Exendin-4 reverses endothelial dysfunction in mice fed a high-cholesterol diet by a GTP cyclohydrolase-1/tetrahydrobiopterin pathway

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Abstract. The present study examined whether exendin-4 (Ex4) can improve the endothelial dysfunction of apolipoprotein E knockout (APOE-KO) mice fed a high-cholesterol diet and the potential mechanism by which it acts. Genetically wild-type (WT) C57BL/6 mice and APOE-KO mice of C57BL/6 background, were each randomly assigned to receive either Ex4 treatment (Ex4-treated, for 8 weeks) or not (control). The 4 groups were fed the same high-cholesterol diet for 8 weeks. The following were measured at the end of the eighth week: Endothelium-dependent vasodilation of the arteries; plasma nitric oxide (NO) and metabolic index; levels of endothelial NO synthase (eNOS); phosphorylated eNOS (p-eNOS; Ser-1,177); guanosine triphosphate cyclohydrolase-1 (GCH1); and tetrahydrobiopterin (THB). Ex4 treatment was associated with higher p-eNOS levels in the WT group and in the APOE-KO group, and higher vascular expression of GCH1 and higher arterial THB content, compared with baseline values. The results of the present study suggested that Ex4 may exert cardioprotective effects by reversing high-cholesterol diet-induced endothelial dysfunction in APOE-KO mice. The protective mechanism is probably associated with the promotion of the expression levels of GCH1 protein and THB that maintain the normal function of eNOS.

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Introduction

The vascular endothelium has an important function in maintaining homeostasis among a number of endothelial-derived factors including nitric oxide (NO), prostacyclin, endothelin-1 and angiotensin II. NO is now considered the most efficient endogenous vasodilator and it can exert vasoprotective effects by inhibiting platelet aggregation, inflammation, oxidative stress, fibrosis, vascular smooth muscle cell migration, proliferation, and leukocyte adhesion (1,2). However these functions of the endothelium can be weakened or eliminated by exposure to atherosclerosis risk factors. Endothelial dysfunction is a key factor in the initiation, progression and complications of atherosclerosis, and is now regarded as a clinical syndrome (3-5). Endothelial NO synthase (eNOS) is the key enzyme for NO generation. Tetrahydrobiopterin (THB) is a required cofactor for eNOS that is primarily regulated by its rate-limiting enzyme, guanosine triphosphate cyclohydrolase I (GCH1) (6,7).

Glucagon-like peptide-1 (GLP-1) is a potent glucose-dependent insulin tropic hormone that is released from intestinal L-cells. It has been observed that GLP-1 also has cardioprotective effects that are distinct from its glucose and weight-loss effects (8,9). The short half-life of GLP-1 and its effects on metabolites limit its clinical application (10).

Exendin-4 (Ex4; Food and Drug Administration approval number BYETTA; NDA; 021773;www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=overview.process&applno=021773), a GLP-1 analog, is a highly potent agonist of the GLP-1 receptor. As a result of its shared effects with GLP-1 and as it resists degradation by dipeptidyl peptidase 4, Ex4 has been approved by the United States Federal Drug Administration as an adjunct therapy to improve glycemic control in patients with type 2 diabetes mellitus.

Previous studies demonstrated that Ex4 has a protective effect against cardiovascular disease. For example, Ex4 exerts cardioprotective effects by improving endothelial function and acting as an antihypertensive, and these effects are independent of its hypoglycemic and weight-loss functions (11-13). d'Uscio *et al* (14) in 2016 demonstrated that exenatide treatment improved the coronary flow velocity reserve of patients

with type 2 diabetes. Further study of human umbilical vein endothelial cells revealed that Ex4 increased NO production, GCH1 levels and eNOS activation via GLP-1R/cyclic adenosine monophosphate (cAMP) signaling pathways (15).

Apolipoprotein E knockout (APOE-KO) mice can develop severe hypercholesterolemia, atherosclerosis and associated endothelial dysfunction (14). Evidence has demonstrated that THB is involved in maintaining the normal function of eNOS (6,7), suggesting that THB and NOS may be associated. Phosphorylation of Ser1177 is associated with the activation of eNOS and enhances NO generation. Several studies have demonstrated that Ex4 improves endothelial function (16,17), but few studies have investigated the involvement of the GCH1/THB signaling pathway in endothelial dysfunction in mice fed a high-cholesterol diet (18).

It was hypothesized that Ex4 treatment may increase GCH1 and THB levels, thereby preserving normal eNOS function, to produce increased NO production (19,20,21). In the present study, the association between THB levels and eNOS function was examined. Furthermore, the effect of Ex4 on dysfunctional endothelium in the thoracic aorta isolated from APOE-KO mice given a high-cholesterol diet was investigated.

Materials and methods

All experiments were performed in accordance with protocols approved by the Guangxi Medical University Animal Ethics Committee (Nanning, China).

Animals. Genetically wild-type (WT) C57BL/6 mice (n=30; weight, 17.22±0.79 g) and APOE-KO mice of C57BL/6 background (n=30; weight, 20.12±0.96 g) were purchased from Charles River Laboratories, Inc., (Beijing, China). All of the mice were male and 6-weeks-old. The WT mice and the APOE-KO mice were each randomly and equally allocated to be treated either with Ex 4 (Ex4-treated) or not (controls). Therefore, 4 groups of 15 mice were created: i) WT control; ii) WT+Ex4; iii) APOE-KO control; and iv) APOE-KO+Ex4. The mice in the two treatment groups (WT+Ex4 and APOE-KO+Ex4) were intraperitoneally injected (IP) with Ex4 (1.0 $nmol \cdot kg^{-1} \cdot day^{-1}$, twice daily) for 8 weeks. The control groups were IP injected with an equal volume of physiological saline.

All the mice in all the groups were fed a high-cholesterol diet (1.25% cholesterol) starting at age 6 weeks and throughout the entire experiment. All mice were provided free access to food and water and atmosphere were maintained at a temperature of 23±2˚C, 40-70% humidity and a 12-h light/dark-cycle.

Tissue procurement. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (22) and was approved by the Laboratory Animal Ethical Committee of Guangxi Medical University.

Following 8 weeks of treatment, the mice were anesthetized by pentobarbital (1%; 80 mg drug/kg animal body weight; IP) and euthanized by exsanguination. Blood samples were harvested from the heart (0.8-1 ml/mouse). The entire length of the aorta of each mouse was carefully dissected, from the aortic valve to the iliac bifurcation. The aorta was immediately put into a dish with ice-cold Krebs buffer (NaCl 120 mM, $NaHCO_3$ 25 mM, KCl 4.8 mM, NaH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, dextrose 11.0 mM and $CaCl_2$ 1.8 mM aerated with 95% O_2, pH=7.4). The fat and loose connective tissue was separated carefully under a light microscope and the gross specimen was observed.

The aorta was fixed in 10% formalin at room temperature of 25˚C for 24 h and embedded in paraffin. Serial 5-μm sections were cut at the aortic arch and descending aorta and stained with hematoxylin/eosin (H&E) 1-3 min at room temperature for histological analysis. Aortas were frozen in liquid nitrogen for subsequent western blotting and high-performance liquid chromatography (HPLC), or prepared for the next vasomotor function experiment.

Pathology and morphological study. The aortic arch of each mouse was employed for the pathological and morphological study. The isolated arteries were observed under a dissecting microscope to study the gross appearance. Photographs of the gross specimen were captured with a NIKON camera (D5000; Nikon Corporation, Tokyo, Japan; Fig. 1). The arteries indicated in Fig. 1 were fixed with 4% paraformaldehyde for 24 h at room temperature and processed for paraffin embedding. Serial 4-μm transverse sections were taken at the aortic arch and stained with H&E for pathological study (Fig. 2) as described previously (23).

Plasma. The plasma glucose of each mouse was measured by the glucose oxidase method performed as described previously (24). (GOD-POD kit, Applygen Technologies Inc., Beijing, China). Insulin levels were monitored by radioimmunoassay as described previously (25) (Insulin RIA Kit, BNIBT, Beijing, China). Plasma triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were determined using an automated clinical biochemistry analyzer (Hitachi 7600; Hitachi, Ltd., Tokyo, Japan).

Artery endothelial function

Measurement of vascular relaxation. The descending thoracic aorta of each mouse was studied. The aorta was crosscut into 3-4 mm vascular rings, avoiding the intercostal artery branches and one side was mounted on a hook in an organ chamber containing 20 ml Krebs buffer (NaCl, 120 mM; NaHCO3, 25 mM; KCl, 4.8 mM; NaH2PO4, 1.2 mM; MgSO4, 1.2 mM; glucose, 11.1 mM; CaCl2, 1.8 mM, aerated with 95% O2 and 5% CO2, pH=7.4.) maintained at 37˚C. The other side was connected with a force transducer to collect isometric tension data. The resting tension of the rings was increased gradually to 5 mN and equilibrated for 60 min. During equilibration, the Krebs buffer in the organ chamber was refreshed every 20 min.

Following equilibration, Krebs buffer with 60 mmol/l KCl was added into the chamber, 3 times, to determine the maximal depolarization-induced contractions. The rings were washed with Krebs buffer 3 times and re-equilibrated for 30 min.

Once vessels were pre-contracted at 37˚C with L-phenylephrine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; 10^{-6} mol/l), the relaxation response to acetylcholine (10^{-5} mol/l; Sigma-Aldrich; Merck KGaA) was tested to

confirm the integrity of the endothelium and then was equilibrated. The rings were pre-contracted with phenylephrine (1 M) and when they reached a stable contraction plateau, endothelium-dependent relaxation responses to cumulative concentrations of acetylcholine (10^{-9} to 10^{-5} mol/l) were measured.

Following another 3 washes with Krebs buffer and a 30 min equilibration, endothelium-independent relaxation responses to sodium nitroprusside (Sigma-Aldrich; Merck KGaA; 10^{-9} to 10^{-5} mol/l) were determined.

Western blot analysis. The snap-frozen aorta of each mouse was lysed with Radioimmunoprecipitation Assay buffer (Beyotime Institute of Biotechnology, Haimen, China) with 1 mmol/l fresh phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology,) and a complete phosphatase inhibitor cocktail tablet ((Roche Diagnostics GmbH, Mannheim, Germany). The protein content was determined by a Bicinchoninic protein assay (Beyotime Institute of Biotechnology). Aorta protein extracts were denatured and 80 μg sample was loaded onto 10%SDS-PAGE gel and transferred. The resolved proteins in the gel were electro-transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% (w/v) nonfat dry milk (dissolved in Tris-buffered saline with 0.05%Tween) for 1 h at room temperature.

Membranes were incubated serially overnight at 4˚C with the following primary antibodies, diluted appropriately in primary antibody dilution buffer: Anti-eNOS (1:1,000; cat. no. sc654 Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-phosphorylated (p)-eNOS ser-1177 (1:1,000; cat. no. sc12972 Santa Cruz Biotechnology, Inc., Dallas, TX, USA); and anti-GCH1 (1:1,000; cat. no. ab236387 Abcam, Cambridge, UK).

The membranes were then washed in TBST 3 times for 10 min, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; ZSGB-BIO; OriGene Technologies, Inc., Beijing, China.) for 1 h at room temperature and washed in TBST 3 times for 10 min. The immunoblots were visualized using a DAB chemiluminescence kit (Beyotime Institute of Biotechnology). Equal loading of protein was confirmed by blotting for GAPDH (1:1,000; cat. no. ta309157; ZSGB-BIO, Beijing, China). Protein levels were normalized with reference to GAPDH. p-eNOS ratios were normalized according to the total eNOS levels of corresponding groups. Relative band densitometry was analyzed by ImageJ software (ImageJ 1.44p; National Institutes of Health, Bethesda, MD, USA).

Measurement of THB and biopterin. Measurement of THB levels in aortas was performed using internal standard HPLC with florescence detection, as described previously (26). A total of three snap-frozen aortas from each group were lysed in ice-cold extraction buffer (50 mmol/l Tris-HCl, pH 7.4, 1 mmol/l dithiothreitol and 1 mmol/l EDTA), centrifuged at 13,000 x g for 10 min at 4˚C and the supernatant was collected.

A protein assay was performed using a Bicinchoninic protein assay (Beyotime Institute of Biotechnology). The proteins from the supernatant were removed by adding 10 μl of a 1:1 mixture of 1.5 mol/l $HClO_4$ and 2 mol/l H_3PO_4, to 90 μl extracts, and centrifuged at 13,000 x g for 10 min at 4˚C. The supernatant was employed for THB measurement.

Total biopterin (THB, dihydropterin and biopterin) was determined by acidic oxidation using 10 μl of 1% iodine and 2% KI dissolved in 1 mol/l HCL, added to 90 μl protein-free supernatant. BH_2+B was determined by alkaline oxidation. This was kept at room temperature in the dark for 1 h. A total of 20 μl, 1 mol/l H_3PO_4 was added to acidify the alkaline-oxidation samples. A total of 5 μl fresh ascorbic acid (20 mg/ml) was added into each sample to remove the iodine. Samples were centrifuged at 13,000 x g for 10 min at 4˚C.

Biopterin (Sigma-Aldrich; Merck KGaA) was used for internal standard. A total of 20 μl of supernatant was loaded into a 250-mm long, 4.6-mm inner diameter Spherisorb ODS-1 column (Guangzhou Research and Creativity Biotechnology, Co., Ltd., Guangzhou, China) with a methanol-to-water (5:95, v/v) mobile phase running at a flow rate of 1.0 ml/min, column temperature was 25˚C and detected fluorometrically at wavelengths of 350 nm for excitation and 440 nm for emission (LC-20 series, Shimadzu Corp., Kyoto, Japan).

Statistical analysis. *In vitro* experiments were repeated 3 times. Data are expressed as the mean ± standard deviation. One-way analysis of variance was used to examine the differences among the 4 groups of mice and the Student-Newman-Keuls post hoc test was used for a further pairwise comparison. All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Plasma analyses and body weight of mice. Plasma TG, HDL-C, glucose and insulin levels were not significantly different among the 4 groups (Table I). The TC in the APOE-KO and APOE-KO+Ex4 groups was ~15-fold higher compared with the WT control and WT+Ex4 groups, and LDL-C levels were also significantly increased in the APOE-KO ($P<0.01$) and APOE-KO+Ex4 ($P<0.01$) groups. However, the TC and LDL-C levels were not statistically different between APOE-KO and APOE-KO+Ex4 groups.

There was a trend in the WT control group toward higher NO levels compared with the APOE-KO group, but the difference was not significant (Table I). However, NO levels in the APOE-KO+Ex4 mice were significantly increased to 37.3±12.6 μmol/l, which was nearly 2-fold higher compared with the APOE-KO group (20.0±7.4 μmol/l; $P=0.015$; both groups). The NO levels were also significantly 2-fold higher in the WT+Ex4 group (65.3±11.7 μmol/l) compared with the WT control group (27.1±7.4 μmol/l; $P<0.001$, both groups).

The body weights of mice were recorded and analyzed (Table II). The data regarding body weights demonstrated no significant difference between the APOE-KO and APOE-KO+Ex4 groups at 6- and 14-weeks-old. The body weights of the WT control and WT+Ex4 groups were also comparable at 6- and 14-weeks-old.

Histopathology and morphological changes of the arteries. Regarding general gross observation of the artery specimens, an atheromatous plaque was undetectable in the WT control (Fig. 1A) and WT+Ex4 (Fig. 1B) groups, and there was no

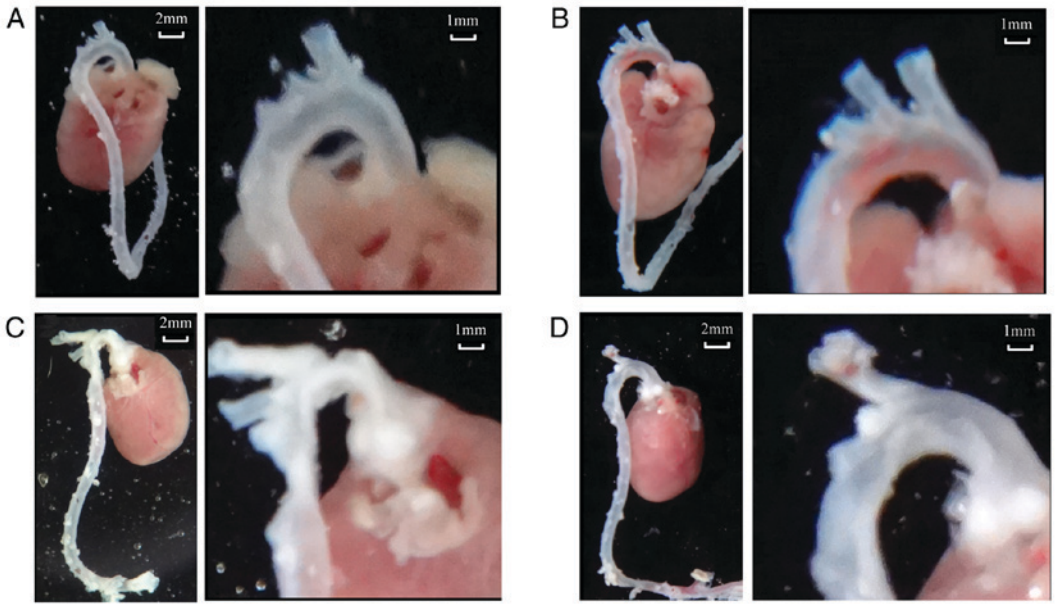

Figure 1. Observations of the morphology of the arteries from C57BL/6 WT and APO-KO mice. (A) WT control group. (B) WT+Ex4 group. (C) APOE-KO group. (D) APOE-KO+Ex4 group. Scale bars, 1 and 2 mm (as indicated). WT, wild-type; APO-KO, apolipoprotein E knockout; Ex4, exendin-4.

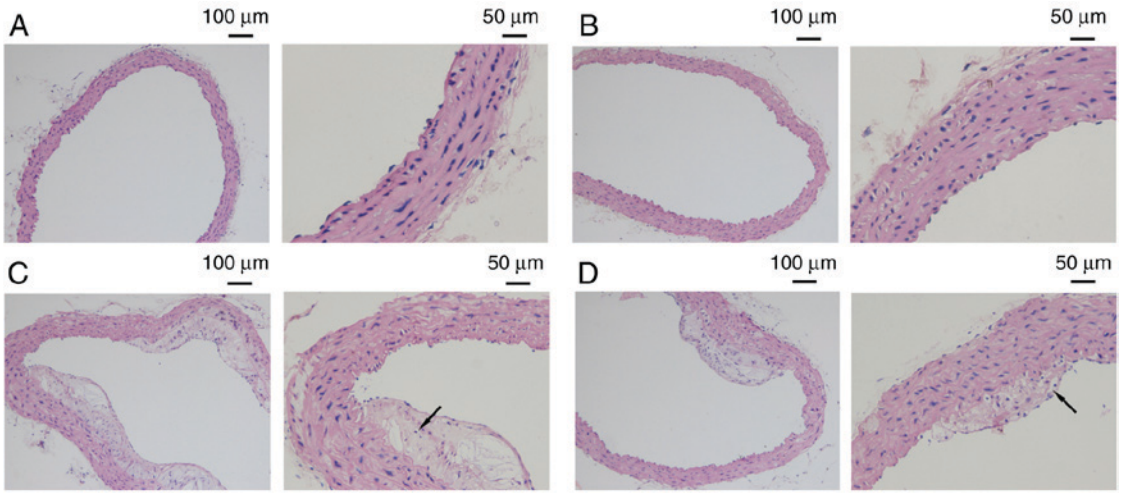

Figure 2. Histopathological hematoxylin and eosin staining of the murine aortic arch, magnification x100 and x200 (scale bars, 50 and 100 μm as indicated). (A) WT control group. (B) WT+Ex4 group. (C) APOE-KO group. (D) APOE-KO+Ex4 group. WT, wild-type; APOE-KO, apolipoprotein E knockout; Ex4, exendin-4. The black arrows indicate macrophages and foam cells.

vascular stenosis; they were normal in morphology. By contrast, in the untreated APOE-KO mice (Fig. 1C) specifically, the histopathology and morphological results demonstrated severe atherosclerosis of the aorta in the APOE-KO mice (Figs. 1 and 2). The arterial lumen was substantially stenosed by an atheromatous plaque, particularly at the aortic arch, the predilection site of arteriosclerotic lesions and also in certain principal branches. However, atherosclerosis was not grossly observed in the APOE-KO+Ex4 (Fig. 1D) group; the lumen and edge of the artery were clearly observed and appeared similar to that of the WT mice.

On microscopic examination of the sections stained with H&E, arteries of the WT control and WT+Ex4 (Fig. 2A and B) groups were similar in morphology. The intimae of the arteries were smooth, and the endothelia and muscle were arranged regularly. Atheromatous lesions could not be observed. Typical atheromatous lesions were demonstrated easily in the arteries of the APOE-KO (Fig. 2C) group, with a number of macrophages and foam cells in atheromatous plaques. The arterial wall was thickened homogeneously. In the APOE-KO+Ex4 (Fig. 2D) group, foam cells were also present in the subendothelium. Atherosclerotic lesions were not diffused so widely and thickening of the arterial wall was inconspicuous when compared with the APOE-KO group.

Endothelium-dependent vasodilation mediated by acetylcholine. Once arterial rings were pre-contracted with 10^{-6} mol/l phenylephrine, concentration-dependent acetylcholine-induced vasodilation was observed (Fig. 3). The maximum acetylcholine-induced-vasodilation of the arteries of the non-treated APOE-KO group (53.9±4.3%) was ~1.5 times that of the WT control group and was significantly different (77.9±8.7%;

Table I. Plasma lipids, glucose, insulin and NO levels.

Factor	WT	WT+Ex4	APOE-KO	APOE-KO+Ex4
TC, mmol/l	2.64±0.46	2.34±0.62	37.61±7.72[b]	33.75±7.38[b]
TG, mmol/l	0.65±0.20	0.87±0.32	1.11±0.24[b]	1.16±0.25[b]
HDL-C, mmol/l	2.19±0.34	2.57±1.37	4.73±2.00	5.13±2.88
LDL-C, mmol/l	0.65±0.14	0.72±0.11	16.87±6.01[b]	13.08±4.55[b]
Glucose, mmol/l	6.59±1.78	6.90±2.25	6.26±1.63	4.14±1.16
Insulin, pmol/l	17.16±4.98	15.06±3.94	12.53±2.74	11.26±2.23
NO, μmol/l	27.11±7.44	65.33±11.72[a]	20.00±7.37	37.33±12.6[c]

Data are presented as the mean ± standard deviation, n=5 per group. [a]P<0.05 and [b]P<0.01 vs. WT mice; [c]P<0.05 vs. the APOE-KO mice. WT, wild-type; APOE-KO, apolipoprotein E knockout; Ex4, exendin-4; HDL-C, high density lipoprotein-cholesterol; LDL-C, low-density lipoprotein; TG, triglyceride; TC, total cholesterol; NO, nitric oxide.

Table II. Body weight of mice in the present study.

Age group (weeks)	WT (g)	WT+Ex4 (g)	APOE-KO (g)	APOE-KO+Ex4 (g)
6	17.22±0.79	17.78±0.83	20.12±0.96	20.48±1.59
14	26.88±1.05	25.78±1.54	29.12±0.97	28.36±0.66

Data are presented as the mean ± standard deviation, n=5 per group. WT, wild-type; APOE-KO, apolipoprotein E knockout; Ex4, exendin-4.

P<0.05; Fig. 3A). The maximum acetylcholine-induced-vasodilation of the arteries of the WT+Ex4 group was 98.9±2.1%, which was significantly higher compared with the WT control group (77.9±8.7%; P<0.05; both groups; Fig. 3B). By contrast, the maximum acetylcholine-induced-vasodilation in the APOE-KO+Ex4 mice was 87.8±3.6%, which was significantly increased compared with the APOE-KO group (53.9±4.3%; P<0.01; Fig. 3C). The 4 groups were comparable regarding the degree of sodium nitroprusside-induced endothelium-independent vasodilation (Fig. 3D).

eNOS and p-eNOS protein levels. Western blotting bands are presented in Fig. 4A. The results indicated that total eNOS protein levels in the arteries of the APOE-KO group were significantly increased compared the WT group (P=0.014). Ex4 treatment was associated with ~2-fold higher total eNOS protein levels in the APOE-KO+Ex4 group compared with the APOE-KO group (P<0.05). However, the total eNOS of the WT+Ex4 group was similar to that of the WT group (P=0.154; Fig. 4B).

The arterial p-eNOS protein levels were also investigated, and the WT control and APOE-KO groups were comparable. Ex4 treatment in the WT+Ex4 group was associated with a 3.5-fold increase in p-eNOS levels compared with the WT non-treated group (P<0.05) and with a 2-fold increase in the APOE-KO+Ex4 mice compared with the non-treated APOE-KO group (P<0.05; Fig. 4C)

GCH1 protein and THB levels. Western blotting analysis was employed to measure the vascular GCH1 protein level (Fig. 5A). GCH1 of the WT+Ex4 group were significantly higher when compared with the WT group (2.5-fold; P<0.05). The GCH1 level of the APOE-KO+Ex4 group were nearly 2-fold higher compared with the APOE-KO group (P=0.002; Fig. 5B).

Production of THB was detected by HPLC (Fig. 5C). The differences in arterial THB levels among the 4 groups were similar to the patterns observed in GCH1 protein and p-eNOS level. In the WT+Ex4 group, THB protein (1.34±0.03 pmol/mg), was significantly higher compared with the WT group (0.37±0.13 pmol/mg; P<0.05). THB protein of the APOE-KO+Ex4 group (0.75±0.14 pmol/mg) was 7-fold higher than that of the APOE-KO group (P<0.05). There was no difference in total biopterin among the 4 groups (Fig. 5C).

The ratio of BH4 to total biopterin was higher in the WT+Ex4 group (59.6±14.6%) when compared with the WT group (35.3±22%; P=0.065), although the difference was not significant. The ratio of BH4 to total biopterin in the APOE-KO+Ex4 group (34.3±9%) was significantly higher when compared with the APOE-KO group (5.4±2.3%; P=0.035; Fig. 5D).

Discussion

In this present study, it was demonstrated that a high-cholesterol diet was associated with higher TC and LDL-C in the APOE-KO mice compared with WT control mice. Also, the results of the present study demonstrated that Ex4 treatment was associated with improved endothelial dysfunction of the aortae in APOE-KO mice fed a high-cholesterol diet, without affecting blood lipid and glucose. The effect of Ex4

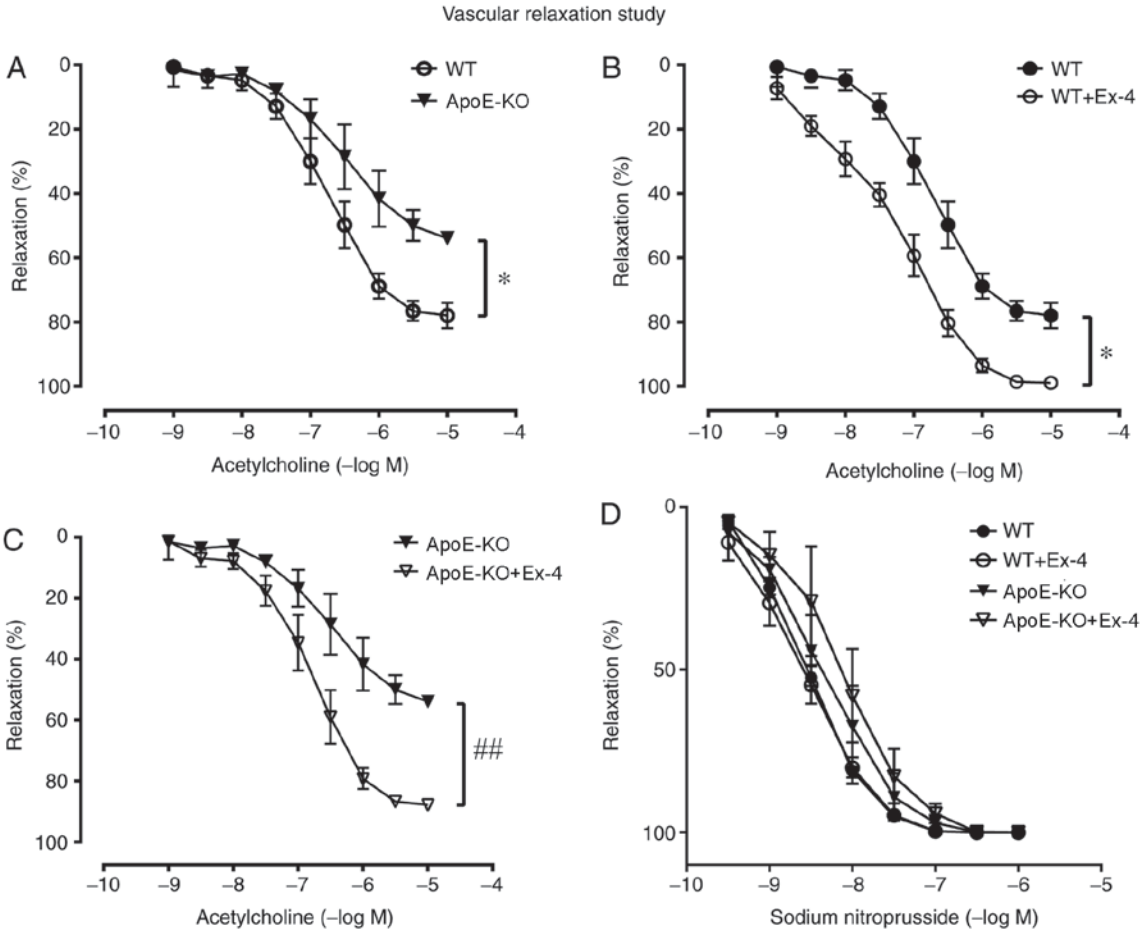

Figure 3. Effect of Ex4 on endothelium-dependent vasodilation responses to cumulative concentrations of acetylcholine. Data of maximum relaxation rate are presented. (A) Relaxation of aortic rings of APOE-KO mice were severely impaired compared with WT control group in acetylcholine-induced (endothelium-dependent vasodilation; n=5 per group). (B) Ex4 improved acetylcholine-induced-endothelium-dependent vasodilation in WT control mice (n=5 per group). (C) Ex4 reversed and markedly improved acetylcholine-induced endothelium-dependent vasodilation in APOE-KO+Ex4 group compared with APOE-KO group. (D) No significant difference between the groups (P>0.05, n=3 per group) in endothelium-independent relaxation in response to sodium nitroprusside. *P<0.05 vs. WT control mice; ##P<0.01 vs. APOE-KO mice. WT, wild-type; APOE-KO, apolipoprotein E knockout; Ex4, exendin-4.

treatment was independent of TC, TG, LDL-C or glucose. A THB-suppressant was not used to demonstrate a link between THB and eNOS directly, since specific studies have investigated this previously (27,28). The acetylcholine-stimulated endothelium-dependent vasodilation was decreased in the APOE-KO mice when compared the WT control group. However, the condition of the aorta of the APOE-KO+ Ex4 group was improved. Similar improvement to the vascular condition was also observed in the WT+Ex4 mice compared with the non-treated WT control mice.

Similar to the results of the present study, a previous study demonstrated that Ex4 treatment directly elicited significant and concentration-dependent vasorelaxation in the rat aortas (29). Ozyazgan *et al* (29) used Ex4 for the long-term treatment of type 2 diabetic rats and demonstrated that Ex4 treatment returned the acetylcholine-induced relaxation response to approximately that of the control group, which is similar to the results of the present study. Different results of endothelial dysfunction of rat arteries demonstrated neither a direct protective effect on endothelial dysfunction nor vasorelaxation effects of Ex4 (30). However, this may be explained by the endothelial protective effect of Ex4 is due to multi-factors *in vivo* rather than rapidly *in vitro*.

In the present study, it was demonstrated that the NO levels in the APOE-KO mice were lower when compared with the WT control mice. This indicated that the production of NO was reduced, or its half-life had been shortened. By contrast, in the Ex4 intervention groups the plasma NO level was higher by 2-fold (WT+Ex4) and 1.5-fold (APOE-KO+Ex4) compared with non-treated groups. The mechanism may involve cAMP/5'-AMP-activated protein kinase-Associated signaling pathways and enhancement of eNOS activity (16). It is hypothesized that the positive effect on endothelial function of Ex4 may involve eNOS.

Acetylcholine-stimulated endothelium-dependent vasodilation was impaired in APOE-KO mice; it was significantly lower than that of the WT control group. However, the aorta condition of the APOE-KO+Ex4 group was better and their arteries performed well in acetylcholine-induced endothelium-dependent vasodilation. Similar improvement of vascular condition was also observed in the WT+Ex4 mice compared with the non-treated WT control mice. A previous isolated vessel study on standard rats demonstrated that Ex4 treatment directly elicited significant and concentration-dependent vasorelaxation in rat aortas (31). However the maximal relaxation only reached ~30%. This may be associated with the healthy

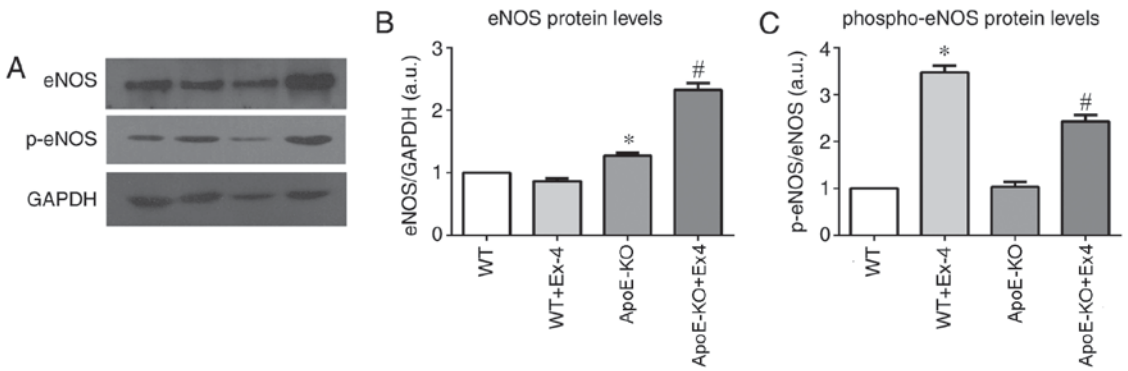

Figure 4. Extracted protein from aortas was subjected to western blotting to determine the eNOS and p-eNOS expression ratios. GAPDH was employed to normalize proteins of interest and p-eNOS ratios were normalized according to the total eNOS levels of the corresponding groups. (A) Western blot analysis for eNOS in the aortas of the four groups. (B) Ratio of total eNOS protein expression relative to WT control group (n=5). (C) Percentage of p-eNOS level relative to WT control group (n=5). Data are presented as the mean ± standard deviation. *P<0.05 vs. WT control mice; #P<0.05 vs. APOE-KO mice. Phospho/p-eNOS, phosphorylated endothelial nitric oxide synthase; WT, wild-type; APOE-KO, apolipoprotein E knockout; Ex4, exendin-4; a.u., arbitrary units.

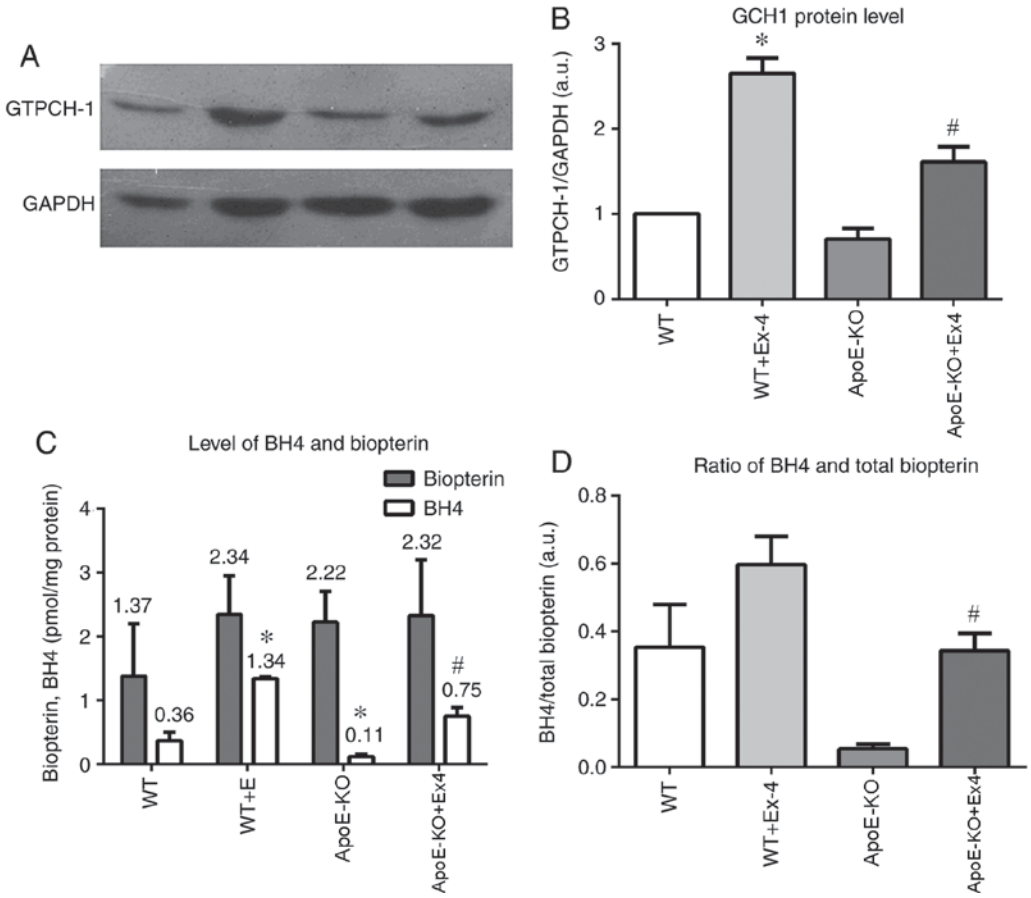

Figure 5. Effects of Ex4 on GCH1 protein and THB levels in arteries. (A) Western blot analysis for GCH1 in the aortas of the four groups. (B) GCH1 protein levels were determined by western blot analysis. GAPDH was employed to normalize the proteins of interest. Ratio of GCH1 protein expression relative to WT control group (n=3). (C) High pressure liquid chromatography was performed to quantify the levels of THB and biopterin content. (D) The ratio of BH4 to total biopterin was compared. Data are presented as the mean ± standard deviation. *P<0.05 vs. WT control mice; #P<0.05 vs. APOE-KO mice. WT, wild-type; APOE-KO, apolipoprotein E knockout; Ex4, exendin-4; THB, tetrahydrobiopterin (BH4); GCH1, guanosine triphosphate cyclohydrolase-1; a.u., arbitrary units.

animal model. the scale of the improvement seems less significant compare with APOE-KO mice which were suffering more severe endothelial injury.

Kuhlencordt *et al* (32) observed the atherosclerotic lesion area of ApoE/eNOS-double knockout mice and demonstrated that atherosclerosis was more severe in eNOS-deficient mice compared with the APOE-KO mice. This may indicate that eNOS has a protective cardiovascular effect. Notably, in the study by Ozaki *et al* (28), the atherosclerotic lesion areas of the aorta were unexpectedly increased in APOE-KO/eNOS-transgene (Tg) mice (eNOS-overexpressing) compared with APOE-KO mice. A previous study demonstrated that

endothelial cells incubated with exenatide can increase eNOS protein levels but did not increase the expression of eNOS mRNA (33). This may indicate that Ex-4 regulates eNOS expression without affecting eNOS gene transcription. It is hypothesized that Ex4 treatment led to a more normalized eNOS function in the APOE-KO+Ex4 group relative to the non-treated APOE-KO mice.

It was established that the phosphorylation of eNOS on serine 1,177 can activate the enzyme and lead to NO production (34,35). The present study demonstrated that p-eNOS levels were increased in mice treated with Ex4, for the APOE-KO and WT control mice, and the treated groups exhibited higher plasma NO. These results suggested that Ex4 may have a protective effect towards eNOS activity. This is similar to previous *in vitro* studies, which suggests that Ex4 upregulated p-eNOS in human umbilical vein endothelial cells and human coronary artery endothelial cells (17,19,33).

THB, a cofactor of NOS regulates eNOS activity (20,36). And GCH1 is the first and rate-limiting enzyme for THB *de novo* synthetic pathway (21), so THB is mainly determined by the content and bioactivity of GCH1. The results of the present study demonstrated that vascular GCH1 and THB protein levels were decreased in the APOE-KO group compared with the WT control group. Cai *et al* (20) demonstrated that GCH1 gene transfer in endothelial cells notably increased THB, enhanced eNOS homodimerization and increased NO production, suggesting that GCH1 may be a reasonable target to augment endothelial THB and normalize eNOS activity in endothelial dysfunction. The present study hypothesized that the severe abnormal function and uncoupling of eNOS in APOE-KO mice was due in part to the absence of THB, and ultimately led to a lower NO level.

Hattori *et al* (37) treated APOE-KO mice with oral THB and observed that continuous THB availability improved NO-mediated endothelial function and limited the progression of atherosclerosis. In the present study, it was also demonstrated that arterial GCH1 protein level were increased in the APOE-KO+Ex4 (~2-fold) and the WT+Ex4 (2.5-fold) groups, compared with their respective non-treated groups.

The THB content of the 2 intervention groups were correspondingly higher and the increase in the APOE-KO+Ex4 group was ~7-fold compared with the non-treated APOE-KO mice. Similar to previous studies, Bendall *et al* (38) demonstrated that eNOS protein was significantly increased in the eNOS-Tg and eNOS/GCH1-transgene mice. However, the ratio of eNOS dimer-to-monomer was significantly decreased in eNOS-Tg mice and its superoxide production was significantly increased. By contrast, with sufficient THB levels of superoxide the function of eNOS was normal in eNOS/GCH-Tg mice. Takaya *et al* (39) reported that atherosclerotic lesion formation was increased in APOE-KO/eNOS-Tg mice compared with APOE-KO mice, while atherosclerotic lesions were reduced in APOE-KO/eNOS-Tg/GCH-Tg mice, which had higher vascular THB compared with APOE-KO/eNOS-Tg mice. The present study demonstrated a similar phenomenon.

Apart from the GCH1-THB-eNOS signaling pathway, one of the important mechanisms of Ex4 on cardiovascular protection, previous studies have demonstrated that Ex4 can improve myocardial function in heart failure by reversing cardiac remodeling (40-42). Evidence from a previous study indicated that Ex4 reduced monocyte/macrophage accumulation in the arterial wall by inhibiting the inflammatory response in macrophages and may contribute to the attenuation of atherosclerotic lesions (43). This effect may also help preserve the bioactivity of THB and eNOS, and is good for endothelial function and cardiovascular disease.

Although the sample size of the present study was small, the statistical power in this study remain valid. Also, the present study, to the best of our knowledge, is the first study using the flow-mediated dilation technique to suggest that long-term administration of Ex4 improves arterial dilation.

In conclusion, models of endothelial dysfunction in APOE-KO mice were established and the results provide strong evidence that systemic application of Ex4 can reverse endothelial dysfunction in APOE-KO mice induced by a high-cholesterol diet. The present study also suggested that the protective mechanisms associated with Ex4 treatment may primarily concern the promotion of GCH1 expression and increase of THB level. Furthermore, adequate THB may help maintain normal eNOS function and preserve eNOS phosphorylation to improve endothelial function and achieve cardiovascular protection.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

ZT, LL, YG, GD and MC performed experiments. LL provided animal experiment technical assistance. ZT analyzed and interpreted data. ZT drafted the manuscript. ZT, YG and JW edited and revised manuscript. JW and ZT contributed to conception and design of research. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were performed in accordance with protocols approved by the Guangxi Medical University Animal Ethics Committee (Nanning, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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