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Adiponectin increases macrophages cholesterol efflux and suppresses foam cell formation in patients with type 2 diabetes mellitus

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ABSTRACT

Objectives: Low levels of blood adiponectin contribute to an increased risk of cardiovascular disease (CVD) in patients with type 2 diabetes mellitus (T2DM). To determine the mechanism through which adiponectin deficiency mediates accelerated cardiovascular disease in patients with diabetes, we investigated the effects of adiponectin on macrophage cholesterol deposition.
Methods and results: 35 diabetic patients and 35 nondiabetic healthy subjects were recruited in this study. Macrophages from patients with diabetes mellitus were cultured in adiponectin-free or adiponectin-supplemented media and exposed to oxidized low-density lipoprotein cholesterol (OxLDL). Adiponectin treatment markedly suppressed foam cell formation in OxLDL-treated macrophages from diabetic subjects only, which was mainly attributed to an increase in cholesterol efflux. Adiponectin treatment significantly increased ATP-binding cassette transporter (ABC) ABCG1 mRNA and protein levels but not ABCA1, without affecting protein expression of scavenger receptors, including scavenger receptor-A (SR-A) and CD36 in diabetics. Pharmacological or genetic inhibition of liver X receptor α (LXRα) blocks the adiponectin-mediated ABCG1 expression, suggesting that LXRα activation is necessary for the attenuation of lipid accumulation of macrophages by adiponectin. In addition, deletion of the adiponectin receptor (adipoR1) in macrophages from diabetic patients accelerated foam cell formation induced by OxLDL. Finally, a strong positive correlation was noted between decreased serum adiponectin levels and impaired cholesterol efflux capacity both before and after adjustment for HDL-C and ApoAI in diabetic patients (both $P < 0.001$).
Conclusions: The present study identifies reduced adiopoR signaling as a critical mechanism underlying increased foam cell formation and accelerated cardiovascular disease in diabetic subjects.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a well-known risk factor for in the initiation and development of atherosclerotic cardiovascular disease, accounting for a high proportion of disability and deaths among diabetics [1,2]. Several studies indicate that in poorly controlled diabetes mellitus, altered insulin signaling and/or hyperglycemia promote unbalanced cholesterol metabolism, which favors oxidized low-density lipoprotein (OxLDL) cholesterol retention and macrophage-derived foam cell formation, a hallmark of the initiation and development of atherosclerosis [3–5].

Cellular cholesterol levels reflect a balance between uptake, efflux, and endogenous synthesis. Under hyperglycemia and/or an insulin-resistant state, macrophages upregulate the expression of scavenger receptors (SR-A, and CD36), which have the ability to take up modified lipoproteins [6,7]. On the contrary, members of the cholesterol reverse transporter family, such as ATP-binding cassette (ABC) mainly ABCG1, are also downregulated in response to high glucose [8,9]. Thus, increased scavenger receptor expression [10] and decreased ABCG1 expression [11] promote macrophage foam cell formation and are considered as a link between diabetes mellitus and atherosclerosis. Therefore, strategies to modulate macrophage cholesterol deposition could have therapeutic potential for limiting the accelerated vascular disease observed in patients with T2DM.

Adiponectin (encoded by *Adipoq*) is an insulin-sensitizing plasma protein expressed in adipose tissue, and it plays an important role in insulin-sensitizing, anti-inflammatory and anti-

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atherogenic properties [12,13]. Plasma adiponectin levels are reduced not only among obese patients [14] but also in disease states frequently associated with insulin resistance and T2DM [14], dyslipidemia [15], hypertension [16] and coronary artery disease (CAD) [17]. The anti-atherogenic effects of adiponectin include the suppression of adhesion molecule expression on vascular endothelial cells [18] and the inhibition of vascular smooth muscle cell proliferation and migration [19]. Adiponectin also stimulates the production of nitric oxide (NO) in endothelial cells [20] and reduces atherosclerosis by suppressing the endothelial inflammatory reaction and macrophage-to-foam cell transformation [21]. Therefore, understanding the molecular mechanism of the accelerated atherosclerosis induced by hypoadiponectinemia may be crucial for treating the epidemic of CVD in diabetics.

This study was designed to explore whether hypoadiponectinemia levels contribute to the increase in macrophage-mediated cholesterol deposition observed in patients with diabetes and investigate the effects of adiponectin on macrophage cholesterol deposition in diabetics and nondiabetic matched controls.

2. Materials and methods

2.1. Materials

The recombinant human and mouse full-length adiponectin were obtained from Alexis (San Diego, CA). CP113818, an acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitor, was purchased from Shanghai Ennopharm Co., Ltd (Shanghai, China). 22-(N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino)-23,24-Bisnor-5-Cholen- 3β-Ol (NBD-cholesterol, Catlog. N-1148), an environment-sensitive probe for investigating lipid transport processes as well lipid−protein interactions, were purchased from Molecular Probes, Inc. Eugene, OR). Human recombinant lipid-free apolipoprotein A-I (ApoAI, Catlog. SRP4693), high density lipoprotein (HDL, L8039) from human plasma, human recombinant macrophage colony-stimulating factor (M-CSF, SRP4237), 8-(4-chlorophenylthio)-cyclic AMP (8-CPT-cAMP, C3912) were obtained from Sigma–Aldrich (St. Louis, MO). 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine percholate (Dil)-labeled OxLDL (Invitrogen, Grand Island, NY). The primary rabbit polyclonal antibodies anti-LXRα, CD36, goat anti-SR-A and horseradish peroxidase (HRP)-conjugated anti-rabbit or goat secondary antibody were all obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Rabbit monoclonal antibody to ABCG1 and mouse monoclonal antibody to ABCA1 were provided by Abcam.

2.2. Study population

Our study population included 35 adult subjects with type 2 diabetes mellitus on medication, with an age of 62 ± 8 years, body mass index of 25.50 ± 2.92 kg/m^2, diabetes duration of 4 ± 1.4 years, and hemoglobin A1c level of 7.94 ± 0.58%. We excluded recently diagnosed diabetes mellitus, pregnancy, known coronary artery disease, and normal adiponectin. This population was compared with 35 normal weight control subjects with no history of diabetes mellitus or hypertension. Subjects were recruited from the outpatient clinic at Guangzhou Military General Hospital in Guangzhou. The study was approved by the ethics committee of Sun Yat-sen University and was conducted in accordance with the Declaration of Helsinki. Participation was voluntary, and each participant provided written informed consent. The ethylenediaminetetraacetic acid samples were immediately centrifuged, aliquoted, and stored at −80 °C until batch analysis. Peripheral blood samples were collected from healthy subjects and patients after an overnight fast between 8:00 AM and 10:00 AM.

2.3. Biochemical measurements

Serum levels of total cholesterol, HDL cholesterol, triglycerides and glucose were measured through an enzymatic method (Wako Pure Chemical Industries) using an automatic analyzer (Hitachi 747 autoanalyzer, Hitachi Ltd, Tokyo). LDL cholesterol was calculated according to the Friedewald formula: LDL cholesterol = total −(triglycerides/5 + HDL cholesterol). Serum levels of apoAI and apo B were measured by immunonephelometry using a BN Prospect analyzer (Dade Behring). Plasma insulin levels were measured with a chemiluminescent enzyme immunoassay (Immulite 1000 Analyzer). The intra-assay and inter-assay coefficients of variation of all measured biochemical parameters were <5%.

2.4. Human serum cholesterol efflux capacity assay

We measured the cholesterol efflux capacity of human serum according to previously established methods [22]. J774 murine macrophages were first radiolabeled with 2 μCi/ml ^{3}H-cholesterol. ABCA1 was upregulated by 6 h incubation with 0.3 mM 8-CPT-cAMP. Subsequently, efflux media from control healthy subjects or diabetic patients containing 2.8% apolipoprotein B (ApoB)-depleted serum were added for another 4 h. All steps were performed in the presence of 2 μg/ml CP113818. Liquid scintillation counting was used to quantify the efflux of radioactive cholesterol from the cells. The quantity of radioactive cholesterol incorporated into cellular lipids was calculated through the isopropanol extraction of control wells not exposed to patient serum. The percentage efflux was calculated by the following formula: [(microcuries of ^{3}H-cholesterol in media containing 2.8% ApoB-depleted serum−microcuries of ^{3}H-cholesterol in serum-free media) ÷ microcuries of ^{3}H-cholesterol in cells extracted before the efflux] × 100. All assays were performed in duplicate.

2.5. Human serum adiponectin concentrations measurement

Adiponectin levels in serum were determined by a commercially available competitive ELISA assay kit (Cat No. AG-45A-0002, AdipoGen, Seoul) according to the manufacturer's instruction. The assay used standards in the range of 0.001–1 μg/ml. The intra-assay and inter-assay coefficients of variation were 4% and 3%, respectively. The normal blood levels of adiponectin range from 8.3 to 13.9 μg/ml.

2.6. Human monocyte isolation and macrophage differentiation

Human monocyte isolation and macrophage differentiation were performed as described previously [23]. Peripheral monocytes were isolated by standard Ficoll isolation techniques according to the manufacturer's protocol and selected by CD14 marker purity (>90% as assessed by flow cytometry). Monocytes were then plated in tissue culture medium in DMEM containing 1.5 mg/ml glucose, 100 ng/ml human macrophage colony-stimulating factor (M-CSF), 10% charcoal/dextran-treated fetal bovine serum (FBS, Hyclone), and 1% antibiotic/antimycotic mixture. Monocytes were allowed to differentiate into macrophages for 3 d in adiponectin-free media before being used in further experiments.

2.7. Mouse peritoneal macrophages

Mouse peritoneal macrophages from *db*/*db* mice and wild-type C57BL/6J mice (n = 8 per each group) were isolated 3 d after the intraperitoneal injection of 4% thioglycollate solution. Cholesterol uptake was evaluated after 6 h of stimulation with Dil-labeled OxLDL cholesterol in mouse macrophages cultured for 24 h in the absence or presence of adiponectin treatment.