Regulatory Effects of HDL on Smooth Muscle Cell Prostacyclin Release
Marisa Viñals, José Martínez-González, Lina Badimon

Abstract—One mechanism by which high density lipoproteins (HDLs) exert their protective effect against coronary artery disease could be related to the induction of prostacyclin (PGI₂) release in the vessel wall. We have recently shown that HDL increases PGI₂ production in rabbit smooth muscle cells (RSMCs) and that this increase is dependent on cyclooxygenase-2 (Cox-2). Here we analyze the mechanism by which rabbit HDL induces PGI₂ release in RSMCs. Our results show that although HDL₂ and HDL₃ share a similar capacity to induce Cox-2 protein levels, HDL₃ stimulates a higher PGI₂ release than does HDL₂, probably because of their relative arachidonate contents. Acetylsalicylic acid pretreatment (300 μmol/L, 30 minutes) significantly reduced the HDL-induced PGI₂ release, suggesting that both preexisting and induced Cox-2 activities were involved in the HDL effect. Ca²⁺-dependent cytosolic phospholipase A₂ (cPLA₂) and Cox-1 protein levels were not altered by HDL. Dexamethasone (2 μmol/L), which also inhibited the HDL-induced PGI₂ release, reduced significantly both Cox-2 mRNA and protein levels without affecting cPLA₂ and Cox-1 protein levels. In addition, methylarachidonyl fluorophosphonate, a potent inhibitor of cPLA₂, did not produce any effect on HDL-induced PGI₂ release. In the presence of cycloheximide, Cox-2 mRNA levels were induced by HDL and inhibited by dexamethasone, suggesting that HDL and dexamethasone work in the absence of de novo protein synthesis. These results indicate an early effect of HDL on PGI₂ biosynthesis, specifically increasing Cox-2. PD98059, an inhibitor of mitogen-activated protein kinase kinase, completely inhibited HDL-induced PGI₂ release, whereas GF109203X, a protein kinase C inhibitor, had no effect. Thus, HDL induces PGI₂ synthesis by a mechanism dependent on the mitogen-activated protein kinase pathway but independent of protein kinase C. (Arterioscler Thromb Vasc Biol. 1999;19:2405-2411.)

Key Words: lipoproteins ■ cyclooxygenase-1 ■ cyclooxygenase-2 ■ cytosolic phospholipase A₂ ■ mitogen-activated protein kinases

Several epidemiological and experimental studies have shown that HDLs have a protective effect against coronary artery disease.¹⁻⁵ The most widely accepted mechanism for this protective effect is through an enhanced reverse cholesterol transport.⁶⁻⁷ However, other mechanisms such as induction of prostacyclin (PGI₁) synthesis in the vessel wall have been postulated. The stimulatory effect of HDL on PGI₁ production by both endothelial and smooth muscle cells (SMCs) has been widely demonstrated.⁸⁻¹² In contrast, the effect of low density lipoprotein (LDL) on the biosynthesis of prostanooids is unclear: they can stimulate, inhibit, or have no effect on prostanooid synthesis.¹¹⁻¹³ This variable effect of LDL has been related to its degree of oxidation.¹⁶⁻¹⁷

PGI₁ is a vasodilator prostaglandin that is synthesized by blood vessels and contributes to the maintenance of vascular tone.¹⁸⁻¹⁹ Other actions of PGI₁ include inhibition of platelet aggregation and adhesion, inhibition of SMC growth, inhibition of leukocyte activation and adhesion, and reduction of cholesterol ester accumulation in vessel wall cells. These biological actions of PGI₁ suggest that it is an endogenous antiatherogenic molecule.²⁰

The rate-limiting step in the conversion of arachidonic acid to prostaglandins and other eicosanoids is at the level of cyclooxygenase (Cox; prostaglandin G/H synthase; E.C. 1.14.99.1). Two isoforms of Cox have been described: Cox-1 and Cox-2. Cox-1 is present in several cells and tissues in relatively stable levels,²¹ although small increases in enzyme content can occur after stimulation with hormones or growth factors.²²⁻²³ Cox-2 is usually absent in resting cells, and its expression is increased by serum, cytokines, mitogens, and different growth factors.²¹⁻²⁶

We have recently shown that HDL increases eicosanoid production in SMCs by a Cox-2–dependent mechanism.²⁷ In the present study, we analyzed the involvement in this process of both preexisting and induced Cox-2 activities, as well as the role of other enzymes implicated in the formation of prostaglandins and leukotrienes, such as cytosolic phospholipase A₂ (cPLA₂).²⁸ In addition, we investigated the intracellular signaling mechanisms by which HDL exerts this effect. Our results indicate that HDL induces PGI₁ biosynthesis by both preexisting and induced enzyme, specifically
Lipoprotein Isolation

Lipoproteins were obtained by sequential ultracentrifugation of normolipemic rabbit plasma in a Beckman 50.2 Ti rotor at densities between 1.019 and 1.063 g/mL (LDL), 1.063 and 1.125 g/mL (HDL1), 1.125 and 1.210 g/mL (HDL2), and 1.063 and 1.210 g/mL (HDL). Lipoproteins were recentrifuged, dialyzed, and assayed for protein and lipid contents. Lipoproteins used in the experiments were <2 weeks old and did not contain detectable thiobarbituric acid–reactive substances.

Isolation of Rabbit Aortic SMCs

RSMCs were obtained by gentle scraping of the medial layer of male New Zealand White rabbit aortas after endothelial layer removal. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in Ham’s F12–Dulbecco’s modified Eagle’s medium (8:2, vol/vol) supplemented with 20% fetal calf serum (FCS). Antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin) were added to the culture medium. RSMCs were identified by their growth behavior, morphology, and immunofluorescence.

Cell Culture and Supernatant Determinations

Cells between the third and seventh passages were grown as described above. At subconfluence fresh medium was added to the wells, and 24 hours later cells were washed 3 times with FCS-free medium and incubated with lipoproteins. When experiments were addressed to inhibit baseline Cox activity, cells were pretreated for 30 minutes with 300 μmol/L ASA, an inhibitor of the 2 Cox isoenzymes. They were then incubated with ASA (300 μmol/L) or dexamethasone (2 μmol/L) for 2 hours; with actinomycin D (1 μg/mL) or cycloheximide (2 μg/mL) for 45 minutes; or with MAFP (1 mmol/L), PD98059 (30 and 50 μmol/L), or GF109203X (0.5 and 20 μmol/L) for 30 minutes before HDL treatment. After lipoprotein incubations (1, 2, 3, 6, 8, or 24 hours), the culture medium was kept frozen at −80°C. PGI₂ in the supernatants was measured as 6-keto-PGF₁α, its stable hydrolysis product, by an ELA kit.

Western Blot Analysis

RSMCs were cultured as described above. Cells were stimulated with lipoproteins for 6 hours. Cell monolayers were washed with PBS and lysed with 50 mmol/L Tris, 1 mmol/L EDTA, and 0.1% Triton X-100. Total protein was separated by SDS–polyacrylamide gel electrophoresis on 4% to 15% gradient polyacrylamide gels with a mini–PROTEAN II slab cell (Bio-Rad). Proteins from polyacrylamide gels were blotted onto nitrocellulose membranes at 40 mA for 1 hour at 4°C. The residual binding capacity of the membranes was blocked with 5% nonfat milk in 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween 20. Blots were incubated with monoclonal antibodies against human Cox-2 (C22420, Transduction Laboratories) and cPLA₂ (sc-454, Santa Cruz Biotechnology, Inc) or with a goat polyclonal antibody against a peptide corresponding to amino acids mapping at the carboxy terminus of the human Cox-1 (sc-1752, Santa Cruz Biotechnology, Inc). Bound antibody was detected using the appropriate horseradish peroxidase–conjugated antibody. Signals were detected with the ECL on a standard x-ray film.

RNA Blot Analysis

RSMCs were cultured as previously described. After lipoprotein incubations, stimulations were halted by the addition of ice-cold isolation reagent Ultraspec RNA (1 mL/21-cm² dish). RNA samples were fractionated in 1.1% agarose gels containing formaldehyde. RNA was transferred by capillarity to Hybond membranes and UV–cross-linked. Filters were prehybridized and hybridized at 42°C in 50% formamide, 1 mol/L NaCl, 50 mmol/L NaPO₄ (pH 6.3), 7.5× Denhardt’s solution, 1% SDS, 10% dextran sulfate, and 200 μg/mL denatured salmon sperm DNA. The human Cox-2 cDNA, kindly provided by Dr Hla, and the rabbit Cox-1 cDNA were used as probes. Washes were carried out under moderate-stringency conditions. Filters were exposed to Agfa Curix RP 2x-ray film at −70°C. Filters were rehybridized with a ribosomal cDNA probe to assess the amount of RNA.

Assay of cPLA₂ Enzymatic Activity

RSMCs were cultured as indicated above and incubated with HDL (150 μg cholesterol per mL) for 1 hour. cPLA₂ activity was assayed by using the cPLA₂ kit from Cayman Chemical Co according to the manufacturer’s instructions. In brief, the cytosolic fraction from RSMC cultures was incubated with the substrate arachidonoylthiophosphatidylcholine (ATPC). Enzymatic hydrolysis of ATPC re-
leaves free thiol, which is then converted into 5-thiol-2-nitrobenzoic acid by Ellman’s reagent [5,5′-dithiobis(2-nitrobenzoic acid)]. 5-Thiol-2-nitrobenzoic acid formation is determined by spectrophotometric analysis at 414 nm.

Statistical Analysis
Data are presented as mean ± SEM. Means were compared by using ANOVA. Differences were considered significant at P < 0.05.

Results
Effect of HDL₂ and HDL₃ Subfraction on PGI₂ Release and Cox-2 Protein Levels
RSMCs were incubated with HDL₂, HDL₃, or the complete HDL fraction (150 µg cholesterol per mL) for 24 hours, and PGI₂ release was measured. Results showed that HDL₃ induced a significantly higher release than did HDL₂ (4.18 ± 0.49 and 2.14 ± 0.26 ng/mL, respectively; P < 0.0001; Figure 1A). In contrast, all of the fractions induced similar Cox-2 protein levels with respect to control cells (Figure 1B). Thus, subsequent studies were performed with the total HDL fraction.

Effect of ASA Pretreatment, Cycloheximide, and Actinomycin D on PGI₂ Release Induced by HDL HDL (150 µg cholesterol per mL) induced a time-dependent increase in PGI₂ production in RSMCs. ASA pretreatment (300 µmol/L, 30 minutes) significantly reduced HDL-induced PGI₂ release (Figure 2A). The ASA-produced inhibition was lost in a time-dependent fashion; it was highest at 2 hours (74.8 ± 1.2%) and decreased after 8 (43.2 ± 9.3%) and 24 (21.4 ± 9.1%) hours of incubation. The effect of ASA pretreatment on control cells was maintained over time.

Both cycloheximide (2 µg/mL), a protein synthesis inhibitor, and actinomycin D (1 µg/mL), an inhibitor of transcription, abolished PGI₂ release induced by HDL (the Table). Taken together, these results suggest that both basal and de novo synthesized Cox activities are involved in PGI₂ production induced by HDL.

Effect of Dexamethasone on PGI₂ Release Induced by HDL
In ASA-pretreated cells, dexamethasone (2 µmol/L), a Cox-2 inhibitor by transcriptional and posttranslational mechanisms,34,35 significantly reduced the HDL-induced PGI₂ release. However, PGI₂ values in the presence of dexamethasone did not reach baseline levels (31.1 ± 7.5% at 2 hours, 40.9 ± 8.6% at 8 hours, and 49.4 ± 12.4% at 24 hours of incubation; Figure 2B). Furthermore, in ASA-pretreated SMCs maintained under ASA treatment for a prolonged incubation time (2, 8, or 24 hours), PGI₂ synthesis induced by HDL was partially inhibited (HDL 3.14 ± 0.69 ng/mL versus HDL plus ASA 0.45 ± 0.08 ng/mL).27 Thus, ASA abolishes the

<table>
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<th>Treatment</th>
<th>Control</th>
<th>Cycloheximide</th>
<th>Actinomycin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.38 ± 0.05</td>
<td>0.14 ± 0.02†</td>
<td>0.19 ± 0.03†</td>
</tr>
<tr>
<td>HDL</td>
<td>2.00 ± 0.14</td>
<td>0.64 ± 0.09†</td>
<td>1.22 ± 0.09†</td>
</tr>
<tr>
<td>20% FCS</td>
<td>44.33 ± 5.65</td>
<td>0.65 ± 0.25†</td>
<td>0.68 ± 0.22†</td>
</tr>
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ASA-pretreated cells were incubated with cycloheximide (2 µg/mL) or actinomycin D (1 µg/mL) for 45 minutes before HDL (150 µg cholesterol per mL) addition. After 8 hours, 6-keto-PGF₁α levels in the supernatants were analyzed by enzyme immunoassay. Results are expressed as ng of 6-keto-PGF₁α per mL/8 h and are shown as mean ± SEM of 3 independent experiments run in triplicate.

P < 0.05: * versus cycloheximide-treated cells; † versus control.
high PGI$_2$ levels induced by FCS (99% inhibition at 24 hours) but only partially inhibits those induced by HDL (61% inhibition at 24 hours).

Role of Cox-2, Cox-1, and cPLA$_2$ on PGI$_2$ Release Induced by HDL

HDL treatment (150 μg cholesterol per mL, 6 hours) significantly stimulated Cox-2 protein levels but did not produce any effect on those of Cox-1 or cPLA$_2$. LDL (150 μg cholesterol per mL) did not affect Cox-1, Cox-2, or cPLA$_2$ protein levels (Figure 3). Dexamethasone treatment (2 μmol/L), which inhibited HDL-induced PGI$_2$ release, blocked Cox-2 protein levels but not those of Cox-1 and cPLA$_2$ (Figure 3). PGI$_2$ release induced by HDL was not abolished by high concentrations (1 mmol/L) of MAFP, a potent inhibitor of cPLA$_2$.

Effect of Cycloheximide and Dexamethasone on Cox-2 mRNA Levels Induced by HDL

Cycloheximide (2 μg/mL, 3 hours), which inhibited PGI$_2$ release induced by HDL (the Table), produced a superinduction of Cox-2 mRNA, which was more significant in HDL than in control cells (saline or LDL-treated cells) (Figure 5A). In the presence of cycloheximide, dexamethasone efficiently inhibited superinduced Cox-2 mRNA levels in both control and HDL-treated cells (Figure 5B). These results indicate that de novo protein synthesis is not a requirement for the modulation of Cox-2 mRNA levels by HDL and dexamethasone. In contrast, cycloheximide prevented the inhibition by dexamethasone of Cox-2 mRNA levels induced by FCS.

Effect of an MAPK Kinase and PKC Inhibitors on PGI$_2$ Release Induced by HDL

PD98059 (30 μmol/L), a specific inhibitor of the activation of MAPK kinase, completely inhibited PGI$_2$ release induced by HDL in SMCs (Figure 6). In contrast, GF109203X (20 μmol/L), a protein kinase C (PKC) inhibitor, had no effect on PGI$_2$ release induced by HDL. However, the effect of PD98059 and GF109203X on HDL-induced Cox-2 mRNA levels was negligible (data not shown).

Discussion

Previous reports have indicated that the levels of PGI$_2$ release by cells treated with HDL depend, at least in part, on HDL activation of Cox-2 and cPLA$_2$. This study provides evidence for the role of Cox-2 and cPLA$_2$ in the regulation of PGI$_2$ release by HDL. The results suggest that the modulation of Cox-2 and cPLA$_2$ protein levels by HDL is mediated, at least in part, by de novo protein synthesis. The inhibition of PGI$_2$ release by dexamethasone suggests that the modulation of Cox-2 and cPLA$_2$ by HDL is not due to the modulation of Cox-2 and cPLA$_2$ by HDL.
We have recently shown that HDL increases eicosanoid production in RSMCs by a Cox-2–dependent mechanism. We now report that in RSMCs, rabbit HDL promotes PGI2 release through both preexisting and induced Cox activity. HDL1 is more effective than HDL2 in inducing PGI2 release, although both HDL fractions induce similar levels of Cox-2 protein. HDL1 has a higher cholesteryl arachidonate content than does HDL2 and, thus, the former may provide a larger supply of substrate for Cox enzymatic activity. On the other hand, ASA pretreatment significantly reduced PGI2 release induced by HDL. Moreover, dexamethasone reduced the superinduction of Cox-2 mRNA levels and other early genes by cycloheximide has been previously shown. Several mechanisms have been proposed to explain the superinduction by cycloheximide of inducible mRNAs, including inhibition of the mRNA degradation machinery.

Dexamethasone can block Cox-2 by transcriptional and posttranslational mechanisms. Our results show that dexamethasone did not inhibit Cox-2 mRNA levels induced by FCS in the presence of a protein synthesis inhibitor (cycloheximide). It has been shown that RNA and protein synthesis inhibitors reverse the effect of dexamethasone, suggesting that dexamethasone inhibition is mediated by 1 or more newly synthesized proteins. However, in the presence of cycloheximide, dexamethasone efficiently inhibited Cox-2 mRNA levels induced by HDL. Thus, dexamethasone could inhibit HDL-induced Cox-2 mRNA levels by acting mainly at the transcriptional level. Indeed, a potential glucocorticoid response element has been described in the Cox-2 gene.

HDL activates a number of signaling pathways whose role in its cellular effects (intracellular cholesterol efflux, PGI2 release, cell proliferation, etc) is not completely understood. We show that the MAPK pathway is involved in the HDL-induced PGI2 release. Our present results with GF109203X and previous data with calphostin C, 2 specific inhibitors of PKC, suggest that HDL-induced PGI2 release is signaled through MAPK activation, independent of PKC. Although PKC and MAPK activations are interrelated, some Cox-2 inducers work by a PKC-independent mechanism. Recently, several authors have shown data linking the MAPK pathway, but not that of PKC, to PGI2 production induced by different molecules. In contrast, PKC seems to play a key role in HDL-induced cholesterol efflux. However, neither GF109203X nor PD98059, a specific inhibitor of MAPK kinase, was able to block the early upregulation of Cox-2 mRNA levels by HDL; thus, alternative signaling could be involved in the process. Taking into consideration that the MAPK pathway is usually linked to different cellular processes triggered by receptors and that purified apo A-I reproduces the binding parameters of HDL and partially mimics the HDL effect on PGI2 synthesis, this HDL effect could be receptor mediated.

Eicosanoids are a widespread lipid-mediator system for intracellular signaling and hence, have multiple cellular actions such as regulation/modulation of vessel tone, platelet and neutrophil function, and fibrinolysis. Thus, it is not surprising that numerous events in the pathogenesis of atherosclerosis are associated with an altered formation of eicosanoids. HDL could exert its protective effect against coronary artery disease, at least in part, through the upregulation of PGI2 biosynthesis. HDL would act through a multiple mechanism: providing substrate to the preexisting...
and/or de novo Cox enzyme, inducing new synthesis of Cox-2 protein, and stabilizing the induced PGI$_2$. Plasma HDLs can contact directly with vascular SMCs when an atherosclerotic plaque or a healthy vessel suffers endothelial denudation. Cox-2 induction may thus contribute to the cardiovascular protection exerted by HDL as part of a defense mechanism triggered to limit the deleterious effect of minimal damage to the vessel wall. Supporting this hypothesis, previous reports have demonstrated that augmenting PGI$_2$ synthesis in angioplasty-injured carotid arteries resulted in inhibition of thrombosis. Although further studies are needed to understand the contribution of the different HDL effects to its protective cardiovascular properties, the present analysis provides new light on the mechanism by which HDL increases PGI$_2$ production in vessel wall cells.

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