platelet/endothelial adhesion molecule 1.

significant differences were observed in MSBP among all experimental groups (Table 1).

HB-EGF expression is decreased under diabetic conditions

Treatment with HB-EGF is known to improve chronic non-healing wounds commonly caused by DM and lasts significantly longer at the injection site than traditional growth factors.[22] However, the expression status of HB-EGF in penis tissue of diabetes-induced ED is not well defined. To understand the expression of HB-EGF in CC tissue under diabetic conditions, we first evaluated the expression of HB-EGF in CC tissues from age-matched control and diabetic mice. Double-immunofluorescence staining with antibodies against PECAM-1 (an endothelial cell marker, green)/HB-EGF (red) and NG2 (a pericyte marker, red)/HB-EGF (green) revealed that the expressions of PECAM-1 (Fig. 1A and D), NG2 (Fig. 1A and E), and HB-EGF (Fig. 1A and F) were significantly reduced in CC tissues. Notably, the expression area of HB-EGF is closely associated with cavernous endothelial cells and pericytes (Fig. 1A). Furthermore, Western blot analysis confirmed that the expression of PECAM-1, NG2, and HB-EGF was significantly reduced in CC tissues from diabetic mice (Fig. 1B and G-I), as well as in MCECs and MCPs under HG conditions (Fig. 1C, J, and K).

HB-EGF promotes angiogenesis and proliferation under high-glucose conditions

To investigate the effects of HB-EGF on angiogenesis under HG conditions, we performed tube formation assays on MCECs and migration assays on MCECs and MCPs under NG (5 mM) and HG (30 mM) conditions.[18] We observed severely disrupted capillary-like structures from the tube formation assays and decreased migration ability from the migration assays (Fig. 2A and B). However, there was a dose-dependent (50, 100, 200, and 500 ng/mL) increase in the formation of capillary-like structures after HB-EGF treatment on MCECs, particularly at the 100 ng/mL

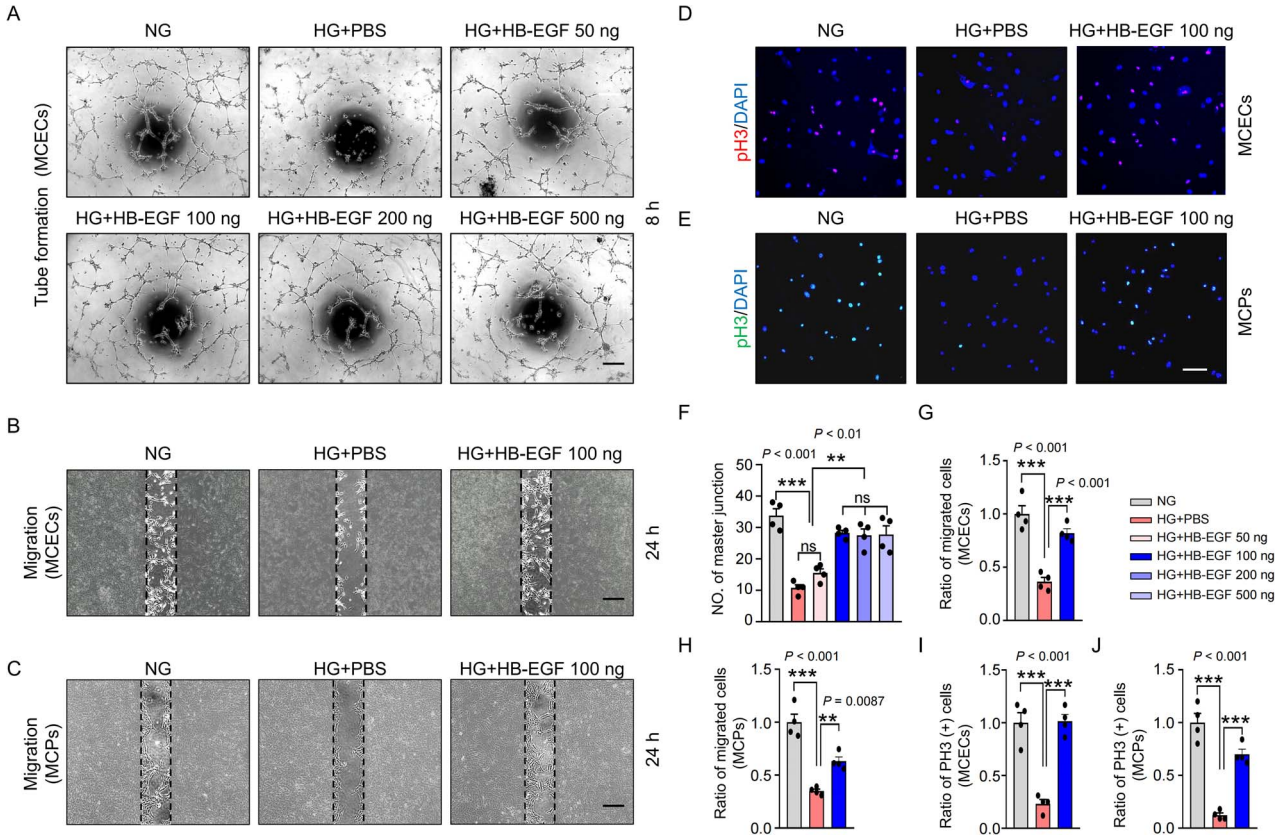

Figure 2. HB-EGF promotes angiogenesis and proliferation under HG conditions. (A) Tube formation assays of MCECs treated with PBS or HB-EGF (50, 100, 200, or 500 ng/mL) under normal-glucose (NG) and high-glucose (HG) conditions. Scale bar = 100 μm. (B and C) Migration assays in MCECs and MCPs treated with PBS or HB-EGF (100 ng/mL) under NG or HG conditions. Scale bar = 100 μm. (D) Representative images of immunofluorescent staining of PH3 (red and green) in MCECs and MCPs treated with PBS or HB-EGF (100 ng/mL) under NG or HG conditions. Nuclei were stained with DAPI (blue). Scale bar, 50 μm. (F) The number of master junctions in MCECs was quantified using ImageJ software (n = 4). (G and H) The number of migrated MCECs (G) and MCPs (H) within the frame line was quantified using ImageJ software (n = 4). (I and J) The number of PH3-positive MCECs (I) and MCPs (J) was quantified using ImageJ software (n = 4). Results are presented as mean ± SEM. **P < .01; ***P < .001. The relative ratio of the NG (G-J) group was arbitrarily set to 1. DAPI, 4′,6-diamidino-2-phenylindole; HB-EGF, heparin-binding epidermal growth factor–like growth factor; HG, high glucose; MCEC, mouse cavernous endothelial cell; MCP, mouse cavernous pericyte; NG, normal glucose; PBS, phosphate-buffered saline; PH3, phosphohistone H3.

concentration, where the effect was very pronounced (Fig. 2A and F). Therefore, we selected 100 ng/mL as the optimal therapeutic dose for our in vitro studies. Subsequent treatment with HB-EGF (100 ng/mL) significantly enhanced the migration abilities of both MCECs (Fig. 2B and G) and MCPs (Fig. 2C and H) under HG conditions. Additionally, we examined the proliferation (using phosphohistone H3) of MCEC (Fig. 2D and I) and MCP (Fig. 2E and J) treated with HB-EGF (100 ng/mL) under HG conditions. We found that proliferation was severely reduced under HG conditions; however, these effects were improved to normal levels after treatment with HB-EGF protein (Fig. 2D, E, I, and J). Taken together, these data suggest that HB-EGF induces angiogenesis and proliferation in MCECs and MCPs under HG conditions.

Exogenous administration of HB-EGF protein improves erectile function in STZ-induced diabetic mice

To determine whether HB-EGF has beneficial effects on erectile function in diabetic mice, we evaluated ICP 2 weeks after administering two intracavernous injections of PBS or various doses of HB-EGF (days −3 and 0; 1, 5, and 10 μg in 20 μL of PBS) to age-matched control and diabetic mice (Fig. 3A). Compared with age-matched controls, the ratios of maximum or total ICP to MSBP were significantly decreased in nontreated (NT) and PBS-treated diabetic mice. However, after two intracavernous injections of HB-EGF, the recovery of erectile function in diabetic mice treated with low-dose HB-EGF (1 μg/20 μL of PBS) was not particularly evident. In contrast, the recovery of erectile function in diabetic mice treated with medium- and high-dose HB-EGF (5 and 10 μg/20 μL of PBS) was significantly improved, reaching more than 91% of the control value (Fig. 3B and C). No detectable differences in MSBP were observed between experimental groups. These results suggest that HB-EGF improves erectile function in diabetic mice.

HB-EGF increases cavernous endothelial cell, pericyte, and neuronal cell content in STZ-induced diabetic mice

To assess the effect of HB-EGF on neurovascular regeneration in diabetic mice, we performed double-immunofluorescence staining with antibodies against PECAM-1/NG2 in the CC tissues and nNOS/neurofilaments-2000 (NF) in dorsal nerve bundles (DNBs) of diabetic mice. We observed that the content of PECAM-1–positive endothelial cells (Fig. 4A and C),

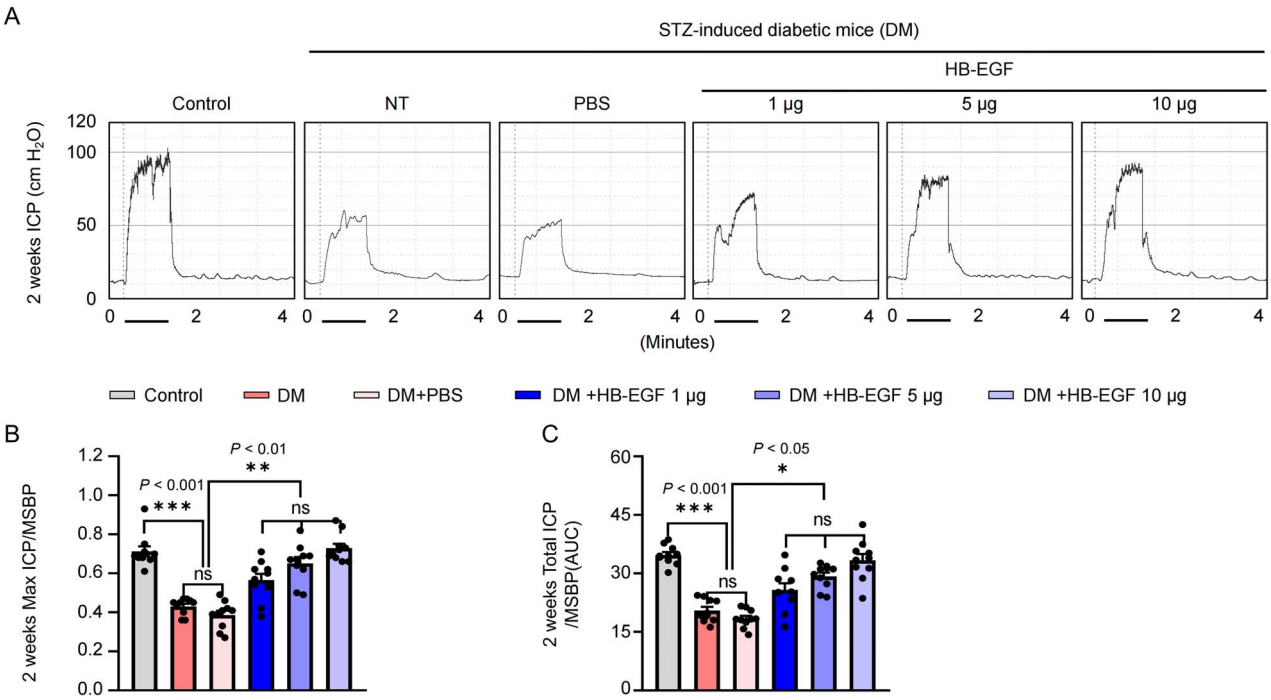

Figure 3. HB-EGF improves erectile function in STZ-induced diabetic mice. (A) Representative ICP responses in age-matched control and diabetic mice 2 weeks after 2 intracavernous administrations of PBS or various doses of HB-EGF (days −3 and 0; 1 μg, 5 μg, and 10 μg in 20 μL of PBS, respectively). The solid bar indicates the stimulus interval. (B and C) ratios of maximum ICP (B) and total ICP (C) to MSBP were calculated for each group (n = 10). Results are presented as mean ± SEM. *P < .05; **P < .01; ***P < .001. HB-EGF, heparin-binding epidermal growth factor–like growth factor; ICP, intracavernous pressure; MSBP, mean systolic blood pressure; PBS, phosphate-buffered saline; STZ, streptozotocin.

NG2-positive pericytes (Figs. 4A and D), and nNOS-, NF-positive neuronal cells (Figs. 4B, E, and F) were lower in PBS-treated diabetic mice than in age-matched controls. However, treatment with HB-EGF (5 μg/20 μL of PBS) significantly enhanced these neurovascular contents in diabetic mice (Fig. 4). In addition, these immunofluorescence results were also validated by Western blot analysis for PECAM-1, NG2, and nNOS (Fig. 4G-J). These results indicate that HB-EGF can improve the content of cavernous endothelial and neuronal cells, hereby ameliorating ED in diabetic mice.

HB-EGF induces tight junction protein levels and reduces permeability in STZ-induced diabetic mice

To assess the expression of tight junction proteins in cavernosum tissues, we performed double-immunofluorescence staining with antibodies against occludin and claudin-5 in the CC tissues of diabetic mice. The expressions of occludin and claudin were lower in PBS-treated diabetic mice, but HB-EGF (5 μg/20 μL of PBS) treatment significantly improved the levels of these endothelial cell-to-cell junction proteins in the CC tissues of diabetic mice (Fig. 5A, C, and D). Additionally, to evaluate the permeability status of penile tissue, we performed immunofluorescence staining of Ox-LDL on the CC tissue of diabetic mice. We observed that Ox-LDL extravasation levels were significantly elevated in PBS-treated diabetic mice. However, HB-EGF (5 μg/20 μL PBS) treatment significantly reduced the leakage of Ox-LDL in diabetic mice (Fig. 5B and E). In addition, these immunofluorescence results was also validated by Western blot analysis for occludin and claudin-5 (Fig. 5F-H). These results suggest that HB-EGF induces tight junction protein levels, thereby reducing the permeability of penile tissue in diabetic mice.

HB-EGF activates the AKT/eNOS signaling pathway in STZ-induced diabetic mice

Previous studies have demonstrated that HB-EGF–induced eNOS activation is dependent on the AKT signaling pathway to promote angiogenesis.[23,24] To determine whether HB-EGF also promotes angiogenesis in the penis of diabetic mice by activating the AKT/eNOS signaling pathway, we performed Western blot analysis using penile tissues from age-matched control and diabetic mice. Interestingly, the phosphorylation levels of AKT and eNOS were lower in PBS-treated diabetic mice than in age-matched controls. Treatment with HB-EGF (5 μg/20 μL PBS) significantly enhanced the phosphorylation of AKT (Ser473) and eNOS (Ser1177) (Fig. 6A-C). Furthermore, through double-immunofluorescence staining with antibodies against PECAM-1 and p-eNOS^{Ser1177} in the CC tissues of diabetic mice, we obtained consistent results (Figs. 6D and E). These findings indicate that HB-EGF mediates the AKT/eNOS signaling pathway and plays a crucial role in the treatment of ED in diabetic mice.

Discussion

Recently, many studies have evaluated the role of angiogenic and neurotrophic factors as potential treatment options for diabetic ED. Such factors include VEGF,[25] angiogenin-1,[26] angiopoietin-4,[27] hepatocyte growth factor,[28] dickkopf2,[29] leucine-rich alpha-2 glycoprotein 1,[30] brain-derived neurotrophic factor,[31] and neurotrophin-3.[32] However, these studies are still in their infancy, and the complex underlying pathology of diabetic ED, particularly the specific mechanisms of angiopathy and neuropathy, remains poorly understood. Therefore, we need more knowledge to expand our treatment

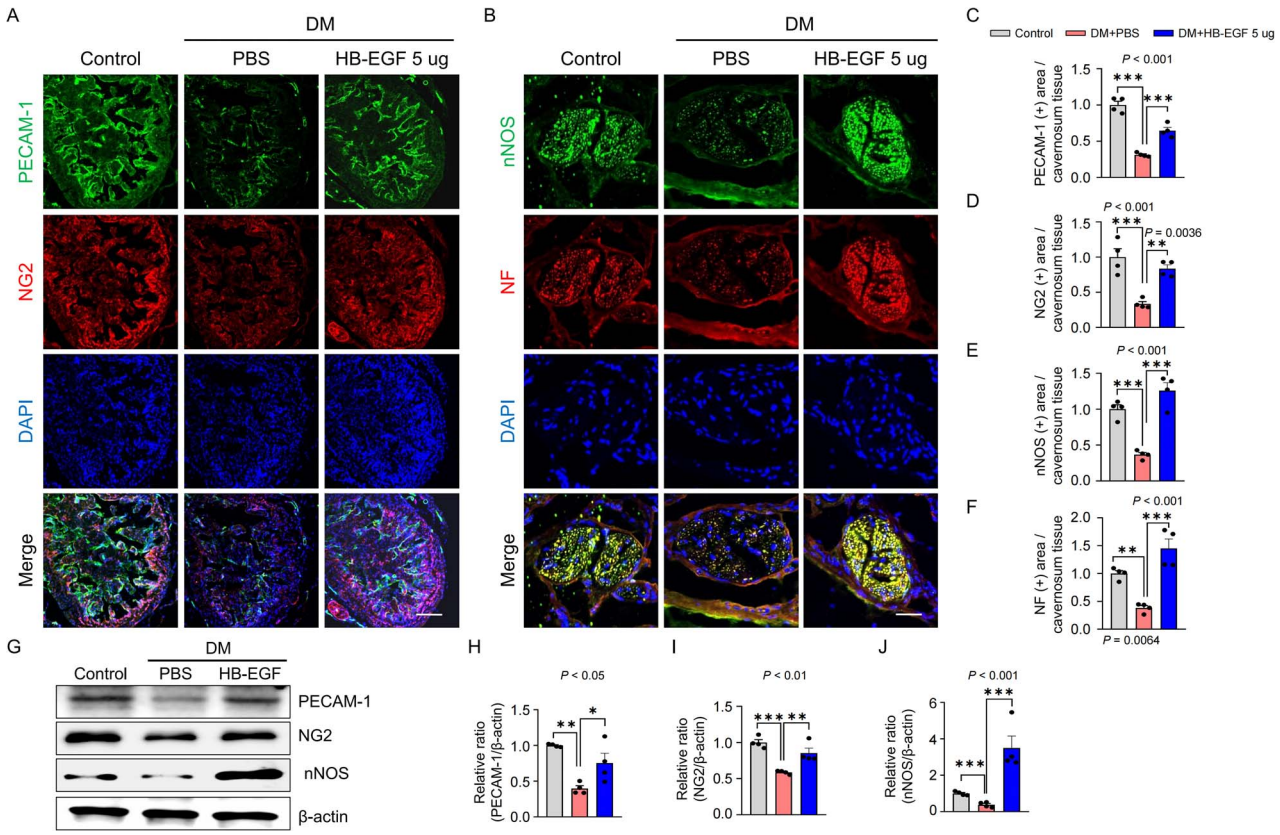

Figure 4. HB-EGF increases cavernous endothelial cell, pericyte, and neuronal cell content in STZ-induced diabetic mice. (A and B) double-immunofluorescence staining of PECAM-1/NG2 (A) and nNOS/NF (B) in penile tissues from age-matched control and diabetic mice 2 weeks after two intracavernous administrations of PBS or HB-EGF (days −3 and 0; 5 μg in 20 μL of PBS). Nuclei were stained with DAPI (blue). Scale bar, 100 μm (A) or 25 μm (B). (C-F) quantification of PECAM-1, NG2, nNOS, and NF immunopositive areas using ImageJ software (n = 4). (G) Representative Western blots for PECAM-1, NG2, and nNOS in penile tissues from age-matched control and diabetic mice 2 weeks after two intracavernous administrations of PBS or HB-EGF (days −3 and 0; 5 μg in 20 μL of PBS). (H-J) normalized band intensity values for indicated targets were quantified using ImageJ software (n = 4). Results are presented as mean ± SEM. **P < .01; ***P < .001. The relative ratio of the control group was arbitrarily set to 1. DAPI, 4′,6-diamidino-2-phenylindole; PECAM-1, platelet/endothelial adhesion molecule 1; HB-EGF, heparin-binding epidermal growth factor–like growth factor; NG2, nerve/glial-antigen 2; STZ, streptozotocin.

options. In this study, we explored whether HB-EGF positively regulates angiogenesis and nerve regeneration, thereby conferring benefits for diabetic ED. Our findings reveal that HB-EGF expression is notably diminished under diabetic conditions, suggesting a potential association between reduced HB-EGF levels and diabetic angiopathy. Therefore, we examined the role of HB-EGF in diabetic ED through the administration of exogenous recombinant HB-EGF protein. We discovered that exogenous HB-EGF effectively diminishes penile permeability by promoting angiogenesis and increasing tight junction protein expression, thereby improving erectile function in diabetic ED. The present study offers crucial insights into the angiogenic properties of HB-EGF and may yield valuable perspectives for the management of diabetic ED.

The angiogenic growth factor HB-EGF, exists in two distinct forms: membrane-bound and soluble HB-EGF,[33] which are ubiquitously expressed across various cell types.[33,34] Prior research has indicated that endothelial-derived platelet-derived growth factor subunit B B and HB-EGF orchestrate pericyte recruitment during vascular development.[35] Our current research demonstrates that HB-EGF is present in MCECs and MCPs, with its expression being compromised in diabetic states. Intriguingly, exogenous HB-EGF enhances the migration and proliferation of MCECs and MCPs, aligning with previous findings.[35] Nonetheless, the specific conditions under which MCECs or MCPs secrete HB-EGF to stimulate angiogenesis and stabilize vessels remain unclear. Particularly, whether MCPs can secrete HB-EGF and, in turn, influence the angiogenesis of MCECs, thereby improving erectile function in diabetic ED, warrants further investigation. This possibility is underscored by recent studies highlighting the pivotal role of pericytes in the recovery of erectile function in diabetic ED.[29,36,37] Moreover, various matrix metalloproteinases and ADAM (a disintegrin and metalloproteinase) metallopeptidases are recognized for converting HB-EGF into its soluble form.[8] Future experiments should examine the expression of these enzymes under diverse physiological and pathological scenarios and the secretion dynamics of HB-EGF by MCECs or MCPs. Such studies will establish a foundational understanding of the role of HB-EGF across different cell types and under varying conditions.

Mounting evidence suggests that diabetic ED is intricately linked to alterations in penile vascular permeability function.[17,38-40] Modulation of cavernous permeability presents a promising therapeutic approach for diabetic ED. Regarding HB-EGF, prior research has demonstrated that its administration can safeguard intestinal barrier function by markedly reducing permeability following radiation therapy–induced

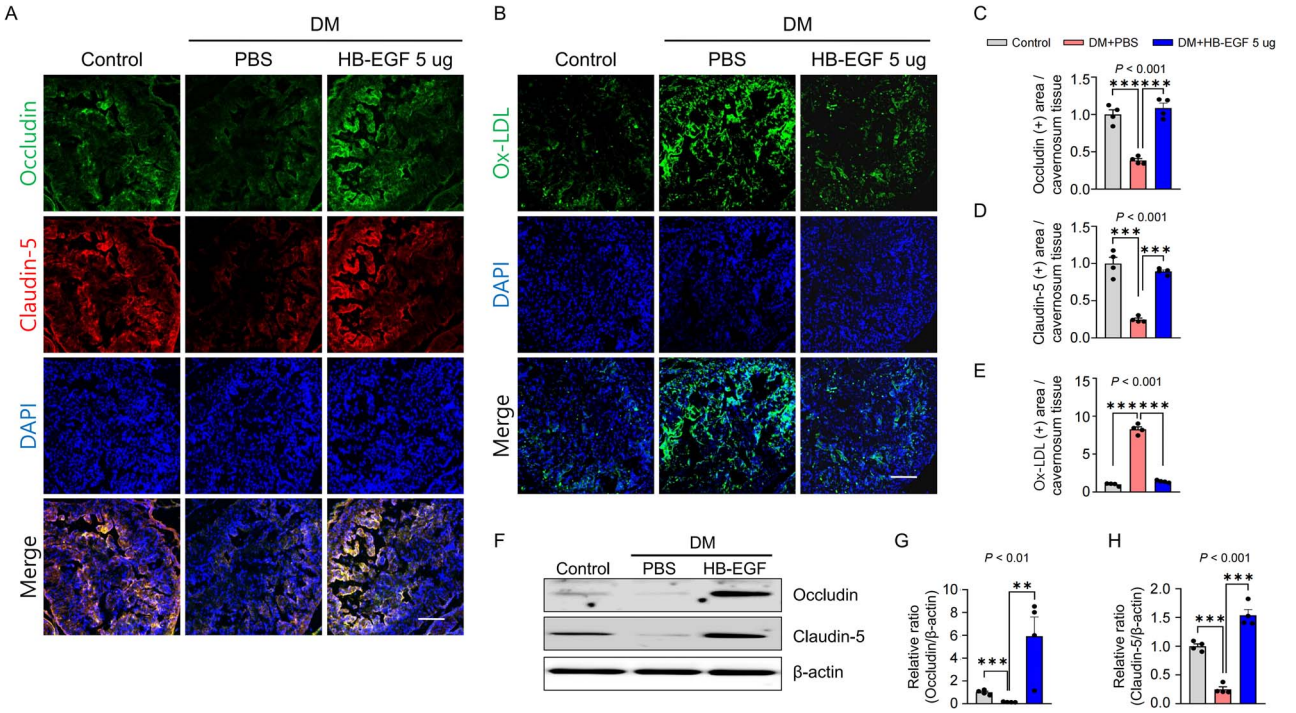

Figure 5. HB-EGF induces tight junction protein levels and reduces permeability in STZ-induced diabetic mice. (A) Double-immunofluorescence staining of occludin (green) and claudin-5 (red) in penile tissues from age-matched control and diabetic mice 2 weeks after two intracavernous administrations of PBS or HB-EGF (days −3 and 0; 5 μg in 20 μL of PBS). (B) Ox-LDL (green) staining in the same samples. Nuclei labeled with DAPI (blue). Scale bar, 100 μm. (C-E) quantitative analysis of occludin, claudin, and ox-LDL immunopositive areas using ImageJ software (n = 4). (F) Representative Western blots for Occludin and Claudin-5 in penile tissues from age-matched control and diabetic mice 2 weeks after two intracavernous administrations of PBS or HB-EGF (days −3 and 0; 5 μg in 20 μL of PBS). (G and H) normalized band intensity values for indicated targets were quantified using ImageJ software (n = 4). Results are presented as mean ± SEM. **P < .01; ***P < .001. The relative ratio of the control group was arbitrarily set to 1. DAPI, 4′,6-diamidino-2-phenylindole; DM, diabetes mellitus; HB-EGF, heparin-binding epidermal growth factor–like growth factor; ox-LDL, oxidized low-density lipoprotein; STZ, streptozotocin.

intestinal injury.[41,42] Our current study corroborates these findings, showing that HB-EGF can decrease penile permeability by upregulating the expression of tight junction proteins. However, the study by Shim et al also posits a potential pathophysiological mechanism where excessive HB-EGF enhances VEGF signaling–induced permeability and mild ventricular dilation in the periventricular region.[43] These results indicate that the role of HB-EGF role in regulating permeability may vary across different organs and under distinct physiological and pathological conditions. Consequently, when employing HB-EGF in the treatment of related diseases, its dosage must be meticulously regulated.

Previous investigations have established that HB-EGF fosters the production of NO in endothelial cells by stimulating the AKT/eNOS signaling pathway, thereby facilitating cell proliferation, survival, migration, differentiation, and ultimately angiogenesis.[8,44-47] In our study, we confirmed these known effects but did not delve into a deeper analysis of HB-EGF mechanisms of action, which warrant further investigation in future research. Notably, HB-EGF is not only an angiogenic factor but is also pervasively present in neurons and glial cells within the brain.[48] The study by Zhou et al has revealed that HB-EGF serves as a potent neurotrophic factor for PC12 cells.[34] Additionally, HB-EGF bolsters the survival of dopaminergic neurons through the activation of the MAPK (mitogen-activated protein kinase) and Akt signaling pathways.[49] In our research, we observed that HB-EGF could enhance the expression of nNOS and NF in the penis of mice with diabetes-induced ED. Presently, there is no comprehensive evidence elucidating the mechanism by which HB-EGF facilitates neurological recovery in diabetes-induced ED. Future studies might systematically analyze its specific mechanisms via whole-gene expression profiling analyses, such as RNA sequencing and single-cell RNA sequencing, in cavernous nerve injury mouse models.

To our knowledge, this is the first study to demonstrate the effect of HB-EGF on improving erectile function in a mouse model of diabetes-induced ED. However, our study has a few limitations. First, our result is similar to those of other published studies, such as studies involving BMP2,[50] IGFBP5,[39] LRG1,[30] VASH1,[51] PC-NVs,[36] DKK3,[52] ESC-NVs,[53] and proNGF antibodies.[54] These studies all used mouse diabetes models, and all showed some effectiveness when treated alone. However, these research results are only in the preclinical stage and there is no further research to facilitate development and clinical application. In particular, protein therapies have many shortcomings, such as high production costs, inability to be used as oral drugs, and inability of large proteins to effectively penetrate tissues to reach their targets. Therefore, there is a need to develop more effective therapeutic targets, especially to study the specific signaling pathways in vascular regeneration and nerve regeneration, so as to determine new strategies for treating ED. Second, high-glucose conditions do not fully mimic the angiopathy caused by diabetes. Furthermore, we were unable to perform protein tracking analysis, which requires large amounts of

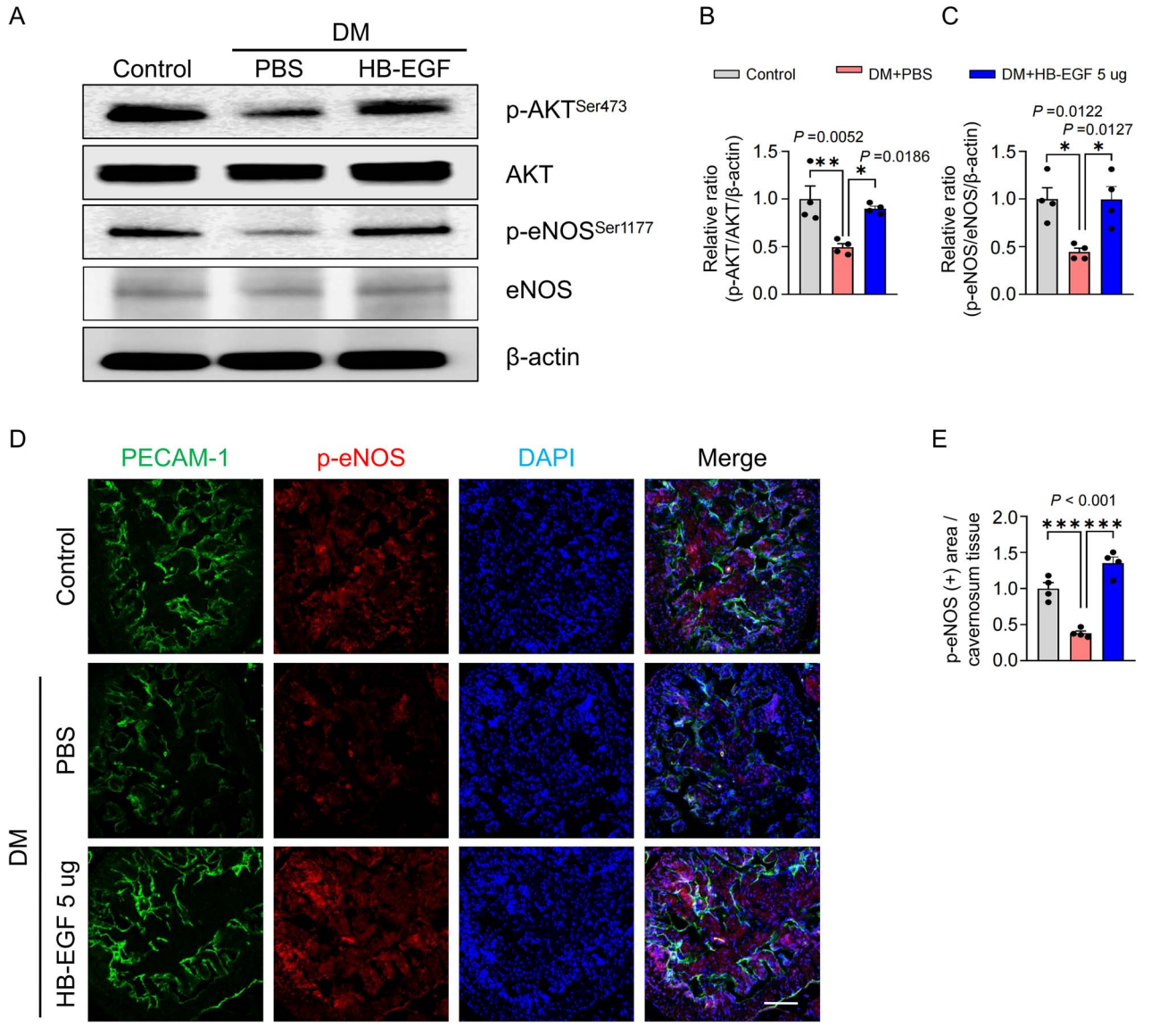

Figure 6. HB-EGF activates the AKT/eNOS signaling pathway in STZ-induced diabetic mice. (A) Representative Western blots for p-AKT^{Ser473}, total AKT, p-eNOS^{Ser1177}, and total eNOS in penile tissues from age-matched control and diabetic mice 2 weeks after two intracavernous administrations of PBS or HB-EGF (days −3 and 0; 5 μg in 20 μL of PBS). (B and C) normalized band intensity values for indicated targets were quantified using ImageJ software (n = 4). (D) Double-immunofluorescence staining of occludin (green) and claudin-5 (red) in penile tissues after ICP study. (E) Quantitative analysis of p-eNOS^{Ser1177} immunopositive areas using ImageJ software (n = 4). Results are presented as mean ± SEM. *P < .05; **P < .01; ***P < .001. The relative ratio of the control group was arbitrarily set to 1. DAPI, 4′,6-diamidino-2-phenylindole; eNos, endothelial nitric oxide synthase; HB-EGF, heparin-binding epidermal growth factor–like growth factor; ICP, intracavernous pressure; PBS, phosphate-buffered saline; p-eNOS, phosphorylated endothelial nitric oxide synthase; STZ, streptozotocin.

protein, as proteins are significantly lost during washing when conjugate to fluorescent agents. Finally, further studies, such as investigation of RNA sequencing arrays, are necessary to evaluate the detailed mechanisms and functions of HB-EGF in other neurovascular diseases.

Conclusion

The use of HB-EGF represents a distinct strategy compared to PDE5 inhibitors, as it seems to improve cavernous endothelial and neuronal cell content by modulating permeability and activating the AKT/eNOS signaling pathway, thereby improving vascular integrity and ultimately enhancing erectile function. Considering these potential mechanisms, HB-EGF may prove beneficial for patients who do not respond to phosphodiesterase type 5 inhibitors.

Author contributions

Drs F.R. Fridayana and J. Ock contributed equally and are considered co–senior authors of this work. In addition, they had full access to all the data in the study and take responsibility for the integrity of data and accuracy of the data analysis.

Conception and design: J.O., G.N.Y., and J.-K.R. Acquisition of data: F.R.F., J.O., F.-Y.L., L.N., M.N.V., Y.H., G.N.Y. Analysis and interpretation of data: F.R.F., J.O., G.N.Y. Drafting the article: F.R.Y., J.O., G.N.Y., J.-K.R. Revising for intellectual content: G.N.Y., J.-K.R. Final approval of the completed article: F.R.F., J.O., F.-Y.L., L.N., M.N.V., Y.H., G.N.Y., J.-K.R.

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Conflicts of interest

None declared.

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