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Vaspin inhibited proinflammatory cytokine induced activation of nuclear factor-kappa B and its downstream molecules in human endothelial EA.hy926 cells

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ABSTRACT

Aims: In this study, we investigated the effects of visceral adipose tissue-derived serpin (vaspin), a newly discovered adipocytokine, on nuclear factor-kappa B (NF-κB) and its downstream molecules in proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukine-1 (IL-1), stimulated human endothelial EA.hy926 cells to elucidate the role of vaspin in the inflammatory states of endothelium.

Methods: A NF-κB luciferase reporter system was constructed and stably transfected into human endothelial cell line EA.hy926. Following transfection, EA.hy926 cells were pretreated with various concentrations of vaspin (0–320 ng/ml) before TNF-α and IL-1 stimulation. The transcription activity of NF-κB was determined using luciferase reporter assay. Expression levels of NF-κB downstream inflammatory cytokines, TNF-α, IL-1 and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). Expressions of adhesion molecules and chemokines, intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1) were determined by quantitative real-time PCR (RT-PCR) and western blot in mRNA and protein levels, respectively.

Results: Results showed that vaspin inhibited TNF-α and IL-1 mediated activation of NF-κB and its downstream molecules in a concentration-dependent manner ($P < 0.05$).

Conclusions: We conclude that vaspin protected endothelial cells from proinflammatory cytokines induced inflammation by inhibition of NF-κB and its downstream molecules.

1. Introduction

Obesity has been considered to be associated with persistent low-grade inflammation which may play a causal role in obesity-induced insulin resistance and its related diseases, including diabetes mellitus, hyperlipidemia and hypertension [1–4]. In obesity, adipocytes, adipose tissue macrophages (ATM) and other immune cells are activated in adipose tissue, especially white adipose tissue and secrete a variety of

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cytokines, including tumor necrosis factor-α (TNF-α), interleukine-1 (IL-1), interleukine-6 (IL-6), leptin, resistin, monocyte chemoattractant protein-1 (MCP-1), visfatin and adiponectin, to regulate metabolic homeostasis [1,5,6]. Among them, visceral adipose tissue-derived serine protease inhibitor (vaspin), a newly discovered adipocytokine, has been found to have insulin-sensitizing and potentially anti-inflammatory effects in condition of obesity and insulin resistance [7–11]. The proinflammatory cytokines, such as TNF-α, IL-1, undoubtedly have local effects, especially on the endothelium which forms the interface between vascular tissues and blood and reacts to these proinflammatory cytokines in the first place [12,13]. Indeed, endothelial cells play an important role in inflammatory responses, leading to diabetes related cardiovascular complications like atherosclerosis [1]. The inflammatory reaction is characterized by extensive production of inflammatory cytokines and excessive infiltration of inflammatory cells by activating the correlated signaling pathways [14,15]. Nuclear factor-kappa B (NF-κB) activation is a major pathway involved in the process of inflammation where TNF-α and IL-1 are canonical players. Activation of NF-κB by TNF-α and IL-1 promotes the release of inflammatory mediators and up-regulate adhesion molecules in endothelial cells, aggravating insulin resistance and vascular disorders [15,16]. Studies have shown that inhibition of NF-κB pathway by pharmaceuticals or genetic techniques protects diet-induced insulin resistance [17,18]. Blockade of NF-κB in endothelium using transgenic technique protected mice from the development of insulin-resistance associated with obesity and reduced infiltration of macrophages into adipose tissue [19].

Vaspin was initially discovered in the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, a spontaneous type 2 diabetes animal model, characterized by insulin resistance, abdominal obesity, dyslipidaemia and hypertension [8,20]. It has been shown that the level of vaspin is obviously increased in white adipose tissue of OLETF rat in the peak of obesity and insulin resistance at 30 weeks of age but decreased with the worsening of diabetes and weight loss at 50 weeks. Administration of vaspin improved insulin resistance and glucose tolerance in high fat and high sucrose fed obese ICR mice and reversed the expression of altered genes related to insulin resistance like leptin, resistin and TNF-α [8]. Resent studies have indicated the protective role of vaspin under diabetes mellitus or high-glucose by inhibition of apoptosis in murine endothelial cells and vascular smooth muscle cells [10,21]. Vaspin also prevent TNF-α induced expression of intracellular adhesion molecule-1 (ICAM-1) by inhibiting NF-κB pathway in rat vascular smooth muscle cells [11]. Recently, a cell-surface GRP78/voltage-dependent anion channel complex is considered as a potential receptor for vaspin in endothelial cells [21]. However, little is known about its role in proinflammatory cytokines induced inflammatory state of endothelial cells.

In this study, we investigated the effects of vaspin on TNF-α and IL-1 stimulated NF-κB activity and its downstream molecules in the human endothelial cell line EA.hy926 to further elucidate the mechanism through which vaspin modulates obesity-associated inflammation in endothelium.

2. Materials and methods

2.1. Cell culture

The human endothelial EA. hy926 cells were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 (Sigma, Shanghai, China) supplemented with 20% fetal bovine serum (FBS, Gibco, Beijing, China), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. The culture medium was replaced every 3 days and cells were passaged as they reach 80–90% confluence. In the experiment, the quiescent endothelial cells were pretreated with recombinant human vaspin protein (Leinco Technologies V112) in the presence of TNF-α (10 ng/ml) and IL-1 (10 ng/ml) (Biovision 1050-50, Shanhai, China). The experiment was repeated at least three times for all the data shown.

2.2. Transfection and NF-κB luciferase reporter assay

The luciferase reporter assay for NF-κB was performed as previously reported [22,23]. The NF-κB luciferase reporter plasmid named pGL4.32 [luc2P/NF-κB-RE/Hygro] (pNF-κB-Luc, Promega) contains five copies of an NF-κB response element (NF-κB-RE), TGGGGACTITCCGC, that drives transcription of the luciferase reporter gene luc2P (Photinus pyralis). luc2P is a synthetically-derived luciferase sequence with humanized codon optimization that is designed for high expression and reduced anomalous transcription. The plasmid backbone contains an ampicillin resistance gene to allow selection in E. coli and a mammalian selectable marker for hygromycin resistance (Fig. S1). 5×10^5 ml^{-1} of EA.hy926 cells at 70–80% confluence were plated in a 60 mm culture dish and stably transfected with 1 μg of NF-κB luciferase reporter plasmid pNF-κB-Luc using 2.5 μl of transfection reagent lipofectamine 2000. After 48 to 72 h, cells were subcultured into 100 mm glass dish at 1/10 density at room temperature with 5% CO_2. For another 24 h, growth medium was replaced with medium containing 200 μg/ml of hygromycin B for positive cell selection. After 7 to 10 days, when most of the untransfected cells died, concentration of hygromycin B was reduced to 100 μg/ml to maintain the selection process until hygromycin B resistant clones formed. Hygromycin B resistant cells were then amplified and stimulated by TNF-α (10 ng/ml) for 2 h. NF-κB luciferase activities were then measured with the luciferase assay system using a luminometer (Promega, China). Positive cells were frozen and preserved for downstream experiments.

2.3. Real-time fluorescence quantitative PCR

mRNA levels of downstream molecules ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and MCP-1 were determined by quantitative RT-PCR. Total RNA was extracted from EA. hy926 cells using Trizol reagent (Invitrogen, USA) following the manufacturer's instruction. 5 μg of each RNA sample was reverse transcribed to cDNA in 10 μl of total volume using the SuperScript First-Strand Synthesis System (Invitrogen, USA).