HDL₃ Induces Cyclooxygenase-2 Expression and Prostacyclin Release in Human Endothelial Cells Via a p38 MAPK/CRE-Dependent Pathway: Effects on COX-2/PGI-Synthase Coupling

G.D. Norata, E. Callegari, H. Inoue, A.L. Catapano

Objective  In endothelial cells, cyclooxygenase-1 (COX-1) and COX-2 both contribute to prostacyclin production. Recent findings suggest that COX-2 contributes significantly to systemic prostacyclin synthesis in humans; whether COX-2 inhibition is related to an increased cardiovascular risk is undergoing debate. HDLs have been shown to increase prostacyclin synthesis, thus in the present study we investigated the molecular mechanisms involved in this effect in endothelial cells.

Methods and Results—HDL₃ (30 µg/mL) induced COX-2 expression in a time- and dose-dependent manner. COX-2 was found mainly in the perinuclear area where it co-localizes with PGI synthase. Transient transfection experiments showed that CRE is required for HDL-induced COX-2 transcription, and we demonstrated that p38 MAPK activation by HDL₃ is involved in COX-2 mRNA transcription and stabilization. As a consequence of COX-2 induction by HDL₃, prostacyclin production increased, incubation with a COX-2 selective inhibitor blocked this effect. Moreover, HDL₃ increased caveolin-1 phosphorylation, thus promoting PGI-synthase shuttling from the membrane to the perinuclear area.

Conclusion—We conclude that in endothelial cells, HDL modulates COX-2/PGI-S activity via both p38 MAPK-dependent COX-2 mRNA stability and transcription and both caveolin-1-dependent PGI-synthase shuttling and COX-2 coupling. The understanding of these mechanisms may provide new insights into the antiatherogenic role of HDL. (Arterioscler Thromb Vasc Biol. 2004;24:871-877.)

Key Words: HDL □ cyclooxygenase-2 □ p38 MAPK □ prostacyclin □ caveolin-1

High-density lipoprotein (HDL) protects from atherosclerotic vascular disease.¹ Beyond reverse cholesterol transport, HDL particles possess several anti-atherosclerotic effects,² including the induction of prostacyclin (PGI₂), a strong vasorelaxant³ that acts also as an inhibitor of platelet and leukocyte activation.⁴ The stimulatory effect on PGI₂ depends mainly on the supply by HDL of endothelial cells with arachidonic acid.³ The rate-limiting step in the conversion of the arachidonic acid to eicosanoids is the activity of cyclooxygenase (COX).⁴ Two major forms of COX, COX-1 and COX-2, have been identified.⁵ Although COX-1 is constitutively expressed in most cell types, COX-2 is induced by various growth factors and cytokines.⁶,⁷ Recent findings suggest that COX-2 contributes significantly for PGI₂ synthesis in endothelial cells,⁸,⁹ whereas COX-1 is mainly involved in TXA₂ synthesis by platelets.⁸,⁹ Whether COX-2 inhibition is related to an increase of cardiovascular risk is uncertain.¹⁰ HDL induces COX-2 expression in rabbit smooth muscle cells¹¹ and cooperates with TNF-alpha to elicit this effect.¹² The molecular mechanisms involved, however, are unclear. COX-2 expression is modulated by growth factors and cytokines via mitogen-activated protein kinase (MAPKs) cascade.¹³,¹⁴ Once activated, the MAPKs may modulate the activity of several transcription factors such as CREB, NFAT, AP-1, and NF-KB,¹⁵–¹⁷ which are involved in COX-2 expression.¹⁸–²¹

In the present study, we investigated the molecular mechanisms involved in the effect of HDL₃ on COX-2 expression and eicosanoid production in cultured human endothelial cells.

Methods HDL₃ (density 1.125 to 1.21 g/mL) was isolated from human plasma and protein content was determined as described.²² HDL₃ was used within 6 hours from isolation. No LPS contamination was detected as assessed by the endotoxin kit (Sigma, Italy).
HUVECs were isolated and cultured as described. In all experiments, cells were preincubated with serum-free medium for 6 hours, then HDL3 was added.

The antibodies to phospho-p38 MAPK, phospho-p44/42 MAPK, phospho-IkB-alpha, phospho-CREB, and phospho-caveolin-1 were from New England Biolabs (Germany). COX-1, COX-2, and PGI and PGE synthase (PGIS, mPGES-1) monoclonal antibodies were from Cayman (USA). β-Actin antibody was from Sigma. Secondary antibodies were from Biorad (Italy). Western blotting analysis was performed as described: all antibodies were diluted 1:1000, except β-actin (1:10000).

The MEK inhibitor, U0126 (New England Biolabs), and the p38 MAPK inhibitor SB203580 (Sigma) were used at a final concentration of 10 μmol/L and 1 μmol/L, respectively. Indomethacin heptyl ester (Cayman), a selective COX-2 inhibitor, was used at 0.1 μmol/L.

**Immunocytochemistry**

Cells were cultured on coverslips in 24-well plates. Fixed cells were incubated with a monoclonal antibody for COX-1 or COX-2 (1:50) overnight at 4°C, followed by incubation with anti-mouse IgG FITC-conjugated (1:100, RD, Italy) for 30 minutes, then propidium iodide (2.5 μg/ml) was added for 30 minutes. For the studies of COX-1 and COX-2 localization with PGIS, mPGES-1 and phospho-caveolin-1 fixed cells were incubated overnight with the antibody, followed by incubation with anti IgG FITC-conjugated (30 minutes), anti-COX-2 phycoerythrin-labeled for 1 hour, and TOPRO 3 (Molecular Probes) (1:500) for 15 minutes. The coverslips were analyzed with a confocal microscope (Nikon Eclipse TE 2000-S; Radiance 2100 Biorad) at 600× magnifications. Sixty sections were captured (0.01 μm each) and a three-dimensional reconstruction was obtained using the software Image ProPlace 4.5 (Media Cybernetics, USA).

**Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction**

Total RNA was extracted and underwent reverse transcription as described. Three μl of cDNA were amplified by real-time quantitative polymerase chain reaction (PCR) with 1× Syber green universal PCR mastermix (Biorad). 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 PCR amplification was related to a standard curve ranging from 10–11 to 10–16 M.

**Transcription Assay**

The construction of various reporter vectors for the human COX-2 gene has been described previously. 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). Because 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. CHO cells were transiently transfected with COX-2 (nucleotide –327/+59), the NF-kB mutated site (KBM), or the CRE mutated site (CRM) luciferase reporter vectors using lipofectamine (Invitrogen, Italy) according to the manufacturer instructions. Luciferase activity was determined and normalized for the cellular protein concentration.

**Detection of Prostaglandin Release by Competitive Enzyme Immunoassay**

Competitive enzyme immunoassay kits for 6-keto PGFα, TXB2, and PGE2 were from Cayman. HUVECs were exposed to HDL3 (30 μg/mL) for 6 hours, washed twice with PBS, and then incubated for 30 minutes with exogenous AA (10 μmol/L); 50 μL for each sample were processed for prostaglandin release according to the manufacturer instructions.

**Statistical Analysis**

Statistical analysis was performed by ANOVA with the use of Statsoft Statistica Package.

**Results**

**HDL3 Induces COX-2 Expression in HUVECs**

COX-2 protein was expressed at low levels in unstimulated cells and was strongly induced 2 hours after exposure to HDL3 (30 μg/mL). In preliminary experiments, this concentration maximally induced COX-2 expression and no further increase was observed up to 600 μg/mL of HDL3. The induction was maximal 4 hours and begun to decrease after 8 hours (Figure 1a). In unstimulated cells, COX-2 expression remained low at all time points (data not shown). Under the same experimental conditions, HDL3 did not affect
COX-1 expression (Figure 1a). These findings were confirmed by immunocytochemistry. COX-2 expression increased after 4 hours in cells incubated with HDL3 without changes of COX-1 expression (Figure 1b). On three-dimensional reconstruction, COX-2 localized in the perinuclear area and in the cytoplasm31 (Figure 1c).

Effects of HDL3 on Intracellular Kinase Pathways and on COX-2 Promoter Activity
HDL3 activated ERK1/2 and p38 MAPK, with a peak of phosphorylation reached after 5 to 10 minutes of incubation (Figure 2). Several transcription factors are activated through MAPK-dependent pathways.17-19 HDL3 activated CREB, with a peak of activity at 10 to 20 minutes (Figure 2), in agreement with the observation that both ERK1/2 and p38 MAPK activate CREB via p90RSK or via MSK-1, respectively. Ik-B alpha phosphorylation results in the release and nuclear translocation of active NF-kB.17 Under our experimental conditions, a basal level of phosphorylation of Ik-B alpha was present, and only a minimal effect on phosphorylation was observed after 5 and 10 minutes of incubation with HDL3 (Figure 2). The human COX-2 promoter region (~327/+59) contains the NF-kB, the NF-IL6, and the CRE sites.20,21 Transient transfection assay showed that HDL3 induced promoter activity by 2.96 ± 0.03-fold, whereas LPS (1 μg/mL), a positive control, induced promoter activity by 4.24 ± 0.02-fold (P < 0.01 for both versus control) (Figure 3).

The promoter activity of the plasmid carrying the mutation at the NF-kB site was 1.87 ± 0.12 fold in HDL3 incubated cells (P < 0.01) and 0.93 ± 0.09 fold in LPS-treated cells, whereas that of the mutant carrying the mutation at the CRE site was 1.15 ± 0.03-fold in HDL3-treated cells and 1.26 ± 0.16-fold in LPS treated cells (Figure 3; P = NS versus control).

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Effects of HDL3 on Eicosanoid Production
The effects of HDL3 on eicosanoids production were assessed in HUVECs exposed to 30 μg/mL of lipoproteins for 6 hours, followed by 30 minutes of incubation with exogenous AA (10 μmol/L).11 In control cells, the production of 6-keto PGFα (PGI2 main metabolite) was 73.14 ± 6.79 pg/mg of cellular protein. Incubation of endothelial cells with HDL3 increased 6-keto PGFα production to 113.38 ± 2.54 pg/mg of cellular protein (P < 0.01) (Table II, available online at http://atvb.ahajournals.org). In the presence of 0.1 μmol/L indomethacin eptyl ester, a selective COX-2 inhibitor,25 HDL3-induced 6-keto PGFα production was reduced to 77.95 ± 10.19 pg/mg of cellular protein and PGE2 resulted in 74.10 ± 3.45 pg/mg of cellular protein and was not affected by HDL3 incubation.
Effects of HDL₃ on PGI Synthase Expression and Cellular Localization

As HDL₃ induces COX-2 expression and increases PGI₂ release, we investigated whether HDL₃ can affect PGI-S or mPGES-1 expression. HDL incubation did not change PGI-S or mPGES-1 expression (1.10 ± 0.2-fold and 1.07 ± 0.5-fold versus control cells, respectively) (Figure 5a). Furthermore, in HDL-treated cells, PGI-S co-localized with COX-2 while mPGES-1 showed a different subcellular distribution (Figure 5b).

PGI-S resides in caveolae in resting cells. Caveolin-1 is the main protein of caveolae, and when phosphorylated it moves into the cytoplasm, shuttling PGI-S in the perinuclear area where it couples to COX-2, thus increasing prostacyclin synthesis. We investigated, therefore, whether HDL can influence caveolin-1 phosphorylation and shuttling in the perinuclear space. After 4 hours of incubation, HDL increased caveolin-1 phosphorylation (Figure 6a), mainly in the area surrounding the nucleus (Figure 6b). Moreover, a three-dimensional reconstruction shows that phosphorylated caveolin-1 localizes near COX-2 in the perinuclear area of HDL-treated cells (Figure 6c), where PGI-S is also located (Figure 6d).

Discussion

The major finding of this study is that HDL₃ induces COX-2 expression and PGI₁ release in human endothelial cells via p38 MAPK activation. The activation of this signaling pathway promotes COX-2 mRNA transcription and stabilization.

On incubation of cells with HDL₃, COX-2 protein localized mainly in the perinuclear area, in agreement with previous findings showing that COX-2 accumulation near the nuclear envelope and in the cytoplasm is required for the increase in COX-2-mediated prostanoid synthesis in vascular endothelial cells. This effect is specific for COX-2; in fact, COX-1 was mainly localized in the cytoplasm and was not modulated by HDL₃, moreover, PGI₁ synthesis was downregulated by a specific COX-2 inhibitor.

The molecular mechanisms by which HDL₃ induces COX-2 are unknown. Here we show that HDL₃ activates 2 of the major kinases pathways involved in COX-2 gene transcription: ERK1/2 and p38 MAPK. HDL can activate ERK1/2 via cell surface S1P receptor in astroglial cells. However, the possibility that MAPK activation results from plasma membrane cholesterol depletion cannot be excluded. In support of this hypothesis, Smith et al showed that increasing concentration of LDL or free cholesterol decreases COX-2 expression and PGI₂ synthesis. As HDL triggers the release of cholesterol from cells, our observation suggests that cellular cholesterol balance plays an important role in determining COX-2 levels.

HDL₃ also activates CREB in a time-dependent fashion, CREB binds to CRE, which serves as an anchor for P300 interaction with upstream transactivators and downstream transcription machinery, thus suggesting that CRE plays a relevant role in COX-2 induction by a number of stimuli. Using transient transfection experiments, we demonstrated that mutation in CRE abrogated the luciferase activity induced by HDL₃, confirming the role of CRE in HDL₃-induced COX-2 gene transcription.

NF-κB has also been suggested to be involved in determining COX-2 gene transcription. We show that a mutation in the NF-κB response element abrogates luciferase activity induced by LPS, used as a positive control, while it slightly decreases HDL₃-induced luciferase activity, suggesting a minor role of this pathway in COX-2 induction by HDL₃.
As transcriptional regulation of the COX-2 gene occurs via activation of MAPKs, we investigated whether inhibition of ERK1/2 or p38 MAPK pathway affected HDL3-induced COX-2 mRNA and protein expression. We show that the p38 MAPK pathway is responsible for the induction of COX-2 by HDL3.

P38 MAPK plays a housekeeping role in maintaining COX-2 mRNA stability via the recognition of the AUUUA motifs present in the 3' untranslated region of COX-2. We therefore studied COX-2 mRNA stability in cells stimulated with HDL3. Simultaneous addition of actinomycin D and SB203580 to the cells resulted in a more rapid decrease in COX-2 mRNA compared with actinomycin D alone. This represents a new mechanism by which HDL can influence gene expression at a posttranscriptional level and is likely to contribute to the increase of COX-2 protein levels in endothelial cells.

COX-2 has been proposed to exert both an antiatherogenic or a proatherogenic role depending on the eicosanoids produced and the arterial wall cells where it is expressed. Eicosanoids are involved in a variety of physiological pro-
cesses in atherosclerosis and thrombosis, including leukocyte–endothelial cell adhesion, vasoconstriction, and platelet aggregation. The dominant prostaglandin produced by endothelial cells is PGI₂. PGI₂ is believed to play a protective role in atherothrombosis. COX-2 contributes significantly to systemic PGI₂ synthesis in humans; therefore, it is possible that COX-2 induced in endothelial cells at lesion-protected areas catalyzes the formation of the anti-atherogenic molecule prostacyclin. This may be the case in the presence of HDL, which increases PGI₂ release mediated by AA in endothelial cells. This effect is dependent mainly on COX-2 as indomethacin epyt ester, a specific COX-2 inhibitor, abolished PGI₂ release induced by HDL. This observation may also be relevant to the recent observation that COX-2 inhibitors may increase CHD risk. In vitro 30 μg/mL of HDL, induces maximally COX-2 expression, and no further increase is observed up to 600 μg/mL (a physiological concentration that constantly bathes arteries in vivo), thus suggesting that low concentrations of HDL are enough to support COX-2 expression, and higher levels may only provide the substrate. Alternatively, the in vitro conditions allow for a better interaction of HDL with cultured endothelial cells compared with in vivo settings, in which proteoglycans may trap lipoproteins and reduce their availability for interactions with the endothelial cells.

The observation that COX-2 induced by HDL does not increase PGE₂, a proatherogenic eicosanoid, synthesized mainly via COX-2, confirms that COX-2 expression in the arterial wall could play both a proatherogenic or anti-atherogenic role, but it is the final eicosanoid produced that is responsible for its proatherogenic or anti-atherogenic properties.

Moreover, HDL-induced COX-2 protein co-localizes with PGI-S in endothelial cells, thus suggesting that in this model, once induced, COX-2 can drive prostacyclin synthesis. PGI-S is associated with caveolae and is activated when shuttled from the plasma membrane in the perinuclear area; moreover, disruption of caveolae organization downregulates prostacyclin production and impairs angiogenesis. Here we demonstrate that HDL induces caveolin-1 phosphorylation, which shuttles with PGI-S from the plasma membrane to the perinuclear area where it co-localizes with COX-2. Furthermore, the possibility that the abundant increase in COX-2 observed can be related to an increase of prostanoids synthesis other than prostacyclin cannot be excluded.

Also, endothelial nitric oxide synthase, the enzyme responsible for nitric oxide synthesis in the endothelium, localizes in the caveolae. Nitric oxide is responsible for several beneficial effects of HDL on endothelial cells, such as helping to maintain endothelial integrity, facilitating vascular relaxation, inhibiting cell adhesion to vascular endothelium, decreasing radical oxygen production, and inhibiting apoptosis. Even if we have not addressed the role of HDL in modulating endothelial nitric oxide synthase shuttling through caveolin-1 phosphorylation, it is conceivable that some of the effects of HDL are mediated via this pathway.

Moreover, the HDL-dependent caveolin-1 phosphorylation favors PGI-S shuttling and COX-2 coupling. These data add further insights into the molecular mechanisms involved in the anti-atherogenic activity of HDL.

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References


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Table I
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Table II