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TRAF6 mediates high glucose-induced endothelial dysfunction

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ABSTRACT

To investigate the role of tumor necrosis factor-associated factor 6 (TRAF6) in high glucose-induced endothelial cell dysfunction. Human aortic endothelial cells (HAECs) were cultured in high glucose medium, and TRAF6 expression was assayed by quantitative real-time Polymerase Chain Reaction (PCR) and western blotting. The effect of TRAF6 on in vitro endothelial cell viability, apoptosis, migration, and endothelial–monocyte adhesion was investigated by gene knockdown. The expression of TRAF6 and related adhesion molecules was assayed in a mouse streptozotocin-induced type I diabetes model. The signaling pathways associated with TRAF6 effects on endothelial cells were investigated in high glucose HAEC cultures. Culture of HAECs in high glucose medium significantly increased TRAF6 mRNA and protein expression in a time dependent manner. High glucose markedly reduced HAEC viability, apoptosis, and migration, and these effects was significantly reversed by TRAF6 knockdown. High glucose significantly increased intercellular adhesion of THP-1 monocytic cells and HAECs via upregulation of ICAM-1 and VCAM-1 expression, and TRAF6 knockdown attenuated the effect on THP-1 cell adhesion. TRAF6, ICAM-1, and VCAM-1 expression were increased in aorta tissue of mice with streptozotocin-induced diabetes. The free radical scavenger N-acetyl-L-cysteine attenuated TRAF6 expression in HAECs cultured in high glucose medium, and TRAF6 knockdown inhibited high glucose-induced IκB-α degradation and JNK phosphorylation. TRAF6 mediated high glucose-induced endothelial dysfunction via NF-κB- and AP-1-dependent signaling. Targeting TRAF6 may delay progression of vascular diseases during diabetes mellitus and atherosclerosis.

1. Introduction

Diabetes mellitus (DM) is characterized by chronic hyperglycemia, and impaired insulin secretion or action, or a combination of both, and is often associated with the development of vascular complications [1]. The prevalence of DM is about 2–5% in most western countries, and is rapidly increasing in Asian countries because of ongoing life style changes [2]. Effective strategies for DM treatment are urgently needed.

The simple endothelium that lines the internal surface of blood vessels is important for maintaining vascular tone and structure [3]. Substantial clinical and experimental evidence suggests that both DM and insulin resistance are responsible for endothelial changes that reduce the anti-atherogenic function of vascular endothelium [4–6], but the underlying mechanisms are not clear.

Previous studies have described TNF receptor associated factor (TRAF) mediated inflammatory responses [7–9]. TRAF6 acts downstream of cytokine and toll-like receptors (TLRs) to regulate the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [10], which plays a key role in the progression of inflammation-related diseases [11–13]. However, the activity of TRAF6 in DM at the molecular has not been determined.

We investigated the role of TRAF6 *in vitro* in a high glucose-induced model of endothelial cell dysfunction and in vivo in a type I diabetes mouse model. We found that TRAF6 mediated high glucose-induced endothelial dysfunction via NF-κB- and activator protein (AP)-1-dependent signaling. Targeting TRAF6 in patients with DM and atherosclerosis might delay progression of vascular diseases.

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Table 1
Primer sequences.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
TRAF6	AGGGTACAATACGCCTCACG	GCTACACGCCTGCATCAGTA
ICAM-1	GGCCGGCCAGCTTATACAC	TAGACACTTGAGCTCGGGCA
VCAM-1	TCAGATTGGAGACTCAGTCATGT	ACTCCTCACCTTCCCGCTC
GAPDH	CAAAGCCAGAGTCCTTCAGA	GATGGTCTTGGTCCTTAGCC

2. Materials and methods

2.1. Ethical approval of the study protocol

All research involving human participants was approved by the Institutional Review Board of Shanghai Jiaotong University School of Medicine, Shanghai, China. All participants gave written informed consent. The study protocol was approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine. The study was conducted following international guidelines for animal experimentation.

2.2. Cell culture

Human aortic endothelial cells (HAECs) were purchased from Lonza Co. Ltd. and cultured at 37 °C in endothelial basal medium-2 (EBM-2) supplemented with endothelial growth medium-2 (EGM-2 SingleQuots Kit, Lonza, Basel, Switzerland). THP-1 cells, a human acute monocytic leukemia cell line, was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium containing 10% fetal bovine serum and 0.05 mM 2-mercaptoethanol (Gibco, CA, USA). HAEC were used between passages four to eight. At 60–70% confluency, the medium was changed to EBM-2 containing 25 mM D-glucose and no supplements for the indicated times. Cells incubated with 5 mM D-glucose + 20 mM D-mannitol or 25 mM L-glucose served as controls.

2.3. Lentiviral short hairpin (sh)RNA vectors

Recombinant lentiviral particles expressing Nox4 or TRAF6 or control short interfering (si)RNA were obtained from GenePharm Co. Ltd (Shanghai, China). HAECs were grown to various degrees of confluency as indicated in figure legends and infected with lentiviral particles in complete medium for 48 h. To increase infection efficiency, cells were co-treated with the cationic polymer Polybrene (8 μg/ml in water). Neither shRNA nor Polybrene affected cell viability. The siRNA and shRNA had no off-target effects, and had no effect on HAEC adherence, shape, and viability at the indicated multiplicity of infection (MOI) and treatment duration.

2.4. Cell proliferation

HAECs (3×10^3 cells) were seeded in 96-well plates in EBM-2 complete medium and infected with TRAF6 or control siRNA lentivirus particles. Two days later, cell proliferation was evaluated by cell counting kit (CCK)-8 assay following the manufacturer's instructions and using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) to measure the absorbance.

2.5. TUNEL staining for cell apoptosis

HAECs were plated at 30,000 cells/well in 24-well plates, infected with lentiviral TRAF6 shRNA (MOI 0.5 for 48 h) and subjected to high glucose treatment for 24 h. The cells were then assayed by TUNEL staining following the manufacturer's instructions (Beyotime Biotechnology institute, Nantong, China) and observed by fluorescence microscopy.

2.6. Wound healing assay

HAECs were plated at 70,000 cells/well in 12-well plates. Cells were grown to 90% confluency, wounded once using a sterile 1 ml pipette tip, and washed twice with complete medium to remove floating cells and cell components. High glucose (25 mM D-glucose) was then added to the culture medium, and incubation continued at 37 °C. Photographs were taken at 0 and 24 h. To investigate the effect of TRAF6 on migration in high glucose medium, we transduced HAECs with 0.5 MOI TRAF6-specific lentiviral shRNA vectors. After 48 h incubation, cells monolayers were wounded and incubated in high glucose as described above. Cultures were photographed at 40× magnification using a Leica inverted phase contrast microscope. The area of the gap at 10 h was subtracted from that at 0 h to quantify cell migration. The assay was repeated at least three times.

2.7. Endothelial-monocyte adhesion assay

A Vybrant Cell Adhesion Assay Kit was used to measure endothelial-monocyte adhesion. Briefly, HAEC were plated in 24-well flat-bottomed plates. At 70–80% confluency, the complete medium was replaced with EBM-2 containing 25 mM D-glucose for 12 h. THP-1 cells were labelled with 5 μM calcein AM in serum-free RPMI 1640 for 30 min at 37 °C, washed twice with prewarmed RPMI 1640, and resuspended in the same medium. The labelled cells (5×10^4 cells) were then added to HAEC cultures and incubated for 1 h at 37 °C. Nonadherent cells were removed carefully and the cell layer was washed three times with ice cold PBS, and 200 μl of PBS was added to each well. Endothelial-monocyte adhesion was quantitated with a fluorimeter by at excitation and emission wavelengths of 485 and 535 nm, respectively. Wells

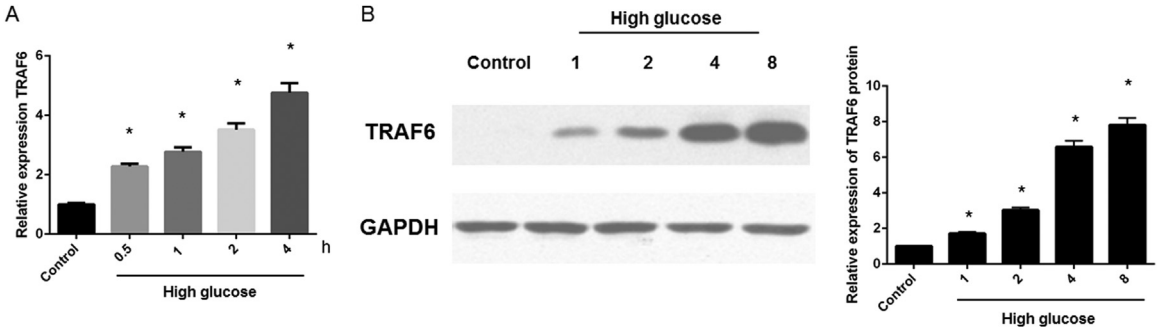

Fig. 1. High glucose enhances TRAF6 mRNA and protein expression. A, At 70% confluency of cultured HAEC, the complete medium was replaced with EBM-2 without supplements for 2 h, and the cultures were then incubated in the presence of 25 mM D-glucose. TRAF6 mRNA was assayed by qPCR, with 18S rRNA as an internal control. B, TRAF6 protein expression in HAEC treated as above was assayed by immunoblotting. Actin served as a loading control. *$P < 0.05$ *vs.* control (normal glucose).