

Effects of Glucagon-Like Peptide-1 on Arteriovenous Fistula Remodelling and Vascular Smooth Muscle Cell Phenotype Switching in Diabetic Rats

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Abstract

This study aimed to determine the potential vascular protective effect of exendin-4, a synthetic glucagon-like peptide (GLP)-1 analogue, on the blood flow and remodelling of arteriovenous (AV) fistulas in a rat model of diabetes. AV fistulas were created in Sprague-Dawley rats with streptozotocin-induced diabetes. The animals were then randomly assigned to receive intraperitoneal injection of vehicle (saline) or exendin-4 for about 2 weeks. The blood flow and vasomotor function of the aortic limb of the fistula were measured. Concentrations of serum norepinephrine and its associate tissue signal proteins, and vascular smooth muscle cell (VSMC) contractile proteins, matrix metalloproteinase (MMP)-2, and collagen expression in the remodelled aorta were examined. Exendin-4 attenuated the contraction response to phenylephrine in isolated arterial segments from treated animals. Treatment with exendin-4 increased serum norepinephrine concentration, reduced aorta tissue α 1-receptor expression, and enhanced downstream signal proteins. Additionally, exendin-4 promoted the switching of VSMCs to the synthetic phenotype, which increased tissue collagen content and suppressed tissue abundances of smooth muscle myosin heavy chain type II and desmin in diabetic animals. In conclusion, our study demonstrated that exendin-4 modulates VSMC transition to the synthetic phenotype and enhances the arterial contraction responses, thereby attenuating blood flow in the AV fistula.

Keywords: Diabetes mellitus; Arteriovenous fistula; Glucagon-like peptide-1; Vascular smooth muscle cell; Phenotypic switching

Introduction

Diabetes mellitus is one of the most common causes of end-stage renal disease (ESRD) requiring renal replacement therapy, with an estimated disease prevalence of 13.2 to 21.9% [1]. Currently, hemodialysis is the primary treatment used for renal replacement therapy, by creating an arteriovenous (AV) fistula or other AV anastomosis. It has been well established that diabetes is an independent risk factor for early loss of AV fistula patency by 6 months (odds ratio 2.3) [1] and increases complication rates in comparison to non-diabetic patients with AV fistulas [2]. Therefore, the maintenance of long-term patent vascular access is beneficial for improving the quality of life of patients with diabetes and ESRD.

Hyperglycemia increases oxidative stress in the vascular wall and causes endothelial dysfunction [3], leading to phenotypic modulation of vascular smooth muscle cells (VSMCs) in the media layer [4]. The VSMCs switch from a quiescent, contractile phenotype to cells expressing synthetic characteristics during high oxidative stress and vascular remodeling [5]. In the synthetic status, VSMCs proliferate and migrate in the vessel wall via increased biosynthesis of extracellular matrix as well as activation of matrix metalloproteinase (MMP) with concomitant decreased expression of contractile proteins such as smooth muscle myosin heavy chain (SM-MHC), desmin, and alpha-actin [5].

Glucagon-like peptide-1 (GLP-1) is a gut-derived hormone, belonging to the incretin family. GLP-1 is secreted by the small intestine under delicate regulation in response to blood glucose concentrations. Since GLP-1 exerts stimulatory effects on insulin release through binding to the GLP-1 receptor (GLP-1R) on pancreatic beta cells, [6,7] GLP-1 analogues have been applied clinically to control hyperglycemia

in type 2 diabetes [8]. GLP-1R is ubiquitously expressed in extra-pancreatic tissues or cells including the stomach, intestine, sinoatrial node myocytes, endothelial and smooth muscle cells [6,9]. GLP-1 and its agonists have also been reported to mediate cardiovascular protective effects in experimental and clinical studies [10-13]. The cytoprotective effect of GLP-1 is attributed to the suppression of programmed cell death including apoptosis and senescence for endothelial cells [14].

The pleiotropic effects of GLP-1 are well documented in certain conditions of endothelial dysfunction [15,16]. Liraglutide, a GLP-1 analogue, reduces the intimal and medial thickness of carotid arteries in patients with diabetes [17]. Comparatively, exenatide, a synthetic GLP-1 receptor agonist, reduces intimal hyperplasia in the carotid arteries of non-diabetic fatty rats [18]. Exenatide has also been reported to prevent venous thrombosis in AV fistulas in a chronic kidney disease rat model [19]. However, the effects of GLP-1 on vascular remodelling and blood flow in the AV fistulas of subjects with diabetes remain undetermined. Since GLP-1 suppresses neointimal formation and mediates vascular protective effects, [20] we hypothesized that administration of the GLP-1 analogue exendin-4 might enhance the blood flow and maturation of AV fistulas in an experimental model of diabetes.

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Method

Animals and AV fistula creation

Age matched young male Sprague-Dawley rats (weight 200–250 g) were maintained in an animal house with 12 hour light and dark cycles. Rats were fed on a standard chow diet and provided with water *ad libitum*. All procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of National Cheng Kung University, Taiwan (IACUC Approval No.102193). The detailed procedures of creating AV fistulas were described previously [21], the rats were anesthetized by isoflurane inhalation (2–3% v/v in oxygen). The inferior vena cava (IVC) and aorta were exposed following a midline abdominal incision. The aorta was punctured with a 20 G disposable needle at the level below the renal vessels. The needle was gradually introduced across the aorta to penetrate the neighbouring wall of the IVC. The needle was then withdrawn and the puncture point was closed using a purse-string suture. The vascular clamps then were removed and the abdominal wall was closed in layers.

Induction of hyperglycemia

The induction of diabetes in rats was performed as described previously [21]. In brief, a single dose of streptozotocin (65 mg/kg, *i.p.*) was injected into the rats. The successful rate of inducing insulin dependent diabetes from a single injection was approximately 60%. The induction of diabetes was confirmed 48 hours later by measuring the blood sugar concentration using an enzymatic kit (Vital Diagnostics Ltd., Thane, India). Hyperglycemia was defined as a random blood glucose level of more than 250 mg/dL [22].

Treatment protocol

Rats were allocated into the following four treatment groups: control, control plus exendin-4, DM, and DM plus exendin-4. Animals in the exendin-4-treated groups received exendin-4 (0.5 µg/kg) *i.p.* injections once a day for 3 days before surgery, and continued for up to 2 weeks after the creation of the AV fistula [16,23] (Figure 1). All experimental animals received a standard chow diet throughout the study period.

Measurement of blood chemistry

Immediately before euthanasia, whole blood was collected via a puncture in the inferior vena cava after deep anesthesia. Blood glucose, serum levels of total cholesterol, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), and triglycerides were analyzed using colorimetric assays (ADVIA 1800 Chemistry

System; Siemens, Munich, Germany) as described previously [22].

Hemodynamic measurements

Intra-arterial blood pressure in the carotid artery was measured directly with a fluid-filled pressure transducing and analyzing system (Power Lab, AD Instruments, Dunedin, New Zealand) [21].

Blood flow in the arterial site of the AV fistula was determined using an ultrasonic flow probe (Transonic Systems, Ithaca, NY) following midline laparotomy. The blood flow tracings were recorded for at least 1.5 minutes and analyzed using Power Lab while the mean arterial pressure was controlled at around 80–85 mm Hg. All hemodynamic measurements were carried out by investigators without knowledge of the treatment groups [24,25].

Organ chamber experiments

Segments of the aorta proximal to the fistula were resected and mounted in organ chambers containing Krebs'-Ringer bicarbonate. Changes in tension were recorded continuously using an isometric force-displacement transducer. After a 45 minute equilibration period, the rings were contracted by the addition of KCl (40 mM) and cumulative addition of phenylephrine (PE, 10^{-9} to 10^{-5} M). Concentration-response curves were obtained by cumulative addition of acetylcholine during pre-contraction of the rings with an effective concentration (EC_{60}) of PE.

Western blot analysis

Fistula tissue homogenates were loaded into polyacrylamide gels (9–12%) and then transferred to nitrocellulose membranes. Mouse monoclonal antibodies were incubated with the membranes for at least 4 hours at room temperature. After washing, the membranes were incubated with a 1:2000 dilution of horseradish peroxidase-linked secondary antibodies, and bands were visualized using enhanced chemiluminescence. The primary antibodies used in this study were anti-iNOS, anti-p47phox, anti-heme oxygenase-1 (all from BD Transduction), anti-α1 receptor (Abcam, Cambridge, UK), anti-phospholipase C (PLC)-β isoform 4 (GeneTex, San Antonio, TX, USA), [26] anti-inositol triphosphate (IP)-3 receptor (Thermo Scientific Pierce, Rockford, IL, USA), anti-matrix metalloproteinase (MMP)-2 (Millipore, Bedford, MA, USA), anti-desmin (Dakocytomation Japan Co. Ltd., Kyoto, Japan), and anti-SM-MHC-II (Santa Cruz Biotechnology, Dallas, TX, USA). Bands were visualized using enhanced chemiluminescence.

Quantification of serum norepinephrine levels

The serum concentrations of norepinephrine were determined using a commercially available ELISA kit (ALPCO Diagnostics, Salem, NH, USA). The analysis was performed according to the manufacturer's instructions.

Measurement of tissue collagen concentration

Frozen fistula tissue were sliced into small cubes and processed in extraction solvent. The reagent was placed in a Sircol Assay Kit (Biocolor Ltd., Carrickfergus, UK) for quantification of tissue collagen levels.

Histological sections

Isolated vascular tissues were immersed in 4% formaldehyde for about 24 hours at room temperature, dehydrated in 30% sucrose overnight, and embedded in paraffin in a core pathology facility. Paraffin-embedded tissues were sectioned and stained with hematoxylin

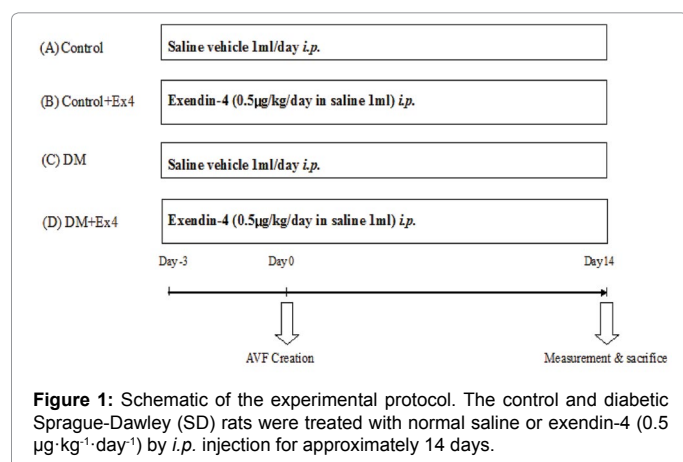


Figure 1: Schematic of the experimental protocol. The control and diabetic Sprague-Dawley (SD) rats were treated with normal saline or exendin-4 (0.5 µg·kg⁻¹·day⁻¹) by *i.p.* injection for approximately 14 days.

and eosin, and Masson's trichrome staining [25].

Immunohistochemical Staining

Immunohistochemical analysis was performed on 5- μ m-thick formalin-fixed paraffin-embedded tissue sections using the Bond-Max Automated IHC stainer (Leica Biosystems Newcastle Ltd., Australia). After de-paraffinization and treatment with Epitope Retrieval Solution 2, the sections were incubated with rabbit anti- α 1 receptor antibody (1:200 dilutions, Abcam) at room temperature for 30 minutes. To enhance polymer binding, tissues were incubated with post primary block (Leica Biosystems) at room temperature for another 8 minutes. Secondary antibody (anti-mouse/rabbit poly-HRP) was applied at room temperature and the tissue sections were finally counterstained with hematoxylin.

Statistical analysis

Results are presented as means \pm SD. Data was compared by the Student's t-test or ANOVA followed by a Dunn's or Dunnett's post-hoc test, as appropriate. Statistical significance was accepted at a level of $P < 0.05$.

Results

Blood biochemistry analysis

Random blood glucose levels were significantly increased in diabetic rats induced by streptozotocin, whereas administration of exendin-4 reduced the blood glucose level of rats with diabetes (Table 1). A significantly higher total cholesterol level was observed in untreated diabetic rats compared with exendin-4 treated controls (92.6 ± 13.6 vs. 52.5 ± 2.2 mg/dL, $P < 0.05$). Exendin-4 did not affect the serum levels of triglyceride, total cholesterol, HDL-c, or LDL-c (Table 1).

Vasomotor function assessments

The contraction response of the AV fistula arterial segments to KCl-induced depolarization was significantly enhanced in diabetic animals treated with exendin-4 (Figure 2A). In contrast, the responses to phenylephrine-mediated α 1-receptor stimulation were significantly attenuated in exendin-4 treated rats (Figures 2B and 2C). Furthermore, the impaired endothelium-dependent relaxation response in diabetic rats was also restored by exendin-4 treatment (Figure 2D).

Expression of inflammation related proteins

The administration of exendin-4 suppressed the abundance of the pro-inflammatory protein iNOS in the AV fistula in comparison to untreated diabetic rats (Figures 3A and 3B). However, only a trend toward expression reduction of p47phox, a major subunit of NADPH

	Control	Con+Ex4	DM	DM+Ex4
Blood glucose (mg/dL)	251.2 \pm 4.0	218.2 \pm 25.3	561.6 \pm 21.5 ^a	404.6 \pm 69.6 ^b
Triglyceride (mg/dL)	52.3 \pm 14.5	50.8 \pm 8.5	160.8 \pm 88.6	164.5 \pm 68.6
Chol-T (mg/dL)	55.9 \pm 3.8	52.5 \pm 2.2	92.6 \pm 13.6 ^c	69.6 \pm 12.5
HDL-c (mg/dL)	39.3 \pm 5.1	29.8 \pm 6.2	45.4 \pm 4.9	41.9 \pm 8.2
LDL-c (mg/dL)	12.3 \pm 1.2	11.5 \pm 0.5	17.8 \pm 2.0	18.6 \pm 3.8

DM: Diabetes Mellitus, Ex4: Exendin-4, Chol-T: Total Cholesterol, HDL-c: High-Density Lipoprotein Cholesterol, LDL-c: Low-Density Lipoprotein Cholesterol. Data were analyzed by one-way ANOVA, and with post hoc Dunn's method, if necessary. These results are shown as mean \pm SD; n=6-9 for each group.

^a $P < 0.05$ vs. control or DM+Ex4

^b $P < 0.05$ vs. control, control+Ex4

^c $P < 0.05$ vs. control+Ex4

Table 1: Serum levels of glucose and lipid profiles in control and diabetic rats.

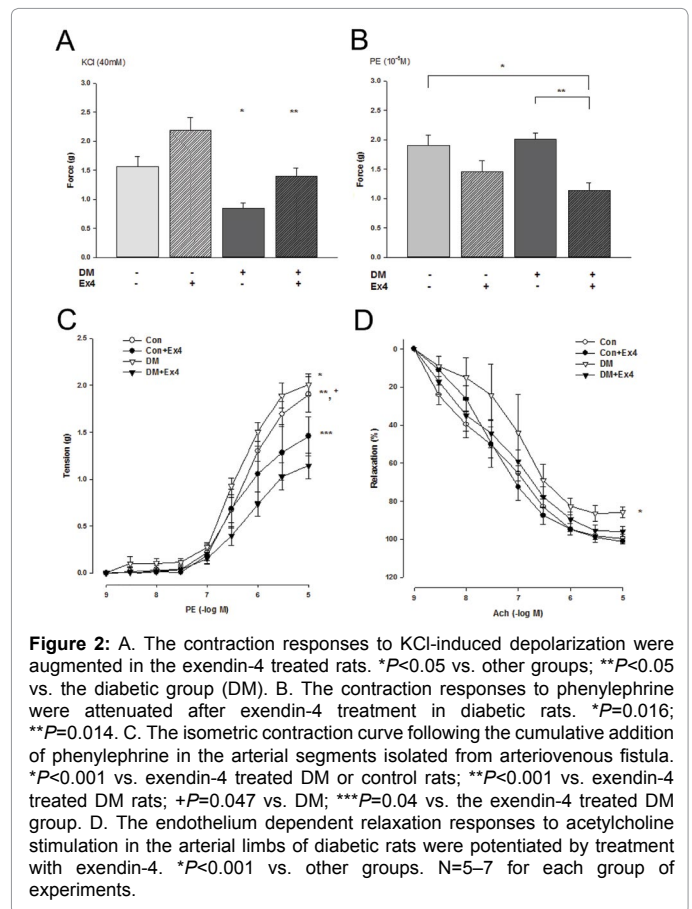


Figure 2: A. The contraction responses to KCl-induced depolarization were augmented in the exendin-4 treated rats. $*P < 0.05$ vs. other groups; $**P < 0.05$ vs. the diabetic group (DM). B. The contraction responses to phenylephrine were attenuated after exendin-4 treatment in diabetic rats. $*P = 0.016$; $**P = 0.014$. C. The isometric contraction curve following the cumulative addition of phenylephrine in the arterial segments isolated from arteriovenous fistula. $*P < 0.001$ vs. exendin-4 treated DM or control rats; $**P < 0.001$ vs. exendin-4 treated DM rats; $+P = 0.047$ vs. DM; $***P = 0.04$ vs. the exendin-4 treated DM group. D. The endothelium dependent relaxation responses to acetylcholine stimulation in the arterial limbs of diabetic rats were potentiated by treatment with exendin-4. $*P < 0.001$ vs. other groups. N=5-7 for each group of experiments.

oxidase, was observed after treatment (Figure 3C). Furthermore, the protein levels of the anti-inflammatory gene heme oxygenase-1 also decreased, indicating that exendin-4 mediated a potent anti-inflammatory response in the vasculature of animals with chronic hyperglycemia (Figure 3D).

Hemodynamic measurements

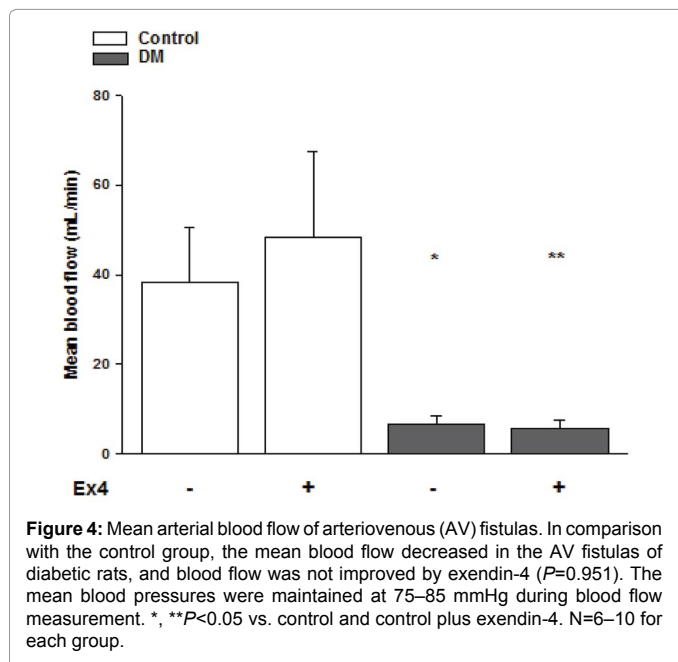
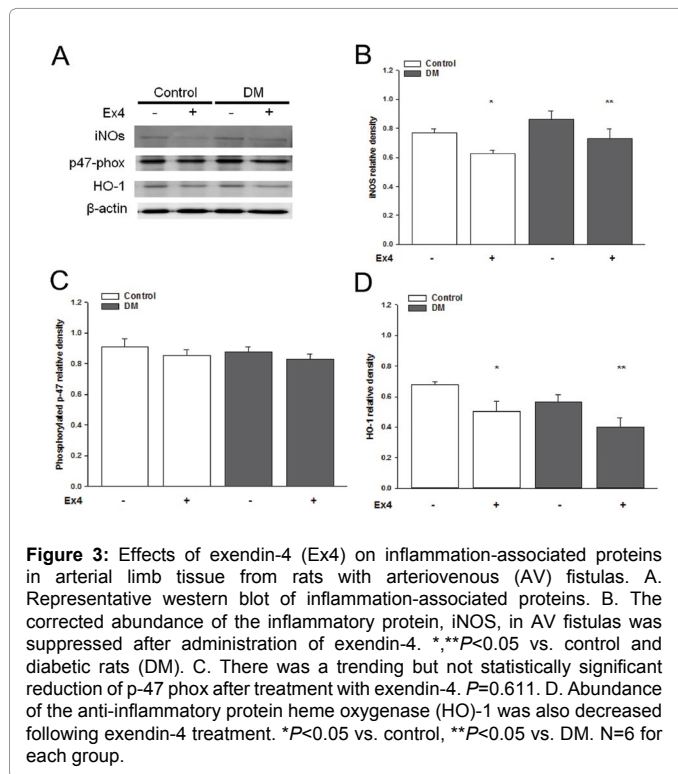
Compared with controls, the blood flow measured in the arterial sites of AV fistulas was significantly decreased in rats with diabetes compared to the rats in the control group, but did not improve following treatment with exendin-4 (Figure 4).

Norepinephrine and α 1-receptor levels

In control and diabetic rats, the serum concentration of norepinephrine significantly increased after treatment with exendin-4 (Figure 5A). Consistent with the observed decreased contraction responses to phenylephrine stimulation, the expression of α 1-adrenergic receptor was suppressed in the aortic fistula segments of diabetic rats treated with exendin-4 (Figure 5B), whereas the downstream signaling proteins PLC- β 4 and IP₃ receptor were up-regulated (Figures 5C and 5D).

Phenotypic modulation of vascular smooth muscle in AV fistulas

The abundance of desmin and SM-MHC-II were reduced in the fistulas of diabetic rats treated with exendin-4 (Figures 6A and 6B). However, the MMP-2 protein level was not affected following exendin-4 treatment in diabetic rats (Figure 6C). Additionally,



exendin-4 enhanced the biosynthesis of collagen in the arterial fistula of diabetic rats (Figure 6D).

Histologic analysis

The expression of $\alpha 1$ -adrenergic receptor in the fistulas of control and diabetic animals was suppressed following treatment with exendin-4 (Figures 7A-7D). Histological sections of vascular rings showed more elongated nuclei in the medial layers in the exendin-4 treated rats (Figures 7E-7H). Masson trichrome staining further

confirmed a greater degree of elastin striae disorganization in the fistula walls of exendin-4 treated animals (Figures 7I-7L).

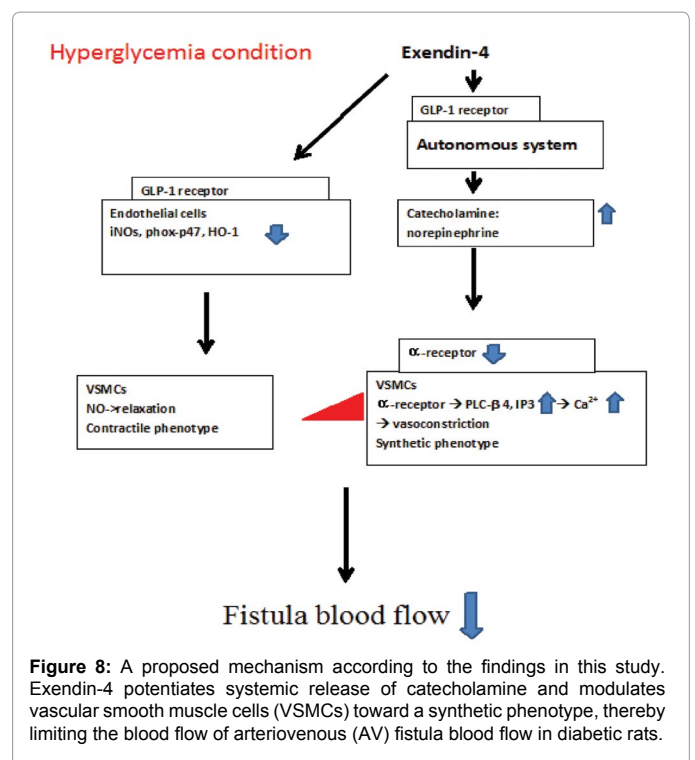
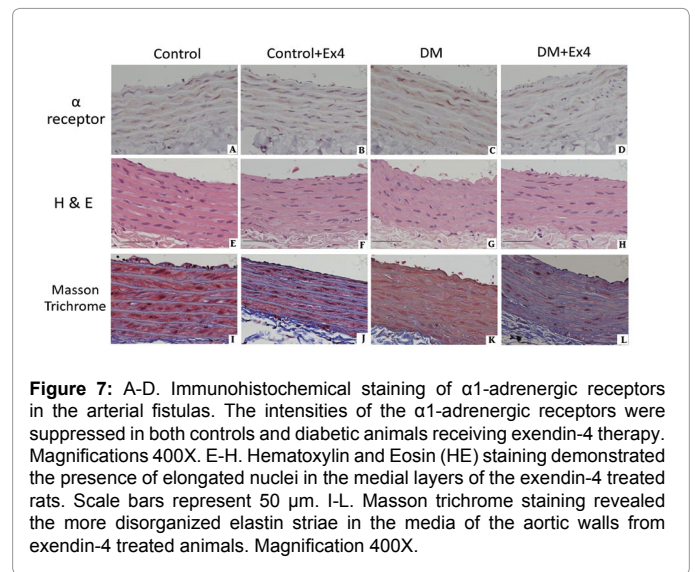
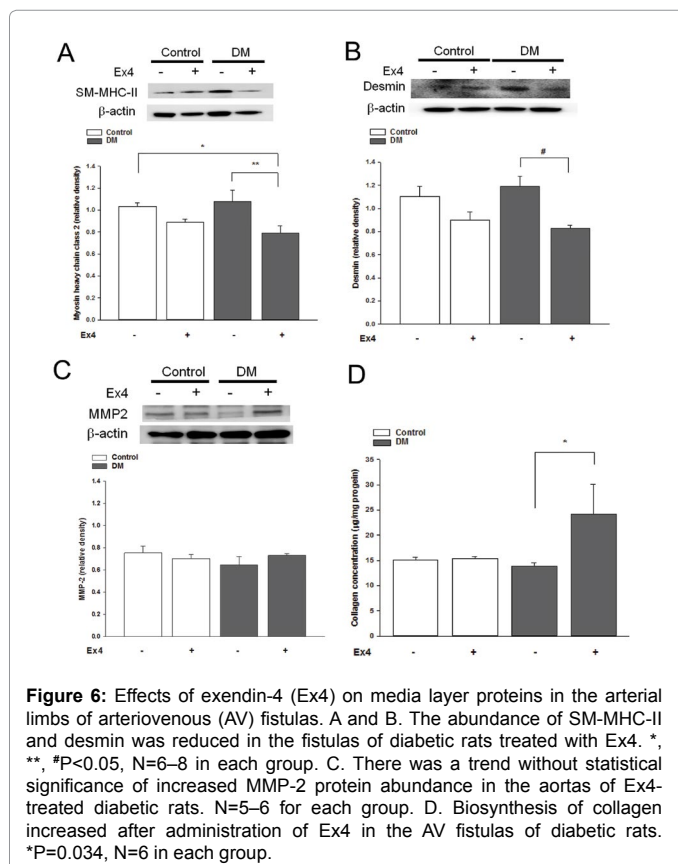
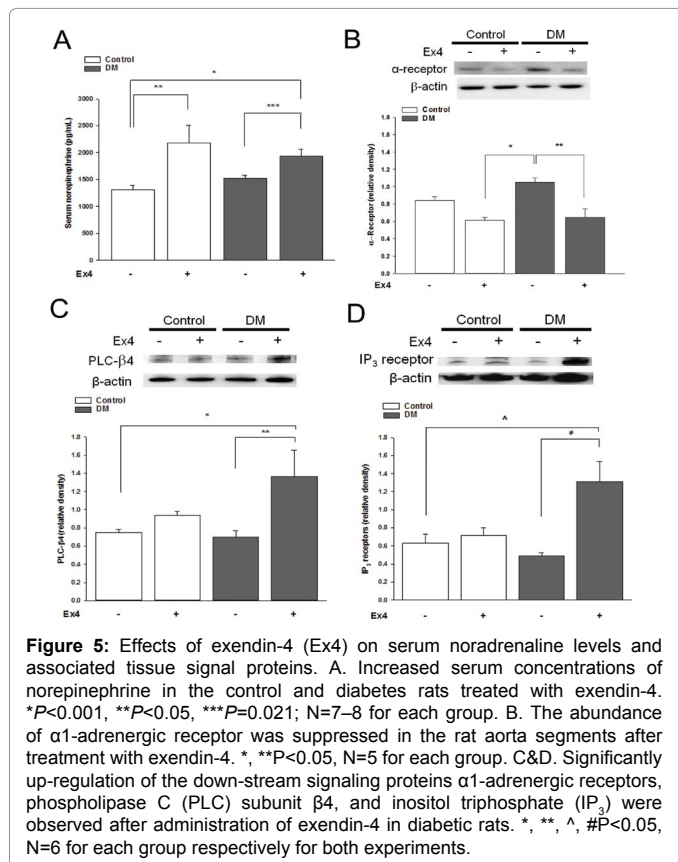
Discussion

Exendin-4 has been extensively reported to mediate vascular protection in diabetic animals and human subjects through the reduction of oxidative stress, up-regulation of NO synthase, and attenuation of monocyte adhesion [10,27]. A cytoprotective response in the senescence of endothelial cells and VSMCs under oxidative stress has also been recently reported [19,28]. Since suppression of vascular oxidative reactions and enhancement of endothelial function improves blood flow and the maturation of AV fistulas, [24] we tested the pleiotropic effects of the novel hypoglycemic agent exendin-4 on the remodeling of AV fistulas by using an experimental model of diabetes in rats.

Our results demonstrated that *i.p.* injection of exendin-4 during the perioperative period of AV fistula creation significantly restored the endothelium-dependent relaxation response and demonstrated a trend toward suppressed abundance of iNOS and NADPH oxidase in the fistula tissue of the diabetic rats, reinforcing the model that exendin-4 mediates an endothelial protective reaction by suppressing the pro-inflammatory response in the vasculature [29]. However, the improvement in vascular endothelial function of AV fistulas following treatment with exendin-4 in diabetic rats did not result in restoration of fistula blood flow. Overall, perioperative administration of exendin-4 augmented blood flow in the fistula of non-diabetic rats, but fistula flow remained low in diabetic rats despite exendin-4 treatment.

Therefore, we further analyzed the isometric contraction responses of the fistulas to KCl and phenylephrine stimulation in an organ bath. The vasomotor function recordings demonstrated that the depolarizing contraction induced by a sub-optimal concentration of KCl (40 mM) was enhanced in control and diabetic animals after exendin-4 treatment, whereas the tensions induced by the cumulative addition of $\alpha 1$ -adrenergic agonist phenylephrine (10^{-9} – 10^{-5} M) were attenuated in the arterial fistulas treated with exendin-4. The abundance of $\alpha 1$ -adrenergic receptors in the fistula tissue was significantly suppressed in exendin-4 treated animals. Furthermore, the down-stream signaling proteins PLC $\beta 4$ and IP $_3$ receptor were reciprocally enhanced. Collectively, these functional and molecular changes suggest that exendin-4 might modulate the reactivity of vascular $\alpha 1$ -adrenergic receptors through the central nervous system or via circulating catecholamine levels [10,30].

In line with previous studies in healthy human volunteers, [31] our results showed that the administration of exendin-4 increased the serum concentrations of norepinephrine in both diabetic and non-diabetic rats with AV fistulas. GLP-1 receptor agonists activate catecholamine neurons in the brainstem, and thus stimulate the sympathetic outflow [30]. High circulating levels of norepinephrine desensitize the vascular response to $\alpha 1$ -adrenergic stimulation and result in hypo-responsiveness and reduced blood flow in the arterial limb of fistulas (Figure 8). On the other hand, Gardiner et al. studied the vasoconstriction response of conscious rats treated with exendin-4, [32,33] and found that pretreatment with phentolamine did not affect the vasoconstriction responses within four experimental days in these non-diabetic animals. However, we consider that the differences in experimental design including length of treatment (4 days vs. 2 weeks), induction of chronic hyperglycemia (non-diabetes vs. diabetes), methods of measurement (*in vivo* vs. *ex vivo*), use of α -adrenergic agents (non-selective antagonist vs. selective 1 agonist) and the presence of AV fistulas preclude a point-wise comparison between these studies.



The medial remodeling of the AV fistula involves phenotypic switching of VSMCs [24]. The differentiation of VSMCs is a dynamic process and is responsive to physiological stimulation, particularly to fluid shear stress [24,34]. In mature blood vessels, VSMCs show an exceedingly low rate of proliferation and extracellular matrix synthesis, and are predominantly committed to contractile function [5]. However, contractile VSMCs might undergo modification to a synthetic phenotype in response to mechanical stress, hormonal, or neural stimulation [5]. In our study, the reduced abundance of smooth muscle cell MHC type II protein and the trend toward reduced desmin following treatment with exendin-4 indicated the switching of VSMCs to the synthetic phenotype in the remodeled fistula [5]. In addition, the

enhanced trend of MMP-2 abundance and significantly increased tissue collagen content confirmed the dominant synthetic VSMC phenotype in the exendin-4 treated fistulas. The elongated nuclei of the VSMCs and the disorganized elastin striae in the media layers of aortic tissue treated with exendin-4 also represented the phenotypic characteristics of synthetic VSMCs. Thus, in response to the high laminar flow conditions, VSMCs became de-differentiated in arterial tissues, thereby enhancing tissue contraction and relaxation responses. These VSMC synthetic characteristics could potentially lead to a limited fistula blood flow, as had been described in our previous study [24].

The mechanism of VSMC phenotype regulation *in vivo* by exendin-4 has not been previously reported, although exendin-4 had been found *in vitro* to suppress platelet-derived growth factor (PDGF)-induced proliferation of aortic VSMCs in mice. In that study, the mechanism was associated with non-canonical GLP-1 signal transduction through the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) [20]. We speculate that the norepinephrine released by exendin-4 treatment might also contribute to the phenotypic transformation of VSMCs, since studies have indicated that norepinephrine triggers cultured VSMCs to switch to the synthetic phenotype [35]. Consistent with this observation, our study supports the hypothesis that an increased serum concentration of norepinephrine promotes the transformation of VSMCs to evince synthetic characteristics *in vivo*.

Conclusion

This study represents the first investigation of the effects of treatment with exendin-4, a novel GLP-1 analogue, during the perioperative period of AV fistula creation in diabetic rats. Our study provides evidence suggesting that exendin-4 potentiates vascular endothelial function by suppressing the pro-inflammatory reaction. However, the improvements in vascular function did not lead to a concomitant restoration of blood flow in the fistula of diabetic rats. We also demonstrated that exendin-4 stimulates the systemic release of norepinephrine, which impairs the responsiveness of the AV fistula and promotes the switching of fistula VSMCs to a synthetic phenotype. These findings suggest that GLP-1 analogues might negatively impact the maturation and blood flow of AV fistulas in diabetic subjects.

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