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Original Article

Exendin-4 protected against critical limb ischemia in obese mice

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Abstract: This study tested the hypothesis that exendin-4 protects against critical limb ischemia (CLI) in obese mice undergoing hypoxic stress (H). B6 mice were categorized into aged-matched control (C)-H (group 1-A), obesity (induced by high-fat diet) (O)-H (group 1-B), C-H-CLI (group 2-A), O-H-CLI (group 2-B), C-H-CLI-exendin-4 (group 3-A) and O-H-CLI-exendin-4 (group 3-B). Animals were sacrificed by day 14 after CLI procedure. By day 14, laser Doppler results showed that blood flow in CLI area was higher in group 3-A than group 2-A, higher in group 3-B than group 2-B, highest in groups 1-A and 1-B, higher in group 2-A than in group 2-B, and higher in group 3-A than in group 3-B (all $p<0.001$), but not significantly different between groups 1-A and 1-B. Furthermore, circulating numbers of endothelial progenitor cells (EPCs) (c-kit/CD31+, Sca-1/KDR+) showed an identical pattern of blood flow in CLI area among groups 2-A, 2-B, 3-A and 3-B, except that these biomarkers were lowest in groups 1-A and 1-B (all $p<0.001$). Protein and cellular levels of angiogenesis factors (VEGF, CXCR4, SDF-1α) exhibited an identical pattern of circulating EPC numbers among all groups (all $p<0.001$). Protein levels of apoptotic (cytosolic cytochrome-C, mitochondrial Bax, cleaved caspase 3 and PARP) and fibrotic (Samd 3, TGF-β) biomarkers showed an opposite pattern of blood flow in CLI area among groups 2-A, 2-B, 3-A and 3-B, but were lowest in groups 1-A and 1-B (all $p<0.001$). This finding suggests exendin-4 protected against CLI in obese mice undergoing hypoxic stress mainly through enhancing angiogenesis and inhibiting apoptosis.

Keywords: Critical limb ischemia, obesity, exendin-4, angiogenesis, apoptosis

Introduction

Obesity is a global health burden with rapidly increasing prevalence. Greater than 10% of the world's adult population is currently obese, defined by a body mass index (BMI) of 30 kg/m^2 or more [1]. The prevalence of obesity in the USA is 34% [2-4], and this is forecast to increase to 42% by 2030 [2]. Obesity is due to excessive fat accumulation that impairs health resulting from social, behavioral, environmental and genetic factors [5, 6].

Cardiovascular disease is the leading cause of morbidity and mortality in obese individuals. Obesity is an important risk factor for the development of type 2 diabetes, musculoskeletal problems (such as osteoarthritis), certain cancers [1], and lung disease [7], and dramatically increases the risk of developing metabolic and cardiovascular disorders [1, 8]. This risk appears to originate from perturbations in adipose-tissue function that elicit a chronic inflammatory state and cause dysregulation of endocrine and paracrine actions of adipocyte-

derived factors. These, finally, impair vascular homeostasis and lead to endothelial dysfunction [9]. Our previous experimental work has consistently demonstrated that obesity suppresses heart function and circulating levels and function of endothelial progenitor cells (EPCs) [10]. Additionally, we have previously shown that obesity suppressed angiogenesis in the setting of critical limb ischemia (CLI), and this was reversed by obesity control [11]. This finding raises the possibility that pharmacomodulation may be an option, other than reducing weight, to repair endothelial dysfunction and increase angiogenesis in the setting of obesity.

Exendin-4 and liraglutide, two GLP-1 analogues, reportedly have multiple cellular protective effects, including protecting endothelial cells from senescence mainly through anti-oxidative [12, 13] and anti-inflammatory [13-15] processes. Additionally, our recent work demonstrated that exendin-4 therapy provided significant protection for the kidneys against acute ischemia-reperfusion injury [16].

Based on these recent reports [9-16], we hypothesized that exendin-4 therapy might protect against CLI in obese mice undergoing intermittent hypoxic stress.

Materials and methods

Ethics

All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at Kaohsiung Chang Gang Memorial Hospital (Affidavit of Approval of Animal Use Protocol No. 2012120303) and performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, National Academy Press, Washington, DC, USA, revised 1996).

Animal model of obesity induction in C57BL/6J mice

The procedure and protocol of obesity induction in C57BL/6J (B6) mice has been detailed in our previous reports [10, 11]. Briefly, 8-week-old male B6 mice (n = 24), weighing 20-22 g, (Charles River Technology, BioLASCO Taiwan Co., Ltd., Taiwan), were fed with a high-fat diet (45 Kcal% fat; Research Diets, Inc) to create a diet-induced obesity model for the purpose of this study. According to the literature [17], our previous experience [11, 12] and the company (Research Diets, Inc), successful obesity induction in mice was defined as a body weight increase of more than 35% after 12 weeks of feeding (high fat diet of 45 Kcal% fat). By the end of 13 weeks of feeding, more than 70% of mice satisfied obesity criteria.

Animal grouping

Obese mice (n = 24) were equally divided into obesity + hypoxia (OH) only, OH+CLI and OH+CLI+exendin-4 (Ex4; 10 μg/kg subcutaneous injection at post-CLI 1 hr, followed by once per day for 13 days).

Other aged-matched B6 mice (n = 24) fed with a control diet for the same duration (i.e., total 13-week control diet), which was also purchased from the same company (Research Diets, Inc), served as age-matched control (C) + hypoxia (CH) only, CH+CLI and CH+CLI+Ex4 (administration was identical to the OH+CLI+exendin-4 group). The dosage of exendin-4 was according to our recent report [16].

Rationale of intermittent hypoxia and procedure and protocol for hypoxia stress

It is well recognized that murine animals have reliable collateral circulation to their limbs. Therefore, in order to ensure consistency of the CLI condition, intermittent hypoxia was applied to all animals as follows: animals were subjected to alternating hypoxia (12 h) and room air (12 h) for three cycles (i.e., three days), before undergoing the CLI procedure. Twenty four hours after the CLI procedure, animals were subjected to 6 hours of hypoxia before returning to room air.

The procedure and protocol for hypoxic stress was detailed in our recent report [18]. Briefly, the mice were kept in a hypoxic chamber that consisted of (1) a plastic chamber with a built-in electric fan for mixing nitrogen and oxygen, a cage of mice with free access to water and animal chow, barium hydroxide lime for absorbing carbon dioxide, and charcoal powder for eliminating ammonia; (2) an oximeter showing the oxygen content inside the chamber (consistently kept at 11 ± 0.2%); (3) gas tanks (i.e. nitrogen and air) connected to the chamber for maintaining the hypoxic environment inside the chamber; (4) transparent glass window for monitoring animal activities.

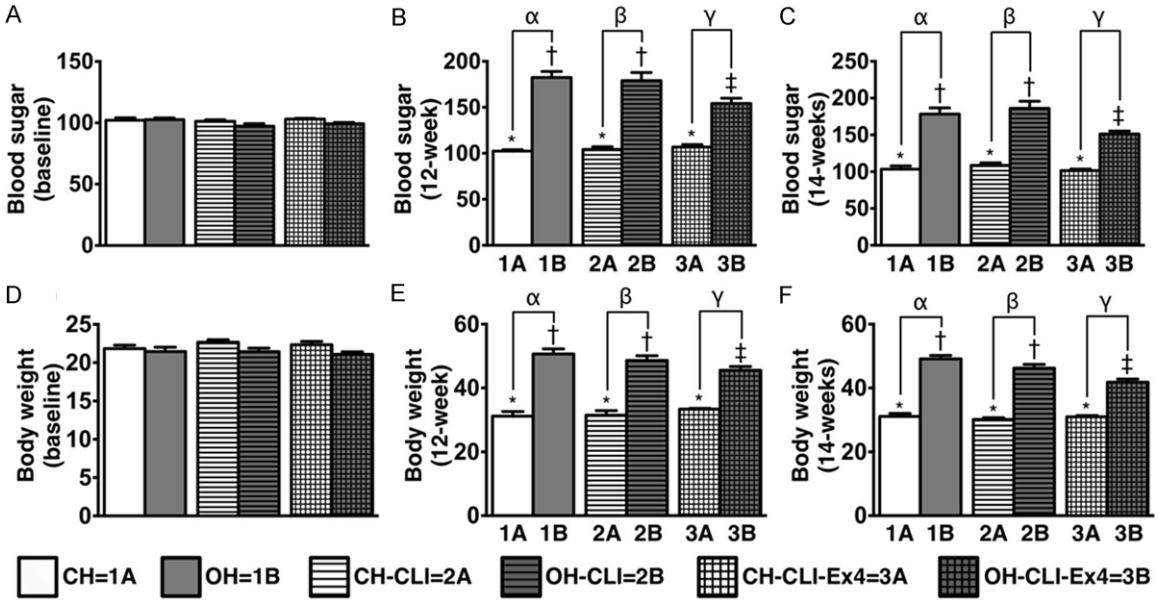

Figure 1. Serial changes of blood sugar and body weight. A. Baseline fasting blood sugar among control animals of thee subgroups (i.e., CH, CH-CLI, CH-CLI-Ex4) and obese animals of three subgroups (OH, OH-CLI, OH-CLI-Ex4), all $p>0.5$. B. Fasting blood sugar at 12 weeks after obesity induction. (1) α indicated 1A vs. 1B, $p<0.001$; β indicated 2A vs. 2B, $p<0.001$; γ indicated 3A vs. 3B, $P<0.01$. (2) † vs. other groups with different black symbols (†, ‡), $p<0.01$. C. Fasting blood sugar at 14 weeks after obesity induction. (1) α indicated 1A vs. 1B, $p<0.001$; β indicated 2A vs. 2B, $p<0.001$; γ indicated 3A vs. 3B, $P<0.01$. (2) † vs. other groups with different black symbols (†, ‡), $p<0.01$. D. Baseline body weight among control animals of thee subgroups and obese animals of three subgroups, all $p>0.5$. E. Body weight at 12 weeks after obese induction. (1) α indicated 1A vs. 1B, $p<0.001$; β indicated 2A vs. 2B, $p<0.001$; γ indicated 3A vs. 3B, $P<0.01$. (2) † vs. other groups with different black symbols (†, ‡), $p<0.05$. F. Body weight at 12 weeks after obese induction. (1) α indicated 1A vs. 1B, $p<0.001$; β indicated 2A vs. 2B, $p<0.001$; γ indicated 3A vs. 3B, $P<0.01$. (2) † vs. other groups with different black symbols (†, ‡), $p<0.05$. Symbols (†, ‡) indicate significance (at 0.05 level). CH = control, hypoxia; OH = obesity control, Ex4 = exendin 4.

Animal model of critical limb ischemia (CLI)

Mice in groups CH+CLI, CH+CLI+Ex4, OH+CLI, and OH+CLI+Ex4 were anesthetized by inhalation of 2.0% isoflurane. The mice were placed in a supine position on a warming pad at 37°C with the left hind limbs shaved. Under sterile conditions, the left femoral artery, small arterioles, circumferential femoral artery and veins were exposed and ligated proximally and distally before removal. To avoid the presence of collateral circulation, branches were removed. Animals in groups 1-A and 1-B only received skin excision and closure without ligation of arteries. After the procedure, the wound was closed and the animal was allowed to recover from anesthesia in a portable animal intensive care unit (ThermoCare®) for 24 hours.

Flow cytometric quantification of endothelial progenitor cells

The assessment of circulating numbers of EPCs has been detailed previously [19]. Briefly, for blood sampling at different time points (days 7 and 14 after CLI procedure), cardiac puncture was adopted for blood sampling using a 30G needle. After treatment with red blood cell-lysing buffer, remaining cells were labeled with appropriate antibodies. Flow cytometric analysis for identification of cell surface markers was performed as previously described [19, 20]. Briefly, the cells were incubated for 30 minutes with primary antibodies, including PE-conjugated antibodies (against CD34, Sca-1, CD31 BD Biosciences), FITC-conjugated antibody against c-kit (BD Biosciences), anti-CXCR4 (Abcam) and anti-KDR (NeoMarkers) antibodies, which were further recognized by Alexa flour 488-conjugated secondary antibodies (Invitrogen). Isotype-identical antibodies (IgG) served as controls. Flow cytometric analyses were performed with a fluorescence-activated cell sorter (Beckman Coulter FC500 flow cytometer).

Determination of blood flow in limbs with laser Doppler

This procedure and protocol have previously been described [11, 19]. Briefly, mice were

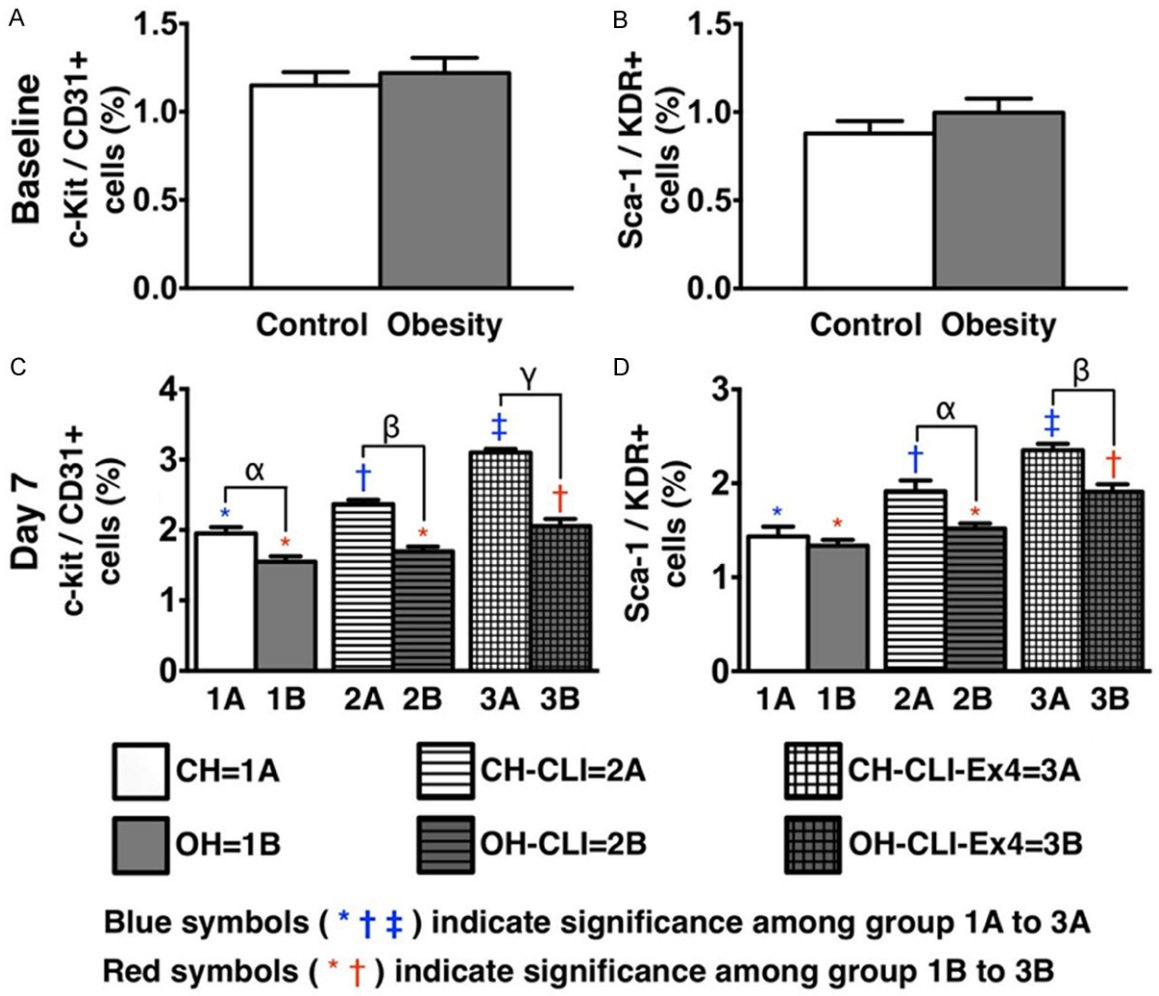

Figure 2. Flow cytometric quantification of circulating level of endothelial progenitor cells (EPCs) at baseline and at day 7 after critical limb ischemia (CLI) procedure (n = 8). (A, B) Baseline circulating numbers of c-kit+/CD31+ (A) and Sca-1+/KDR+ (B) did not differ between the CH (control + hypoxia) and OB (obesity + hypoxia) groups, all p value >0.1. (C) Circulating level of c-kit+/CD31+ cells at day 7 after CLI procedure. (1) α indicated 1A vs. 1B, $p<0.05$; β indicated 2A vs. 2B, $p<0.05$; γ indicated 3A vs. 3B, $P<0.01$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.001$. (3) * vs. other groups with different red symbols (*, †), $p<0.01$. (D) Circulating level of Sca-1+/KDR+ at day 7 after CLI procedure. (1) α indicated 2A vs. 2B, $p<0.05$; β indicated 3A vs. 3B, $p<0.05$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.01$. (3) * vs. other groups with different red symbols (*, †), $p<0.05$. Statistical analysis in (A to D) using one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). CH = control, hypoxia; OH = obesity control, Ex4 = exendin 4.

anesthetized by inhalational 2.0% isoflurane prior to CLI induction and on days 2 and 14 after CLI procedure prior to being sacrificed. The mice were placed in a supine position on a warming pad at 37°C and blood flow was detected in both inguinal areas by a Laser Doppler scanner (moorLDLS, Moor, Co. UK). All data were collected for further analysis. The mice were sacrificed at day 14 after CLI induction and the quadriceps muscle collected for individual study. Blood was drawn to measure EPCs with flow cytometry prior to sacrifice.

Western blot for protein expression

Equal amounts (10-30 μg) of protein extracts from ischemic quadriceps from the animals (n = 8 for each group) were loaded and separated by SDS-PAGE using 7% or 12% acrylamide gradients. Proteins were transferred to nitrocellulose membranes which were then incubated in the primary antibodies against vascular cell adhesion molecule CXCR4 (1:1000, Abcam), VEGF (1:1000, Abcam), stromal cell-derived growth factor (SDF)-1α (1:1000, Cell Signaling),

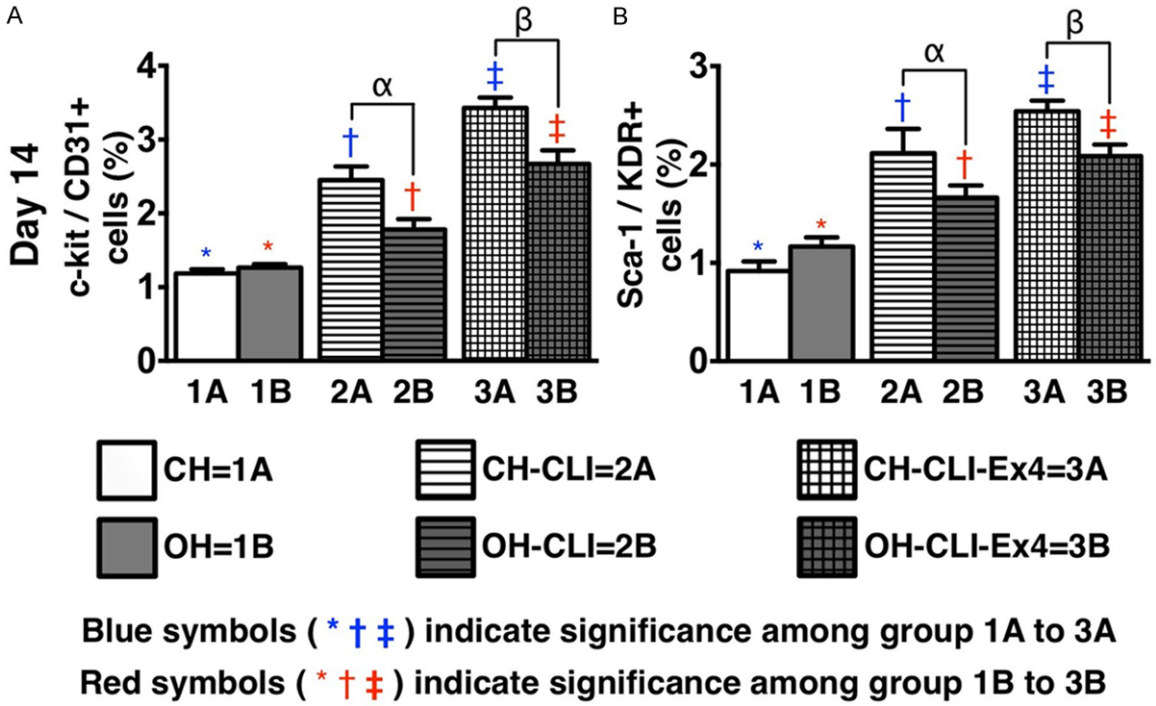

Figure 3. Flow cytometric quantification of circulating level of endothelial progenitor cells (EPCs) at day 14 after critical limb ischemia (CLI) procedure (n = 8). (A) Circulating level of c-kit+/CD31+ cells at day 14 after CLI procedure. (1) α indicated 2A vs. 2B, $p<0.03$; β indicated 3A vs. 3B, $p<0.03$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.001$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.001$. (B) Circulating level of Sca-1+/KDR+ at day 14 after CLI procedure. (1) α indicated 2A vs. 2B, $p<0.05$; β indicated 3A vs. 3B, $p<0.05$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.01$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.05$. Statistical analysis in (A and B) using one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). CH = control, hypoxia; OH = obesity control, Ex4 = exendin 4.

angiopoietin (1:1000, Millipore), CD31 (1:1000, Abcam), endothelial nitric oxide synthase (eNOS) (1:1000, Abcam), Bax (1:1000, Abcam), caspase 3 (1:1000, Cell Signaling), PARP (1:1000, Cell Signaling), cytochrome C [(Cyt C) 1:2000, BD), phosphorylated (p)-Smad3 (1:1000, Cell Signaling), p-Smad1/5 (1:1000, Cell Signaling), bone morphogenetic protein (BMP)-2 (1:500, Abcam), transforming growth factor (TGF)-β (1:500, Abcam) and actin (1:10000, Chemicon). Signals were detected with HRP-conjugated goat antimouse or goat anti-rabbit IgG (1:2000, Cell Signaling). The washing procedure was repeated eight times within 40 minutes. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences), which was then exposed to Biomax L film (Kodak). For quantification, ECL signals were digitized using Labwork software (UVP).

Immunofluorescent (IF) staining

IF staining was performed to examine CD31+, VEGF, smooth muscle actin (SMA)+, CXCR4+, and SDF-1α+ cells (n = 8 for each group) using respective primary antibodies based on our recent study [18, 19]. Irrelevant antibodies were used as controls in the current study.

Statistical analyses

Quantitative data are expressed as mean ± SD. Statistical analysis was performed by ANOVA followed by Bonferroni multiple-comparison *post hoc* test. All analyses were conducted using SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC). A probability value <0.05 was considered statistically significant.

Results

*Serial changes of eight-hour fasting sugar and body weight in the animals (**Figure 1**)*

The baseline eight-hour fasting blood sugar and body weight did not differ among the control animals or among the obese animals.

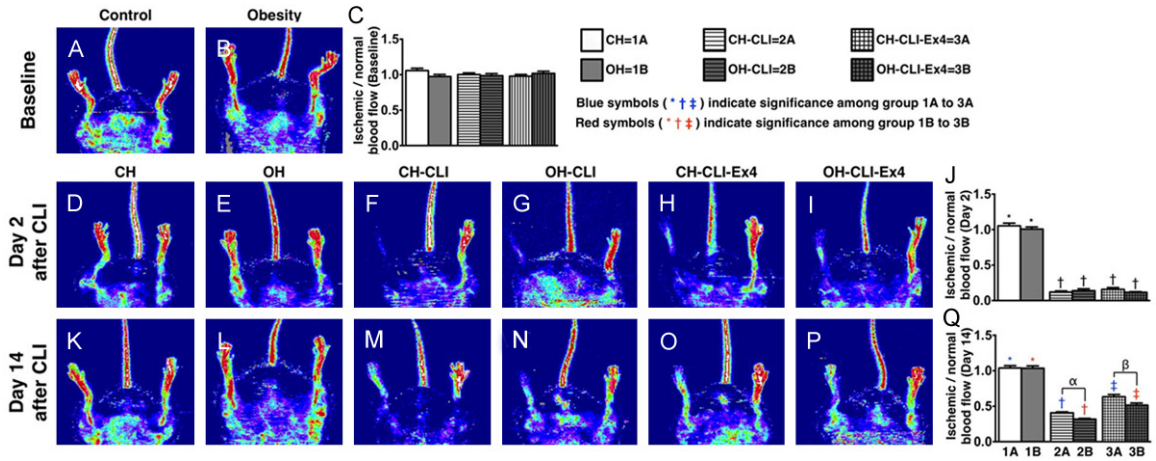

Figure 4. Time courses of laser Doppler analysis of blood flow (n = 8). (A & B) Illustration of laser Doppler of normal blood flow in both hind limbs in each group of control hypoxia (CH) and obese hypoxia (OH) mice prior to the procedure. (C) Statistical analysis showing no difference of ratio of left to right hind-limb blood flow in with respective group (i.e., CH vs. OH, CH-CLI vs. OH-CLI, or CH-CLI-Ex4 vs. OH-CLI-Ex4) among the animals prior to CLI induction, all *p* value = 1.0. (D to I) Illustration of laser Doppler of ratio of left to right blood flow among six groups at day 2 after CLI procedure. (J) Analytic results: * vs. other groups with different red symbols (*, †), p<0.0001. (K to P) Illustration of laser Doppler of ratio of left to right blood flow among six groups at day 14 after CLI procedure. (Q) Analytical results: (1) α indicated 2A vs. 2B, p<0.05; β indicated 3A vs. 3B, p<0.05. (2) * vs. other groups with different blue symbols (*, †, ‡), p<0.0001. (3) * vs. other groups with different red symbols (*, †, ‡), p<0.0001. Statistical analysis in (C, J & Q) using one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). CH = control, hypoxia; OH = obesity control, Ex4 = exendin 4.

These two parameters also did not differ between the control and the obese animals.

In control animals, by the end of 12 and 14 weeks after obesity induction, the fasting blood sugar and the body weight did not differ among the three groups [i.e. CH (control + hypoxia), CH-CLI, CH-CLI-Ex4].

In obese animals, by the end of 12 and 14 weeks after obesity induction the fasting blood sugar and the body weight were significantly higher in OH (i.e., obesity + hypoxia) and OH-CLI groups than OH-CLI-Ex4 group, but it showed no difference between the former two groups.

When comparing between control and obese animals, these two parameters were significantly higher in obese mice than in control mice with respective to any group.

*Circulating endothelial progenitor cells at baseline and by days 7 and 14 after CLI procedure (**Figures 2** and **3**)*

Flow cytometric analyses showed that the circulating levels of EPCs (i.e., c-kit/CD31+ and Sca-1/KDR+ cells) did not differ between control and obese mice (**Figure 2**).

In control animals: by day 7 after CLI procedure, the numbers of c-kit/CD31+ and Sca-1/KDR+ cells were significantly lower in CH (i.e., control + hypoxia) group than in CH-CLI and CH-CLI-Ex4, but were significantly different between the latter two groups. Additionally, by day 14 after CLI, these biomarkers were significantly higher in CH-CLI-Ex4 group than in CH and CH-CLI groups and significantly higher in CH-CLI group than in CH group (**Figure 3**).

In obese animals: by days 7 and 14 after CLI procedure, circulating expressions of these EPCs (i.e., c-kit/CD31+ and Sca-1/KDR+ cells) showed an identical pattern as in control animals among OH (i.e., obesity + hypoxia), OH-CLI and OH-CLI-Ex4 groups (**Figure 3**).

Interestingly, when comparing between control and obese animals, the circulating numbers of these EPCs were significantly lower in obese than in control mice within respective groups (i.e., OH vs. CH, OH-CLI vs. CH-CLI, and OH-CLI-Ex4 vs. CH-CLI-Ex4) (**Figure 3**).

*Serial changes of laser doppler analysis of blood flow (**Figure 4**)*

To determine the effect of exendin-4 therapy on improving blood flow in the ischemic region, the ratio of ischemic/normal blood flow (INBF) was measured in both control and obese animals.

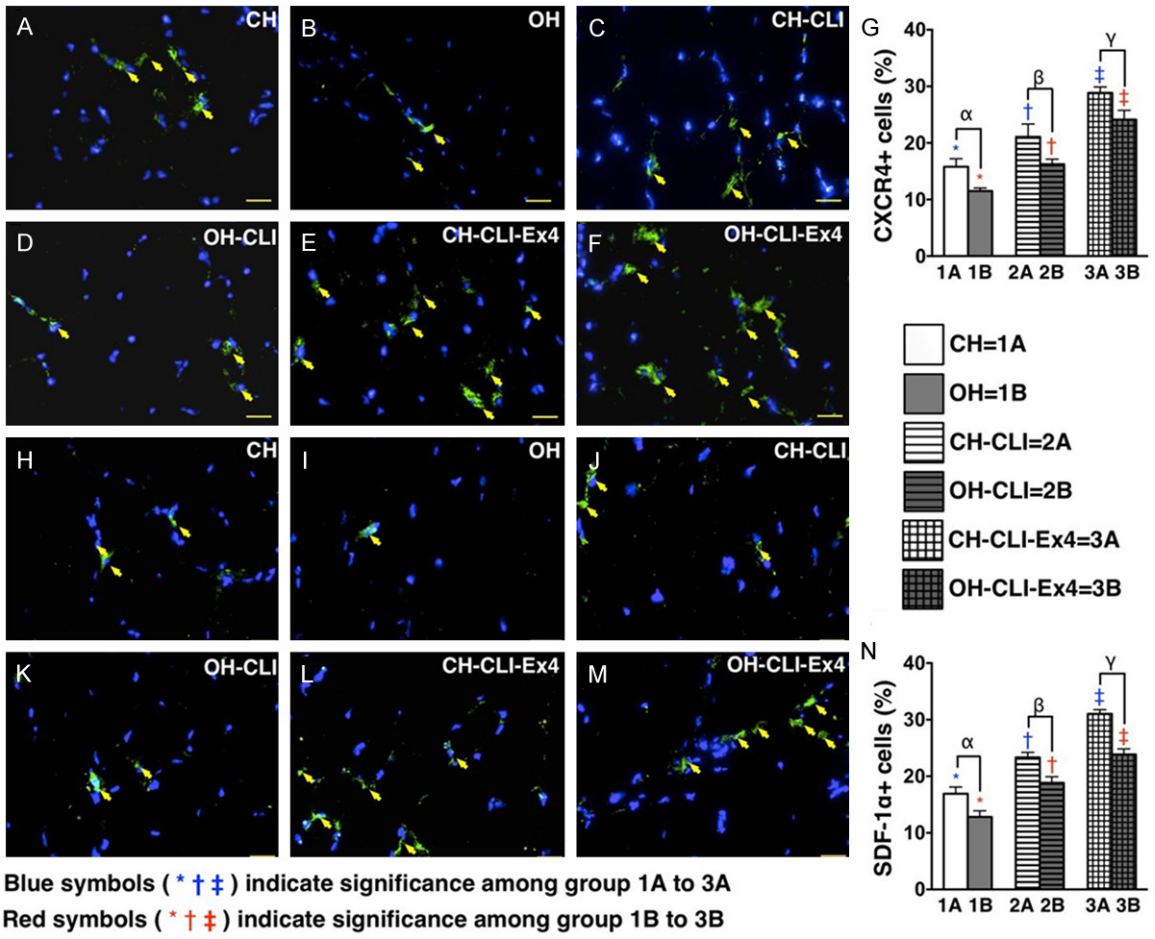

Figure 5. Immunofluorescent (IF) staining of CXCR4+ and stromal cell-derived factor (SDF)-1α+ cells in ischemic quadriceps at day 14 after critical limb ischemia (CLI) procedure (n = 8). (A to F) Illustrating IF microscopic findings (400x) of CXCR4+ cells (yellow arrows) in ischemic quadriceps of six group animals, respectively. (G) Statistical analysis of CXCR4+ cells. (1) α indicated 1A vs. 1B, p<0.01; β indicated 2A vs. 2B, p<0.01; γ indicated 3A vs. 3B, P<0.01. (2) * vs. other groups with different blue symbols (*, †, ‡), p<0.001. (3) * vs. other groups with different red symbols (*, †, ‡), p<0.001. (H to M) Illustrating IF microscopic findings (400x) of SDF-1α+ cells (yellow arrows) in ischemic quadriceps of six group animals, respectively. (N) Statistical analysis of SDF-1α+ cells (%). (1) α indicated 1A vs. 1B, p<0.01; β indicated 2A vs. 2B, p<0.01; γ indicated 3A vs. 3B, P<0.01. (2) * vs. other groups with different blue symbols (*, †, ‡), p<0.001. (3) * vs. other groups with different red symbols (*, †, ‡), p<0.008. Statistical analysis in G) and N) using one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). The scale bars in right lower corner represent 20 μm. CH = control, hypoxia; OH = obesity control, Ex4 = exendin 4.

In control animals: prior to CLI procedure, the ratio of INBF was similar among the three groups. However, by days 2 and 14 after CLI procedure, this parameter was significantly lower in CH-CLI and CH-CLI-Ex4 groups than in CH group, but not significantly different between the former two groups. However, by day 14 after CLI procedure, this parameter was significantly increased in CH-CLI-Ex4 compared with CH-CLI.

In obese animals, this parameter exhibited an identical pattern to control animals among the three groups (i.e., OH, OH-CLI, OH-CLI-Ex4) at the three time intervals.

Intriguingly, when comparing control and obese animals, the ratio of INBF was significantly lower in obese mice than in controls within respective groups (i.e., CH-CLI vs. OH-CLI; CH-CLI-Ex4 vs. OH-CLI-Ex4).

*Immunofluorescent staining for identification of cellular expressions of angiogenesis factors in CLI area by day 14 after CLI procedure (**Figures 5-7**)*

In control animals: the IF microscopic finding showed that the cellular expressions of CXCR4, SDF-1α (**Figure 5**), two indicators of EPCs, and VEGF (**Figure 6**), an indicator of endothelial cell

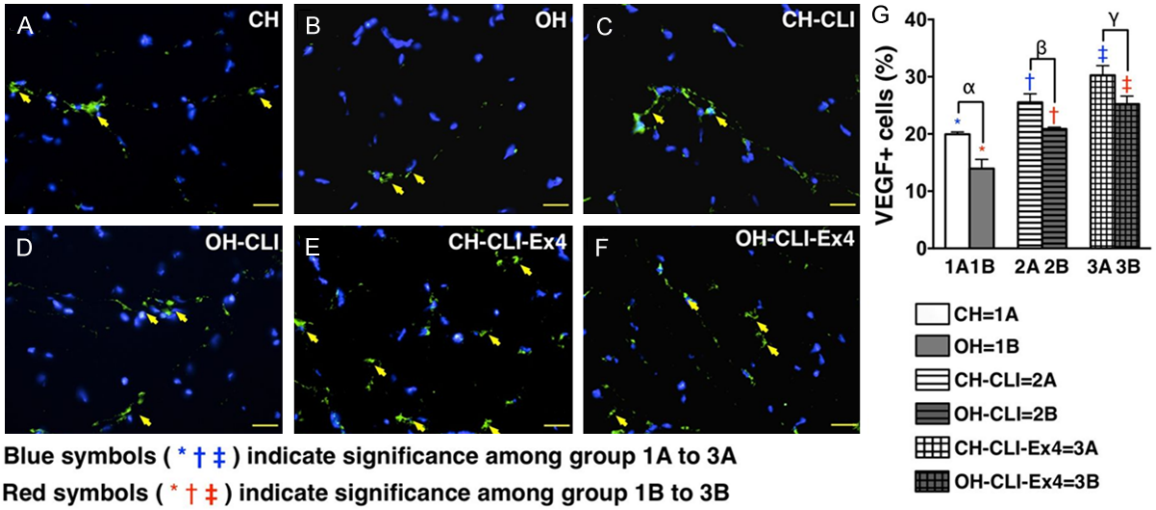

Figure 6. Immunofluorescent (IF) staining of vascular endothelial growth factor (VEGF)+ cells in ischemic quadriceps at day 14 after critical limb ischemia (CLI) procedure (n = 8). (A to F) Illustrating IF microscopic findings (400x) of VEGF+ cells (yellow arrows) in ischemic quadriceps of six group animals, respectively. (G) Statistical analysis of VEGF+ cells. (1) α indicated 1A vs. 1B, $p<0.01$; β indicated 2A vs. 2B, $p<0.01$; γ indicated 3A vs. 3B, $P<0.01$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.001$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.001$. Statistical analysis in (G) using one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). The scale bars in right lower corner represent 20 μm. CH = control, hypoxia; OH = obesity control, Ex4 = exendin 4.

(EC), were significantly progressively increased from CH to CH-CLI-Ex4 by day 14 after CLI procedure. Additionally, IF staining identified that the cellular expressions of CD31+ cells (i.e., EC) and α-SMA+ cells (i.e., for identification of small vessels/capillaries) (**Figure 7**), two indicators of angiogenesis, were significantly lower in CH-CLI than in CH and CH-CLI-Ex4, and significantly lower in CH-CLI-Ex4 than in CH.

In obese animals, these biomarkers (i.e., CXCR4+, SDF-1α+, VEGF+, CD31+, α-SMA+ cells) displayed an identical pattern to control animals among the three groups.

When comparing between control and obese animals, these biomarkers were significantly lower in obese than in control animals within respective groups.

*Protein expressions of angiogenesis factors in CLI area by day 14 after CLI procedure (**Figure 8**)*

In control animals, protein expressions of angiopoietin, CXCR4, SDF-1α and VEGF, four indicators of angiogenesis factors, significantly and progressively increased from CH to CLI-Ex4. Additionally, protein expressions of CD31 and eNOS, two additional angiogenesis factors, were significantly higher in CH than in CH-CLI and CH-CLI-Ex4, and significantly higher in CH-CLI-Ex4 than in CH-CLI.

In obese animals, these biomarkers showed an identical pattern to control animals among the three groups of obese animals.

When comparing between the control and the obese animals, these biomarkers were significantly lower in obese than in control animals within the respective groups.

*The protein expressions of apoptotic biomarkers in CLI Area by Day 14 after CLI procedure (**Figure 9**)*

In control animals, the protein expressions of mitochondrial Bax and cleaved (i.e., active form) caspase 3 and PARP, three indices of apoptosis, and cytosolic cytochrome C, an indicator of mitochondrial damage, were significantly higher in CH-CLI than in CH and CH-CLI-Ex4, and significantly higher in CH-CLI-Ex4 than in CH. Conversely, the protein expression of mitochondrial cytochrome C, an indicator of mitochondrial preservation, showed an opposite pattern among the three groups.

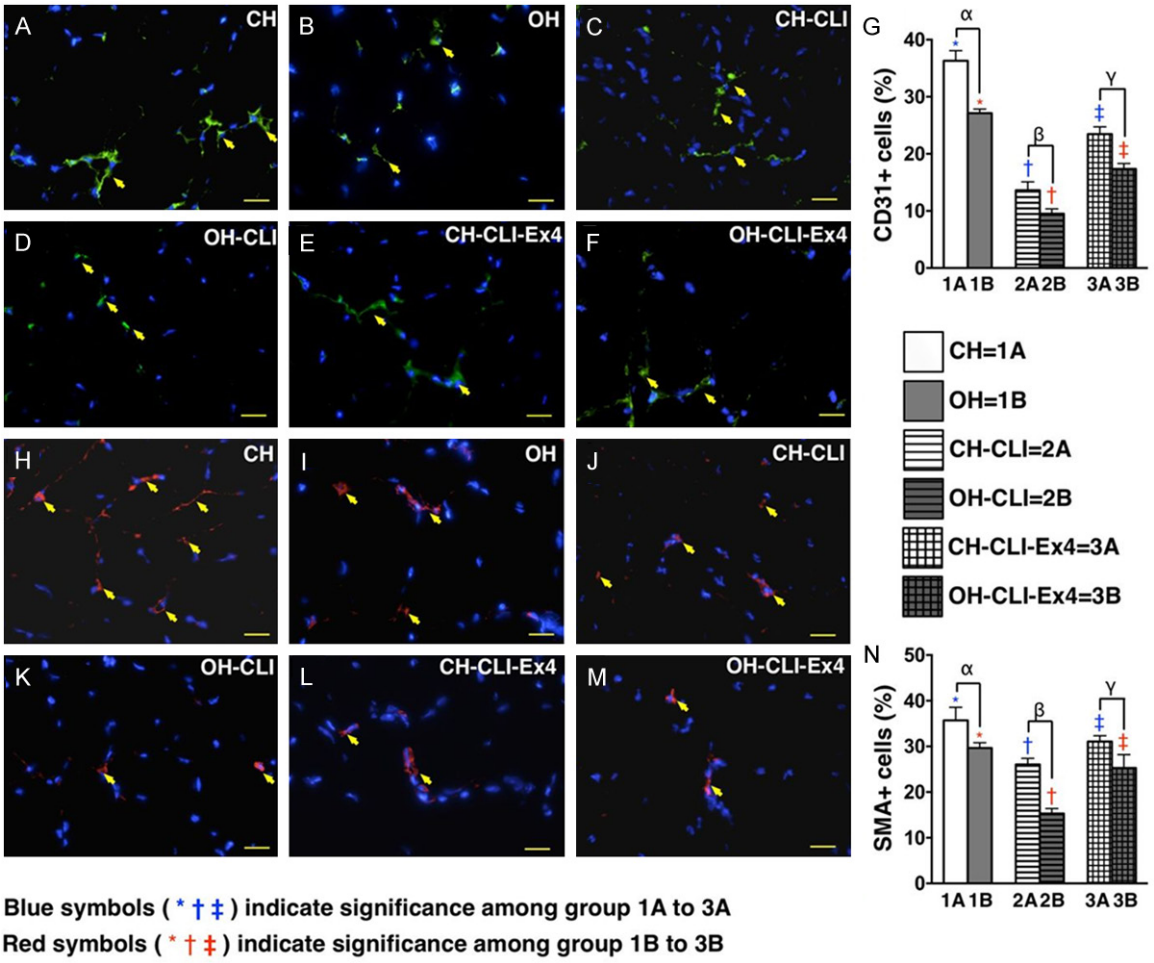

Figure 7. Immunofluorescent (IF) staining of CD31+ and α-smooth muscle actin (SMA)+ cells in ischemic quadriceps at day 14 after critical limb ischemia (CLI) procedure (n = 8). (A to F) Illustrating IF microscopic findings (400x) of CD31+ cells (yellow arrows) in ischemic quadriceps of six group animals, respectively. (G) Statistical analysis of CD31+ cells. (1) α indicated 1A vs. 1B, p<0.001; β indicated 2A vs. 2B, p<0.01; γ indicated 3A vs. 3B, P<0.01. (2) * vs. other groups with different blue symbols (*, †, ‡), p<0.0001. (3) * vs. other groups with different red symbols (*, †, ‡), p<0.0001. (H to M) Illustrating IF microscopic findings (400x) of α-SMA+ cells (yellow arrows) in ischemic quadriceps of six group animals, respectively. N) Statistical analysis of α-SMA+ cells (%). (1) α indicated 1A vs. 1B, p<0.01; β indicated 2A vs. 2B, p<0.001; γ indicated 3A vs. 3B, P<0.01. (2) * vs. other groups with different blue symbols (*, †, ‡), p<0.001. (3) * vs. other groups with different red symbols (*, †, ‡), p<0.0001. Statistical analysis in (G and N) using one-way ANOVA, followed by Bonferroni multiple comparison post hoc test. Symbols (*, †, ‡) indicate significance (at 0.05 level). The scale bars in right lower corner represent 20 μm. CH = control, hypoxia; OH = obesity control, Ex4 = exendin 4.

In obese animals, these biomarkers also showed an identical pattern to the control animals among the three groups.

When comparing between the control and obese animals, these apoptotic biomarkers (i.e., Bax, cleaved caspase 3 and PARP, cytosolic cytochrome C) were significantly higher in obese than in control animals within the respective groups. However, mitochondrial cytochrome C showed an opposite pattern to apoptosis when comparing obese mice with control mice within respective groups.

*Protein expressions of fibrotic and anti-fibrotic biomarkers in CLI Area by day 14 after CLI procedure (**Figure 10**)*

In control animals, protein expressions of Smad3 and TGF-β, two indicators of fibrosis, were significantly higher in CH-CLI than in CH-CLI-Ex4 and CH, and significantly higher in CH-CLI-Ex4 than in CH. Conversely, protein expressions of Smad1/3 and BMP-2, two indicators of anti-fibrosis, exhibited an opposite pattern to fibrotic biomarkers among the three groups.

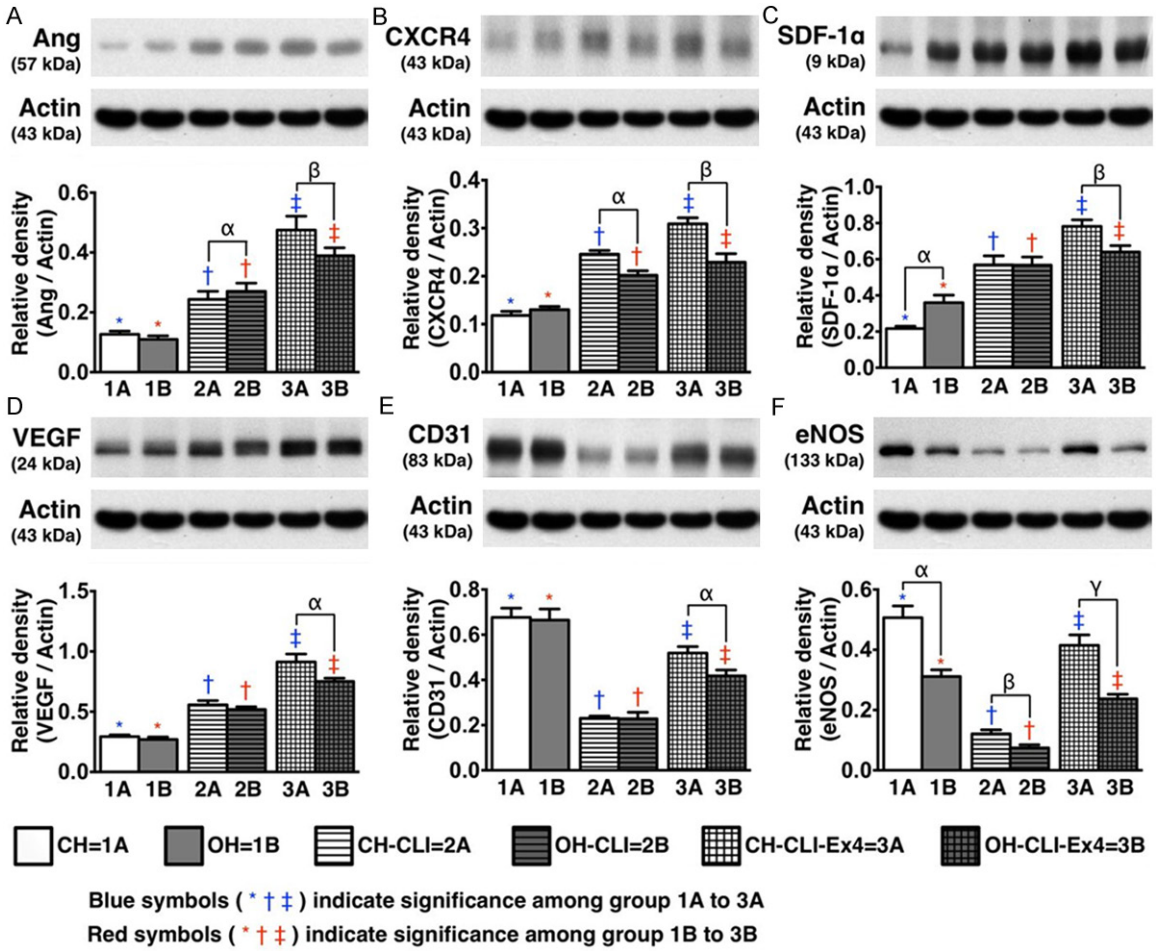

Figure 8. Protein expressions of angiogenesis in ischemic quadriceps at day 14 after critical limb ischemia (CLI) procedure (n = 8). A. Protein expression of angiopoietin (Ang). The statistical results: (1) α indicated 2A vs. 2B, $p<0.05$; β indicated 3A vs. 3B, $p<0.03$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.001$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.001$. B. Protein expression of CXCR4. The statistical results: (1) α indicated 2A vs. 2B, $p<0.05$; β indicated 3A vs. 3B, $p<0.05$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.001$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.01$. C. Protein expression of stromal cell-derived factor (SDF)-1α. The statistical results: (1) α indicated 1A vs. 1B, $p<0.05$; β indicated 3A vs. 3B, $p<0.05$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.001$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.01$. D. Protein expression of vascular endothelial growth factor (VEGF). The statistical results: (1) α indicated 3A vs. 3B, $p<0.05$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.001$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.01$. E. Protein expression of CD31. The statistical results: (1) α indicated 3A vs. 3B, $p<0.05$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.001$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.001$. F. Protein expression of endothelial nitric oxide synthase (eNOS). (1) α indicated 1A vs. 1B, $p<0.01$; β indicated 2A vs. 2B, $p<0.05$; γ indicated 3A vs. 3B, $P<0.01$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.0001$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.001$.

In obese animals, the fibrotic and anti-fibrotic biomarkers showed an identical pattern to control animals among the three groups.

When comparing between control and obese animals, fibrotic biomarkers were significantly higher in obese than in control mice within respective groups. However, anti-fibrotic biomarkers showed an opposite pattern to fibrotic biomarkers when comparing obese with control mice within respective groups.

Discussion

This study investigated the impact of obesity on the recovery of blood flow after CLI. The therapeutic impact of exendin-4 on improving angiogenesis and blood flow in the CLI zone yielded several striking implications. First, circulating levels of EPCs was remarkably lower in obese than in control mice in the setting of ischemia. Second, as compared with control mice, obese mice had poor recovery of blood flow in the CLI

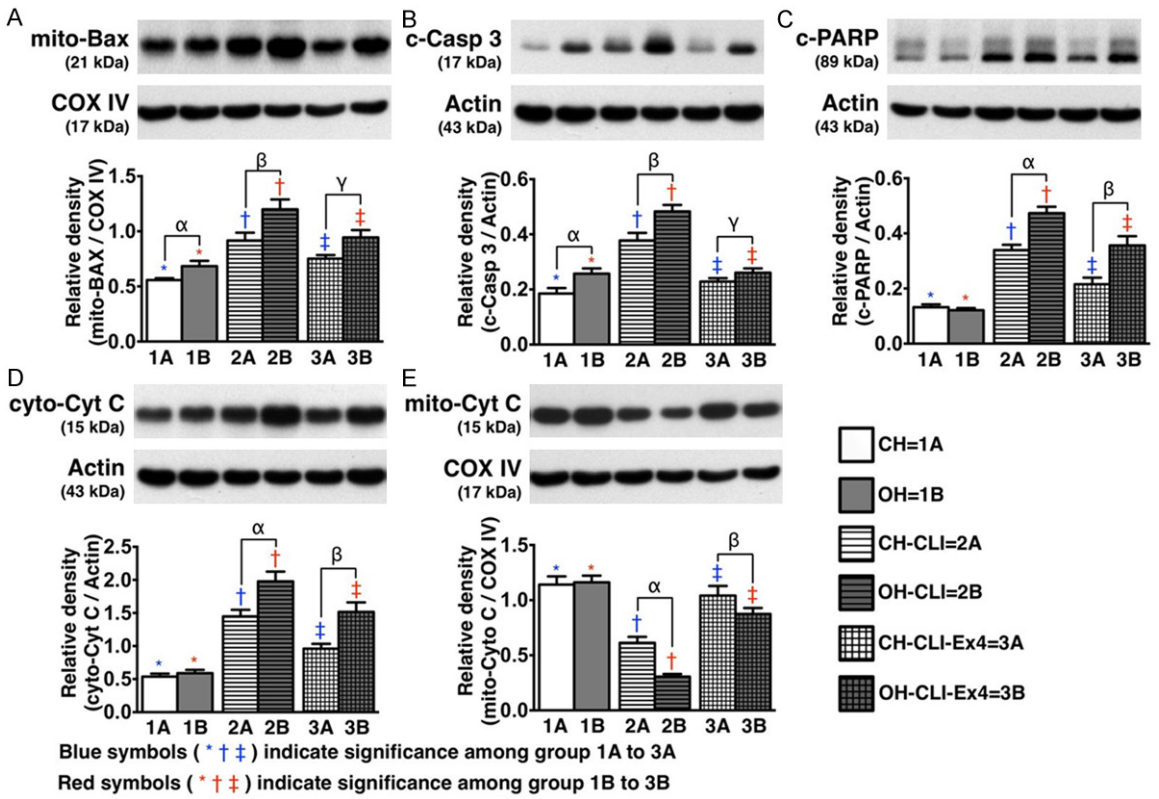

Figure 9. Protein expressions of apoptosis in ischemic quadriceps at day 14 after critical limb ischemia (CLI) procedure (n = 8). A. Protein expression of mitochondrial Bax (mito-BAX). The statistical results: (1) α indicated 1A vs. 1B, p<0.05; β indicated 2A vs. 2B, p<0.01; γ indicated 3A vs. 3B, P<0.01. (2) * vs. other groups with different blue symbols (*, †, ‡), p<0.01. (3) * vs. other groups with different red symbols (*, †, ‡), p<0.001. B. Protein expression of cleaved caspase 3 (c-Casp 3). The statistical results: (1) α indicated 1A vs. 1B, p<0.05; β indicated 2A vs. 2B, p<0.01; γ indicated 3A vs. 3B, P<0.05. (2) * vs. other groups with different blue symbols (*, †, ‡), p<0.01. (3) * vs. other groups with different red symbols (*, †, ‡), p<0.001. C. Protein expression of cleaved poly (ADP-ribose) polymerase (c-PARP). The statistical results: (1) α indicated 2A vs. 2B, p<0.05; β indicated 3A vs. 3B, p<0.05. (2) * vs. other groups with different blue symbols (*, †, ‡), p<0.01. (3) * vs. other groups with different red symbols (*, †, ‡), p<0.001. D. Protein expression of cytosolic cytochrome C (Cyto-Cyt C). The statistical results: (1) α indicated 2A vs. 2B, p<0.05; β indicated 3A vs. 3B, p<0.05. (2) * vs. other groups with different blue symbols (*, †, ‡), p<0.01. (3) * vs. other groups with different red symbols (*, †, ‡), p<0.001. E. Protein expression of mitochondrial cytochrome C (mito-Cyt C). The statistical results: (1) α indicated 2A vs. 2B, p<0.05; β indicated 3A vs. 3B, p<0.05. (2) * vs. other groups with different blue symbols (*, †, ‡), p<0.01. (3) * vs. other groups with different red symbols (*, †, ‡), p<0.001.

area. Third, exendin-4 therapy significantly enhanced circulating levels of EPCs, and it improved blood flow and angiogenesis in the CLI area. Finally, control animals had a better response to Ex-4 treatment than obese mice for the recovery of blood flow in the setting of CLI.

It is well recognized that obesity is a risk factor for endothelial dysfunction, arterial atherosclerosis and cardiovascular disease [1, 8, 9, 21]. One important finding in the present study was that without ischemic stress (i.e., at baseline) circulating levels of EPCs did not differ between obese and control animals. However, in the setting of CLI (i.e. ischemic stress), circulating numbers of EPCs were remarkably lower in obese than in control animals, whether with or without exendin-4 treatment. Interestingly, previous work has also revealed that the circulating number of EPCs did not differ among controls, obesity and obesity-control animals [11]. Additionally, this study [11] has further identified that in the setting of CLI, the circulating numbers of EPCs were notably reduced in obese animals than in control and obesity-control animals. Accordingly, our results corroborate the findings of previous work [11], and

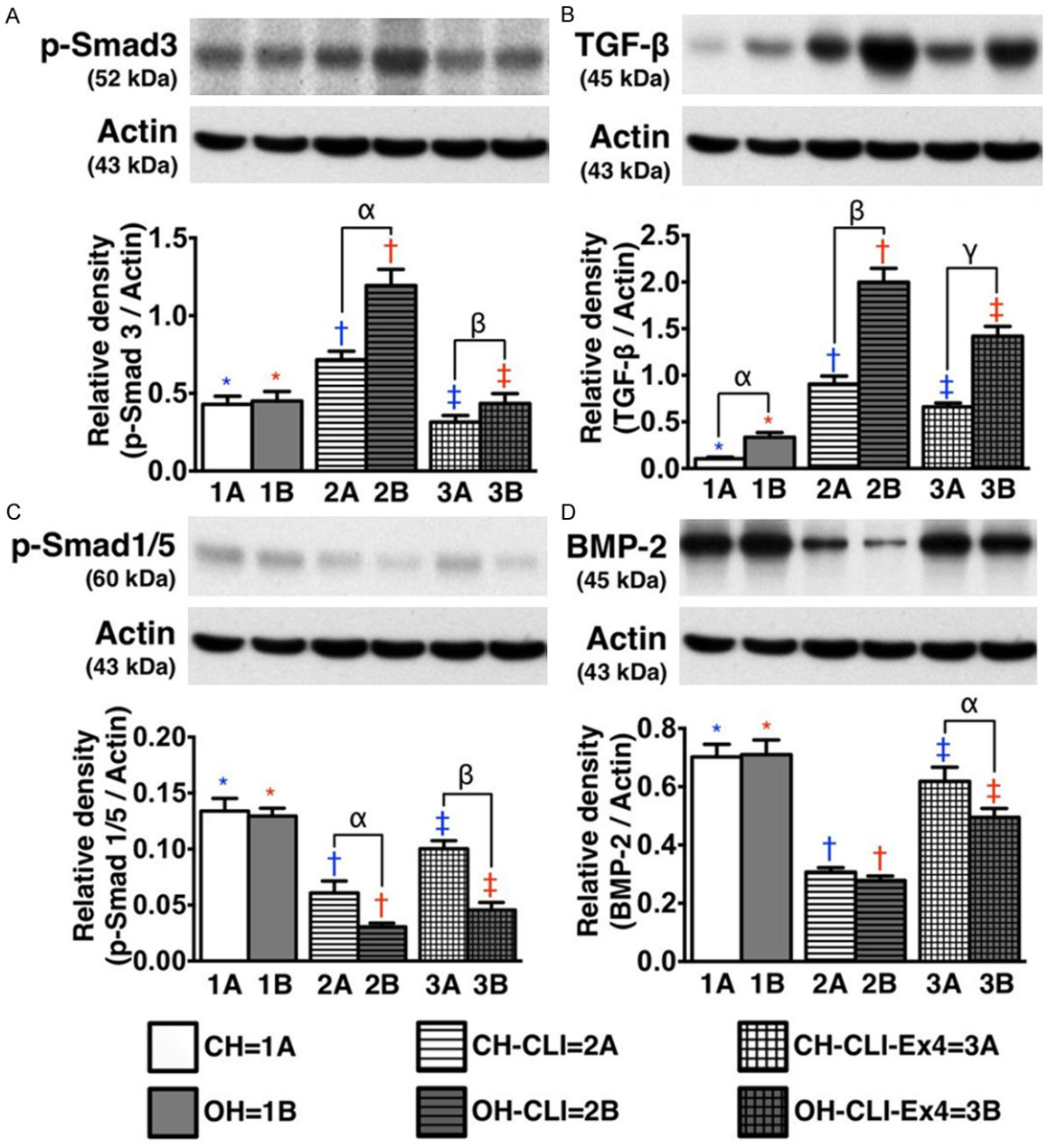

Figure 10. Protein expressions of fibrotic and antifibrotic biomarkers in ischemic quadriceps at day 14 after critical limb ischemia (CLI) procedure (n = 8). A. Protein expression of phosphorylated (p)-Smad3. The statistical results: (1) α indicated 2A vs. 2B, $p<0.01$; β indicated 3A vs. 3B, $p<0.05$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.01$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.001$. B. The protein expression of transforming growth factor (TGF)-β. The statistical results: (1) α indicated 1A vs. 1B, $p<0.05$; β indicated 2A vs. 2B, $p<0.001$; γ indicated 3A vs. 3B, $P<0.01$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.01$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.001$. C. The protein expression of phosphorylated (p)-Smad1/5. The statistical results: (1) α indicated 2A vs. 2B, $p<0.05$; β indicated 3A vs. 3B, $p<0.05$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.01$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.001$. D. The protein expression of bone morphogenesis protein (BMP)-2. The statistical results: (1) α indicated 3A vs. 3B, $p<0.05$. (2) * vs. other groups with different blue symbols (*, †, ‡), $p<0.001$. (3) * vs. other groups with different red symbols (*, †, ‡), $p<0.001$.

highlight that the intrinsic capacity to respond to ischemic stress is significantly impaired in obese compared to control mice.

A principal finding in the present study was that blood flow in the CLI region that was measured by laser Doppler was significantly lower in OH-CLI animals than in CH-CLI animals by day 14 after the CLI procedure. This recovery defect of blood flow in the ischemic region reflects the finding of circulating numbers of EPCs among the obese animals. The most important finding in the present study was that the circulating numbers of EPCs and blood flow in the ischemic zone was remarkably increased in OH-CLI and yet further increased in CH-CLI animals after receiving exendin-4 therapy. This finding implicates that exendin-4 therapy is effective for improving angiogenesis and the ischemic situation. Intriguingly, previous studies [22, 23] have shown that dipeptidyl peptidase-4 (DDP-4) inhibitor improves neovascularization by increasing circulating EPCs. The underlying mechanism for improving circulating EPCs was displayed through increasing plasma glucagon-like peptide-1 (GLP-1) levels that, in turn, reduced plasma DPP-4 concentration [22, 23]. The results from these previous studies [22, 23] support our findings that exendin-4, a GLP-1 analogue, augmented blood flow in the setting of CLI. Accordingly, our findings highlight the potential use of exendin-4 for obese and diabetic patients, particularly those who have overt endothelial dysfunction/cardiovascular disease.

That DDP-4 inhibitor therapy augmented the expression of ischemia-induced increases in SDF-1α within the ischemic zone has previously been established [22, 23]. Additionally, an increased concentration of SDF-1α in the CLI area is well recognized to cause EPC homing to the area for angiogenesis/neovascularization [22, 24]. An essential finding in the present study was that the protein and cellular expressions of angiogenesis biomarkers were remarkably higher in OH-CLI-Ex4 than in OH-CLI animals and notably higher in CH-CLI-Ex4 than in CH-CLI animals. These findings offer other reasons that could explain why blood flow was substantially increased in both OH-CLI and CH-CLI animals after receiving exendin-4 treatment. Importantly, even when undergoing exendin-4 therapy, the levels of angiogenesis factors remained significantly lower in obese than in control animals. These findings, in addition to strengthening the findings of previous studies [22, 24], imply that more aggressive treatment should be recommended for those patients with coexistent obesity and diabetes mellitus.

An association between upregulation of apoptosis and fibrosis and CLI has been identified in our previous experimental studies [11, 18, 23-25]. In the present study, another essential finding was that apoptotic and fibrotic biomarkers were significantly higher, and anti-apoptotic and anti-fibrotic biomarkers were significantly lower, in OH-CLI and CH-CLI animals than in those of both obese and control mice. These findings reinforce the findings of our previous studies [11, 18, 23-25]. A particularly important finding was that these biomarkers were markedly reversed in OH-CLI animals and further remarkably reversed in CH-CLI animals after receiving exendin-4 treatment. Interestingly, our previous study has shown that exendin-4 therapy protects kidney from ischemia-reperfusion injury by suppressing oxidative stress, inflammation, cellular apoptosis and fibrosis [16]. In this regard, our findings, in addition to being comparable with the findings of our previous study [16], could, at least in part, explain why exendin-4 therapy protected against CLI in both obese and control animals.

Study limitations

This study has limitations. First, this study did not determine whether the elevated number of circulating EPCs mobilized from bone marrow or elsewhere to circulation. Second, although the number of circulating EPCs was increased in CLI obese animals after receiving exendin-4 treatment, the function of these EPCs was not directly assessed in the study.

In conclusion, circulating numbers of EPC and angiogenesis capacity were remarkably impaired in obese mice, but were significantly improved after exendin-4 treatment. The results of our study encourage the use of exendin-4 for diabetic patients with obesity and cardiovascular diseases.

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Disclosure of conflict of interest

The authors report no conflict of interest.

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