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CIKS (Act1 or TRAF3IP2) mediates high glucose-induced endothelial dysfunction

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ABSTRACT

Hyperglycemia-induced endothelial dysfunction is characterized by enhanced inflammatory cytokine and adhesion molecule expression, and endothelial–monocyte adhesion. The adapter molecule CIKS (connection to IKK and SAPK/JNK; also known as Act1 or TRAF3IP2) is an upstream regulator of NF-κB and AP-1, and plays a role in inflammation and injury. Here we show that high glucose (HG; 25 mM *vs.* 5 mM D-glucose)-induced endothelial–monocyte adhesion and inhibition of endothelial cell (EC) migration were both reversed by CIKS knockdown. In EC, HG induced CIKS mRNA and protein expression *via* DPI-inhibitable Nox4-dependent ROS generation. Further, HG induced *CIKS* transcription and enhanced *CIKS* promoter-dependent reporter gene activation *via* Nox4, ROS, AP-1 and C/EBP. Coimmunoprecipitation and immunoblotting revealed CIKS/IKKβ/JNK physical association under basal conditions that was enhanced by HG treatment. Importantly, CIKS knockdown inhibited HG-induced (i) IKKβ and JNK phosphorylation, (ii) p65 and c-Jun nuclear translocation, and (iii) NF-κB- and AP-1-dependent proinflammatory cytokine, chemokine, and adhesion molecule expression. Similar to HG, the deleterious metabolic products of chronic hyperglycemia, AGE-HSA, AOPPs-HSA and oxLDL, also induced CIKS-dependent endothelial dysfunction. Notably, aortas from streptozotocin-induced and the autoimmune type 1 diabetic NOD and Akita mice showed enhanced DPI-inhibitable ROS generation and CIKS expression. Since CIKS mediates high glucose-induced NF-κB and AP-1-dependent inflammatory signaling and endothelial dysfunction, targeting CIKS may delay progression of vascular diseases during diabetes mellitus and atherosclerosis.

1. Introduction

Diabetes mellitus (DM) is characterized by chronic hyperglycemia with impaired secretion of insulin, insulin action or a combination of both, and is often associated with the development of vascular failure [1]. Chronic hyperglycemia activates the polyol pathway, increases the formation of advanced glycation end products (AGEs), advanced oxidation protein products (AOPPs), and oxidatively-modified LDL (oxLDL), and stimulates anti-oxLDL antibody generation [2–5]. High glucose (HG), AGEs, AOPPs, and oxLDL all contribute to robust activation of oxidative stress, and increase the expression and secretion of various adhesion molecules including intercellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1. Both ICAM-1 and VCAM-1 serve as ligands for integrins on activated monocytes, promoting firm adhesion of monocytes to endothelial cells, monocyte rolling and migration into sub-endothelial matrix, and the development and progression of vascular diseases, including atherosclerosis [3,6].

Both *ICAM1* and *VCAM1* are transcriptionally regulated by the ubiquitously expressed oxidative stress-responsive transcription factors nuclear factor (NF)-κB and activator protein (AP)-1 [7]. Addition

Abbreviations: Act1, activator of NF-κB; AOPPs, advanced oxidation protein products; AGE, advanced glycation end product; AP-1, activator protein-1; C/EBP, CCAAT/enhancer-binding protein; CIKS, connection to IKK and SAPK/JNK; DCFH-DA, 2′,7′-dichlorofluorescin-diacetate; DCF, dichlorofluorescein; dn, dominant negative; DPI, diphenylene iodonium; copGFP, copepod green fluorescent protein; GST, glutathione-S-transferase; HSA, human serum albumin; IκB, inhibitory κB; IKK, IκB kinase; IL, interleukin; IP/IB, co-immunoprecipitation/immunoblotting; IRF, IFN regulatory factor; JNK, c-Jun amino-terminal kinase; kd, kinase deficient; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MOI, multiplicity of infection; MMP, matrix metalloproteinase; NEMO, NF-κB essential modulator; NF-κB, nuclear factor kappa B; Nox, NADPH oxidase; NADPH, nicotinamide adenine dinucleotide phosphate; oxLDL, oxidized low density lipoprotein; RAGE, receptor for AGE; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; siRNA, small interfering RNA; shRNA, small hairpin RNA; TRAF, TNF Receptor Associated Factor; TRAF3IP2, TRAF3 interacting protein 2; TNF, tumor necrosis factor; UTR, untranslated region; WT, wild-type.

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of an SOD mimetic inhibited TNF-α-induced NF-κB and AP-1 activation, and ICAM-1 and VCAM-1 expression in human aortic endothelial cells [8]. Further, inhibition of NF-κB attenuated IL-18-induced ICAM-1 and VCAM-1 expression on human dermal microvascular endothelial cells [9]. Conversely, forced expression of constitutively active IKKβ in the absence of other proinflammatory stimuli has been shown to induce ICAM-1 and VCAM-1 expression in endothelial cells [10]. Similarly, AP-1 has been shown to play an equally critical role in adhesion molecule expression. For example, IL-1β has been shown to induce ICAM-1 and VCAM-1 in mouse Sertoli cells *via* c-Jun amino terminal kinase (JNK) activation [11]. Moreover, IL-1β induces ICAM-1 expression in 549 cells *via* JNK and NF-κB [12] and AP-1 in endothelial cells [13], emphasizing the critical roles of NF-κB and AP-1 in adhesion molecule expression.

In the resting cell, NF-κB is localized to the cytoplasm complexed with inhibitory κB (IκB) proteins, thus remaining inactive [14]. Activation of NF-κB requires phosphorylation of IκB on specific serine residues by the IκB kinase (IKK) complex and subsequent proteasome-dependent degradation. The multi-component IKK complex is composed of two kinases IKKα and IKKβ, and a regulatory subunit IKKγ [14]. The transcriptional activity of AP-1 is regulated by JNK, a member of the mitogen activated protein kinase family. The AP-1 family composes of Jun, Fos, and activating transcription factor (ATF) [15]. By homo or heterodimerizing with other members of their family, NF-κB and AP-1 induce ICAM-1 and VCAM-1 expression. Of note, a crosstalk between AP-1 and NF-κB subunits has been reported in *ICAM1* transcription [16].

Recently, the novel adaptor protein CIKS (connection to IκB kinase and stress-activated protein kinase/c-Jun N-terminal kinase) was identified, and shown to play an important role in the activation of NF-κB and JNK signaling [17]. CIKS is also known as NF-κB activator 1 (Act1) and TRAF3-interacting protein 2 (TRAF3IP2) [18]. As its name implies, CIKS lies upstream of IKK and JNK, and activates IKK/NF-κB and JNK/AP-1-dependent signaling [17]. Its critical role in interleukin (IL)-17 mediated autoimmune and inflammatory signaling has been extensively described. In autoimmune encephalomyelitis, astrocyte specific deletion of CIKS inhibited proinflammatory cytokine and adhesion molecule expression, and attenuated disease progression [19]. CIKS deficient mice exhibited less severe allergic airway inflammation, pulmonary inflammation, and dextran sodium sulfate-induced colitis, suggesting a causal role for CIKS in autoimmune and inflammatory disorders [20–22]. Furthermore, the recent demonstration that human pancreatic islet cells express TRAF3IP2 (CIKS), and this expression is enhanced by inflammatory cytokines [23], raises the intriguing possibility that CIKS may be involved in the pathogenesis of type 1 diabetes. However, the role of CIKS in endothelial dysfunction, a hallmark of DM and atherosclerosis, is not known.

In the current study, we investigated the effects of HG on CIKS expression and determined its role in NF-κB and AP-1 activation, ICAM-1 and VCAM-1 expression, and endothelial–monocyte adhesion and endothelial migration *in vitro*. Further, we also investigated the effects of AGE-HSA, oxLDL and AOPPs-HSA on CIKS expression *in vitro*. Finally, CIKS levels were analyzed *in vivo* in the aortas of three different type 1 diabetic animal models. Our results show for the first time that CIKS is a critical mediator of HG-induced endothelial dysfunction. HG-induced IKK and JNK phosphorylation, NF-κB and AP-1 activation, and cytokine and adhesion molecule expressions were markedly attenuated by CIKS knockdown. Interestingly, HG also enhanced CIKS nuclear translocation. Further, CIKS knockdown reversed HG-inhibited endothelial cell migration. Notably, CIKS expression was markedly increased in the aortas of NOD, Akita and streptozotocin-induced type 1 diabetic mice. Thus targeting CIKS may have a protective effect in the pathogenesis of vascular diseases by ameliorating the endothelial cell dysfunction resulting from diabetes mellitus and excessive oxidative stress.

2. Materials and methods

2.1. Materials

The materials used are detailed in the Supplementary data section.

2.2. Animals

The investigations conform to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, and all protocols were approved by the Institutional Animal Care and Use Committees at Tulane University in New Orleans, LA and the University of Texas Health Sciences Center in San Antonio, TX. All animals were housed in temperature (22 °C) and light-controlled (12 h light and 12 h dark) atmosphere with *ad libitum* access to water and food. To induce type 1 diabetes, 10 week-old male C57Bl/6 mice (Charles River Laboratories International, Wilmington, MA; n = 4/group) were administered once daily for 4 days with streptozotocin in sodium citrate buffer (pH 4.5; IP, 60 mg/kg bodyweight). The control group received sodium citrate buffer alone. Blood glucose levels were quantified at 3, 5, 10, and 17-day post-STZ and animals euthanized. Type 1 diabetes prone male Akita mice on C57Bl/6 background (Stock# 003548) and control C57Bl/6 mice (Stock #000664) were purchased from the Jackson Laboratories. Akita mice develop hyperglycemia as a consequence of a single base pair substitution in the *Ins2* gene, resulting in improper folding of proinsulin, aggregation in endoplasmic reticulum (ER), ER stress, and loss of β-cells of islets of Langerhans. Akita mice develop severe hyperglycemia as early as 5–6 weeks of age. Akita mice were sacrificed at 10 weeks of age. Type 1 diabetes-prone female Non-Obese Diabetic (NOD) mice (NOD/ShiLtJ, Stock# 001976) and insulitis-resistant diabetes-free NOR/LtJ control mice (Stock# 002050) were purchased from the Jackson Laboratories (Bar Harbor, ME). At 18 weeks of age, animals were euthanized. Blood glucose levels were monitored using Contour glucometer (Bayer Healthcare, Mishawaka, IN). Aortas were collected, snap frozen, and stored at −80 °C for not more than 3 days prior to protein and mRNA extraction.

2.3. Cell culture

Clonetics® human aortic endothelial cells (HAEC, #CC-2535; Lonza) were cultured at 37 °C in endothelial basal medium-2 (EBM-2, #CC-3156) supplemented with EGM-2 SingleQuots (Lonza, #CC-4176). THP-1 cells (human acute monocytic leukemia cell line) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum and 0.05 mM 2-mercaptoethanol. HAEC were used between passages 4 to 8. At 60%–70% confluency the medium was changed to EBM-2 (without supplements) containing 25 mM D-glucose for the indicated time periods. Cells incubated with 5 mM D-glucose + 20 mM D-mannitol or 25 mM L-glucose served as controls.

2.4. Preparation of AGE-HSA and AOPPs-HSA

AGE-HSA was prepared as previously described [24] by exposing fatty acid and globulin-free human serum albumin (HSA) to 1 M D-glucose in 100 mM sodium phosphate buffer (pH 7.4) containing, 200 U/ml of penicillin, 200 μg/ml streptomycin, 80 μg/ml of gentamicin, and 1.5 mM of PMSF at 37 °C in the dark for 60 days, and dialyzed for 16 h against PBS. As a control, HSA was subjected to the same procedure, but without exposure to D-glucose. Fluorescence was measured at excitation/emission wavelengths of 370/440 nm in a spectrofluorophotometer, and the concentrations of AGE-HSA and HSA were determined by the method of Bradford. AOPPs-HSA was prepared as previously described [25] by exposing fatty acid and globulin-free HSA to HOCl/PBS for 30 min at room temperature and dialyzed for 16 h against PBS. AOPP concentration was