Biochemical and Biophysical Research Communications 428 (2012) 230–233

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Upregulation of lectin-like oxidized low density lipoprotein receptor 1 (LOX-1) expression in human endothelial cells by modified high density lipoproteins

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ARTICLE INFO

Article history:
Received 2 October 2012
Available online 13 October 2012

Keywords:
Endothelial cells
High density lipoprotein
15-Lipoxygenase
LOX-1

ABSTRACT

Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) is the main endothelial receptor for oxidized low density lipoprotein (OxLDL). LOX-1 is highly expressed in endothelial cells of atherosclerotic lesions, but also in macrophages and smooth muscle cells. LOX-1 expression is upregulated by several inflammatory cytokines (such as TNF-α), by oxidative stress, and by pathological conditions, such as dyslipidemia, hypertension, and diabetes.

High density lipoprotein (HDL) possess several atheroprotective properties; however under pathological conditions associated with inflammation and oxidative stress, HDL become dysfunctional and exhibit pro-inflammatory properties. *In vitro*, HDL can be modified by 15-lipoxygenase, an enzyme overexpressed in the atherosclerotic lesions. Here we report that, after modification with 15-lipoxygenase, HDL_3 lose their ability to inhibit TNFα-induced LOX-1 expression in endothelial cells; in addition, 15LO-modified HDL_3 induce LOX-1 mRNA and protein expression and bind to LOX-1 with increased affinity compared to native HDL_3. Altogether these findings confirm that 15LO-modified HDL_3 possess a pro-atherogenic role.

1. Introduction

Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) is the main endothelial receptor for oxidized low density lipoprotein (OxLDL) [1]. Endothelial dysfunction induced by Ox-LDL, whose presence in the plasma of subjects with atherosclerosis-related diseases has been widely confirmed [2,3], is considered a key process in the pathogenesis of atherosclerosis [4,5]. LOX-1 has been detected in atherosclerotic plaques, where it is overexpressed by endothelial cells but also by other cell types, including macrophages and smooth muscle cells [6,7]. The binding of OxLDL to LOX-1 initiates multiple intracellular signaling cascades that, in turn, induce endothelial damage and dysfunction, promote foam cell formation, and support migration, proliferation and transformation of smooth muscle cells [7]. Several factors can upregulate *in vitro* and *in vivo* LOX-1 expression, including pro-inflammatory cytokines (such as TNF-α and IL-1ß), reactive oxygen species and pathological conditions, such as dyslipidemia, hypertension, and diabetes [8–10].

The contribution of LOX-1 to the pathogenesis of atherosclerosis is demonstrated in transgenic and knockout mice models. LOX-1 knockout mice fed a high cholesterol diet have a reduced binding of OxLDL to the aortic endothelium, with a consequent preserved endothelial function [11]; similarly, the double knockout LOX-1/LDLR had a reduced atherogenesis and very low levels of inflammatory markers compared with LDLR knockout mice [11]. On the contrary, LOX-1 transgenic mice showed a significant increase in lesion area [12]. Several observations suggest also an involvement of LOX-1 in the destabilization and rupture of atherosclerotic plaques [13–15].

High density lipoprotein (HDL) exhibit a protective activity toward the vascular endothelium, as they have anti-oxidant, anti-inflammatory and anti-thrombotic properties [16]; HDL stimulate nitric oxide production [17] and promote endothelial cell migration and re-endothelialization through SR-BI (scavenger receptor class B type I), the main HDL receptor [18]. However, under pathological conditions associated with inflammation and oxidative stress, HDL lose their anti-atherogenic functions [19,20], becoming dysfunctional and exhibiting pro-inflammatory characteristics.

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.10.020

We have previously shown that *in vitro* HDL can be modified by 15-lipoxygenase (15LO), an enzyme overexpressed in the atherosclerotic lesions [21,22], thus resulting in a dysfunctional lipoprotein with impaired ability to promote cholesterol efflux from macrophages [23] and to protect endothelial cells from inflammation [24]. Here we report the effect of 15LO-mediated modification of HDL_3 on LOX-1 expression in the endothelial cells. Furthermore, as the ligand-LOX-1 interaction can contribute to a switch of endothelial cell phenotype to a pro-atherogenic state, we studied the possible interaction of 15LO-modified HDL_3 with LOX-1.

2. Materials and methods

2.1. Materials

MEM, fetal bovine serum (FBS), bovine serum albumin (BSA), penicillin–streptomycin, glutamine, TNF-α, DiO (3,3′-dioctadecyloxacarbocyanine perchlorate) were from Sigma–Aldrich (St. Louis, MO, USA); PD10 columns and ECL were from Amersham Biosciences (Uppsala, Sweden); endothelial cell growth factor (ECGF) was from Boehringer Mannheim. Antibodies were as follows: anti-LOX-1 from R&D Systems, anti-ß-actin and anti-mouse IgG peroxidase-conjugate from Sigma–Aldrich.

2.2. Cell culture

HUVEC were isolated according to established procedures [25] and cultured in the medium M199 supplemented with 20% FBS, ECGF (20 μg/ml), heparin (15 U/ml), penicillin–streptomycin (1%) and glutamine (1%). Cells were used between the 3rd and 5th *in vitro* passage. Wild type and LOX-1-overexpressing EA.hy-926 cells [26] were grown in MEM containing 10% FBS, 1% streptomycin, 1% penicillin, 2% tricine, 1% glutamine, 1% non-essential amino-acids and 1% HAT.

2.3. Isolation of plasma lipoproteins

The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki. HDL_3 (d = 1.125–1.21 g/ml) was isolated from fresh plasma of normolipidemic healthy volunteers by sequential ultracentrifugation [27]. Protein content was determined by the method of Lowry using BSA as standard [28]. Modification with 15-lipoxygenase was carried out as described [23,24].

For the lipid labeling, native and 15LO-modified HDL_3 were incubated with the fluorescent dye DiO (300 μg DiO/mg HDL_3 protein) for 18 h at 4 °C, passed on a PD10 column to remove excess unbound DiO, then centrifuged in a TL100 centrifuge at d = 1.21 g/ml for 4.5 h at 4 °C. DiO-labeled lipoproteins were then passed through a PD10 column and protein content was determined by the method of Lowry.

2.4. Real time quantitative PCR (RT-PCR)

Total RNA was extracted and reverse transcribed [29]. Three microliters of cDNA were amplified by real-time quantitative polymerase chain reaction (PCR) with 1× SYBR green universal PCR mastermix (BioRad) [30]. The sequences of the primers used for amplification were as follows: RLP-13A (housekeeping gene), 5′-TAGCTGCCCCACAAAACC-3′ (fw) and 5′-TGCCGTCAAACACCCTTGAGA-3′ (rev); LOX-1: 5′-GAGAGTAGCAAATTGTTCAGCTCCTT-3′ (fw) and 5′-GCCCGAGGAAAATAGGTAACAGT-3′ (rev). Each sample was analyzed in duplicate using the IQ™-Cycler (BioRad). For quantification, the target sequence was normalized to the RLP-13A content.

2.5. Immunoblotting

To analyze the expression of LOX-1, cell proteins were separated on a 10% SDS–PAGE, then transferred onto a nitrocellulose membrane. Protein expression was analyzed by immunoblotting using a mouse anti-human LOX-1 antibody (1:1000); a mouse anti-ß-actin antibody (1:1000) was used to normalize the protein loading. After incubation with an anti-mouse IgG peroxidase-conjugated as secondary antibody, immuno-complexes were detected by ECL followed by autoradiography.

2.6. Lipoprotein-cell association studies

For lipoprotein-cell association studies, wild type and LOX-1-overexpressing EA.hy cells were incubated at 37 °C for 1 h with DiO-labeled 15-LO-HDL_3. Cells were then washed with PBS, detached by trypsinization, fixed in 1% paraformaldehyde and immediately subjected to fluorescence flow cytometry using a FACScan (Becton Dickinson). For each sample 10.000 events were analyzed; data were processed using the CellQuest program (Becton Dickinson).

2.7. Measurement of ICAM-1 expression at the cell surface

EA.hy-LOX-1 cells were incubated for 18 h in the presence of native or 15LO-modified HDL_3 (100 μg/ml). At the end of the incubation, cells were harvested by trypsinization, washed in PBS-BSA (1%) and incubated for 20 min at 4 °C with an anti-CD54 (ICAM-1) monoclonal antibody, followed by incubation for 20 min at 4 °C with a goat anti-mouse IgG-FITC, as described [26]. After washing, antigen expression was measured by flow cytometry (FACScan, Becton Dickinson). A total of 10.000 events were analyzed; data were processed using the CellQuest program.

2.8. Statistical analysis

Values are expressed as mean ± S.D. The statistical significance of the differences between groups was determined by the Student's t-test and values of $P < 0.05$ were considered to be significant.

3. Results and discussion

LOX-1 mediates some effects induced by oxidized LDL in endothelial cells as well as in other cell types [7]. LOX-1 expression is upregulated by a number of inflammatory and pro-atherogenic stimuli [8–10]. As TNFα is a well-studied inducer of this scavenger receptor [31], we tested the ability of 15LO-HDL_3 to modulate the expression of LOX-1 expression at the mRNA level in endothelial cells exposed to TNFα. While native HDL_3 significantly reduced the TNFα-induced LOX-1 mRNA expression, 15LO-HDL_3 had no inhibitory effect (Fig. 1), thus reinforcing the concept that modification impaired the protective function of HDL_3.

We previously showed that modification of HDL_3 with 15LO not only reduces the lipoprotein ability to inhibit TNFα-induced adhesion molecule expression, but also confers pro-atherogenic properties to HDL_3, as the modified lipoprotein induces adhesion molecule expression [24]. Thus, we studied the effect of 15LO-HDL_3 on LOX-1 mRNA expression in endothelial cells. As expected, while native HDL_3 had no effect on the transcription of LOX-1 gene, 15LO-HDL_3 significantly increased the expression of LOX-1 mRNA, strengthening the finding that modification of HDL_3 with 15LO confers pro-inflammatory properties to this class of lipoproteins (Fig. 2).

We then analyzed the expression of LOX-1 at the protein level. Western blotting analysis of cells incubated with lipoproteins for

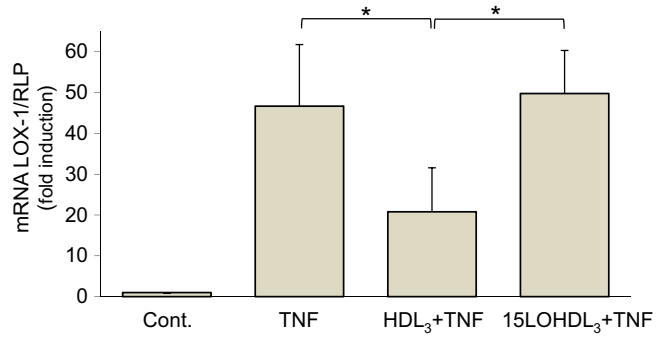

Fig. 1. 15LO-modified HDL_3 failed to inhibit TNFα-induced LOX-1 mRNA expression. HUVEC were pre-incubated with HDL_3 or 15LO-modified HDL_3 (100 μg/ml) for 6 h, then exposed to 10 ng/ml TNF-α for 18 h. Total mRNA was isolated and the expression of LOX-1 was evaluated by real time PCR. RLP13A was used as an internal control. Results are given as mean ± SD from 4 independent experiments. $^*P < 0.05$.

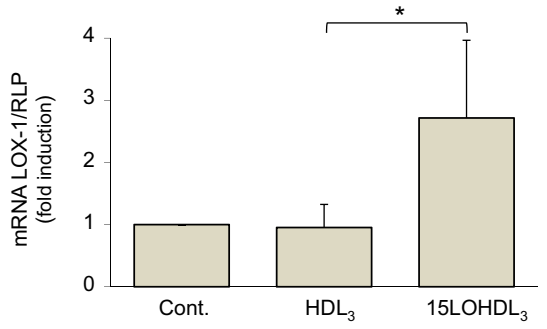

Fig. 2. 15LO-modified HDL_3 induced LOX-1 mRNA expression. HUVEC were incubated with HDL_3 or 15LO-modified HDL_3 (100 μg/ml) for 18 h. Total mRNA was isolated and the expression of LOX-1 was evaluated by real time PCR. RLP13A was used as an internal control. Results are given as mean ± SD from 6 independent experiments. $^*P < 0.005$.

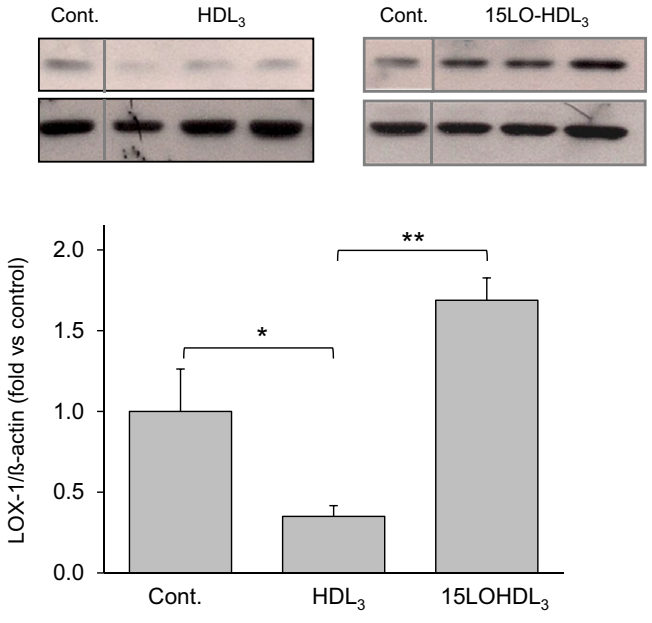

Fig. 3. 15LO-modified HDL_3 induced LOX-1 protein expression. HUVEC were incubated with HDL_3 or 15LO-modified HDL_3 (100 μg/ml) for 24 h. LOX-1 protein expression was evaluated by Western blotting. $^*P < 0.05$; $^{**}P < 0.00005$.

24 h showed that 15LO-HDL_3 significantly increased the expression of LOX-1 level, when compared to native HDL_3 (Fig. 3).

We previously showed that after modification with 15LO, LDL were not efficiently recognized by the LDL receptor, while the binding to LOX-1 increased sharply [26], thus suggesting that 15LO-LDL could be a ligand for LOX-1. To investigate whether LOX-1 might also be a receptor for 15LO-HDL_3, a human LOX-1-overexpressing cell line generated by infection of EA.hy-926 cells with a plasmid encoding for human LOX1 was used for lipoprotein-cell association studies [26]. The association of DiO-labeled 15LO-HDL_3 was higher in LOX-1 overexpressing cells, compared to wild type EA.hy cells (Fig. 4A). This finding is in agreement with the previous observation that LOX-1 is a possible receptor for hypochlorite-modified HDL_3 [32].

To evaluate whether the increased binding of 15LO-HDL_3 to LOX-1 might translate into a pro-atherogenic response, the surface expression of ICAM-1 was assessed in LOX-1-overexpressing cells exposed to native or modified HDL_3. We found that 15LO-HDL_3 increased ICAM-1 surface expression, while native HDL_3 did not (Fig. 4B). This finding suggested that LOX-1 might mediate some deleterious effects of 15LO-HDL_3 in endothelial cells.

In summary, here we identified 15LO-modified HDL_3 as a new inducer and ligand for LOX-1; the main finding of our study is that 15LO-mediated modification of HDL_3 impairs the ability of the lipoprotein to protect endothelial cells from TNFα-induced LOX-1 expression, and at the same time 15LO-modified HDL_3 induce the expression of LOX-1 in endothelial cells. Finally, while 15LO-modified HDL_3 interacted less efficiently with the main HDL receptor, SR-BI [23], LOX-1 was identified as a possible receptor for 15LO-

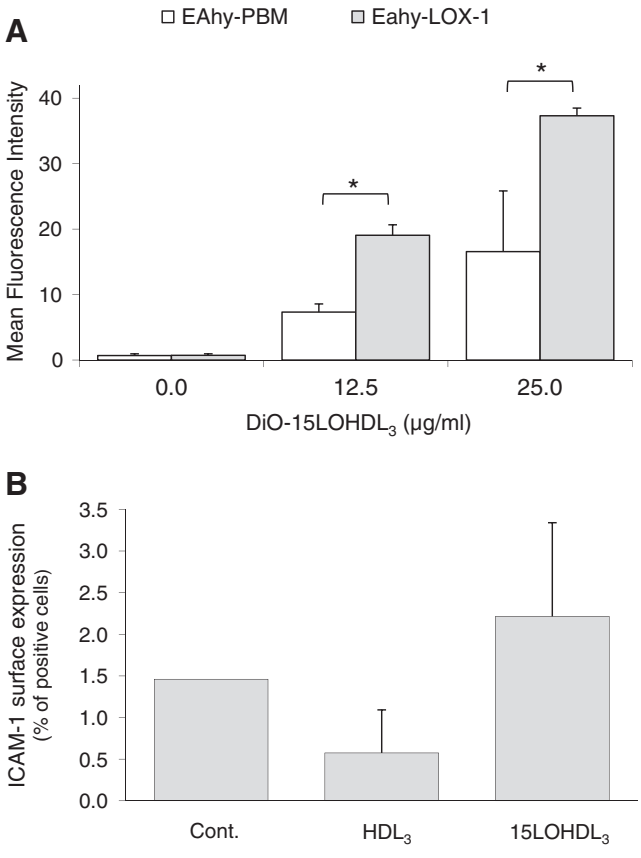

Fig. 4. Interaction of 15LO-HDL_3 with LOX-1. (A) Wild type or LOX-1-overexpressing EA.hy cells were incubated with DiO-labeled 15LO-HDL_3 (12.5 and 25 μg/ml) for 1 h at 37 °C. Cell-associated fluorescence was evaluated by flow cytometry. Data are mean ± SD of 3 independent experiments performed in duplicate. $^*P < 0.005$. (B) ICAM-1 surface expression was determined by FACS analysis in EA.hy-LOX-1 cells exposed to 100 μg/ml HDL_3 or 15LO-HDL_3 for 18 h.

modified HDL_3 and was involved in the induction of ICAM-1 expression. Altogether these findings confirm that modifications induced by 15LO reduce the anti-inflammatory properties of HDL_3 while conferring pro-inflammatory characteristics, contributing to the activation and dysfunction of endothelial cells.

Conflict of interest

The authors have no financial conflict of interest regarding this work.

Acknowledgement

This work was supported by the SISA (Società Italiana per lo Studio dell'Aterosclerosi, Lombardia section).

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