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Metformin restores impaired HDL-mediated cholesterol efflux due to glycation

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ABSTRACT

High-density lipoprotein (HDL) mediates cholesterol efflux, which is the initial and rate-limiting step of reverse cholesterol transport. The present study was undertaken to evaluate the effect, on macrophage cholesterol efflux, of functional modification of HDL by its glycation. We also investigated the effects of the glycation-inhibitors metformin (MF) and aminoguanidine (AG) on glycated HDL-mediated cholesterol efflux. Human plasma HDL (5 mg protein/mL) was glycated by incubation with 3-deoxyglucosone (3-DG). Glycation was monitored by measuring carboxymethyl-lysine (CML). HDL-mediated cholesterol efflux was determined using human THP-1-derived macrophages pre-labeled with [^{3}H]-cholesterol. To measure expression of potential factors related to the efflux in the macrophages, ATP-binding cassette transporter (ABC) G1 was analyzed by real-time quantitative RT-PCR and Western blot. Glycation of HDL significantly reduced the HDL-mediated cholesterol efflux from THP-1-derived macrophages (87.7 ± 4.2% of control, $n = 9$, $p < 0.0001$). In the presence of metformin or aminoguanidine (100 mM), glycated HDL-mediated cholesterol efflux was restored to 97.5 ± 4.3% and 96.9 ± 3.1%, respectively. Exogenous HDL reduced ABCG1 mRNA and protein expression in THP-1-derived macrophages, but glycation deprived HDL of this effect. We conclude that glycated HDL particles are ineffective as acceptors of ABCG1-mediated cholesterol efflux; and this may explain, at least in part, accelerated atherosclerosis in diabetic patients. Metformin serves as a possible candidate to restore impaired cholesterol efflux and reverse cholesterol transport.

1. Introduction

Diabetic dyslipidemia is associated with an increased risk of developing atherosclerosis and coronary artery disease. A reduced high-density lipoprotein (HDL) level, one of the cardinal features of diabetic dyslipidemia, serves as an independent risk factor for cardiovascular diseases and is implicated in metabolic syndrome. HDL is known to possess various anti-atherogenic potentials such as anti-inflammatory, anti-oxidant, anti-thrombotic and vasodilating effects [1]. Recently, HDL was reported to play a pivotal role in the reverse cholesterol transport system [2]. Apolipoprotein A-I (apoA-I), a major apolipoprotein in HDL, transports intracellular free cholesterol in peripheral tissues through ATP-binding cassette transporter (ABC) A1, and then nascent HDL, HDL_2 and HDL_3 act as acceptors of ABCG1-mediated cholesterol efflux [3]. HDL cholesterol is finally excreted in bile through the liver. ABCA1 and ABCG1 function cooperatively in the reverse cholesterol transport system [4] to regulate plasma HDL [5]. ABCG1 is expressed at high levels in macrophages, and this also suggests the major role of ABCG1 in cholesterol efflux in macrophages [6]. ABCG1 is reported to promote cholesterol efflux to HDL particles but not to apoA-I in vivo [4,7].

We previously reported that human monocyte-derived macrophages in type 2 diabetics secreted lower levels of apolipoprotein E [8]. We also demonstrated therapeutic effectiveness of anti-dyslipidemic agents such as clofibrate [9], simvastatin [10] and a combination of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ agonists [11] in improving HDL metabolism.

In diabetic patients, hyperglycemia-induced advanced glycation end products (AGEs) contribute largely to the pathogenesis of diabetic complications (microangiopathy) and atherosclerosis (macroangiopathy). Also, the reverse cholesterol transport system can be impaired by glycation in a non-enzymatic reaction between reducing sugars and proteins in lysine, arginine or other amino terminal residues, nucleic acids or phospholipids. Glycation of apoA-I and HDL also results in functional changes in their composition because lysine residues play an important role in cholesterol efflux and reverse cholesterol transport. Passarelli et al. [12] recently demonstrated that intracellular glycoxydation contributes to cellular lipid accumulation due to the reduced cellular cholesterol removal by apoA-I in an ABCA1-dependent pathway. Hoang et al.

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reported that AGE-induced modification of HDL impairs reverse cholesterol transport [13].

In the present study, we examined the inhibitory effect of glycation of HDL on cholesterol efflux from THP-1-derived macrophages and the molecular pathway that regulates the efflux. Effects of AGE inhibitors metformin and aminoguanidine were also studied.

2. Materials and methods

2.1. Materials

Human HDL was purchased from CALBIOCHEM (Darmstadt, Germany); apoA-I was from Sigma–Aldrich (St. Louis, MO, USA). 3-Deoxyglucosone (Dojindo Laboratories, Kumamoto, Japan), metformin (Nippon Shinyaku, Kyoto, Japan) and aminoguanidine (Sigma–Aldrich) were dissolved in phosphate-buffered saline (PBS).

2.2. Cell culture

THP-1 cells (Riken Cell Bank, Tokyo, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco) at 37 °C in 5% CO_2. THP-1 cells were differentiated into macrophages by treatment with 200 nM phorbol 12-myristate 13-acetate (PMA; Biomol, Plymouth Meeting, PA, USA) for 72 h. The cells were cultured in a 12-well plate at a density of 1.0×10^5 cells/well (for cholesterol efflux) or in a six-well plate at a density of 1.0×10^6 cells/well (for RT-PCR and Western blotting).

2.3. Glycation of HDL with 3-deoxyglucosone

Human HDL (5 mg protein/mL) was glycated by incubation with 3-deoxyglucosone (3-DG; 0, 30 or 100 mM) at 37 °C for 7 days. Glycation of HDL was confirmed by measuring the concentration of carboxymethyl-lysine (CML), using an ELISA kit (CycLex, Nagano, Japan).

2.4. Determination of HDL-mediated cholesterol efflux from THP-1-derived macrophages

Macrophages were labeled with 0.6 μCi/mL [^{3}H]-cholesterol (PerkinElmer, Boston, MA, USA) for 24 h in DMEM containing 10% FBS. The cells were washed twice with PBS and incubated for 24 h in DMEM containing 0.2% bovine serum albumin (BSA, Sigma). The macrophages were then incubated in the presence of non-glycated or glycated HDL (50 μg protein/mL) for 24 h and cholesterol efflux was determined by measuring radioactivity in the culture medium and cells. The effects of the AGE inhibitors metformin and aminoguanidine on HDL-mediated cholesterol efflux were studied by using HDL preparations glycated by incubating with 3-DG (100 mM) in the presence of metformin or aminoguanidine (0–100 mM) for 7 days. The percentage of cholesterol efflux was calculated by dividing radioactivity (dpm) in culture medium by the sum of the radioactivity in the medium and cells; [(medium)/(medium + cells) × 100%].

2.5. Real-time quantitative RT-PCR

Macrophages were exposed to glycated or non-glycated control HDL and the levels of mRNA for ABCG1 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) in the cells were analyzed by real-time quantitative RT-PCR (ABI PRISM 7700 Sequence Detection System; Applied Biosystems, Foster City, CA, USA). The mRNA expression levels were normalized to GAPDH as an internal standard. Total RNA was extracted from macrophages using an RNeasy Mini Kit (QIAGEN, Japan). Double-stranded cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems). The TaqMan probes for ABCG1 (Hs00245154_m1) and GAPDH (Hs99999905_m1) were Assay-on-Demand gene expression products (Applied Biosystems).

2.6. Western blot analysis

Cells were lysed using Laemmli's reducing sample buffer. The lysate was subjected to electrophoresis on a 6–9% gradient polyacrylamide gel, and the proteins were transferred to an Immobilon-P PVDF membrane (Millipore Corporation, Billerica, MA, USA). ABCG1 protein was detected using a rabbit anti-ABCG1 antibody (1:2500; Novus Biologicals, Littleton, CO, USA). Immunodetection was performed using a HRP-labeled anti-rabbit IgG antibody and a SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL, USA). Actin levels were analyzed in a similar manner using an anti-actin rabbit IgG (1:200; Santa Cruz, Santa Cruz, CA, USA). Pictures of the membranes were downloaded to a computer and the intensity of each band was quantified with an NIH image (National Institutes of Health, Bethesda, MD, USA).

2.7. Statistical analysis

Values are expressed as mean ± standard deviation (S.D.). Differences between groups were determined using ANOVA with Bonferroni/Dunn post hoc correction. Two groups were compared using the Mann–Whitney U test. Statistical analyses were performed using StatView 5.0 (SAS Institute, Cary, NC, USA). $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Cholesterol efflux mediated by glycated or non-glycated HDL

The treatment with 3-DG resulted in increased CML levels in HDL preparations in a concentration-dependent manner as shown in Fig. 1A ($n = 6$).

Glycation of HDL suppressed its ability to induce cholesterol efflux from macrophages (Fig. 1B). Cholesterol efflux from the cells incubated with HDL glycated with 100 mM 3-DG was 23.8 ± 1.4%, which was significantly lower as compared to that from the cells incubated with non-glycated HDL: 26.0 ± 0.9% ($n = 6$, $p = 0.0082$).

The following experiments were carried out using HDL preparations glycated by incubating with 100 mM 3-DG for 7 days.

3.2. Effects of AGE inhibitors on glycation of HDL

The effects of the AGE inhibitors metformin and aminoguanidine on 3-DG-induced glycation of HDL are summarized in Fig. 2. CML levels in HDL preparation glycated in the presence of metformin (0.76 ± 0.12 ng/mL, $n = 6$, $p < 0.0001$) or aminoguanidine (0.17 ± 0.15 ng/mL, $n = 6$, $p < 0.0001$) were significantly lower when compared to HDL glycated without any inhibitor (1.30 ± 0.12 ng/mL, $n = 6$). The inhibitory effect of aminoguanidine was much higher than that of metformin (Fig. 2).

3.3. Effects of AGE inhibitors on glycated HDL-mediated cholesterol efflux

AGE inhibitors metformin and aminoguanidine were found to inhibit the increase in CML in HDL preparation incubated with 3-DG, and the effect of aminoguanidine was more prominent (Fig. 2). These inhibitors reversed the inhibition of HDL-induced cholesterol efflux in response to HDL glycation (Fig. 3). Cholesterol efflux mediated by glycated HDL (87.1 ± 2.9% of control, $n = 9$) was