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Exendin-4 protects hindlimb ischemic injury by inducing angiogenesis

Hye-Min Kang, Inkyung Sohn, Junyang Jung, Joo-Won Jeong, Chan Park*

Department of Anatomy and Neurobiology, Biomedical Science Institute, School of Medicine, Kyung Hee University, Republic of Korea

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ABSTRACT

Exendin-4, an analog of glucagon-like peptide-1, has shown to have beneficial effects on endothelial function, and was recently approved for the treatment of diabetes. In previous studies, we showed that exendin-4 induces angiogenesis in *in vitro* and *ex vivo* assays; in this study, we assessed the proangiogenic effects of exendin-4 *in vivo* using a mouse hindlimb ischemia model. Treatment with exendin-4 for three days mitigated hindlimb and gastrocnemius muscle fiber necrosis. Hindlimb perfusion was determined using indocyanine green fluorescence dynamics that showed, significantly higher blood flow rate to the ischemic hindlimbs in an exendin-4–treated group. Immunohistochemistry assay showed that exendin-4 increased CD31-positive areas in the gastrocnemius muscle of ischemic limbs. Furthermore, treatment of the hindlimbs of ischemic mice with exendin-4 increased vascular endothelial growth factor (VEGF) and phospho-extracellular signal-related kinase (ERK) on western blot analysis. Our data demonstrate that exendin-4 prevents hindlimb ischemic injury by inducing vessels via VEGF angiogenic-related pathways. These findings suggest that exendin-4 has potential as a therapeutic agent for vascular diseases that stimulate angiogenesis.

1. Introduction

Peripheral arterial disease (PAD) is a typical consequence of insufficient angiogenesis [1] and is caused by atherosclerosis that results in obstructions in the arteries that limit blood supply to organs other than the heart. The lower extremity is the most common site for PAD [2]. Although long under-recognized and, therefore, underdiagnosed, PAD is now considered an important public health problem with a prevalence nearly equal to that of coronary artery disease (CAD) [3,4]. The prevalence of PAD increases with age, affecting approximately 6% of individuals aged 50–60 years, and 10–20% of individuals aged >70 years [2,4]. Although smoking and diabetes mellitus are causative for both PAD and CAD, these risk factors have a disproportionately stronger role in PAD than in CAD [5]. Given the persistence of smoking, and the anticipated rise in the prevalence of diabetes following the increased obesity rates in the Western world, PAD is likely to be an increasingly important issue [6]. The hindlimb ischemia model involves acute interruption of arterial supply and remains the most commonly used pre-clinical *in vivo* method of assessing the angiogenic and arteriogenic potential of agents and cells [7,8].

Exendin-4 is a GLP-1 receptor (GLP-1R) agonist that shares 53% amino-acid sequence identity to GLP-1. Exendin-4 can induce pancreatic β-cell proliferation and inhibition of β-cell apoptosis, similar to GLP-1 [9,10]. Consequently, exendin-4 was recently approved for the treatment of type 2 diabetes mellitus (DM). Apart from its effects in diabetic patients, exendin-4 also has been shown to exert beneficial actions on endothelial function [11]. Moreover, several studies in various animal models have shown that exendin-4 may reduce myocardial infarct size [12,13]. Endothelial cells express GLP-1R, and acute administration of GLP-1 improves endothelial dysfunction in type 2 diabetes patients with CAD [14], demonstrating the importance of GLP-1 in regulating endothelial function. Interestingly, exendin-4 stimulates the proliferation of human coronary artery endothelial cells through cAMP-dependent protein kinase (PKA) and phosphoinositide 3-kinase (PI3K)/Akt-dependent pathways [15]. Although some studies have investigated how exendin-4 affects the proliferation of endothelial cells, its effects on angiogenic processes in an *in vivo* vascular disease model have not been elucidated. Therefore, in this study we evaluated the

Abbreviations: BFI, blood flow index; CAD, coronary artery disease; DAB, 3,3-diaminobenxidine; GC, gastrocnemius; GLP-1R, GLP-1 receptor; MAPK, mitogen-activated protein kinase; MTT, mean transit time; PAD, peripheral arterial disease; PKA, cAMP-dependent protein kinase; ROI, region of intensity; TBS-T, Tris-buffered saline Tween 20; VEGF, vascular endothelial growth factor.

* Corresponding author. Department of Anatomy and Neurobiology, Biomedical Science Institute, School of Medicine, Kyung Hee University, #1 Hoeki-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea.
E-mail address: psychan@khu.ac.kr (C. Park).

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angiogenic effects of exendin-4 through mouse hindlimb ischemia.

2. Materials and methods

2.1. Animals

Eight-week-old male Balb/c mice (Dae Han Bio Link Co, Ltd., Chungbuk, Korea) were used in the animal experiments. All animals were housed in groups in temperature-controlled (20 ± 2 °C) environments with unlimited access to food and water (12-h light/dark cycle). All experiments were carried out in accordance with the animal care guidelines of the National Institutes of Health and the Korean Academy of Medical Sciences. The animals were divided into two groups treated with 0.9% saline or 1 μg/kg exendin-4 (Sigma–Aldrich, St. Louis, MO, USA) for three days. Serial indocyanine green (ICG) perfusion imaging was performed immediately after surgery and on postoperative days 3 and 7. The animals were then sacrificed on day 3 or day 7 post-surgery and underwent immunohistochemistry or western blot analysis.

2.2. Hindlimb ischemia surgery

Surgery was carried out, as described by Niiyama [16]. Animals were anesthetized with an intraperitoneal injection of 30 mg/kg Zoletil (Virbac Korea, Seoul, Korea) and 10 mg/kg Rompun (Bayer Korea, Seoul, Korea); incisions were made with fine forceps and surgical scissors. Next, the subcutaneous fat tissue was transversely incised by Change-A-tip™cautery (Bovie Medical Corporation, Purchase, NY, USA) to reveal the underlying femoral artery. The neurovascular bundle was exposed from the membranous femoral sheath. Then, using a clean set of fine forceps and cotton swab, the femoral artery was separated from the femoral vein and nerve at the proximal location near the groin. After dissection, a strand of 7-0 silk suture was passed underneath the proximal end of the femoral artery, which was occluded with double knots. Then, in the proximal region to the knee, the femoral artery was occluded with silk suture, the segment of femoral artery was carefully transected between the distal and proximal knots with a cautery. The retractor was removed and the incision was closed using 4-0 silk sutures. During surgery, body temperatures were monitored by rectal probe and maintained at 37 °C using a temperature-controlled Homeothermic Blanket Systems (Harvard Apparatus, Holliston, MA).

2.3. Ischemia score

Ischemia score was determined, as described by Westvik [17]. Scoring was designed to detect less severe levels of ischemia (0 = auto amputation of leg; 1 = leg necrosis; 2 = foot necrosis; 3 = discoloration of two or more toes; 4 = discoloration of one toe; 5 = discoloration of two or more toenails; 6 = discoloration of one toenail; 7 = no necrosis).

2.4. Histology

Mice were anesthetized with Zoletil and Rompun, transcardially perfused with 0.05 M PBS followed by cold 4% paraformaldehyde (Sigma–Aldrich) in 0.1 M phosphate buffer, pH 7.4. The muscle sectioning was carried out with modifications, as described by Limbourg [18]. The excised gastrocnemius (GC) muscles were post-fixed at 4 °C for 2 h in the same paraformaldehyde solution, transferred to 15% sucrose in 0.05 M PBS and incubated at 4 °C for 2 h, and then moved to a solution of 30% sucrose in 0.05 M PBS at 4 °C overnight. The next day, the muscles were removed from the sucrose solution and submerged in optimal cutting temperature (O.C.T) compound and snap frozen in liquid nitrogen. The GC muscles were cut into 20-μm frozen sections at −20 °C using a freezing microtome (Leica, Nussloch, Germany) and stored at −70 °C until use.

2.5. Indocyanine green imaging

After the mice were anesthetized, fur from the hindlimbs was removed using an electric shaver and hair removal cream. Each mouse was placed under an 830-nm band-pass filter CCD camera (Vieworks Co., Ltd., Anyang, Korea). ICG (1 μg/g wt) (Dongin-Dang Co., Ltd., Siheung, Korea) was injected into the tail vein and 760-nm lights were used to illuminate the hindlimbs. Time-series ICG fluorescence signals were acquired every 500 ms for 7 min. The initial 200 frames were used to generate blood flow maps using software provided by the manufacturer (Vieworks Co., Ltd.); calculations were performed according to a previous report by Kang et al. [19].

2.6. Immunohistochemistry

For immunohistochemical detection of CD31, sections were incubated for 15 min in 1% H_2O_2 and then overnight at 4 °C in 0.3% Triton X-100 containing 0.5 mg/mL bovine serum albumin and 1:200 diluted anti-rat CD31 (BD Bioscience Korea, Seoul, Korea) primary antibody. After one day, sections were incubated with 1:200 diluted anti-rat secondary (Vector Laboratories, Burlingame, CA, USA) antibodies for 2 h. Then, sections were incubated with 1:100 diluted avidin–biotin–peroxidase complex (Vector Laboratories) for 1 h at room temperature. Peroxidase activity was visualized by incubating the section with 0.02% 3,3-diaminobenxidine (DAB) (Sigma–Aldrich); the sections were mounted on gelatin-coated slides.

2.7. Western blot analysis

The GC muscles were homogenized in ice-cold RIPA buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with a protease inhibitor cocktail (Sigma–Aldrich), Na_3VO_4 (Sigma–Aldrich), and NaF (Sigma–Aldrich). After centrifugation (12,000 rpm, 4 °C for 20 min), the supernatant was used for immunoblotting. Proteins were separated by SDS–PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk or 5% bovine serum albumin in Tris-buffered saline Tween 20 (TBS-T) for 1 h at room temperature and incubated with the primary antibody at 4 °C overnight; anti-rabbit GLP-1R (1:1000 dilution; Abcam, Cambridge, MA, USA), anti-rabbit VEGF (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse phospho-ERK (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), and anti-mouse α-tubulin (1:10,000 dilution; Sigma–Aldrich) in TBS-T. After washing, the membrane was incubated with peroxidase-labeled antibody against rabbit or mouse immunoglobulin (Vector Laboratories) at room temperature for 1 h. Western blotting was then conducted using the ECL western detection system (Thermo Fisher Scientific Inc.). Densitometric analysis of immunoblots for 56 kDa GLP-1R, 42 kDa VEGF, 44 kDa phospho-ERK, and 55 kDa α-tubulin levels was performed. Images were scanned and analyzed semi-quantitatively using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Image analysis and statistics

Assays were performed in duplicate and three independent experiments were performed unless otherwise stated. ImageJ was used to analyze cell counts or stained areas. Statistical significance was analyzed using Student's *t*-test using Graph Pad Prism, version

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5.0 (Graph Pad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

3. Results

3.1. Exendin-4 reduces regional tissue damage after hindlimb ischemia

To determine whether exendin-4 reduced tissue damage, we evaluated regional tissue damage after hindlimb ischemia using a modified ischemic score [17] at postoperative day 7. This method assigned a lower score to more serious damage. Scoring provided a detailed injury report such as loss, edema, or discoloration of toes. The exendin-4–treated group registered a significantly higher score than the saline group, indicating that exendin-4 decreased the need for amputation of the foot or leg ($P < 0.05$) (Fig. 1A). To evaluate the histological differences among the exendin-4–treated group, we performed hematoxylin and eosin staining of transactional GC. The saline-treated group developed a large area of muscle necrosis with significantly fewer regenerating myocytes (Fig. 1B).

3.2. Exendin-4 changed blood flow dynamics after hindlimb ischemia after three days

To examine whether exendin-4 induced blood flow changes, serial ICG perfusion imaging was performed immediately postoperatively and on postoperative days 3 and 7. After intravenous ICG injection, raw images were acquired and streamed to a computer. In the raw fluorescent images, blood vessels appeared as bright areas. From the serial images, we created a filtered time-series stack image. At postoperative day 3, blood flow dynamics were conjugated for I_{max}, T_{rising}, blood flow index (BFI), and mean transit time (MTT). The I_{max} mean maximal fluorescence intensity values and the T_{rising} values were extracted from the first peak as a representative parameter of the status of the tissue blood supply. Based on the T_{rising} values and I_{max} values, BFI as the slope of the peak time was calculated; BFI represents overall blood volume information with respect to time. To reduce variations, BFI was calculated ipsilateral with a contralateral region of intensity (ROI) ratio (Fig. 2A). The exendin-4–treated group had a significantly increased BFI ratio (Fig. 2B, $P < 0.05$). This result indicates that the exendin-4–treated group had a rich vascular network, much more so than the saline group. MTT calculates whole blood supply, including delayed collateral flow and as the center of gravity of the dynamic curve. MTT was calculated as the ipsilateral ROI (Fig. 2C). The MTT values of the exendin-4–treated group slightly decreased versus those of the saline group, but not significantly (53.77 ± 1.83 s versus 54.71 ± 2.90 s, respectively).

3.3. Exendin-4 enhanced angiogenesis in a mouse hindlimb ischemia model

Immunohistochemistry analysis for CD31, an endothelial cells marker, was performed on the mouse GC muscles to determine the angiogenic effects of exendin-4. The CD31-stained area significantly increased in the exendin-4 treatment group compared to the control group (2.23-fold of control; $P < 0.05$, Fig. 3A). To investigate the signal pathway of exendin-4–induced angiogenesis, western blot analysis of the mouse GC muscles was performed three days after treatment with exendin-4 (Fig. 3B). GLP-1R, a receptor for exendin-4, was confirmed in mouse GC muscles at first. With or without exendin-4, GLP-1R was analogously expressed. VEGF, the representative pro-angiogenic factor, increased in the exendin-4–treated group compared with the control group. Given VEGF stimulates mitogen-activated protein kinase (MAPK) signaling pathways [20], phospho-ERK in the mouse GC muscles increased in the exendin-4 group.

4. Discussion

In a previous study, we reported that exendin-4 has angiogenic effects *in vitro* and *ex vivo*. In this study, we extensively investigated whether exendin-4 has angiogenic effects and protects against ischemic injury in an *in vivo* model. We assessed the histological change in hindlimbs, blood perfusion rate, and molecular changes

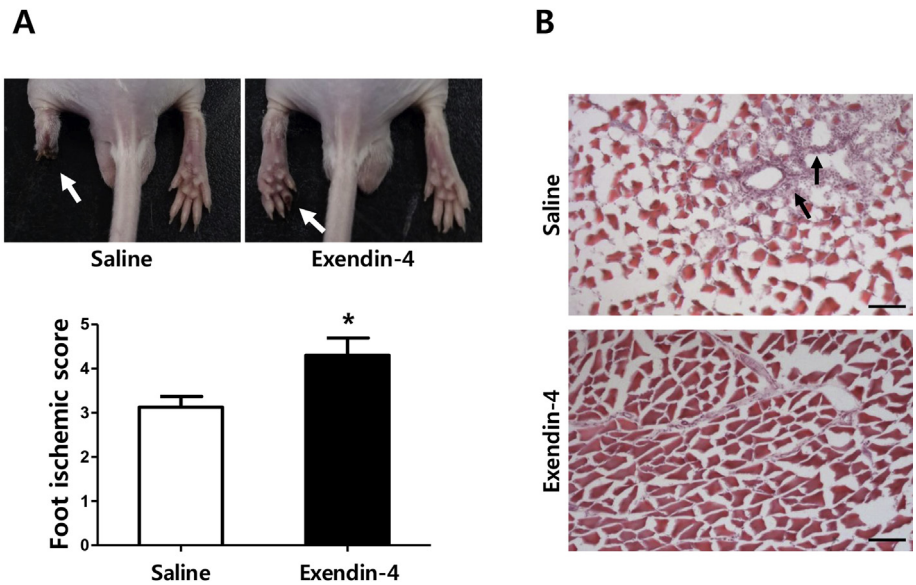

Fig. 1. Exendin-4 reduced mouse hindlimb ischemia-induced injury. (A) Representative photographs of the ischemic foot (*top*) and evaluated injury (*bottom*) using ischemic score at seven days after injury. The exendin-4–treated group was significantly protected from loss-of-foot necrosis. $^{*}P < 0.05$. (B) Hematoxylin-and-eosin stained cross-section of gastrocnemius muscle at seven days after injury. *Arrows* indicate necrotic muscle fibers, characterized by eosinophilia, loss of muscle architecture, and inflammatory infiltrate. Scale bar = 10 μm.

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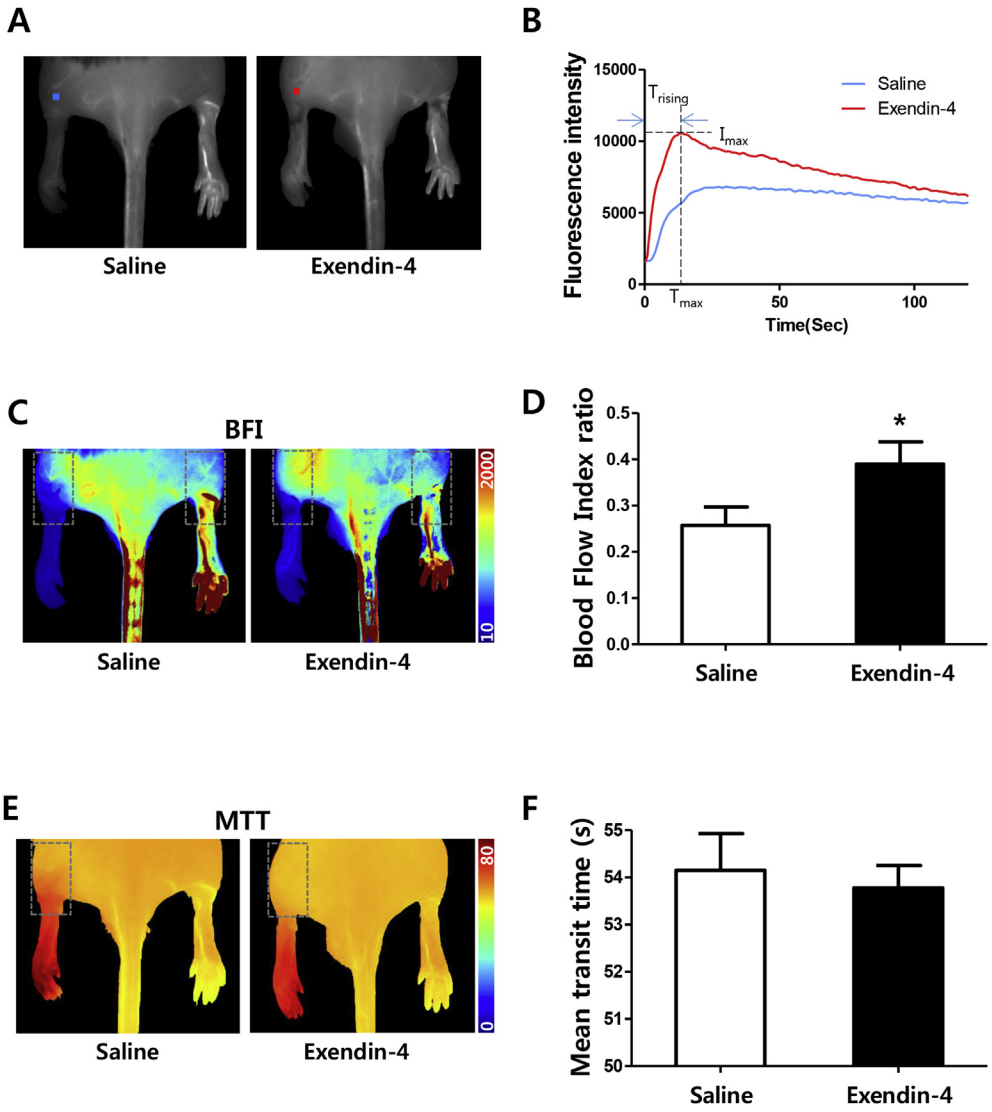

Fig. 2. Exendin-4 changed blood flow dynamics at three days after hindlimb ischemia. (A) Representative time-series stack image after tail vein injection of indocyanine green (ICG). Selected pixels are indicated by colored squares. (B) Graph showing the dynamics of selected pixels. Interpolated dynamics were plotted, and $T_{arrival}$ and T_{max} (for the calculation of T_{rising}) are indicated in the time axis. I_{max} is indicated on the fluorescence intensity axis. (C) Representative images of blood flow index (BFI) analysis. BFI represents overall blood volume information with respect to time. BFI was calculated ipsilateral with a contralateral region of intensity (*grey square*) ratio. Exendin-4 significantly increased BFI ratio. (D) Quantification of BFI. $^{*}P < 0.05$. (E) Representative images of mean transit time (MTT) analysis. MTT was used to calculate whole blood supply, including delayed collateral flow. The MTT values of mice receiving exendin-4 were slightly decreased compared with the control group, but not significant. (F) Quantification of MTT.

in a clinically similar ischemic hindlimb model. The results demonstrated that exendin-4 decreases the need for limb amputation, which may due to increased blood flow and angiogenesis via VEGF, a phospho-ERK pathway.

In peripheral arterial disease models, the degree of ischemia relies on the location and length of the artery occlusion. Many research groups have used a hindlimb ischemia model that features arterial occlusion in mice to mimic peripheral artery disease [21]. We used a model of complete excision of the femoral artery and its side branches that was originally developed in mice, which resulted in deep distal ischemia. This condition may have arisen because a collateral network cannot be formed because of preexisting arterioles that were disconnected from the vessels [21]. Excision reduces the blood flow to an ischemic limb, even at rest, and would therefore be more similar to the clinical situation of a patient with severe limb ischemia [21]. Dissection of the femoral artery and vein also results in severe ischemia, often leading to auto-amputation [22]. However, this model does not seem clinically relevant because the problem in patients with ischemic disease consists of arterial obstructions, rather than venous complications.

Symptoms of ischemia, such as necrosis of the toes and limb paralysis, were observed in our model. Calculation of an ischemic score based on the area of necrosis is useful for evaluation of treatment on recovery from ischemia [17]. Seven days after operation, we determined the ischemic scores. Foot amputations processed from toe to ankle were significantly reduced with three days of exendin-4 treatment. Coincidently, we observed that histological necrosis in the GC muscle was reduced by exendin-4 treatment.

To evaluate blood flow in the ischemic hindlimb, we adopted a sensitive optical imaging method using the near infrared fluorescence dye, ICG, combined with a time-series analysis of ICG molecular dynamics. To avoid measuring necrosis in an ongoing area of the paw, we exclusively selected the calf area, which usually shows changes in capillary density in a hindlimb ischemia model [23]. ICG

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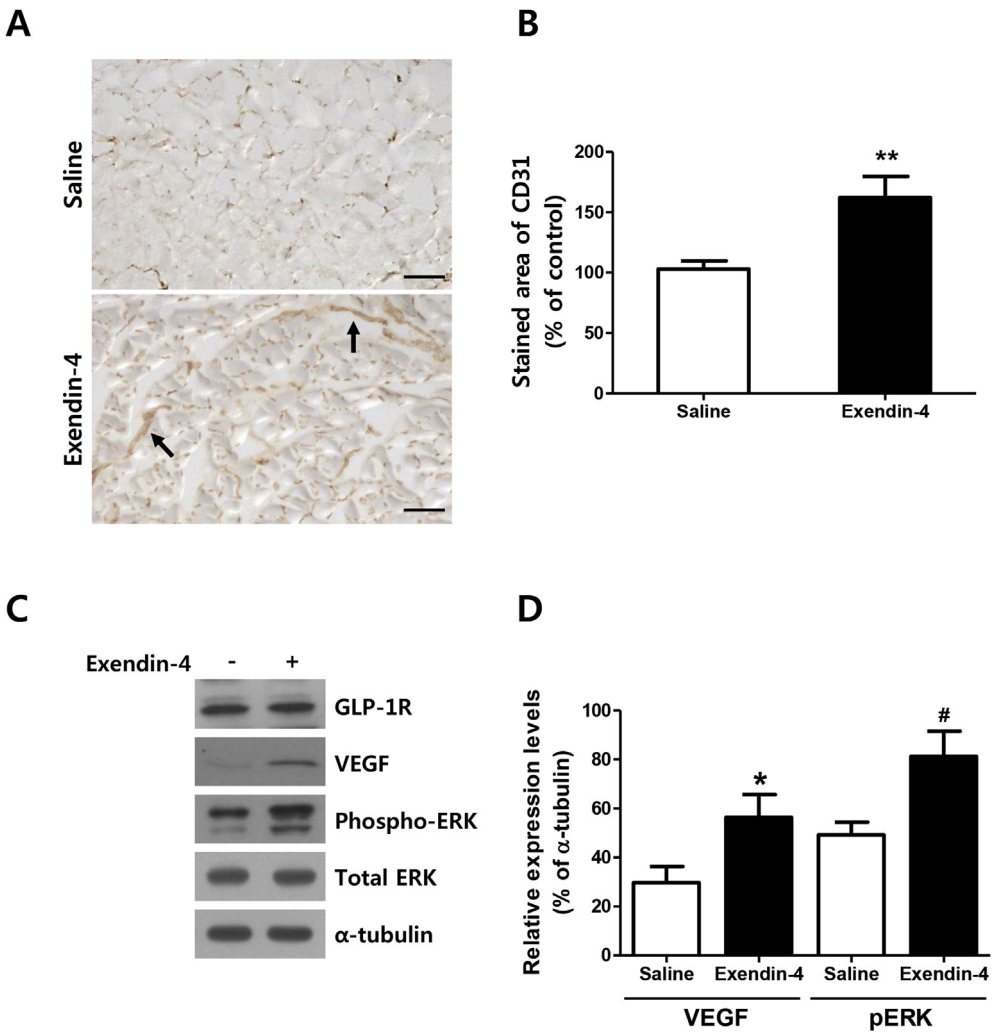

Fig. 3. Exendin-4 improved angiogenesis in the gastrocnemius muscle after hindlimb ischemia. (A) Immunostaining for CD31 showed that the exendin-4–treated group had significantly increased formation of capillaries (*arrows*) in the gastrocnemius at seven days after injury. Scale bar = 10 μm (B) Quantification of immunostaining. $^{**}P < 0.01$. (C) Western blot analysis pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and phospho-ERK (pERK), showed increased expression levels in gastrocnemius in the saline versus the exendin-4–treated group three days after injury. (D) Quantification of VEGF and pERK. $^{*}P < 0.05$; $^{\#}P < 0.05$.

dynamics were distinct in each group, and the subtle differences were successfully expressed using four dynamic parameters, including BFI and MTT. However, the BFI, or the slope of the first peak, did significantly differ between the groups. Consequently, the infusion rate of blood was significantly higher in the exendin-4 group, which raised the possibility of an increase in blood vessels in the exendin-4 group. In addition, our data showed that the MTT of the GC muscle area was not significantly different for the two groups. This indicates that the time to take back systemic circulation is not significantly different, which may have been caused by an intact vein in our ischemic model.

Capillary density remains the most commonly assessed histological measure of angiogenesis. The correlation between capillary density and perfusion changes was demonstrated to be good in multiple experimental conditions [23,24]. In the present study, we observed histological changes in the GC muscles. In the ischemic model, histology showed increased capillary density in GC muscle [24,25], and treatment with exendin-4 increased the sprouting of new capillaries in GC muscles. Coincidently, a recent study shows that PEGylated exendin-4 mediates cardio-protective action by promoting angiogenesis after myocardial infarction [26].

The upregulation of chemokines leads to attachment of monocytes to the activated endothelium, invasion into the arteriolar wall, and infiltration of the peri-adventitial space [25]. This angiogenic process is very rapid in otherwise healthy rodents, as indicated by angiographic evidence of collateral formation at seven days after femoral artery ligation. Among the many different growth factors implicated in angiogenesis, VEGF remains the most intensively investigated and is the key cytokine mediating endothelial cell proliferation and angiogenesis. A recent report demonstrated that a PEGylated exendin-4, modified GLP-1 analog increased VEGF expression [26]. Our study also showed that treatment with exendin-4 increases VEGF expression in ischemic hindlimb. In addition, we showed the phosphorylation of ERK by exendin-4 treatment that demonstrated that exendin-4 executed its angiogenic effect by mediating the VEGF signaling pathway.

In a previous study, we demonstrated that exendin-4 directly regulates endothelial cells (EC) migration, sprouting and tube formation, and also induces ECs proliferation [27]. In the present study, we focused on the angiogenic effects in an *in vivo* model and the therapeutic availability of exendin-4. Findings indicate that exendin-4 induces the formation of capillaries and increases blood flow in ischemic limbs, suggesting that it may be of potential use in treating diabetic vascular complications as well as diabetes itself.

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