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Up-regulation of 14-3-3β plays a role in intimal hyperplasia following carotid artery injury in diabetic Sprague Dawley rats by promoting endothelial cell migration and proliferation

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ABSTRACT

Purpose: The objective of this study was to determine whether diabetes mellitus (DM)-induced up-regulation of 14-3-3β (YWHAB) in endothelial cells enhances intimal hyperplasia in carotid artery-injured DM Sprague-Dawley (SD) rats.

Methods: YWHAB expression and rat aortic endothelial cell (RAOEC) vitality were examined using Cell Counting Kit-8 (CCK-8), quantitative reverse transcription PCR (qRT-PCR), and western blot analysis in cells treated with different glucose concentrations (5.6, 10, 15, 25, or 35 mM). For *in vivo* experiments, a YWHAB small interfering (si) RNA recombinant lentiviral vector (YWHAB-LV) or Mock siRNA recombinant lentiviral vector (Mock-LV) were injected into streptozotocin-induced DM SD rats via the tail vein. YWHAB expression and carotid artery morphology were assessed 7 days post injury using immunofluorescence (IF) and hematoxylin-eosin (HE) staining. The proliferation and migration of Mock-LV and YWHAB-LV-infected RAOECs treated with 25 mM glucose were examined using cell scratch tests and flow cytometry. BCL2-Associated X (BAX) distribution in RAOECs treated with 25 mM glucose was examined using IF staining and western blot analysis.

Results: Western blot, qRT-PCR, and CCK-8 analyses demonstrated that both YWHAB expression and cell vitality increased with increasing glucose concentration ($p < 0.05$). YWHAB IF staining was increased in DM rats compared with the normal group ($p < 0.05$). HE staining showed that intimal hyperplasia is alleviated in YWHAB-silenced DM rats ($p < 0.05$). YWHAB silencing suppressed the proliferation and migration of RAOECs treated with 25 mM glucose ($p < 0.05$). Moreover, western blot analyses and IF staining demonstrated that YWHAB silencing increased the translocation of BAX from the cytoplasm to mitochondria in RAOECs treated with 25 mM glucose ($p < 0.05$).

Conclusions: Our results indicate that hyperglycemia-induced up-regulation of YWHAB in endothelial cell plays a significant role in intimal hyperplasia following carotid artery injury by enhancing endothelial cell proliferation and migration. YWHAB inhibition in hyperglycemic patients may constitute a potential target for therapeutic interventions via restenosis prevention.

1. Introduction

Diabetes mellitus (DM) is a serious chronic metabolic disorder resulting from an absolute or relative insufficiency of insulin [1,2]. Approximately 60–80% of DM patients present with hypertension and the vascular complications of DM patients account for approximately 60% of all DM-related deaths [3,4]. Of the various vascular complications, DM-related excessive proliferation and migration of endothelial cells are thought to underlie the pathogenesis of artery intimal hyperplasia, which may result in pathologies such as atherosclerosis or arterial restenosis following interventional treatment [5,6].

Hyperglycemia in DM is thought to play important roles by increasing the expression of mitogenic growth factors and inflammatory mediators [7,8]; endothelial cells perceive these mechanical signals and then convert them into biological events affecting cell proliferation and apoptosis [9,10]. During this process, vascular complications occur because of excessive endothelial cell

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proliferation and migration. For example, vascular endothelial growth factor (VEGF) and its receptors activate pathways leading to endothelial cell proliferation and eventually capillary tube formation [11].

14-3-3 proteins are phospho-serine/phospho-threonine (pS/T) binding proteins that can be classified into seven isoforms, namely β, γ, ε, δ, θ, η, and ζ, all of which adopt a similar horseshoe-like structure capable of binding pS/T residues in a sequence specific context [12]. 14-3-3 β, encoded by the YWHAB gene [13], has been identified as a key regulatory component in many vital cellular processes such as signal transduction, protein synthesis, protein folding and degradation, cell cycle, cytoskeleton rearrangement, cellular trafficking, DNA replication, apoptosis, and survival [14]. A number of studies have suggested that expression of the 14-3-3 protein YWHAB might be up-regulated under high glucose concentrations [15,16] and that YWHAB is associated with growth disorders as well as several types of cancer including lung, breast, neck, and brain tumors [17]. Takihara et al. [18] found that overexpression of 14-3-3 β in NIH 3T3 cells could stimulate cell growth and promote tumor formation in nude mice; Clapp et al. [19] identified 14-3-3β/α as a specific inhibitor of apoptosis following the accumulation of reactive oxygen species in human cells; and Zheng et al. [20] confirmed the interaction between 14-3-3 β and Big Mitogen-activated Protein Kinase 1 (BMK1), an important factor that promotes cell proliferation and inhibits cell apoptosis, using yeast two-hybrid analysis. Moreover, Cavet et al. [21] identified 14-3-3β as a binding protein of p90 Ribosomal S6 Kinase (RSK), whose family members are involved in mitogen-activated cell growth and proliferation, differentiation, and cell survival. However, although an association between diabetes and cardiovascular complications has been identified, to date, no studies have examined the association between high glucose, 14-3-3β expression, and intimal hyperplasia in diabetics following injury.

Recently, Haque et al. [15] showed that down-regulation of microRNA (miR)-152 may induce the proliferation of human retinal endothelial cells (hRECs), while Zhao et al. [22] demonstrated that high glucose may suppress the expression of miR-152 in hepatocytes, which may induce the up-regulation of YWHAB [16]. Based these findings, we hypothesized that YWHAB might also be involved in the development of high glucose-induced vascular complications. Therefore, we investigated the role of YWHAB in the proliferation and migration of rat aortic endothelial cells (RAOECs). In addition, to further elucidate the possible mechanism underlying the development of DM-related intimal hyperplasia, we examined the effect of YWHAB on the translocation of BCL2-Associated X (BAX), a proapoptotic member of the Bcl-2 family, which localizes mainly in the cytoplasm but redistributes to the mitochondria in response to apoptotic stimuli by inducing cytochrome C release [23].

2. Materials and methods

2.1. Rat aortic endothelial cell culturing and infection

RAOECs were cultured in 5.6, 10, 15, 25, or 35 mM glucose Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). Once cells reached at least 70% confluence, they were harvested and subjected to western blot and quantitative real-time polymerase chain reaction (qRT-PCR) analysis or the CCK-8 test. RAOECs treated with 25 mM glucose were infected with either a YWHAB small interfering (si) RNA recombinant lentiviral vector (YWHAB-LV) or corresponding control expression vector (Mock-LV) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The YWHAB-LV and Mock-LV were provided by the laboratory of Prof. Wheeler (Toronto, Canada).

2.2. Cell vitality

Once cells reached at least 70% confluence they were incubated with 10 μl Cell Counting Kit-8 (CCK8; Yeasen, Shanghai, China) working solution for 1 h prior to measuring cell optical density (OD) at 450 nm. The OD value represented cell vitality.

2.3. Animal protocols

Twenty-four SD male rats (200 ± 20 g), were randomly separated into four groups: uninjured (n = 6), normal (n = 6), diabetes mellitus (DM; n = 6), and DM + YWHAB silencing group (n = 6). Three rats from each group were used for Hematoxylin-eosin (HE) staining and the other three rats were used for Immunofluorescence (IF) staining. All rats were fed a standard diet ad libitum. Room temperature was maintained at 23–25 °C, with 50–60% humidity and an 8 h light period daily. Animals and forage were purchased from the Model Animal Research Centre of Nanjing University (Jiangsu, China). This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.4. Establishment of the carotid artery injury diabetes mellitus rat model and injection of the lentivirus in vivo

Following an intraperitoneal injection of 65 mg/kg streptozotocin to establish the DM rat model, 100 μl of either Mock-LV or (108 TU/ml titer) or YWHAB-LV (108 TU/ml titer) were injected into the tail vein of rats in the DM and DM + YWHAB silencing groups, respectively. Rats in the normal group served as the negative control. Two weeks post tail lentivirus injection, rats were anesthetized with isofluorane and a 2-French balloon catheter (Edwards Lifesciences, Irvine, CA, USA) was inserted through the left external carotid artery into the common carotid artery and insufflated three times with 2 atm of pressure. Following injury, the external carotid artery was quickly ligated and blood flow was resumed. All DM rats were sacrificed at 7 days post injury.

2.5. Hematoxylin-eosin and immunofluorescence staining

Specimens were incubated in 4% paraformaldehyde for 24–48 h and then embedded in paraffin. Paraffin sections (3–5 mm thick) were dewaxed, stained, examined microscopically, and photographed. Images were captured using a fluorescence microscope (Jenoptik, Jena, Germany). YWHAB expression levels were determined using an immunofluorescence assay; 3–5 mm frozen carotid artery sections were cultured for 24 h in complete medium, fixed with 4% paraformaldehyde for 10 min, and permeabilized in 0.01 M phosphate buffered saline (PBS)/0.5% Triton X-100 for 5 min. Intima were then incubated with an anti-YWHAB primary antibody (1:200 dilution; Abcam, Cambridge, UK) for 1 h at room temperature and then with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA). Following washes in PBS, the intima were incubated for 3 min with 0.25 mg/ml 4′,6-diamidino-2-phenylindole (DAPI). A similar protocol was used for the *in vivo* experiments; briefly, the RAOECs were washed with PBS prior to lysis in RIPA buffer supplemented with protease inhibitors (Roche Applied Sciences, Laval, QC, Canada) and then incubated with a primary antibody against YWHAB or BAX (anti-BAX, 1:200 dilution; Sigma-Aldrich, St. Louis, MO, USA). Mitochondria were visualized with mitochondria-targeted dsRed (Yeasen). Images were captured using a fluorescence microscope (Jenoptik).

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