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Effect of thymosin beta 4 in the presence of up-regulation of the insulin-like growth factor-1 signaling pathway on high-glucose-exposed vascular endothelial cells

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ABSTRACT

Thymosin beta 4 ($T\beta_4$), which regulates vascular cell growth, can ameliorate some of the problems associated with diabetes. However, the precise signaling mechanisms by which $T\beta_4$ protects against hyperglycemia-induced damage to endothelial cells have not been investigated in detail. Thus, the aim of this study was to elucidate the role of $T\beta_4$ in diabetes and the possible involvement of insulin-like growth factor-1 (IGF-1), which affects cellular survival, metabolism, and glucose homeostasis in high-glucose (HG)-injured human umbilical vein endothelial cells (HUVECs). Immunoblotting assays revealed that under HG blockade conditions, $T\beta_4$ did not alter the insulin-signaling pathway, but induced overexpression of IGF-1 protein, leading to activation of factors in alternative signaling pathway. Small interfering RNA of $T\beta_4$ and IGF-1 were studied to clarify relationship between Tβ4 and IGF-1. These findings suggest that IGF-1 induction by $T\beta_4$ ameliorates the damage in HG-injured HUVECs which manifest as diabetic vascular disorder.

1. Introduction

The vascular disorder induced by hyperglycemia (high concentrations of glucose in the blood), which affects the systemic vasculature (both the micro- and macrovasculature), is the primary cause of morbidity and mortality in diabetic patients (Ruderman et al., 1992). The vascular endothelium is a major component of blood vessel walls, and is the site at which diabetes-induced vascular disorder is initiated; in the progression of diabetes, hyperglycemia affects the vascular endothelium, leading to apoptosis of the vascular cells. This hyperglycemia-induced endothelial apoptosis plays a major role in the pathogenesis of diabetes (Meng et al., 2008; Yoon et al., 2005). Various mechanisms for the cytotoxic effect of high glucose (HG) have been proposed in the literature, such as oxidative stress and cellular morphological abnormalities (Chen et al., 2007; Yu et al., 2008). Accordingly, recent studies have shown that the association between the morphological and functional abnormalities induced by hyperglycemia are reflected by increased apoptosis in those systemic organs that are affected by diabetes, including the eye (Kern et al., 2000; Li et al., 1997), heart (Fiordaliso et al., 2000; Frustaci et al., 2000), and vascular endothelium (Frustaci et al., 2000). Thus, hyperglycemia-induced vascular disorder is a major consideration when establishing therapeutic strategies to treat diabetic complications.

Insulin-like growth factor-1 (IGF-1) is one of the growth factors involved in the development of embryos and somatic cells (Chitnis et al., 2008). It promotes cell proliferation and differentiation, and suppresses apoptosis in various cell types, in collaboration with other growth factors (Jones and Kazlauskas, 2001). It has been suggested that there is a significant association between IGF-1 and diabetes, and a link has been found between low circulating levels of IGF-1 and adverse risk markers and increased incidence of cardiovascular disease in the late stages of diabetes (Ekman et al., 2000). IGF-1 exerts antiapoptosis effects on various cell types, and its function is triggered in response to IGF-1 receptor (IGF-1R). IGF-1R, when activated by IGF-1, mediates intracellular signaling pathways such as phosphotidyl inositol 3-kinase/protein kinase B (AKT) (Liu et al., 2001), leading to inactivation of the downstream targets of glycogen synthase kinase-3 beta (GSK3β) (Fukushima et al., 2012), ultimately modulating cell proliferation and differentiation (Delafontaine et al., 2004; Li et al., 2003). Moreover, a recent study demonstrated that IGF-1 prevents apoptosis in human umbilical vein endothelial cells (HUVECs) that have been exposed to HG (Li et al., 2009).

Abbreviations: $T\beta_4$, thymosin beta 4; IGF-1, insulin-like growth factor-1; IGF- 1R, IGF-1 receptor; IRS-1, insulin receptor substrate 1; AKT, phosphotidyl inositol 3-kinase/protein kinase B; GSK3β, glycogen synthase kinase-3 beta; HUVECs, human umbilical vein endothelial cells; HG, high-glucose.

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Thymosin β_4 ($T\beta_4$) is the major actin-sequestering molecule in eukaryotic cells; it binds to actin to prevent actin polymerization (Safer and Nachmias, 1994; Sanger et al., 1995). $T\beta_4$ plays a pivotal role in wound healing and tissue remodeling via its actin-sequestering function (Crockford et al., 2010; Hinkel et al., 2010; Shrivastava et al., 2010). Considerable evidence has recently been published on the beneficial effects of $T\beta_4$ in diabetic complications. Indeed, it has been demonstrated that $T\beta_4$ is involved in protection and recovery from diabetic injury, and that exerts its effects via antiapoptotic, anti-inflammatory, and angiogenic functions both *in vitro* and *in vivo* (Malinda et al., 1999). Moreover, $T\beta_4$ attenuates hyperglycemia and improves insulin resistance in diabetic mice (Zhu et al., 2012). A comparative analysis in a clinical study revealed that the level of $T\beta_4$ in the cornea was significantly lower in diabetic patients than in healthy controls (Saghizadeh et al., 2005). Accordingly, it is believed that exogenous $T\beta_4$ administration could promote wound healing and recovery from peripheral neuropathy in human patients, as it appears in diabetic mice (Philp et al., 2004; Wang et al., 2012). Our previous study which $T\beta_4$ improves dermal burn wound healing in diabetic mice also suggested a good therapeutic possibility in diabetic vascular disorder (Kim and Kwon, 2014). Although, these evidences demonstrated beneficial functions of $T\beta_4$ in diabetic complications, underlying mechanisms played by $T\beta_4$ in diabetes have not yet been investigated well.

In the present study, we investigated whether $T\beta_4$ protects vascular endothelial cells against hyperglycemia via the IGF-1-signaling pathway, and clarified the association between $T\beta_4$ and IGF-1 in HUVECs. Based on the findings of this study, a potential mechanism underlying the effects of $T\beta_4$ is presented, which may serve as a novel basis for a therapeutic strategy for the treatment of diabetic vascular disorder.

2. Materials and methods

2.1. Chemicals

$T\beta_4$ was purchased from Tocris Bioscience (Bristol, UK). D-glucose, saline, LY294002, sodium nitroprusside (SNP), and other standard reagents were purchased from Sigma (St. Louis, MO, USA). Primary antibodies raised against insulin, phosphorylated (p) insulin receptor (IR), IR, p-AKT, AKT, p-GSK3β, GSK3β, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies raised against vascular endothelial growth factor (VEGF), p-IGF-1R, IGF-1R, p-insulin receptor substrate 1 (IRS-1), and IRS-1 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). IGF-1 antibody was purchased from Abcam (Cambridge, UK), and $T\beta_4$ antibody was purchased from Millipore (Temecula, CA, USA). Secondary antibodies (i.e., antirabbit, antigoat, or antimouse IgG antibodies conjugated with horseradish peroxidase) were obtained from Millipore. All other chemicals and reagents were of analytic grade.

2.2. Cell culture and treatments

HUVECs as primary cells (Lifeline Cell Technology, Frederick, MD, USA) were cultured at 37 °C under a humidified, 5% CO_2 atmosphere in VascuLife complete medium (Lifeline Cell Technology). For this experiment, the HUVECs under 4 passages were incubated for 4 days until confluence, after which they were starved in serum-free basal medium for 24 h. Cells in the HG group were incubated in 30 mM glucose for different periods (24, 48, and 72 h), while cells in the normal glucose (NG) group were incubated in 5 mM glucose for the same periods. Cells in the experimental groups were first pretreated for 1 h with 10 μM LY294002 and 50 μM SNP, and then for 1 h with or without $T\beta_4$ (0.01, 0.1, 0.2, 0.3, and 0.4 μg/ml), after which the media were replaced by HG.

2.3. Small interfering RNA transfection

The DharmaFECT 1 small interfering RNA (siRNA) Transfection Reagent (Dharmacon, Denver, CO, USA) was used to transfect the cells with 50 nM $T\beta_4$ siRNA, IGF-1 siRNA, or scrambled siRNA oligonucleotides (Dharmacon) according to the manufacturer's instructions and as reported previously.

2.4. Cell viability assay and intracellular ROS assay

Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit from Sigma according to the manufacturer's instructions. The level of ROS was quantified by fluorescence using 2′,7′-dichlordihydrofluorescin diacetate (Invitrogen, CA, USA) according to manufacturer's instructions.

2.5. Immunoblotting analysis

Immunoblotting was performed by following our previous study (Kim and Kwon, 2013). The immunoreactions were visualized using the SuperSignal West Dura Extended Duration Substrate (Thermo, CA, USA) and analyzed using the ChemiImager system (Alpha Innotech, CA, USA).

2.6. Tube formation assay

The formation of vascular-like structures by HUVECs on growth factor reduced Matrigel (BD Biosciences, Bedford, MA, USA) was confirmed according to manufacturer's protocol. Briefly, Matrigel were thawed at 4 °C for overnight, and each well of pre-chilled 96 well culture plates was coated with 50 μl Matrigel and incubated at 37 °C for 1 h. HUVECs (5×10^3 cells) were added in 100 μl culture medium with various conditions. After 12 h of incubation at 37 °C and 5% CO_2 atmosphere, tube formation was observed and photographed using an inverted phase contrast microscope (Observer.A1, Carl Zeiss, Germany). The degree of tube formation was quantified by measuring the length of tubes in 3 randomly chosen low power fields (50×) from each well using the Image Pro analysis program.

2.7. Statistical analyses

The data were analyzed using Student's *t*-test (for two groups), one-way ANOVA, and Tukey's test (for more than two groups), and are presented as mean and SEM values. The cutoff for statistical significance was set at $p < 0.05$. All analyses were performed using the Statistical Package for Social Sciences (version 13.0 for Windows, SPSS, Chicago, IL, USA).

3. Results

3.1. $T\beta_4$ attenuates HG-induced vascular toxicity in HUVECs

The presence of HG-induced cellular toxicity in HUVECs was first confirmed by examining the cell viability following incubation in 30 mM HG for different incubation periods (24, 48, and 72 h; Fig. 1A). Previous studies have shown that 30 mM HG is adequate for inducing cellular toxicity (Chen et al., 2007; Ido et al., 2002; Yu et al., 2006). As shown in Fig. 1A, the cell viability was only reduced after 72 h of HG treatment, with a significant reduction of approximately 72%. Thus, an incubation period for HG-induced cellular toxicity of 72 h was considered adequate. The cell viability of $T\beta_4$-pretreated (0.2, 0.3, and 0.4 μg/ml) HUVECs after incubation with HG (30 mM for 72 h) increased in a dose-dependent manner, with significant increments of approximately 104%, 107%, and 113% when using 0.2, 0.3, and 0.4 μg/ml $T\beta_4$, respectively (Fig. 1A).