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Thymosin β4 promotes the recovery of peripheral neuropathy in type II diabetic mice

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ABSTRACT

Peripheral neuropathy is one of the most common complications of diabetes mellitus. Using a mouse model of diabetic peripheral neuropathy, we tested the hypothesis that thymosin β4 (Tβ4) ameliorates diabetes-induced neurovascular dysfunction in the sciatic nerve and promotes recovery of neurological function from diabetic peripheral neuropathy. Tβ4 treatment of diabetic mice increased functional vascular density and regional blood flow in the sciatic nerve, and improved nerve function. Tβ4 upregulated angiopoietin-1 (Ang1) expression, but suppressed Ang2 expression in endothelial and Schwann cells in the diabetic sciatic nerve. In vitro, incubation of Human Umbilical Vein Endothelial Cells (HUVECs) with Tβ4 under high glucose condition completely abolished high glucose-downregulated Ang1 expression and high glucose-reduced capillary-like tube formation. Moreover, incubation of HUVECs under high glucose with conditioned medium collected from Human Schwann Cells (HSCs) treated with Tβ4 significantly reversed high glucose-decreased capillary-like tube formation. PI3K/Akt signaling pathway is involved in Tβ4-regulated Ang1 expression on endothelial and Schwann cells. These data indicate that Tβ4 likely acts on endothelial cells and Schwann cells to preserve and/or restore vascular function in the sciatic nerve which facilitates improvement of peripheral nerve function under diabetic neuropathy. Thus, Tβ4 has potential for the treatment of diabetic peripheral neuropathy.

Introduction

Peripheral neuropathy is one of the most common and disabling complications of diabetes mellitus. Studies of diabetic peripheral neuropathy from experimental animals and humans indicate that the development of diabetic neuropathy is closely associated with marked neurovascular dysfunction (Cameron et al., 2001; Ebenezer et al., 2011; Tesfaye et al., 1993). Vascular dysfunction precedes the appearance of nerve conduction velocity deficits, leading to nerve damage (Cameron and Cotter, 1999; Cameron et al., 2005; Ebenezer et al., 2011). Therapies targeting neurovascular function have been shown to restore nerve function in experimental diabetic peripheral neuropathy (Ii et al., 2005; Kusano et al., 2004; Schratzberger et al., 2001).

Thymosin Beta4 (Tβ4), a small 4.9 kDa polypeptide of 43 amino acids, is a major intracellular G-actin-sequestering peptide (Goldstein et al., 1996) and is present in almost all cell types (Crockford et al., 2010). Tβ4 has multiple biological functions that include promoting diabetic wound healing by repairing and regenerating damaged tissues (Philp et al., 2003) and enhancing angiogenesis after myocardial infarction and vasculogenesis during development (Crockford, 2007; Smart et al., 2007a). Tβ4 is currently under a phase II clinical trial for the treatment of patients with acute myocardial infarction (ClinicalTrials.gov, http://clinicaltrials.gov/ct2/show/NCT01311518; Ruff et al., 2010). In the central nervous system, preclinical studies show that Tβ4 has neuroprotective and neurorestorative effects by reducing neuronal damage and enhancing oligodendrogenesis, which lead to improvement of neurological outcomes after stroke, traumatic brain injury and multiple sclerosis (Morris et al., 2010; Philp et al., 2003; Xiong et al., 2011; Zhang et al., 2009). However, the effect of Tβ4 on peripheral nerves, such as diabetic peripheral neuropathy, has not been invetigated.

The angiopoietins (Ang1 and Ang2) and their receptor Tie-2 regulate vascular development and homeostasis (Suri et al., 1996; Teichert-Kuliszewska et al., 2001). Ang-1 promotes vascular stabilization and maturation whereas Ang2 acts as a partial agonist or antagonist of Ang1 signaling, depending on vascular endothelial growth factor (VEGF) bioavailability (Maisonpierre et al., 1997; Suri et al., 1996; Thebaud et al., 2005). The Ang/Tie2 signaling pathway plays an important role in mediating vascular function under diabetes (Chen and Stinnett, 2008b; Tuo et al., 2008). Hyperglycemia downregulates Ang1 and upregulates Ang2, which exacerbates myocardial infarction (Morris et al., 2010; Tuo et al.,

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2008). Increases in Ang1 levels normalize diabetes induced immature vasculature (Chen and Stinnett, 2008a). Patients with peripheral diabetic neuropathy have elevated levels of circulating Ang2 (Rasul et al., 2011). However, the effect of the Ang/Tie2 signaling pathway on peripheral diabetic neuropathy has not been extensively studied.

In the present study, using a mouse model of type II diabetes, we tested the hypothesis that treatment of diabetic peripheral neuropathy with Tβ4 ameliorates neurovascular dysfunction and improves peripheral nerve function. In addition, we investigated the effect of Tβ4 on the Ang/Tie2 signaling pathway under conditions of diabetic peripheral neuropathy.

Material and methods

Animals

All experimental procedures were carried out in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the institutional Animal Care and Use Committee of Henry Ford Hospital. Male BKS.Cg-*m*+/+*Lepr*^{db}/*J* (db/db) mice (Jackson Laboratories, Bar Harbor, Maine) aged 20 weeks were used. Age-matched heterozygotes mice (db/m), a non-penetrant genotype (Jackson Laboratories), were used as the control animals.

Tβ4 treatment

db/db mice at age 20 weeks were treated with Tβ4 at a dose of 6 mg/kg or 24 mg/kg (RegeneRx, Inc, intraperitoneal injection, i.p.), every 3 days for 4 weeks (n = 10/group). db/db mice (n = 10/group) at the same age treated with the same volume of saline were used as a control group. Age-matched db/m mice treated with Tβ4 (6 mg/kg i.p. every 3 days, n = 10/group) or saline (n = 10/group) were used as additional control groups. All mice were sacrificed 8 weeks after the initial treatment. Doses of Tβ4 were selected based on published studies (Mora et al., 1997; Morris et al., 2010).

Blood glucose levels were measured from the mouse tail vein by using an instant check meter (Roche Diagnostics, Indianapolis, IN). Blood glucose levels, body weight and functional tests were measured at baseline (before treatment) and then every 2 weeks until sacrifice. Electrophysiological measurements were performed before treatment and then every 4 weeks until sacrifice. All procedures and analyses were performed by investigators who were blinded to the treatment administered.

Measurement of regional sciatic nerve blood flow by laser Doppler flowmetry

Regional sciatic nerve blood flow was measured at the end of the experiments (8 weeks after the initial treatment) using laser Doppler flowmetry (LDF PeriFlux PF4, Perimed AB, Järfälla, Sweden) (Zhang et al., 1997). Briefly, under anesthesia (ketamine/xylazine, i.p., 100/10 mg/kg, JHP Pharmaceuticals LLC. MI; LLOYD Inc. IA), the mouse was mounted on a Koft stereotaxic apparatus. The left sciatic nerve was exposed in the mid-thigh region and animal rectal temperature was kept at 37 ± 1.0 °C during the measurement period using a feedback controlled water bath. Using a micromanipulator, an LDF probe was placed at the surface of the sciatic nerve and relative flow values expressed as perfusion units were recorded every 5 min for a total of 3 records. Regional sciatic nerve blood flow values from db/m mice were used as base line values and data are presented as a percentage of baseline values.

Neurophysiological measurements

Sciatic nerve conduction velocity was assessed with orthodromic recording techniques, as previously described (Ii et al., 2005; Schratzberger et al., 2000; Wang et al., 2011a). Briefly, mice were anesthetized with ketamine/xylazine (i.p., 100/10 mg/kg). The stimulating electrodes were plated at the knee and sciatic notch. Trigger single square wave current pulses were delivered using an isolated pulse stimulator (Model 2100, A-M Systems, Everett, WA). The simultaneous electromyographies were recorded by two sterilized electrodes placed in the dorsum of the foot with a Grass Amplifier (Model P5, Grass Instruments, Quincy, MA). During the measurements, animal rectal temperature was kept at 37 ± 1.0 °C using a feedback controlled water bath. Motor nerve conduction velocity (MCV) and sensory nerve conduction velocity (SCV) were calculated according to a published study (Ii et al., 2005).

Tail-flick and hot plate tests

To examine thermal hyperalgesia, tail-flick test (the water immersion method) and hot plate test (the IITC Hot Plate Analgesia test) were employed according to published methods (Janssen et al., 1963; South and Smith, 1998; Vanderah et al., 2001; Wang et al., 2011a). Briefly, for tail-flick test, a mouse was restrained in a conical polypropylene tube with an opening through which its tail was exposed. Approximately 2 cm of the mouse's tail was immersed into a 52 °C ± 0.2 water bath and the time until the rodent flicks or removes its tail was recorded (Vanderah et al., 2001). For hot plate test, a mouse was placed within a plexiglass chamber on a transparent glass surface and allowed to acclimate for at least 20 min. A thermal stimulation meter (IITC Model 39 Hot Plate Analgesia Meter, IITC Life Science, CA) was used with floor temperature at 55 °C. The latency of paw withdrawal in response to the radiant heat was recorded (South and Smith, 1998). Cut-off periods of 10 and 15 s were employed to avoid tissue damage for the tail-flick and hot plate tests, respectively. In both tests, at least three readings per animal were taken at 15 min intervals, and the average was calculated.

Immunohistochemistry

The left and right side sciatic nerves were isolated at the mid-thigh level, fixed in 4% paraformaldehyde, and embedded in paraffin according to published protocol (Wang et al., 2011a). Three cross sections (6 μm thick) or three longitudinal sections (6 μm thick) in the one in ten series (60 μm apart) for each animal were used for immunostaining according to our published protocols (Wang et al., 2011a). The following primary antibodies were used: polyclonal rabbit anti-Ang1 (1:2000; Abcam, Cambridge, MA), monoclonal mouse anti-CD31 antibody (1:500, BD Biosciences, San Jose, CA), polyclonal rabbit Anti-Von Willebrand Factor (vWF) (1:300, Dako, Carpenteria, CA), monoclonal mouse anti-occludin (1:200, Zymed, San Francisco, CA) and polyclonal rabbit anti-S100 (1:400, Abcam). Rabbit or goat IgG was used as a negative control. Sections were counterstained with 4′,6-Diamidino-2-phenylindole (DAPI) (1:5000, Thermo Scientific, Rockford, IL).

Image analysis and quantification

To examine microvascular perfusion in the sciatic nerve, fluorescein isothiocyanate (FITC)-dextran (2×10^6 molecular weight, Sigma; 0.2 mL of 50 mg/mL) was administered intravenously to the mice 10 min before sacrifice (Zhang et al., 1999). The sciatic nerves were rapidly removed and placed in 2% of paraformaldehyde for 2 h. The nerves were whole mounted and imaged under a 4× microscope objective (Zeiss Axiophot). Thereafter, the nerves were embedded in OCT compound and cross cryosections (20 μm thick) prepared. Three sections at 60 μm intervals from each mouse were used for further image analysis.

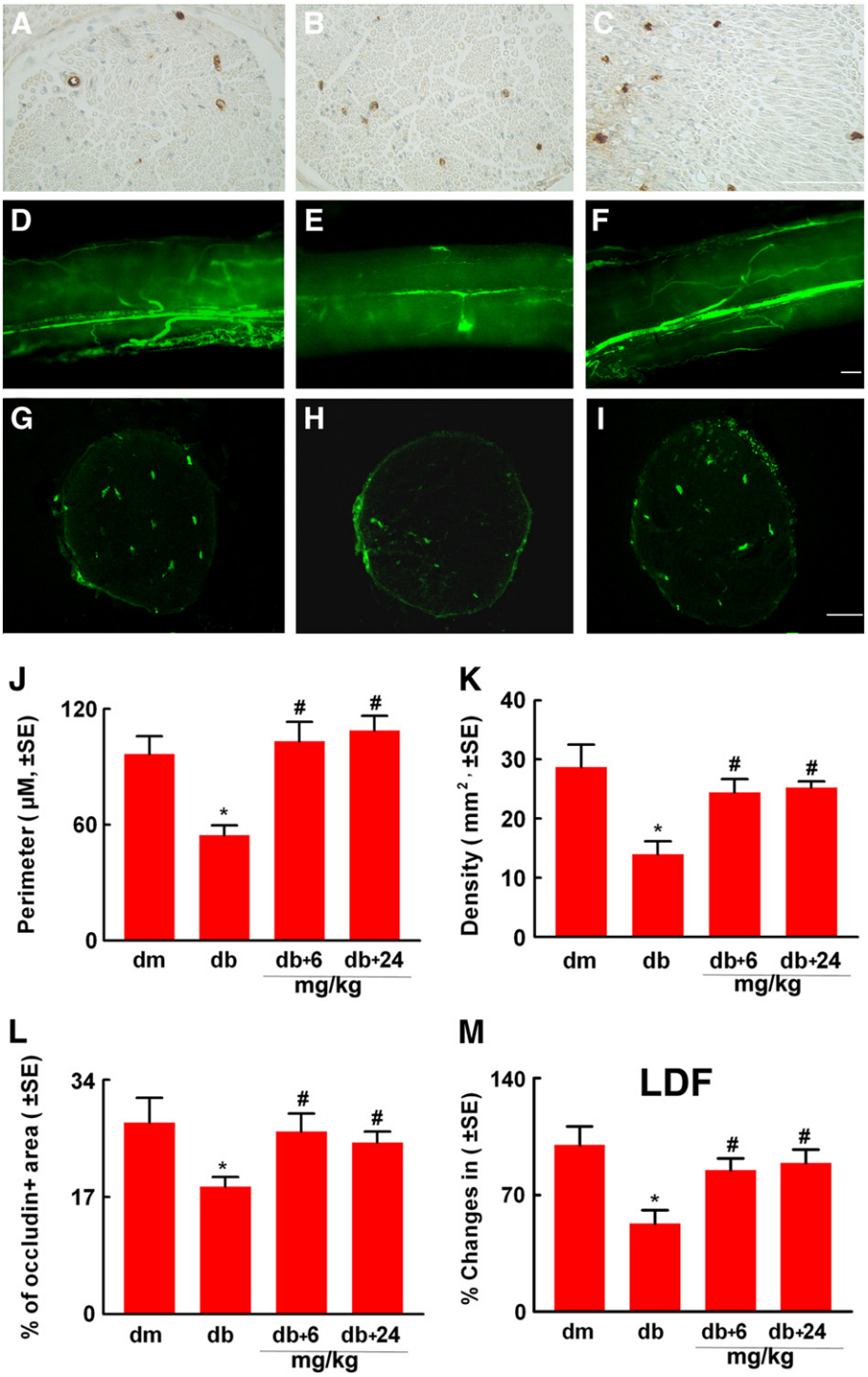

Fig. 1. Tβ4 improves vascular function in the sciatic nerve. Panels A to C show vWF immunoreactive blood vessels at the cross section of the sciatic nerve from a representative db/m mouse (A), db/db mouse treated with saline (B), and db/db mouse treated with Tβ4 (24 mg/kg, C). Whole mounted (D to F) and cross sections (G to I) of the sciatic nerve show FITC-dextran perfused vessels from a representative db/m mouse (D, G), db/db mouse treated with saline (E, H), and db/db mouse treated with Tβ4 (24 mg/kg, F, I). Panels J to M show quantitative data of vWF immunoreactive vascular perimeters (J, n = 6/group), and density of FITC-dextran perfused vessels in cross section (K, n = 4/group), percentage of occludin immunoreactive area (L, n = 6/group) and percentage changes of sciatic nerve blood flow with a reference of db/m mice at 100% (M, n = 4/group). *P<0.05 and #P<0.05 versus the db/m mouse and the saline treated db/db mouse, respectively. Bar = 100 μm. dm = db/m mouse; db = db/db mouse.

The cross sections were digitized under a 20× microscope objective (Zeiss Axiophot) via a Micro Computer Imaging Device (MCID) system (Imaging Research Inc, St. Catharines, ON, Canada) (Zhang et al., 1999). The total number of FITC-dextran perfused vessels was counted and divided by the total tissue-area to determine vascular density.

For analysis of vWF immunoreactive vascular morphology and density, three cross sections spaced at 60 μm intervals from each

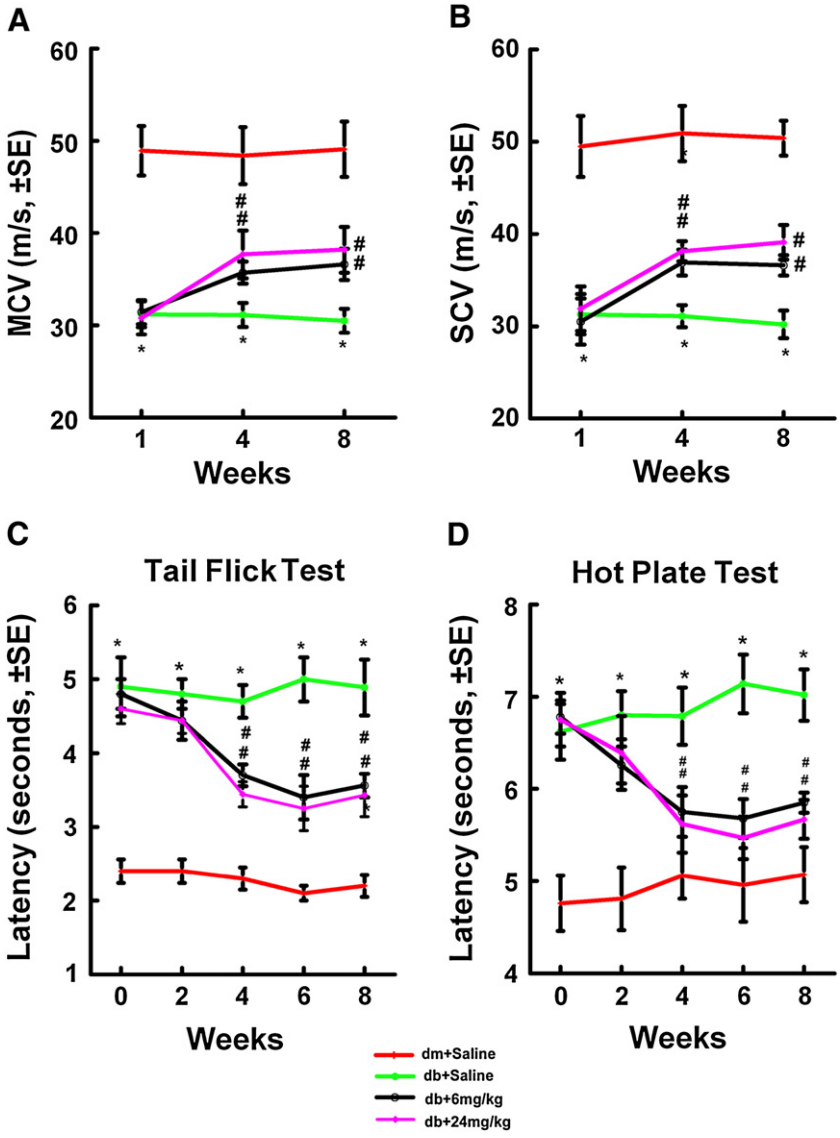

Fig. 2. The effect of Tβ4 on neurological function. Treatment of db/db mice with Tβ4 improves neurological function measured by MCV (A), SCV (B), Tail flick test (C) and Hot plate test (D). *P<0.05 and #P<0.05 versus the db/m mouse and the db/db mouse treated with saline, respectively. n = 10/group. dm = db/m mouse; db = db/db mouse.

mouse were used. Three fields of the view per section were randomly imaged under a 20× objective and vWF immunoreactive vascular perimeter and total number of vWF positive vessels were measured using MCID system.

All analysis was conducted with the examiner blinded to the identity of the samples being studied.

Cell culture

In the present study, we defined a normal glucose medium (NG) as a medium containing 5 mM glucose, while a high glucose medium (HG) is referred to a medium containing 30 mM glucose, which was chosen to match glucose levels prevalent in uncontrolled diabetic patients (Wu et al., 1999). These glucose concentrations for the in vitro hyperglycemia experiments have been used by others (Kim et al., 2008; Perrone et al., 2008).

Human Schwann Cells (HSCs, ScienCell Research Laboratories, Carlsbad, CA) derived from primary culture and Human Umbilical Vein Endothelial Cells (HUVEC, American Type Culture Collection, ATCC, Manassas, VA) were cultured according to the manufactures' instructions (ScienCell Research Laboratories and ATCC). To examine the effect of Tβ4 on HSCs, HSCs were cultured under the normal glucose or high glucose condition in the presence of different concentrations of Tβ4 (0, 25, 50 and 100 ng/ml) for 24 or 72 h. The cells were harvested for real-time RT-PCR and Western blot analysis. To collect conditioned medium from HSCs, 2.5×10^6 HSCs were plated onto a 35-mm-diameter dish in 1.2 ml of defined medium. The cells were cultured under the normal glucose or high glucose conditions in the presence or absence of Tβ4 (100 ng/ml) for 24 h. HSCs were then washed three times with PBS and a fresh medium was added to avoid excessive Tβ4 contamination. The cells were cultured for an additional 48 h, and the supernatant (conditioned medium) was collected, centrifuged for 10 min at 1000 rpm, and stored at −80 °C.

To assess the effect of Tβ4 on in vitro angiogenesis, a capillary-like tube assay was used (Lee et al., 1999; Zhang et al., 2003, 2004). Briefly, HUVECs (2×10^4 cells) were cultured on 96-well plate coated by Matrigel (BD Biosciences, Rockville, MD) in the conditioned medium

Table 1
Effect of Tβ4 on bodyweight.

Weight, g					
Groups	0 w	2 w	4 w	6 w	8 w
dm-Saline	30.3 ± 0.8*	30.8 ± 0.7*	30.4 ± 0.9*	30.1 ± 0.7*	30.9 ± 0.8*
db-Saline	61.0 ± 0.8	61.2 ± 0.7	59.3 ± 1.2	57.6 ± 2.2	57.1 ± 2.8
db-TB4(6 mg/kg)	59.5 ± 0.9	57.1 ± 1.1	56.2 ± 1.2	53.7 ± 1.5	53.0 ± 1.7
db-TB4 (24 mg/kg)	58.9 ± 1.1	58.1 ± 1.2	55.7 ± 1.5	56.0 ± 1.4	54.8 ± 1.6

Values are mean ± SE. *P<0.01 versus db + Saline group. n = 10/group. W = week, 0 w represents before the treatment, while other numbers indicate after the treatment. dm = db/m mouse; db = db/db mouse.

or Dulbecco's Modified Eagle Medium (DMEM) in the presence or absence of Tβ4 (0, 25, 50 and 100 ng/ml) for 5 h. Total length of tubes was measured in 3 random fields from each well using MCID (Wang et al., 2004).

Real-time RT-PCR

Total RNA samples from cells were isolated using the Stratagene Absolutely RNA MicroRNA isolation kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. The complementary DNA (cDNA) was reversely transcribed from the same concentrations of total RNA products using random hexamers and M-MLV reverse-transcriptase (Invitrogen, Carlsbad, CA). Using the SYBR Green real-time PCR method (Wang et al., 2005), quantitative PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA) by means of three-stage program parameters provided by the manufacturer, as follows; 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. Each sample was tested in triplicate, and samples obtained from three independent experiments were used for analysis of relative gene expression using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001). The following primers for real-time PCR were designed using Primer Express software (ABI): Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (FWD, AGA ACA TCA TCC CTG CAT CC; REV, CAC ATT GGG GGT AGG AAC AC) and Ang1 (FWD GAA GGG AAC CGA GCC TAT TC; REV, GCT GAA ATC AGC ACC GTG TA), Ang2 (FWD, CAG ATC CGG GCT CTA GAC AG; REV, TCC GGA AAT CGT TCT TCA TC).

Western blot analysis

Western blot was performed according to published methods (Wang et al., 2005). Briefly, equal amounts of proteins were loaded on 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes, and the blots were subsequently probed with the following antibodies: polyclonal rabbit anti-Ang1 (1:1000; Abcam, Cambridge, MA), polyclonal rabbit anti-Ang2 (1:1000; Abcam), Phospho-Akt (1:1000, Cell Signaling Technology, Inc. Danvers, MA) and Akt (1:1000, Cell Signaling Technology). For detection, horseradish peroxidase-conjugated secondary antibodies were used (1:2000) followed by enhanced chemiluminescence development (Pierce, Rockford, IL). Normalization of results was ensured by running parallel Western blot with β-actin antibody. The optical density was quantified using an image processing and analysis program (Scion Image, Ederick, MA).

Statistical analysis

For functional tests, data were evaluated for normality. Tail flick test data were not normal, and nonparametric Kruskal–Wallis Test was considered. The repeated measure analysis of variance (ANCOVA) was considered with dependent factor of time and independent factor of groups. The analysis started testing for group by time interaction, followed by testing the main effect of group and subgroup analyses. Two-sample *t*-test or analysis of variance (ANOVA) was used to study the group difference on LDF, immunostaining, biochemistry, Western blot, and tube formation analysis, respectively. Regression model was used to study the dose responses. The data are presented as mean ± SE. A value of $P<0.05$ was taken as significant.

Results

Tβ4 improves diabetes-induced vascular dysfunction in the sciatic nerve

To examine whether db/db mice develop impairment of neurovasculature, we examined microvessels of the vasa nervora in the sciatic nerve of the mice at the age of 28 weeks. Analysis of vWF immunoreactive vessels of the vasa nervora revealed that blood vessel perimeter in the db/db mice was significantly reduced compared to that in the age-matched db/m mice, although vascular density was not significantly different between these two groups (73.5 ± 10.5 vs.56.9 ± 6.4 in db/m mice, $P>0.05$) (Fig. 1). In addition, the db/db mice exhibited substantial reduction of occludin, a tight junction protein, immunoreactive vessels (Fig. 1). To examine whether the reduced perimeter in the db/db mice affects vascular function, we measured plasma-perfused microvessels, which represents functional vessels (Ii et al., 2005; Zhang et al., 1997) and regional blood flow. To measure plasma-perfused vessels, we intravenously injected FITC-dextran into the mice and then sacrificed them 10 min after injection, which provides sufficient time for the FITC-dextran to circulate throughout the entire vascular system under physiological conditions. Whole mount and cross sections showed that db/db mice exhibited overt reduction of FITC-dextran perfused vessels (Fig. 1). Quantitative analysis of FITC-dextran perfused vessels on cross sections of the sciatic nerves revealed that the db/db mice had a significant reduction in microvascular densities perfused by FITC-dextran compared to the db/m mice (Fig. 1). In parallel, sciatic nerve blood flow measured by LDF was significantly reduced in the db/db mice compared to that in the db/m mice (Fig. 1). Together, these data indicate that diabetes induces vascular dysfunction in the sciatic nerve, which is consistent with published studies (Ii et al., 2005; Jeong et al., 2009;

Table 2
Effect of Tβ4 on blood glucose.

Blood glucose(g/dl)					
Groups	0 w	2 w	4 w	6 w	8 w
dm-Saline	141.6 ± 14.5*	138.6 ± 6.4*	141.4 ± 7.7*	140.1 ± 8.0*	141.3 ± 4.6*
db-Saline	513.8 ± 14.0	516.6 ± 18.6	517.6 ± 10.7	516.4 ± 32.3	524.2 ± 23.3
db-TB4(6 mg/kg)	503.4 ± 24.0	513.4 ± 17.5	512.8 ± 24.8	517.6 ± 21.4	503.3 ± 18.0
db-TB4(24 mg/kg)	517.6 ± 15.0	515.9 ± 13.1	504.7 ± 16.5	521.6 ± 16.0	515.8 ± 19.3

Values are mean ± SE. *P<0.01 versus db + Saline group. n = 10/group. W = week, 0 w represents before the treatment, while other numbers indicate after the treatment. dm = db/m mouse; db = db/db mouse.

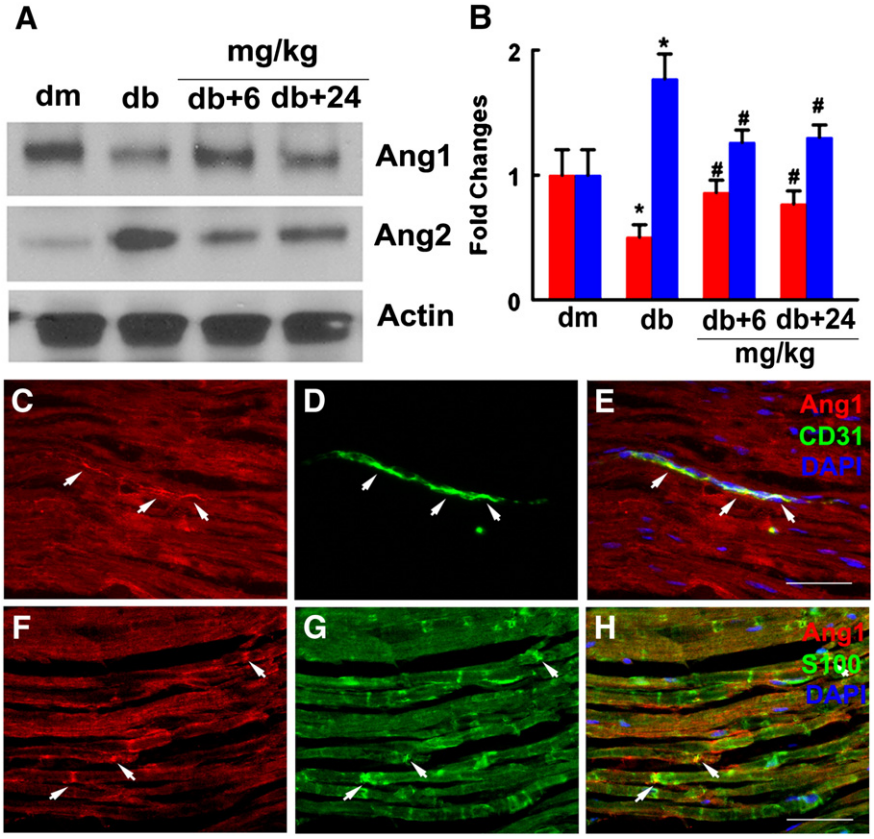

Fig. 3. The effect of Tβ4 on Ang1 and Ang2 expression in endothelial cells and Schwann cells of the diabetic mouse. Western blot analysis (A and B) of Ang1 and Ang2 levels in sciatic nerve tissue and β-actin was used as an internal control. Representative images of double immunofluorescent staining show that Ang1 immunoreactivity (C, F, E, H, red, arrows) was colocalized to CD31 positive vessels (D, E, green, arrows) and S100 positive Schwann cells (G, H, green, arrows). *P<0.05 and #P<0.05 versus the db/m mouse and the saline treated db/db mouse, respectively. n = 6/group. Bar = 100 μm. dm = db/m mouse; db = db/db mouse.

Kusano et al., 2004). However, the db/db mice treated with Tβ4 at doses of 6 and 24 mg/kg for 4 weeks starting at animal age of 20 weeks exhibited significant increases in vascular perimeter, occludin immunoreactive vessels, the density of FITC-dextran perfused vessels and sciatic nerve blood flow at age of 28 weeks compared to the db/db mice treated with saline, which were close to levels measured in the db/m mice (Fig. 1). These data indicate that Tβ4 ameliorates diabetes-induced vascular dysfunction in the sciatic nerve.

Tβ4 improves neurological function in the diabetic mouse

Impairment of peripheral nerve conduction is a key indicator for diabetic patients having peripheral neuropathy (Said, 2007; Tesfaye et al., 2010) and vascular dysfunction affects nerve conduction (Cameron and Cotter, 1999; Cameron et al., 2005). We therefore investigated whether augmentation of regional blood flow by Tβ4 affects motor and sensory conducting velocity (MCV and SCV) in the sciatic nerve. Electrophysiological recordings showed that MCV and SCV were significantly slowed in db/db mice compared to those of the age matched db/m mice (Fig. 2), which are comparable to values reported by others (Ii et al., 2005; Pande et al., 2011). Treatment of the db/db mice with Tβ4 at doses of 6 and 24 mg/kg for 4 weeks showed marked improvement in both MCV and SCV at the end of Tβ4 treatment and at 4 weeks after termination of the treatment compared with saline-treated db/db mice (Fig. 2). We then examined the effect of Tβ4 treatment on sensory function by measuring the thermal latency with tail flick and hot plate tests. Treatment of the db/db mice with Tβ4 markedly improved the thermal latency starting at the end of Tβ4 treatment, which persisted for at least 4 weeks after termination of the treatment (the end of experimental period) (Fig. 2). To examine the effect of Tβ4 on non-diabetic mice, we treated db/m mice with Tβ4 at a dose of 6 mg/kg and did not detect any functional changes as measured by the methods listed above (data not shown). These data suggest that Tβ4 improves peripheral nerve function in the diabetic mouse.

Treatment of the db/db mouse with Tβ4 did not significantly alter blood glucose levels and animal body weight (Tables 1 and 2).

Tβ4 regulates pro-angiogenic genes in the sciatic nerve

Alterations of Ang1 and Ang2 levels have been detected in diabetic patients and experimental diabetes (Rasul et al., 2011; Tuo et al., 2008). To examine the effect of Tβ4 on these angiogenic genes, we measured protein levels of Ang1 and Ang2. Western blot analysis of the sciatic nerve showed substantial reduction of Ang1 levels and an increase in Ang2 levels in the db/db mouse (Fig. 3), whereas treatment of the db/db mouse with Tβ4 significantly increased Ang1 expression, but decreased Ang2 expression (Fig. 3). Double immunostaining revealed that Ang1 immunoreactive cells were CD31 (a marker of endothelial cells) and S100 (a marker of Schwann cells) positive (Fig. 3). Collectively, these data suggest that Tβ4 upregulates Ang1 expression on endothelial cells and Schwann cells in the diabetic mouse.

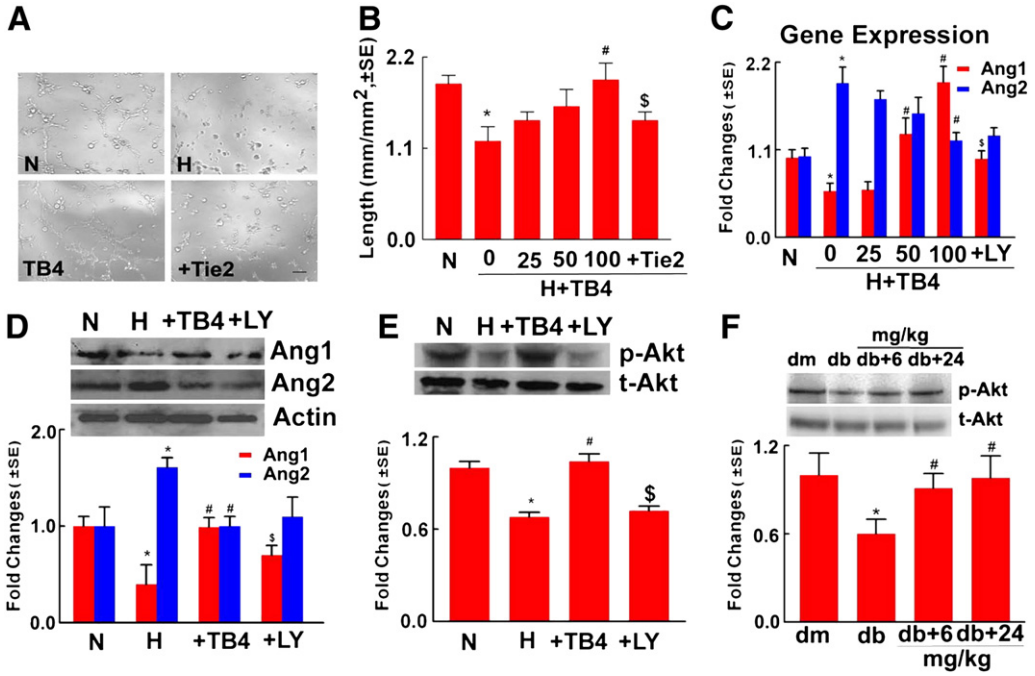

Fig. 4. The effect of Tβ4 on the Ang/Tie2 and PI3K/Akt signaling pathways. Representative microscopic images (A) and quantitative data (B) show capillary-like tube formation in HUVECs cultured in normal glucose (N), high glucose (H), high glucose with Tβ4 (H + TB4, 100 ng/ml), and high glucose with Tβ4 in the presence of a neutralizing antibody against Tie2 (+Tie2, 5 μg/ml). Real-time RT-PCR (C) and Western blot (D) data show mRNA and protein, respectively, levels of Ang1 and Ang2 in HUVECs cultured with normal glucose (N), high glucose (H), high glucose with Tβ4 (+TB4), and high glucose with Tβ4 in the presence of LY294003 (+LY, 10 μM). Panel E shows Western blot analysis of pAkt and total Akt in HUVECs cultured under different conditions listed above. GAPDH and β-actin were used as internal controls for mRNA and proteins, respectively. *P<0.05, #P<0.05 and $P<0.05 versus the normal glucose (N), high glucose (H) and high glucose with Tβ4 (100 ng/ml) groups, respectively. n = 6/group. Bar = 50 μm. Western blot analysis of sciatic nerve tissue (F) shows pAkt and total Akt levels in db/m (dm) and db/db (db) mice, db/db mice treated with Tβ4 at 6 mg/kg (db + 6) or 24 mg/kg (db + 24). *P<0.05 vs db/m mice and #P<0.05 vs db/db mice. n = 6/group.

The Ang/Tie2 signaling pathway mediates the effect of Tβ4 on endothelial function

The aforementioned in vivo data suggest that Ang1 and Ang2 genes mediate Tβ4-improved vascular function in the diabetic mouse. To examine a cause–effect of these genes on endothelial cells under hyperglycemia condition, we performed in vitro experiments using a capillary-like tube formation assay, which is widely used to examine in vitro endothelial function (Grant et al., 1995; Lee et al., 1999; Wang et al., 2004; Zhang et al., 2003). Incubation of HUVECs under high glucose conditions decreased capillary-like tube formation compared to HUVECs cultured under normal glucose conditions (Fig. 4). However, Tβ4 suppressed the effect of high glucose on reduction of capillary-like tube formation (Fig. 4). Quantitative RT-PCR and Western blot analysis showed that incubation of HUVECs with high glucose substantially decreased and increased Ang1 and Ang2 expression, respectively (Fig. 4), whereas Tβ4 abolished high glucose-induced expression of Ang1 and Ang2 (Fig. 4). Blockage of Tie2, a receptor of Ang1 and Ang2, with a neutralizing antibody against Tie2 inhibited Tβ4-increased capillary-like tube formation (Fig. 4). These data indicate that the Ang/Tie2 signaling pathway plays an important role in mediating Tβ4-imrpoved endothelial function under hyperglycemia.

Ang1 secreted by Tβ4-treated Schwann cells improves endothelial function

In addition to endothelial cells, our in vivo double immunostaining data showed that Schwann cells expressed Ang1. We therefore, examined the effect of Tβ4 on angiopoietin expression in Schwann cells. High glucose substantially downregulated Ang1 and upregulated Ang2, while Tβ4 at a dose of 100 ng/ml suppressed the high glucose effect on the angiopoietin expression in HSCs (Fig. 5), suggesting that Tβ4 also regulates angiopoietin expression in Schwann cells. We then investigated whether Schwann cells secrete Tβ4-upregulated Ang1 that consequently improves vascular function under high glucose condition. Using an ELISA specific to detect human Ang1, we measured Ang1 levels in supernatants harvested from HSCs cultured for 48 h. ELISA showed that supernatants from HSCs cultured with high glucose had a significant reduction of Ang1 levels compared to levels in supernatants collected from normal glucose condition, while Tβ4 reversed the effect of the high glucose on Ang1 levels (Fig. 5).

Next, we examined the effect of the supernatants on capillary-like tube formation by culturing HUVECs with conditioned medium harvested from HSCs. Compared to the conditioned medium collected from HSCs cultured with normal glucose, the conditioned medium from HSCs cultured under high glucose condition resulted in a significant decrease of capillary-like tube formation (Fig. 5). In contrast, the conditioned medium collected from HSCs treated with Tβ4 under high glucose condition significantly increased capillary-like tube formation. In the presence of the neutralizing antibody against Tie2, the effect of Tβ4 conditioned medium on capillary-like tube formation was inhibited (Fig. 5). Together, these data indicate that in addition to endothelial Ang1, soluble Ang1 secreted by Tβ4-treated Schwann cells improves endothelial cell function.

The PI3K/Akt signaling pathway mediates the effect of Tβ4 on Ang 1 expression

To further investigate whether intracellular signaling pathways are involved in Tβ4-upregulated Ang1/Ang2 on endothelial cells and Schwann cells, we examined the PI3K/Akt signaling pathway that may mediate the effect of Tβ4 on endothelial progenitor cell migration (Qiu et al., 2009). Western blot analysis of HUVECs and HSCs showed that the high glucose condition markedly decreased

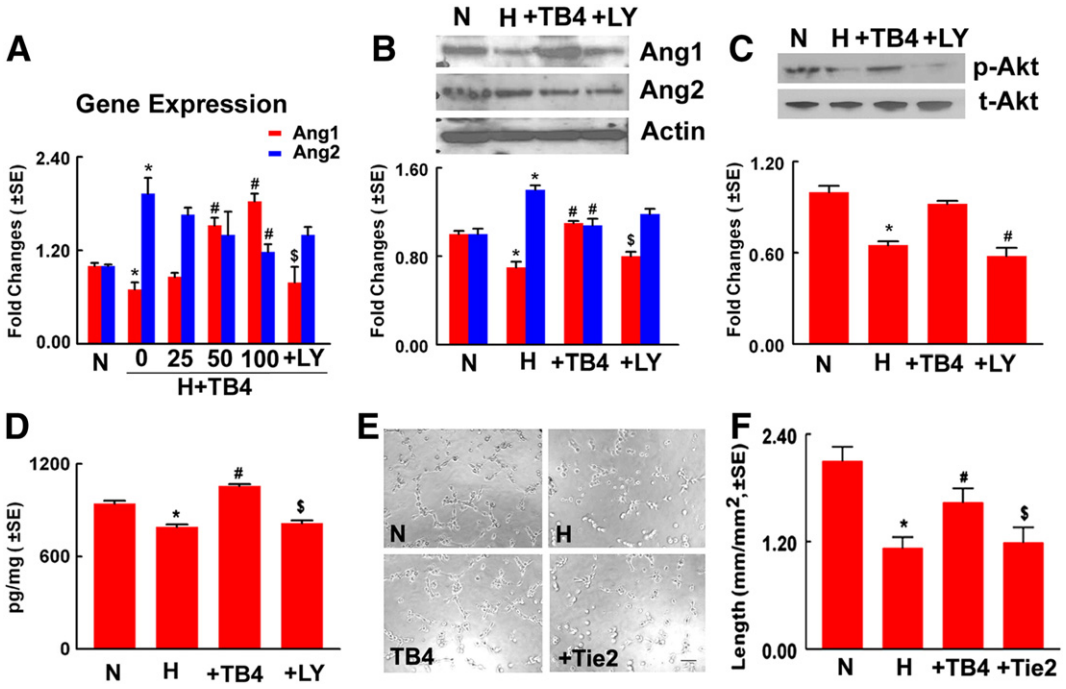

Fig. 5. Ang1 secreted by Tβ4-treated Schwann cells improves endothelial function. Real-time RT-PCR (A) and Western blot (B) data show mRNA and protein levels of Ang1 and Ang2 in HSCs cultured with normal glucose (N), high glucose (H), high glucose with Tβ4 (+TB4), and high glucose with Tβ4 in the presence of LY294003 (+LY). Panel C shows Western blot analysis of pAkt and total Akt in HSCs cultured under different conditions listed above. GAPDH and β-actin were used as internal controls for mRNA and proteins, respectively. Panel D shows ELISA data of Ang1 levels in supernatants harvested from HSCs cultured with normal glucose (N), high glucose (H), high glucose and Tβ4 (+TB4, 100 ng/ml), and high glucose and Tβ4 in the presence of LY294003 (+LY). Representative microscopic images (E) and quantitative data (F) show capillary-like tube formation in HUVECs cultured with the conditioned medium collected from HSCs in normal glucose (N), high glucose (H), high glucose and Tβ4 (+TB4, 100 ng/ml), and high glucose and Tβ4 in the presence of the antibody against Tie2 (+Tie2). *P<0.05, #P<0.05 and $P<0.05 versus the normal glucose (N), high glucose (H) and high glucose with Tβ4 (100 ng/ml) groups, respectively. n = 6/group. Bar = 50 μm.

pAkt levels compared to the normal glucose (Figs. 4 and 5). Treatment of HUVECs and HSCs with Tβ4 under the high glucose condition significantly increased pAkt levels, which was fully blocked by a PI3K inhibitor LY294002 (Figs. 4 and 5), indicating that Tβ4 activates the PI3K/Akt signaling pathway. Moreover, LY294002 completely suppressed the effect of Tβ4 on Ang1, but not Ang2 expression on HUVECs and HSCs under high glucose condition (Figs. 4 and 5). These data suggest that the PI3K/Akt signaling pathway is involved in Tβ4-regulated Ang1 expression on endothelial and Schwann cells. To verify these in vitro findings, we examined activation of Akt in the sciatic nerve of diabetic mice. Western blot analysis of the sciatic nerve tissue showed that diabetes substantially reduced pAkt compared to non-diabetic db/m mice, whereas treatment of db/db mice with Tβ4 robustly elevated pAkt levels compared to db/db control mice (Fig. 4), suggesting that Tβ4 also regulates the PI3K/Akt signaling pathway in vivo.

Discussion

The present study for the first time demonstrates that Tβ4 significantly improved sciatic nerve vascular function and peripheral nerve function in a mouse model of diabetic peripheral neuropathy. The Ang/Tie2 signaling pathway likely mediates the effect of Tβ4 on improved vascular function.

Tβ4, a naturally occurring peptide, enhances heart regeneration after myocardial infarction and promotes brain repair after stroke (Bock-Marquette et al., 2004; Morris et al., 2010). However, the effect of Tβ4 on diabetic peripheral neuropathy has not been investigated. Using a well established mouse model of type II diabetes, the present study indicates that Tβ4 ameliorates diabetic peripheral neuropathy, evidenced by reduction of sciatic nerve conduction velocity deficits, a key parameter for diabetic peripheral neuropathy, and improves responses to thermal stimuli. These data suggest that Tβ4 has potential for the treatment of diabetic peripheral neuropathy.

Studies of diabetic peripheral neuropathy from experimental animals and human indicate that the development of diabetic neuropathy is closely associated with marked neurovascular dysfunction (Cameron et al., 2001; Ebenezer et al., 2011; Tesfaye et al., 1993). Vascular dysfunction precedes appearance of nerve conduction velocity deficits, leading to nerve damage (Cameron and Cotter, 1999; Cameron et al., 2005; Ebenezer et al., 2011). Our data demonstrated that Tβ4 substantially increased plasma-perfused vessels and regional blood flow in the sciatic nerve, concomitantly with improvement of neurological function of diabetic neuropathy, suggesting that normalization of vascular function by Tβ4 may contribute to observed reduction of nerve conduction velocity deficits. Our findings are consistent with prior reports that restoration of vascular function by either pro-angiogenic factors or cell therapies enhances neurological function in diabetic peripheral neuropathy (Ii et al., 2005; Jeong et al., 2009; Kusano et al., 2004). However, further studies on the direct effects of Tβ4 on nerve fiber morphology and its relationship to improvement in neurological function are warranted. Our data show that Tβ4 at doses of 6 and 24 mg/kg significantly improved neurological outcome. Currently, we do not know why a dose of 24 mg/kg is not superior to a lower dose of 6 mg/kg to improve vascular function and neurological outcomes in diabetic mice with peripheral neuropathy. We previously demonstrated that Tβ4 at a dose of 30 mg/kg significantly reduces brain injury and improves neurological outcome compared to a dose of 6 mg/kg (Xiong et al., 2012). A clinical phase 1A and 1B study demonstrates that Tβ4 at a dose range of 42 to 1260 mg daily for 2 weeks did not have adverse effects, suggesting that Tβ4

has a large dose range in human (RegeneRx Biopharmaceuticals Inc). Further studies on the effects of doses at 30 mg/kg and higher on diabetic peripheral neuropathy are warranted.

The Ang/Tie2 signaling pathway regulates vascular homeostasis (Suri et al., 1996; Teichert-Kuliszewska et al., 2001). Ang1 promotes vascular maturation, while Ang2 acts as a competitive inhibitor of Ang1 for Tie2 binding and destabilizes blood vessels (Maisonpierre et al., 1997; Suri et al., 1996; Thebaud et al., 2005). Hyperglycemia downregulates Ang1 and upregulates Ang2 (Tuo et al., 2008). Increases in Ang1 levels normalize diabetes induced immature vasculature (Chen and Stinnett, 2008a). Ang1 by increasing angiogenesis reduces myocardial infarction, whereas an elevation of Ang2 levels exacerbates the infarction in diabetic rats (Tuo et al., 2008). Patients with diabetic peripheral neuropathy have significantly elevated levels of circulating Ang2 (Rasul et al., 2011). Our data show that hyperglycemia downregulated Ang1 and upregulated Ang2 on endothelial cells and Schwann cells, whereas Tβ4 reversed expression of Ang1 and Ang2. Tβ4 is a potent angiogenic factor and regulates angiogenesis and vasculogenesis during development by promoting progenitor cell differentiation and by directing endothelial cell migration (Bock-Marquette et al., 2004; Smart et al., 2007a, b). The effect of Tβ4 on the Ang/Tie2 pathway has not been investigated. Our data that blockage of Tie2 with a neutralizing antibody suppressed the effect of Tβ4 on in vitro angiogenesis implicate the Ang/Tie2 signaling pathway in mediating Tβ4-improved vascular function observed in vivo. Our data further suggest that Tβ4 regulates Ang1 through the activation of the PI3K/Akt pathway. A recent study shows that Tβ4 interacts with ATP-responsive P2X4 receptor to regulate endothelial cell migration (Freeman et al., 2011). Angiogenesis involves endothelial cell proliferation and migration (Goukassian et al., 2001; Wang et al., 2011b). Therefore, further studies are warranted for investigating whether purinergic signaling is involved in the beneficial effects of Tβ4 observed in the present study.

Schwann cells secrete numerous factors that regulate degeneration and regeneration peripheral nerves (Campana, 2007; Frostick et al., 1998; Sobue, 1990). The present study showed that Ang1 and Ang2 secreted by Schwann cells affected endothelial function under hyperglycemia condition, while Tβ4-elevated Ang1 levels on Schwann cells lead to enhancement of in vitro angiogenesis. We speculate that Tβ4 acts on endothelial cells and Schwann cells to preserve and/or restore vascular function in the sciatic nerve, which facilitates improvement of peripheral nerve function under diabetic neuropathy.

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