HDL_3 DECREASE THE OXIDATIVE AND INFLAMMATORY STRESS INDUCED BY AGE-LDL IN HUMAN ENDOTHELIAL CELLS

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In diabetes, hyperglycemia determines the formation of irreversibly glycated low density lipoproteins (LDL). In a previous study, we demonstrated that advanced glycation end products- modified-LDL (AGE-LDL) increase the oxidative and inflammatory stress in human endothelial cells (HEC). Epidemiological studies indicate that plasma high density lipoproteins (HDL) concentration is negatively correlated with the cardiovascular risk. The aim of the present study was to determine if a specific anti-atherosclerotic fraction of HDL, HDL_3, is capable to restore the endothelial function disturbed by the interaction with AGE-LDL. HEC from EA.hy926 line were incubated for 24h with AGE-LDL in the presence or absence of HDL_3. Following, the NADPH oxidase activity, its subunits $p22^{phox}$ and NOX4 gene expression, and the expression of the monocyte chemoattractant protein-1 (MCP-1) were determined. Results showed that HDL_3 inhibits NADPHoxidase activity and $p22^{phox}$ gene expression, without significantly modifying NOX4 mRNA level. Co-incubation of HDL_3 and AGE-LDL with HEC determined the decrease of MCP-1 gene expression. In conclusion, the present study demonstrates that HDL_3 may partially restore the endothelial dysfunction induced by AGE-LDL by reducing the oxidative and inflammatory stress.

Key words: Human endothelial cells; AGE-LDL; HDL_3; Oxidative stress; Inflammation.

INTRODUCTION

Hyperglycemia in type-1 and type-2 diabetes is considered a powerful and independent risk factor for cardiovascular disease (CVD), stroke, and peripheral arterial disease – clinical manifestations of atherosclerosis[1]. The primary causal factor leading to the pathophysiologic alterations of the diabetic patients vasculature is the chronic exposure to high levels of blood glucose. Although the effects of glucose in adversely modulating cellular properties occurs by a variety of mechanisms[2,3], one of the most important pathway involved in the pathogenesis of the accelerated atherosclerosis in diabetes is the increase in nonenzymatic glycation of proteins and lipids, with the formation of advanced glycation end products[4] (AGE). Among the proteins known to be glycated in diabetes is apolipoprotein B (ApoB), the specific protein of LDL. Indeed, it was demonstrated that the level of glycated ApoB in diabetic patients is about two-fold higher than in healthy individuals[5,6].

Diabetes related changes in plasma lipid levels are also key factors involved in the progression of accelerated atherosclerosis. The characteristic features of a diabetic lipid profile are: high plasma triglycerides concentration, low high density lipoproteins (HDL) cholesterol concentration and increased concentration of small dense low density lipoproteins (LDL) particles. Epidemiological studies demonstrate that increased plasma levels of HDL protect against the development of atherosclerosis[7]. The anti-atherosclerotic mechanism of action of HDL is under investigation. The inverse correlation between HDL levels and CVD

risk might be explained by the ability of HDL to remove cholesterol from the extra-hepatic tissues and deliver it to the liver for excretion in the bile, in a process known as reverse cholesterol transport[8]. However, HDL have additional functions; they modulate endothelial function by stimulating endothelial nitric oxide production, and act as antioxidant or anti-inflammatory agent[9]. Circulating HDL particles are a class of lipoproteins characterized by a high heterogeneity in physicochemical properties and intravascular metabolism[10]. By ultracentrifugation in a gradient density, two HDL subfractions can be isolated: HDL_2 and HDL_3. Data from literature demonstrate that ultracentrifugally isolated small dense HDL_3 (with density 1.125–1.21 g/ml) exert greater inhibition of adhesion protein expression in endothelial cells than HDL_2[11]. In addition, it is known that HDL_3 subfraction exibits potent antioxidant activity[10].

Reactive oxygen species (ROS) play an important role in the development of CVD, including atherosclerosis and diabetes. The increase of the oxidant stress is due, in large part, to excess production of oxidant molecules, and to a decreased antioxidant capacity of the vasculature. NADPH oxidase (NADPHox), a multi-subunit enzyme located in the cytoplasm, is considered to be the main source of superoxid anion in the vasculature. Endothelial cell express this protein complex, $p22^{phox}$ being the main regulatory subunit. In addition, the NADPHox complex contains one of the different isoforms of catalytic subunits: NOX-1, NOX-2, NOX-4, and NOX-5. It is known that NOX-4 is highly expressed in HEC[12].

It is well-accepted that atherosclerosis is a chronic inflammatory disorder characterized by an accumulation of macrophages and T lymphocytes in the intima of medium and large arteries. The macrophages accumulating in the atherosclerotic plaques are derived mainly from blood monocytes that adhere to endothelial cells before migrating into the subendothelial space. Monocyte chemoattractant protein-1 (MCP-1), also known as CCL2, is a key chemokine implicated in the recruitment of circulating monocytes to the inflammation sites. MCP-1 is expressed in vessels from diabetic patients[13,14]. Increased levels of circulating MCP-1 are present in type1[15], type2[16], or gestational diabetes[17].

In a previous study, we demonstrated that irreversibly glycated LDL generate HEC dysfunction by augmenting the NADPHox activity and inducing the up-regulation of MCP-1 chemokine, by a mechanism involving NF-kB activation[18]. The aim of the present study was to determine the ability of HDL_3 to restore the endothelial cell function altered by the incubation with advanced glycation end products modified LDL (AGE-LDL).

To this purpose, we evaluated the effect of HDL_3 on the oxidative and inflammatory status of HEC incubated with AGE-LDL by measuring the NADPHox activity and the gene expression of $p22^{phox}$, NOX4 and MCP-1.

MATERIALS AND METHODS

LDL isolation and in vitro glycation

Plasma was obtained from the blood of healthy donors from the Haematology Center, Bucharest and LDL was isolated by ultracentrifugation in potassium bromide gradient density. LDL fraction was collected and extensively dialyzed over night, at 4°C, against phosphate buffer saline (PBS) supplemented with 2.5mM ethylenediaminetetraacetic acid (EDTA), pH 7.4. Native LDL (nLDL) was used within 1 week from its isolation. A fraction of LDL was non-enzymatically glycated by incubation of LDL with D(+) glucose, in a final concentration of 0.2 M, for 4 weeks at 37ºC, under sterile conditions. Incubation of LDL with D(+) glucose was done in the presence of antioxidants: 2.5 mM EDTA and 1 μM Butylated hydroxytoluene (BHT). The resulting product was characterized as AGE-LDL[19]. Prior to the incubation with cells, AGE-LDL was extensively dialyzed against PBS, pH 7.4 over night, at 4°C, to eliminate excess glucose.

HDL isolation

HDL_3 (with density between 1.125–1.21 g/ml) were isolated by sequential ultracentrifugation at 4°C from the plasma of healthy volunteers, as previously described[20].

Cell culture

A human umbilical vein endothelial cells line, EA.hy926, was employed. Cells were grown in Dulbecco's modified Eagle Medium (DMEM), supplemented with 5mM glucose and 10% fetal calf serum (FCS). At confluence, the cells were starved by incubation with DMEM without phenol red and ferrous nitrate, supplemented with 0.2% FCS and normal glucose concentration (5mM glucose). After 24h starvation, cells were incubated for another 24h with 100μg protein/ml nLDL or AGE-LDL in the presence or absence of 50 μg protein/ml HDL_3 in DMEM supplemented with 0.2 % FCS and 5mM glucose.

Determination of NADPH oxidase activity

After incubation, the NADPHox activity was determined in a HEC suspension by the lucigenin-enhanced chemiluminescence assay**,** using a low concentration of lucigenin to minimize artifactual O_2^- production due to redox cycling[21]. Briefly, the reaction started by addition of the cell suspension to the mixture (PBS without Ca or Mg ions,

1mol/l, pH 7.0, 5 µmol/L lucigenin, 100 µmol/L NADPH) and the light emission was recorded every second for 15 min in a luminometer (Berthold Detection Systems, Germany). The activity was expressed as relative light units (RLU)/mg protein.

Gene expression of $p22^{phox}$, NOX4 and MCP-1

Total RNA was isolated from the cells and 1 µg of the total RNA was reverse transcribed using commercial kits from Sigma (Sigma-Aldrich Co., St Louis, MO, USA), according to the manufacturer instructions. After the reverse transcription, the complementary DNA (cDNA) was subjected to amplification using specific primers for human $p22^{phox}$, NOX4, MCP-1 and β-actin (as internal standard), using an Applied Biosystem Real-Time PCR with StepOne soft. The specific primers used were: for $p22^{phox}$-5'-GTT TGT GTG CCT GCT GGA GT-3' (forward) and 5'-TGG GCG GCT GCT TGA TGG T-3' (reverse); NOX4 - 5'-TGGCTG CCCATCTGGTGAATG-3' (forward) and 5'-CAGCAGCCCTCCTGAAACATGC-3' (reverse); MCP-1 - 5'-AGC ATG AAA GTC TCT GCC GCC CTT CTG -3' (forward) and 5'-ATT ACT TAA GGC ATA ATG TTT CAC A-3' (reverse) and β-actin- 5'-GGG AAA TCG TGC GTG ACA TTA AG-3' (forward) and 5'- TGT GTT GGC GTA CAG GTC TTT G-3' (reverse).

The amplification products were detected by continuously monitoring the SyBr green fluorescence with high affinity for double stranded DNA. The quantification of the PCR products was performed by the "Fit Point Method". The cDNA was amplified through 40 cycles, with the annealing temperature at 60°C, followed by a melting curve program (from 55°C to 94°C, with fluorescence read at every 1°C), and finally a cooling step at 30°C. The results are presented as histograms, in which the gene expression of the targets are expressed relative to the values corresponding to nLDL, considered as controls.

Protein assay

Protein concentration of each sample was measured with a modified Lowry method, or BCA assay, using as standard bovine serum albumin.

Statistical analysis

Statistical evaluation was done by t-test with two tailed distribution and $p \leq 0.05$ was considered statistically significant. Data were expressed as means ± SD.

RESULTS

HDL_3 decrease NADPHox activity

After 24h incubation with AGE-LDL, the NADPHox activity increased significantly ($p<0.05$) in HEC, compared to cells incubated with nLDL (1.43 ± 0.13 *vs*. 1.00 ± 0.04). The concomitant presence of HDL_3 in the culture medium determined the decrease of NADPHox activity in cells incubated with AGE-LDL (0.87 ± 0.11 in the presence of HDL_3 *vs*. 1.43 ± 0.13 in the absence of HDL_3) (Fig.1)

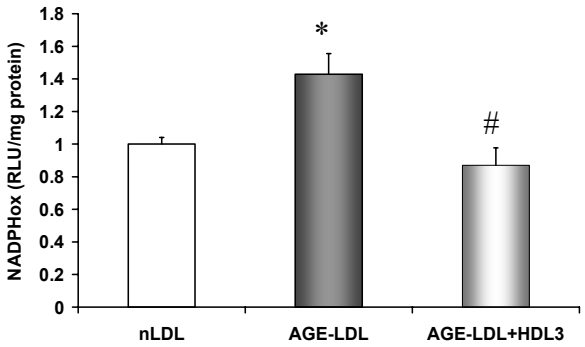

Fig. 1. NADPHox activity in EA.hy926 cells incubated with 100 ug/ml nLDL or AGE-LDL in the presence/absence of 50 ug/ml HDL_3. Data are expressed relative to nLDL (considered 1) and presented as means ± S.E.M., *$p<0.05$ for AGE-LDL *vs* nLDL, #$p<0.05$ for AGE-LDL+HDL_3 *vs*. AGE-LDL.

HDL_3 decrease the $p22^{phox}$ gene expression

To determine the molecular mechanism by which HDL_3 decrease the NADPHox activity in cells incubated with AGE-LDL, we evaluated by Real-Time PCR the mRNA levels of $p22^{phox}$ and NOX4, two of the NADPHox subunits. After 24h incubation with AGE-LDL, the gene expression of $p22^{phox}$ and NOX4 increased statistically significant compared to nLDL-treated cells (1.17± 0.05 *vs*. 1.00 ± 0.04, respectively 1.47 ± 0.15 *vs*. 1.00 ± 0.16) (Fig 2 and Fig 3). The presence of HDL_3 determined a statistically significant decrease of $p22^{phox}$ subunit gene expression (Fig. 2). NOX4 gene expression was not modified by HDL_3, although a tendency of decrease was observed (Fig. 3).

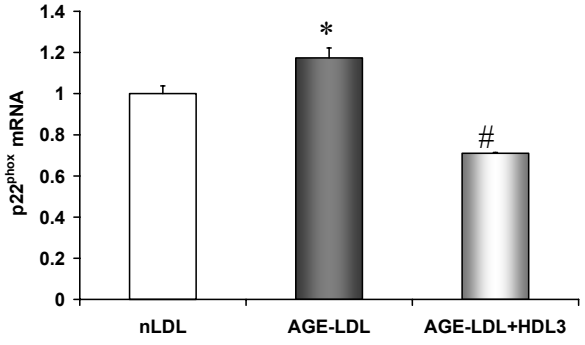

Fig. 2. Quantification of $p22^{phox}$ gene expression in EA.hy926 cells incubated with nLDL or AGE-LDL in the presence or absence of HDL_3. Data are expressed relative to nLDL (considered 1) and presented as means ± S.E.M., *$p<0.05$ for AGE-LDL *vs.* nLDL, #$p<0.05$ for AGE-LDL+HDL_3 *vs.* AGE-LDL.

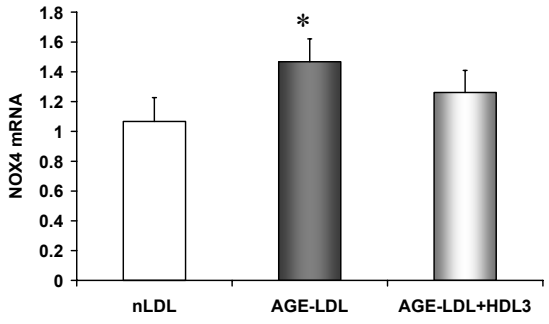

Fig. 3. Quantification of NOX4 gene expression in EA.hy926 cells incubated with nLDL or AGE-LDL with/without HDL_3. Data are expressed relative to nLDL (considered 1) and presented as means ± S.E.M., *p<0.05 for AGE-LDL *vs* nLDL.

HDL_3 decrease the MCP-1 gene expression

Incubation of EA.hy926 cells with AGE-LDL determined a notable increase of MCP-1 gene expression as compared with nLDL incubated cells (2.02 ± 0.16 *vs.* 1.00 ± 0.067). Co-incubation with HDL_3 determined a statistically significant decrease of MCP-1 mRNA compared to AGE-LDL incubated cells (1.30 ± 0.15 in the presence of HDL_3 *vs.* 2.02 ± 0.16 in the absence of HDL_3) (Fig.4).

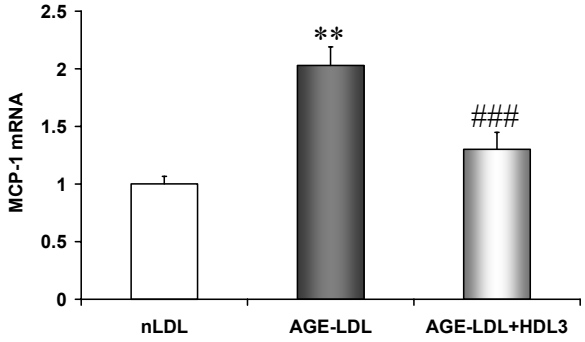

Fig. 4. MCP-1 gene expression determined by Real-Time PCR in EA.hy 926 cells incubated with nLDL or AGE-LDL in the presence or absence of HDL_3. Data are expressed relative to nLDL (considered 1) and presented as means ± S.E.M., **p<0.01 for AGE-LDL *vs*. nLDL, $^{###}$p<0.001 for AGE-LDL+ HDL_3 *vs* AGE-LDL.

DISCUSSION

Despite advances made in the prevention and management of cardiovascular diseases, people with diabetes mellitus continue to have an alarmingly high morbidity and mortality due to cardiovascular complications. Beside alterations of the lipid profile, it was shown that various components of plasma are modified by glycation in diabetes due to the high plasma glucose concentration. Glycated LDL is a modified form of LDL which circulates in the plasma of diabetic patients [22,23]. Existing data demonstrate that glycated LDL levels are increased in hyperlipidemic patients and further increased in hyperlipidaemic-diabetic patients, being positively correlated with the plasma glucose, total cholesterol, and LDL cholesterol levels, and negatively with HDL-cholesterol levels.

The data presented in this study demonstrate that HDL_3 inhibits NADPHox activity in human endothelial cells exposed to AGE-LDL. We assume that the decrease in NADPHox activity might be induced by the decrease of $p22^{phox}$ gene expression. The effect of HDL on NADPHox activity was very recently demonstrated using a diabetic rat model[24]. Using ApoAI gene transfer, Van Linhout *et al.* showed that the increase in HDL-cholesterol levels is associated with the decrease of NADPHox activity and of the gene expression of $p22^{phox}$ and NOX4 in the aorta of the diabetic rat. It was also demonstrated, that HDL inhibits NADPHox activity in vascular smooth muscle cells in culture activated with thrombin[25].

In this study, we demonstrated that HDL_3 inhibit MCP-1 gene expression in human endothelial cells incubated with AGE-LDL. Our results add to the studies of Mackness *et al*.[26] who demonstrated that HDL is capable to inhibit MCP-1 expression in HEC incubated with oxidized LDL by a mechanism dependent on paraoxonase-1. In addition, Pirillo A *et al.* demonstrated that HDL_3 (100ug/ml) determine the downregulation of MCP-1 gene expression in cultured endothelial cells stimulated with TNF-alpha[27].

In conclusion, the present study demonstrates that the HDL_3 subfraction can restore the proper endothelial function by decreasing the oxidative and inflammatory stress. Thus, the decreased levels of HDL, characteristic for the diabetic condition, are a good therapeutic target ment to impede the accelerated atherosclerosis complication of diabetes mellitus.

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