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A novel mechanism of diabetic vascular endothelial dysfunction: Hypoadiponectinemia-induced NLRP3 inflammasome activation

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ABSTRACT

It has been well documented that hypoadiponectinemia is associated with impaired endothelium-dependent vasodilation. However, the exact molecular mechanism which mediates this process has not been fully described. The current study aimed to investigate the role of hypoadiponectinemia-induced NLRP3 inflammasome activation in diabetic vascular endothelial dysfunction and its molecular mechanism. Male adult adiponectin knockout mice and wild type mice were fed with a high fat diet to establish a type 2 diabetic mellitus model. In addition, human umbilical vein endothelial cells (HUVECs) were cultured and subjected to high glucose/high fat (HG/HF). The NLRP3 inflammasome activation was increased in type 2 diabetic mice and treatment of diabetic aortic segments with MCC950, a potent selective inhibitor of NLRP3 inflammasome ex vivo improved endothelial-dependent vasorelaxation. NLRP3 inflammasome activation and vascular endothelial injury were significantly increased in APN-KO mice compared with WT mice in diabetes and MCC950 decreased diabetic vascular endothelial dysfunction to comparable levels in APN-KO mice and WT mice. Adiponectin could decrease NLRP3 inflammasome activation and attenuate endothelial cell injury, which was abolished by NLRP3 inflammasome overexpression. Inhibition of peroxynitrite formation preferentially attenuated NLRP3 inflammasome activation in APN-KO diabetic mice. The current study demonstrated for the first time that hypoadiponectinemia-induced NLRP3 inflammasome activation was a novel mechanism of diabetic vascular endothelial dysfunction.

1. Introduction

Cardiovascular complications are the leading cause of death for patients with type 2 diabetic mellitus, a disease affecting >94 million people in China [1,2]. Inflammation in endothelial cells plays a critical role in the pathogenesis of vascular disease in obesity-related type 2 diabetes [3,4]. It is therefore crucial to clarify the mechanism of inflammatory vascular injury in diabetic mellitus to search for novel therapeutic strategies.

Adiponectin is an abundant adipocyte-derived plasma protein involved in myocardial protection and insulin sensitivity [5,6]. The anti-inflammatory and vascular protective effects of adiponectin have been recognized, for example, it has been reported that adiponectin decreases TNF-α-induced ICAM-1 expression and NF-κB activation in endothelial cells [7,8]. However, many fundamental questions remain unanswered. What's more, numerous epidemiological studies have shown that plasma adiponectin levels were negatively correlated with cardiovascular disease in obesity and diabetes [9,10]. Moreover, several clinical observations and basic studies have demonstrated that hypoadiponectinemia is associated with impaired endothelium-dependent vasodilation [11–13]. However, the exact molecular mechanism which mediates this process has not been fully described.

Inflammasomes are large multiprotein complexes that consist of caspase-1, apoptosis-associated speck-like protein (ASC), and NLRP (nucleotide-binding oligomerization domain-like receptor with a pyrin domain). The ASC protein bridges the interaction between NLRP and caspase-1, making it essential for inflammasome activation and subsequent interleukin-1β (IL-1β) and IL-18 secretion [14,15,17,18]. To date, four additional inflammasome proteins have been identified: NLRP1, NLRP3, NLRC4, and AIM2 [15,17,18]. The NLRP3 inflammasome is the most fully characterized and has been associated

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with a wide range of diseases, including infections, auto-inflammatory and autoimmune diseases,and metabolic disorders [18,19,21]. The NLRP3 inflammasome, activated by endotoxins, K^+ channel openers, uric acid and reactive oxygen species (ROS) [22], plays a crucial role in insulin resistance and the pathogenesis of diabetes [23]. Our laboratory has previously demonstrated that the NLRP3 inflammasome is activated in cardiac microvascular endothelial cells (CMECs), but not in cardiomyocytes, playing a critical role in the pathophysiology of MI/R injury [24]. However, the question of whether the NLRP3 inflammasome also plays an important role in diabetic vascular endothelial injury has not yet been addressed. It also remains unclear whether the NLRP3 inflammasome activation is involved in hypoadiponectinemia associated diabetic vascular endothelial injury. Our previous data has indicated that adiponectin can reduce ROS production – an important activator of the NLRP3 inflammasome. Therefore, it is possible that the activation of the NLRP3 inflammasome may be linked with loss of adiponectin in diabetes mellitus.

The aims of the current study were: (1) to identify the role of NLRP3 inflammasome activation in diabetic vascular endothelial injury; (2) to determine the role of hypoadiponectinemia in NLRP3 inflammasome activation in diabetes; (3) to investigate the mechanism of hypoadiponectinemia-induced NLRP3 inflammasome activation.

2. Materials and methods

2.1. Materials

APN knockout mice were provided by Professor Xinliang Ma in Thomas Jefferson University, in USA. They were global knockout. Phenotypic characteristics of male APN knockout mice and WT control mice were same and had been previously described [25,26]. Adult male adiponectin knockout mice (APN-KO) and their wild type littermates (WT) were used in this study. All experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care. Dihydroethidium (DHE) was from Molecular Probes (Eugene, OR, USA). ELISA kits for IL-1β, IL-18,ICAM-1 and VCAM-1 were purchased from Nanjing Institute of Jiancheng Bioengineering (Nanjing, China). Antibodies against NLRP3, Caspase-1, Akt and eNOS were from Cell Signaling Technology (Boston, USA). MCC950 was pruchased from Med Chem Express.

2.2. Animal care

Male APN knockout (APN-KO) mice and their wild type (WT) C57BL/6 littermates were maintained in a temperature-controlled barrier facility. Mice aged 8 to 9 weeks were used for these studies. Thirty-two APN-KO and thirty-two WT mice were divided into four groups respectively ($n = 8$ for each group): WT + Normal diet (WT), APN-KO + Normal diet (APN-KO), WT + High fat diet (WT + HFD), APN-KO + High fat diet group (APN-KO + HFD), WT + High fat diet + EUK134 group (WT + HFD + EUK134), APN-KO + High fat diet + EUK134 group (APN-KO + HFD + EUK134), WT + High fat diet + 1400W (WT + HFD + 1400W), and APN-KO + High fat diet + 1400W (APN-KO + HFD + 1400W). Besides WT and APN-KO groups, the type 2 diabetes model was established by feeding the rest of mice with high-fat diet (D12492, Research Diets, Inc; NJ; USA) containing (kcal) 20% protein, 20% carbohydrate, and 60% fat for 8 weeks. Mice in WT and APN-KO groups were fed with a chow diet (D12450B, Research Diets, Inc; NJ; USA) containing (kcal) 20% protein, 70% carbohydrate, and 10% fat. Mice were anesthetized with 3% isoflurane and sacrificed with an overdose injection of pentobarbital, and serum was harvested and aortic segments were surgically dissected from the heart to the abdominal aortic bifurcation.

2.3. Cell culture

Experiments on Human umbilical vein endothelial cells (HUVECs) were carried out on the same batch at passages 3–7. After serum starvation for 3 h, cells were pretreated with globular adiponectin (gAd; 2 μg/mL) for 2 h before exposure to high glucose/high fat (HG/HF) medium, which contained glucose (25 mmol/L) and free fatty acid (FFA) palmitate (500 μmol/L) for 48 h. Controls were incubated with 5 mmol/L glucose and equal concentrations of palmitate-free BSA. Cells were collected after treatment and activation of the NLRP3 inflammasome, endothelial diastolic function, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression were determined as described in detail below.

2.4. Evaluation of vasorelaxation

Aortas were placed in cold physiological saline solution (PSS) and aortic segments were cut into 4 rings of 1 mm in length. These aortic rings were inatalled on a multi wire myograph system, which linked to transducers to recorded data. The aortic rings were then stretched to initial length, stable for 60 min. The rings were treated with 1 μmol/L phenylephrine (PE) before acetylcholine (ACh) and nitroprusside (SNP) were added to test the diastolic function. Diastolic extent of each concentrations were measured and results were expressed as the percentage of exposure to PE.

2.5. Small interfering RNA and plasmid transfection

Small interfering RNA (siRNA) duplexes against NLRP3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Predesigned NLRP3-specific siRNA or control scrambled siRNA (Santa Cruz, Thermo Scientific, respectively) were diluted in 5% glucose and mixed with jet PEI (polyethyleneimine; Genesee Scientific, San Diego, CA). Each scrambled siRNA contained a sequence that is not predicted to target any known cellular mRNA. HUVECs (80% confluent) were transfected with Lipofectamine 2000 Reagent (final siRNA concentration: 100 nmol/L). The NLRP3 overexpression plasmid was purchased from Hanbio Biotechnology (Shang Hai, CHINA), and was transfected into HUVECs using LipoFiter.

2.6. Inflammatory cytokine detection by ELISA

The levels of inflammatory cytokines (IL-1β, IL-18,sICAM-1 and sVCAM-1) in mouse serum and cell culture supernatant were measured using a commercially enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology Co, Wuhan, China).

2.7. Apoptosis assay

Apoptosis of HUVECs was assessed rely on caspase-3 activity by using a caspase-3 activity assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Caspase-3 activity was showed as nmol pNA/h/mg protein.

2.8. Western blotting

Aortic vessels were surgically dissected and harvested from the heart to the abdominal aortic bifurcation. Collection of arterial and cellular proteins to carry on Western blot analysis. Equal amounts of protein was separated by 10% SDS-PAGE and transferred to a nitrocellulose membranes. Blocking with 5% skim milk before the membrane was incubated with primary antibodies with a 1:1000 dilution at 4 °C overnight. Then the membrane incubated with sheep anti-rabbit or anti-mouse IgG HRP for 60 min at room temperature and visualized using ChemiDocXRS.