SPANDIDOS PUBLICATIONS

Oxidized-HDL_3 modulates the expression of Cox-2 in human endothelial cells

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Abstract. Modified high density lipoprotein (HDL) has been suggested to modulate endothelial expression of pro-inflammatory genes. Since oxidised HDL (Ox-HDL) has been found in atheromatous plaques and receptors for modified HDL are present on endothelial cells, we investigated the effect of Ox-HDL_3 on the expression of Cox-1 or Cox-2. Ox-HDL_3, increased Cox-2 mRNA and protein expression in endothelial cells while no effect on Cox-1 expression was observed. The intracellular pathways involved in this effect were investigated. The incubation with specific inhibitors of intracellular kinases showed that PI3K is mainly involved in the Ox-HDL_3-dependent Cox-2 induction. Transient transfection experiments suggested that the NF-IL6 response element in the proximal promoter (-327 to 59) is involved in Ox-HDL_3-mediated Cox-2 expression. These data suggest that Ox-HDL induce Cox-2 expression in endothelial cells through a PI3K/NF-IL6-dependent pathway.

Introduction

Numerous clinical and epidemiological studies have demonstrated the inverse relationship between HDL cholesterol and the risk of atherosclerosis (1). Beyond the ability of HDL to remove cholesterol from peripheral tissue, HDL particles have additional beneficial effects on the vascular wall (1-3). HDL can inhibit the chemotaxis of monocytes, the adhesion of leukocytes to the endothelium, the LDL oxidation, the endothelial dysfunction and apoptosis. HDL can also act on the vascular tone through the release of vasorelaxant molecule like NO and prostacyclin (PGI2) (3). We have demonstrated previously that HDL increases the release of PGI2 through the induction of cyclooxygenase 2 (Cox-2) and the coupling with PGI syntase in endothelial cells (4). It has been shown that modification of HDL could affect their function (5,6). Oxidised (Ox-) and modified HDL are present in atherosclerotic plaques (7-9) and human analogues of SR-B1 and LOX-1 have been identified as receptors for modified HDL on endothelial cells. *In vitro*, HDL is readily modified using a variety of oxidants (10), with kinetics similar to that reported for LDL (11). Ox-HDL loses its ability to promote cholesterol efflux (5), to induce nitric oxide release (12) and to modulate the expression of matrix-degrading protease and PAI-1 in endothelial cells (13,14).

In the present study, we have investigated the effect of Ox-HDL_3 on the expression of Cox-2 in endothelial cells and the molecular mechanisms involved.

Materials and methods

Materials. HDL subfraction 3 (d 1.125-1.21 g/ml) was obtained from freshly isolated human plasma by preparative ultracentrifugation and dialyzed in PBS containing 0.01% EDTA (15). HDL_3 (1 mg protein/ml) was oxidized with 20 μM Cu_2SO_4 for 24 h at 37˚C, as described (13). The oxidation was blocked by the addition of 40 μM butylated hydroxytoluene (BHT). Under these conditions, we have previously shown that lipoprotein oxidation does not proceed further at 4˚C. The LPS content of HDL_3 was measured using an endotoxin kit from Sigma. No contamination was detected (data not shown). Native HDL_3 and Ox-HDL_3 were used within 6 h of preparation.

The MEK inhibitor, U0126 (New England Biolabs, Germany), the p38 MAPK inhibitor SB 203580 (Sigma, Italy), and the PI3K inhibitor Ly 294002 (Alexis, Italy) were used at a final concentration of 10 μmol/l, 0.5 μmol/l and 50 μmol/l, respectively (15). At these concentrations the inhibitors effectively decreased the phosphorylation of the downstream targets (data not shown).

HUVECs were isolated as described (16) and cultured under standard conditions in medium M-199 containing 20% FCS (fetal calf serum), heparin (15 U/ml) and ECGF (endothelial cell growth factor, 20 μg/ml) (Roche, Italy). The cells

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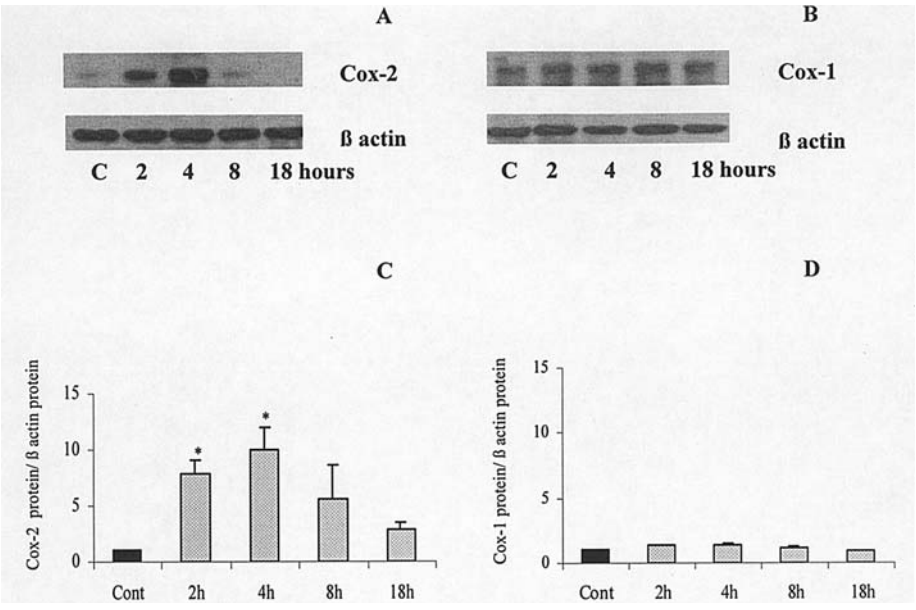

Figure 1. Effect of Ox-HDL_3 on Cox-2 and Cox-1 protein expression in endothelial cells. HUVECs were incubated with Ox-HDL_3 30 μg/ml for 2, 4, 8, 18 h. Cells were lysed and the lysates were analysed by immunoblotting using anti Cox-2 (A) and anti Cox-1 (B). The blot was stripped and reprobed with anti ß-actin to confirm equal expression (A and B). Results from 3 different experiments are presented in (C) and (D) (mean ± SD; $^*p<0.05$ vs control cells).

were used within the 4th passage. Cells were plated in 6-well plates and used after 48 h as subconfluent cultures. In all experiments, cells were preincubated with serum-free medium for 6 h, and then native or Ox-HDL_3 were added for different times. Cells were incubated in the presence or absence of compounds with appropriate chemicals or vehicle additions (DMSO, 0.1% vol/vol).

Real-time quantitative RT-PCR. Total RNA was extracted and underwent reverse transcription as described (4,17). Three μl of cDNA was amplified by real-time quantitative PCR with 1X SYBER Green universal PCR mastermix (Bio-Rad) (4). The specificity of the SYBER Green fluorescence was tested by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon. The melting peaks of the amplicons were as expected (data not shown). The primers used, the amplicon size and the melting temperature have been described previously (4). Each sample was analyzed in duplicate using the IQ™-Cycler (Bio-Rad). The PCR amplification was related to a standard curve ranging from 10^{-11} M to 10^{-14} M.

Immunoblotting. Cox-1 and Cox-2 expression was investigated as described (18). Briefly, cells were plated in 6-well plates and treated with Ox-HDL_3 or native HDL_3 for 5-40 min, then lysed using a Tris-glycine buffer (0.25 M Tris, 0.173 M glycine) containing 3% SDS and 1 mM PMSF (phenylmethylsulfonil fluoride). Aliquots of the samples (15 μg) were diluted in a 2% ß-mercaptoethanol buffer containing glycerol and bromophenol blue and electrophoresed on a 12% SDS-PAGE, then transferred onto a nitrocellulose membrane using a Trans Blot Cell (Hoefer Scientific Instrument, San Francisco, CA) (19). The membrane was saturated at room temperature in PBS containing 3% BSA for 1 h, washed with PBS-T (PBS containing 0.1% Tween-20), then incubated overnight at 4˚C with a mixture of primary antibody (1:1000 incubation at room temperature for 1 h for Cox-1 and Cox-2 antibodies;

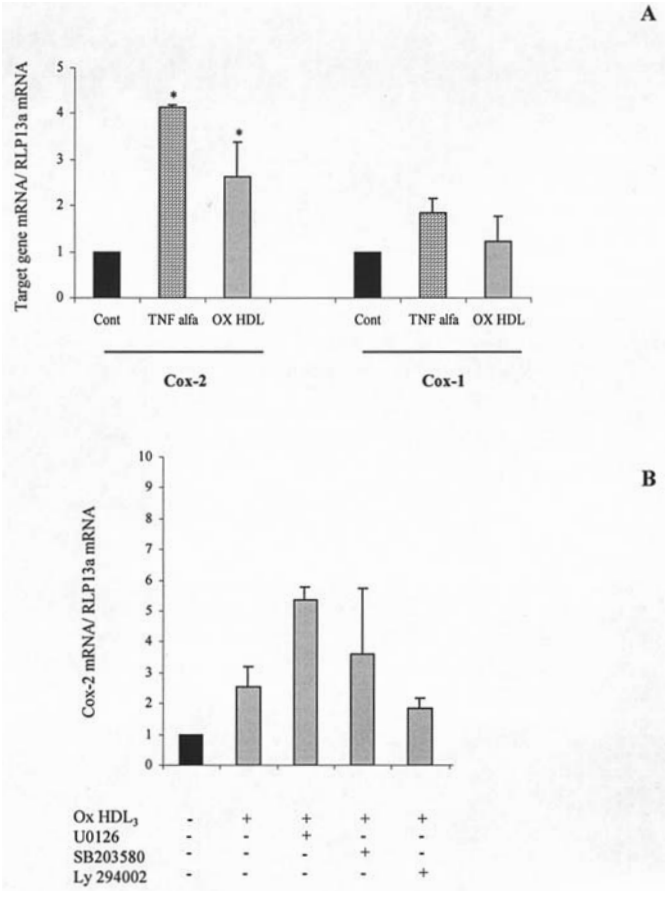

Figure 2. Effects of kinase inhibitors on Ox-HDL_3 induced Cox-2 mRNA expression in endothelial cells. (A) HUVECs were incubated for 2 h for mRNA detection with Ox-HDL_3 (30 μg/ml), or TNF-α (10 ng/ml) used as positive control. Cox-2 and Cox-1 mRNA expression was measured by quantitative real-time PCR and normalised to RLP13a mRNA expression. (B) HUVECs were incubated for 2 h with Ox-HDL_3 (30 μg/ml) alone or in presence of U0126 (10 μM) or SB 203580 (0.5 μM), Ly 294002 (50 μM). Cox-2 mRNA expression was measured by quantitative real-time PCR and normalised to RLP13a mRNA expression. The mean ± SD from 3 different experiments is shown. $^*p<0.05$ vs control cells.

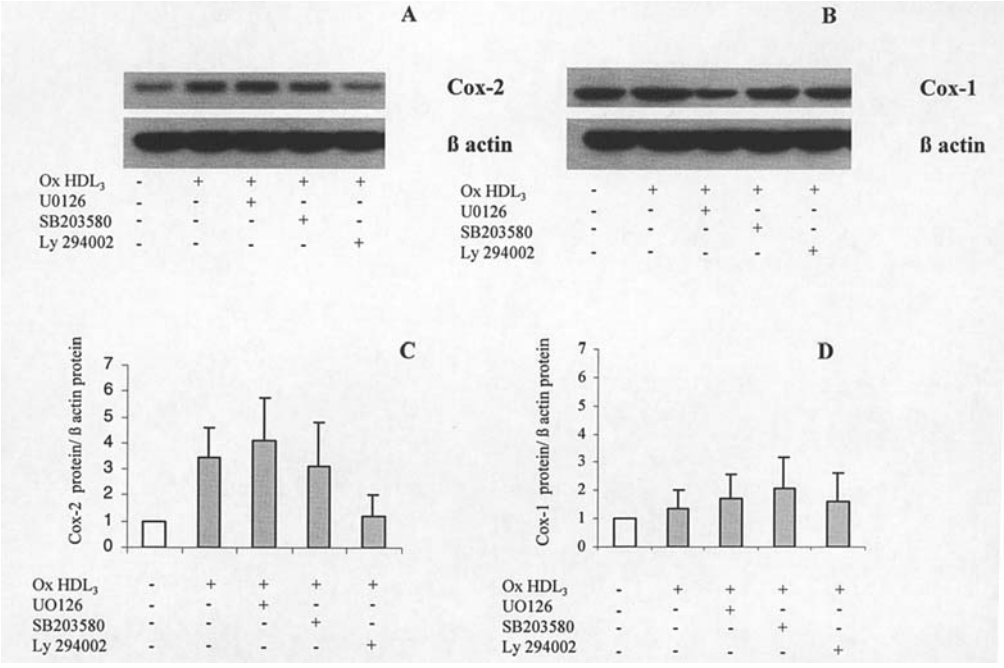

Figure 3. Effects of kinase inhibitors on Ox-HDL_3 induced Cox-2 and Cox-1 protein expression in endothelial cells. HUVECs were incubated for 6 h with Ox-HDL_3 (30 μg/ml) alone or in presence of U0126 (10 μM) or SB 203580 (0.5 μM), Ly 294002 (50 μM) and SH-5 (10 μM). Cells were lysed and the lysates were analysed by immunoblotting using anti Cox-2 (A) and anti Cox-1 (B). The blot was stripped and reprobed with anti ß-actin to confirm equal expression (A and B). Results from 3 different experiments are presented in (C) and (D) (mean ± SD).

1:1000 incubation at room temperature for 1 h for ß-actin antibody) followed by a 1:1000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma) or peroxidase-conjugated anti rabbit IgG (Bio-Rad, Italy). Immunocomplexes were detected by an enhanced chemiluminescence method (ECL, Amersham, Italy), followed by autoradiography and quantified by the Image program (NIH 1.52).

Transfection assay. Transient transfection experiments were first performed using HUVECs and EAhy 926 cells; however, the efficiencies reached were very low with a high degree of cytotoxicity (data not shown). As human Cox-2 promoter regulation is similar in a wide number of cell types we performed transfection experiments in CHO cells, a cell line widely used for studies involving the effects of HDL *in vitro* (4,20). CHO cells were transiently transfected with Cox-2 (nucleotide -327/+59), the NF-κB mutated site (KBM) or the NF-IL6 mutated site (ILM) luciferase reporter vectors using a calcium phosphate precipitation method as described (4). ß-galactosidase activity was assayed as described (14). Luciferase activity was determined and normalized to the ß-gal activity of the cotransfected pSV-ß-galactosidase construct.

Statistical analysis. Data presented in the text and figures are mean ± SD and are representative of 4 different experiments. Statistical analysis was performed by ANOVA with the use of Statsoft Statistica Package.

Results

The experimental set-up was designed to analyse endothelial cell expression of Cox-1 and Cox-2 in the presence of Ox-HDL_3 in relation to basal conditions. The cells were kept in a serum-free medium for 6 h; then HDL_3 (30 μg/ml) or Ox-HDL_3 (30 μg/ml) were added for 4 h. Control cells were incubated for 4 h with the experimental medium containing the same percentage of PBS that was added with the stimulus.

Cox-2 protein was expressed at low levels in unstimulated cells and was strongly induced 2 h after exposure to Ox-HDL_3 (30 μg/ml); the induction was maximal after 4 h and decreased after 8 h (Fig. 1). Cox-2 expression in unstimulated cells remained low at all time points (data not shown). Ox-HDL_3 did not affected Cox-1 protein expression (Fig. 1). Next we examined the effects of Ox-HDL_3 on the Cox-1 and Cox-2 mRNA expression. The cells were kept in a serum-free medium for 6 h; then Ox-HDL_3 (30 μg/ml) or TNF-α (10 ng/ml), used as positive control, were added for 2 h. We observed the induction of Cox-2 mRNA expression by Ox-HDL_3 (30 μg/ml) or TNF-α (10 ng/ml), while no effect was observed on Cox-1 mRNA expression.

To investigate the role of ERK1/2, p38 MAPK and PI3K/Akt pathways in Cox-2 expression induced by Ox-HDL_3, cells were preincubated with the MEK1 inhibitor U0126, the p38 MAPK inhibitor SB 203580 and the PI3K inhibitor Ly 294002 for 1 h; Ox-HDL_3 (30 μg/ml) were then added for 2 and 4 h to evaluate Cox-2 mRNA and protein expression (Figs. 2 and 3). U0126 was unable to block Ox-HDL-induced Cox-2 expression. SB 203580 partially inhibited Ox-HDL-induced Cox-2 expression while Ly 294002 completely abolished Ox-HDL-induced Cox-2 mRNA expression and partially inhibited protein expression. No effect of SB 203580 and Ly 294002 was observed on Cox-1 protein expression.

Next we examined the effects of Ox-HDL on the Cox-2 promoter activity. The human Cox-2 promoter region (-327/+59) contains the NF-κB, the NF-IL6 and the CRE sites

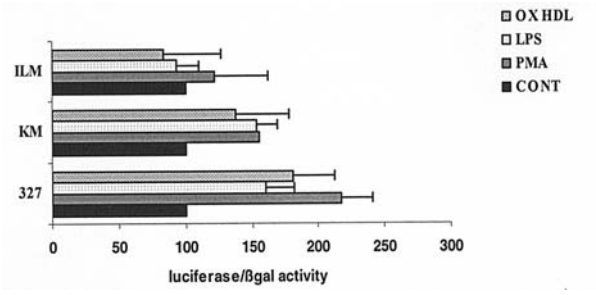

Figure 4. Identification of the regions responsible for Ox-HDL_3-induced promoter activity of the human Cox-2 gene. Following transfection, CHO cells were incubated for 6 h with LPS (1 μg/ml) or PMA (50 ng/ml) both used as positive control, and with Ox-HDL_3 (30 μg/ml). The results are presented as relative luciferase activity normalized to ß-galactosidase activity. Each experiment was carried out in triplicate.

(4,18). Transient transfection assay showed that Ox-HDL induced promoter activity by 199±27% (Fig. 4). PMA, used as positive control, induced promoter activity by 218±35% while LPS induced promoter activity by 160±29% (Fig. 4). Upon incubation with Ox-HDL, the promoter activity of the construct carrying the mutation at the NF-κB site was 140±48%, that of the construct carrying the mutation at the NF-IL6 site was 71±26% (Fig. 4). Upon incubation with PMA the promoter activity of the construct carrying the mutation at the NF-κB site was 155±14%, that of the construct carrying the mutation at the NF-IL6 site was 93±13% (Fig. 4); finally upon incubation with LPS the promoter activity of the construct carrying the mutation at the NF-κB site was 156±24%, that of the construct carrying the mutation at the NF-IL6 site was 83±32% (Fig. 4), thus suggesting a major role of the NF-IL6 site on the effect observed.

Discussion

Several lines of evidence have accumulated indicating that oxidative modification of HDL can occur *in vivo* (10,11,21,22). Furthermore, investigations using the Ox-HDL-specific 9F5-3a antibody have indicated the presence of Ox-HDL in the intima of atheromatous plaques in the human abdominal aorta located specifically in aortic endothelial cells (7), and in sera from patients with chronic renal failure (23). In addition, oxidative modification of HDL not only attenuates its beneficial properties, such as stimulation of cholesterol efflux from foam cells (21), endothelium-dependent vasoreactivity (24) and anti-oxidative activity (10), but also generates a pro-atherogenic species that inhibits nitric oxide synthesis in endothelial cells (25) and induces production of reactive oxygen species and apoptosis via NF-κB activation (26). Several genes involved in the inflammatory response observed during atherogenesis are modulated through the activation of the NF-κB pathway, including Cox-2 (27).

Here we demonstrated that Ox-HDL could play an harmful role by inducing Cox-2 expression. This effect was specific for Cox-2 as no effect on Cox-1 induction was observed and is dependent upon the activation of intracellular signalling pathways regulating the transcription machinery. We have previously shown that Ox-HDL could modulate PAI-1 expression through the activation of the p38 MAPK pathway promoting RNA stabilization (14). For Ox-HDL-induced Cox-2 expression the p38 MAPK or the ERK1/2 pathway seems to play a minor role, while the activation of the PI3K pathway is fundamental for Ox-HDL-dependent Cox-2 expression in agreement with previous observation with a different experimental setting (28).

To determine the specific transcription factor that is involved in the Ox-HDL mediated Cox-2 expression, we examined the effects of Ox-HDL on the luciferase activity of plasmids containing the Cox-2 promoter defective in the NF-κB or the NF-IL6 binding site (4,18).

Our data clearly demonstrated that the NF-IL6 site is implicated is the response observed. NF-IL6 is a member of the C/EBP family of transcription factors and is involved in inducing several acute-phase protein genes in response to immune and inflammatory stimulation. NF-IL6 also plays a mayor role in inducing the expression of Cox-2 by cytokines and endotoxin (29) thus suggesting that Cox-2 induction by Ox-HDL via NF-IL6 could depend on a pro-inflammatory activated endothelium.

What are the physiological implication(s) of our data? The possibility that Cox-2 plays a harmful role by catalysing the biosynthesis of pro-inflammatory prostanoids has been suggested. Cox-2 immunostaining has been observed in macrophages/foam cells, intimal and medial smooth muscle cells and endothelial cells in atherosclerotic arteries, whereas normal arteries contained no Cox-2 protein (27). Similarly, Schonbeck *et al* (30) found expression of both Cox-1 and Cox-2 by endothelial cells, smooth muscle cells and macrophages in atherosclerotic arteries, while normal arteries only expressed Cox-1. Furthermore, Cox-2 and prostaglandin synthase E colocalize in symptomatic lesions and are possibly involved in metalloprotease activation via PGE_2 production (31) suggesting a role for PGE_2 in plaques instability.

On the other hand, Cox-2 might play an atheroprotective role (27). *In vitro* laminar shear stress upregulates Cox-2 mRNA and protein in HUVEC, turbulent shear stress does not have this effect (32). Prostacyclin is a potent vasodilator, inhibits platelet aggregation and blocks leukocyte adhesion and activation (33). It is therefore possible that Cox-2 induced in endothelial cells at lesion-protected areas catalyses the formation of the anti-atherogenic molecule prostacyclin. These finding suggest the possibility that Cox-2 induction alone is not enough to drive prostaglandin production toward a pro-atherogenic or anti-atherogenic profile, but the prostanoids produced account for this effect. Future studies are thus warranted to identify the prostanoid profile induced by Ox-HDL in the endothelium.

In summary, we have shown that Ox-HDL induces Cox-2 expression in human endothelial cells through a PI3K, NF-IL6-dependent pathway, suggesting a new mechanism by which Ox-HDL could modulate the inflammatory response in the arterial wall.

Acknowledgements

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