Exendin-4 protects hindlimb ischemic injury by inducing angiogenesis

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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.

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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

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