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Humanin prevents high glucose-induced monocyte adhesion to endothelial cells by targeting KLF2

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ABSTRACT

Endothelial dysfunction and vascular complications induced by hyperglycemia play an important role in the pathological development of atherosclerosis in diabetes. Humanin, a 24-amino acid mitochondria-derived polypeptide, has displayed its cytoprotective effects in diverse cell types and tissues. In the current study, we aimed to characterize the effects of humanin on high glucose-induced endothelial dysfunction. Firstly, we found that humanin treatment induced the expression of Krüppel-like factor 2 (KLF2), an essential transcriptional regulator of endothelial function, at the transcriptional level in human umbilical vein endothelial cells (HUVECs). Additionally, our results indicate that humanin treatment regulated the expression of KLF2 target genes such as endothelial nitric oxide synthase (eNOS) and endothelin-1 (ET-1). Evidence demonstrated that the effects of humanin on KLF2 expression was mediated by the phosphorylation of extracellular signal regulated kinase 5 (ERK5). Furthermore, humanin restored high glucose-induced reduction of KLF2 expression. We also showed that humanin significantly reduced the expression of vascular cell adhesion molecule 1 (VCAM-1) and E-selectin. Notably, humanin treatment markedly prevented high glucose-induced attachment of the monocyte THP-1 cells to HUVECs. However, knockdown of KLF2 abolished these effects. Lastly, we report that humanin treatment inhibited high glucose-induced secretion of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). These findings suggest that humanin may have therapeutic potential for the treatment of hyperglycemia-associated endothelial dysfunction.

1. Introduction

Hyperglycemia, one of the main pathological symbols in diabetes, is defined as high levels of blood glucose and associated with macrovascular complications (Tabit et al., 2010). Diabetes-related macrovascular complications are one of the major causes of morbidity and mortality in patients with type 2 diabetes mellitus (Marín-Peñalver et al., 2016). Hyperglycemia is linked to endothelial cell dysfunction in diabetes and is an important cause of premature atherosclerosis (Shah and Brownlee, 2016). High glucose-induced endothelial dysfunction plays a key role in the development of diabetes-associated cardiovascular complications. Increasing evidence has shown that high glucose (HG) directly results in vascular dysfunction. Upon exposure to high glucose, vascular endothelial cells (ECs) produce high levels of reactive oxygen species (ROS) and proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Lenna et al., 2014). Importantly, under high glucose conditions, endothelial cells generate high levels of vascular cell adhesion molecule 1 (VCAM-1) and E-selectin, which mediate the adhesion of circulating leucocytes to the endothelium, a key step in the initiation of atherosclerosis. Abnormal expression of endothelial pro-inflammatory pathways and molecules have been found in hyperglycemia. Interestingly, reduced expression of Krüppel-like factor 2 (KLF2) has been associated with high glucose-induced endothelial dysfunction in recent studies (Zhong et al., 2015). KLF2 is an essential regulator of endothelial functions (Feinberg et al., 2004). KLF2 exerts a variety of protective actions in endothelial cells by regulating the endothelial pro-inflammatory pathway, thrombotic activation, and angiogenesis (Novodvorsky and Chico, 2014a). KLF2 has been considered as an important therapeutic target for various diseases, including macrovascular complications induced by hyperglycemia.

Humanin (HN) was originally identified as a 24-amino acid mitochondrial derived peptide in 2001 (Hashimoto et al., 2001). Previous

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studies have demonstrated that humanin possesses various protective capacities in diverse cell types and tissues (Sreekumar et al., 2017). For example, humanin can suppress apoptosis by binding to BAX, tBID, and BimEL (Yen et al., 2013). Humanin also exhibits a protective function against oxidative stress, senescence, and mitochondrial dysfunction in human retinal pigment epithelial cells by regulating the Janus kinase 2 (Jak2)/signal transducer and activator of transcription 3 (Stat3) signaling pathway (Sreekumar et al., 2016). Interestingly, humanin has been shown to be expressed in the endothelial cell layer of the vascular wall in internal mammary arteries, atherosclerotic coronary arteries, and sections of the greater saphenous vein (Bachar et al., 2010). Additionally, humanin can attenuate renal microvascular remodeling, endothelial inflammation and cell death in hypercholesterolemic ApoE−/− mice by reducing the expression of MCP-1 and TNF-α (Zhang et al., 2012). The physiological function of humanin as a survival factor suggests that humanin may have the potential to protect endothelial cells under conditions of high glucose levels.

2. Materials and methods

2.1. Cell culture, treatment, and transfection

Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC, USA. Cells were cultured in EGM-2 endothelial cell growth medium (Lonza, USA) supplemented with 10% fetal bovine serum (FBS) and gentamycin/amphotericin. Cells were treated with 50, 100, and 200 μM humanin (#89143-468, Enzo Life Sciences, USA) for 24 h followed by high glucose (HG, 25 mM) or normal glucose (7 mM) exposure for another 24 h. Simvastatin (2 μM) (#S6196, Sigma-Aldrich, USA) was used as a positive control. HUVECs were cultured with humanin (100 μM) for 24 h in the presence or absence of the specific ERK5 inhibitor XMD8-92 (10 nM) (#SML1382, Sigma-Aldrich, USA).

2.2. Transfection of KLF2 siRNA

Double-stranded small interfering RNA (siRNA) oligonucleotides targeting KLF2 (5′-GGGCCUUAAUUUGUACUGUTT-3′) and the negative control siRNA were purchased from Ambion, USA. It was verified that the sequence of this siRNA did not display the off-target effect. HUVECs were seeded onto 6-well plates and grown to 70–80% confluence. Then KLF2 siRNA at a final concentration of 10 nM was transfected into HUVECs using Lipofectamine RNAi-MAX reagent in accordance with the manufacture's protocols. Twenty-four h after transfection, cells were exposed to humanin and high glucose. The efficacy of gene silencing of KLF2 was evaluated by western blot analysis.

2.3. Luciferase assays

DNA fragments of -1.7-kb human KLF2 gene promoter were amplified by polymerase chain reaction (PCR) using human genomic DNA as previously described (Sen-Banerjee et al., 2005). The fragments were subsequently cloned to a pGL3-basic reporter vector (Promega, USA) and transfected into HUVECs. Briefly, HUVECs at the confluence of 80% were placed in 10-cm dish and transfected with 6 μg KLF2-luc plasmid using Lipofectamine 2000 (Life Technologies, USA) in Opti-MEM (Gibco, USA) for 6 h. A plasmid with renilla luciferase gene (phRL-TK) was co-transfected as a control. Transfected HUVECs were cultured in growth medium and treated with 50, 100, and 200 μM humanin simvastatin (2 μM) for 24 h. Luciferase reporter gene activity was detected using a Promega dual-luciferase reporter 1000 assay system in a microplate spectrophotometer.

2.4. RNA isolation and real-time PCR analysis

Total intracellular RNA was isolated from HUVECs using an miRNeasy mini kit (Qiagen, USA). Contaminating DNA was removed from RNA with DNAse I (Life Technology, USA). 1 μg of purified RNA was used for a reverse transcription PCR to synthesize first-strand cDNA with a iScript cDNA reverse transcription kit (Bio-Rad, USA). Real-time PCR (qRT-PCR) was carried out on an ABI StepOne Plus instrument with SYBR green qPCR master mix (Life Technology, USA). Target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta CT}$ method.

2.5. Western blot analysis

Cell lysates were prepared using cell lysis buffer (Cell Signaling Technology, USA). Proteins were denatured by boiling at 95℃ for 5 min in a sample buffer. Thirty μg of intracellular protein was separated using 8–12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA). After blocking using 5% non-fat milk in TBS-0.1% Tween 20, membranes were sequentially incubated with primary antibodies and HRP-conjugated secondary antibodies. Blots were developed with enhanced chemiluminescence (ECL) detection (Amersham Biosciences, UK).

2.6. Enzyme linked immunosorbent assay (ELISA) for TNF-α and IL-1β detection

Secreted amounts of TNF-α and IL-1β were determined using a standard ELISA kit (Sigma-Aldrich, USA) in accordance with the manufacturer's protocols. Absorbance measured at 450 nm was used to index the levels of TNF-α and IL-1β.

2.7. Monocyte-endothelial cell adhesion assay

Human THP-1 monocytes from ATCC, USA were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 g/ml streptomycin, and 2 mM L-glutamine. HUVECs were seeded and allowed to grow to confluent monolayers. Cells were treated with 50, 100, and 200 μM humanin for 24 h followed by exposure to high glucose (HG, 25 mM) or normal glucose (7 mM) for another 24 h.

THP-1 cells were labeled with 20 μM calcein acetoxymethyl ester for 30 min. Then 2×10^5 cells/ml THP-1 cells were added to HUVEC layers and incubated for 2 h. Unattached THP-1 cells were washed away and fluorescent signals were captured by a fluorescent microscope with excitation at 485 nm and emission at 528 nm.

2.8. Statistical analysis

The experimental data are presented as the means ± SEM. Statistical analysis of the results was performed using the analysis of variance (ANOVA) test. A P value less than 0.05 was considered statistically significant.

3. Results

KLF2 has been defined as a key transcriptional regulator of endothelial function. Firstly, we investigated the effects of humanin in HUVECs. Real-time PCR results in Fig. 1A indicate that humanin significantly increased the expression of KLF2 at the gene level in a dose-dependent manner from 50 to 200 μM. Consistently, western blot results revealed that humanin treatment markedly increased the expression of KLF2 at the protein level in a dose-dependent manner (Fig. 1B). Notably, we also reported that humanin significantly increased the promoter luciferase activity of KLF2 (Fig. 1C), suggesting that humanin increased the expression of KLF2 at the transcriptional level. Simvastatin (2 μM), a well-known activator of KLF2, was used as a positive control. KLF2 regulates endothelial function by promoting the expression of eNOS but inhibiting the expression of ET-1. To further confirm the effects of humanin on the expression of KLF2, we measured the