Cyclooxygenase-2–Dependent Prostacyclin Formation Is Regulated by Low Density Lipoprotein Cholesterol In Vitro

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Abstract—Reduction of plasma low density lipoprotein (LDL) levels is associated with a reduced risk of myocardial infarction, stroke, and death. Some of this clinical benefit may be derived from an improvement in endothelium-dependent vasodilation. In the present study, we examined the effects of LDL reduction on cyclooxygenase (COX) activity and prostacyclin (PGI₂) production. Human umbilical vein endothelial cells exposed to reduced concentrations of LDL demonstrated increased PGI₂ production in a dose-dependent manner (from 0.75±0.2 to 2.6±0.2 ng/mL, P<0.0001). This alteration in PGI₂ production did not result from LDL-induced changes in PGI₂ synthase expression. However, selective inhibition of COX-2, but not COX-1, blocked PGI₂ production under low cholesterol conditions. Addition of exogenous cholesterol induces dose-dependent reductions in endothelial COX-2 expression as measured by reverse transcription–polymerase chain reaction and by Western blotting. Pretreatment of cells with actinomycin D, a transcription inhibitor, reduced COX-2–derived PGI₂ production by 45.9% (from 0.55±0.09 to 0.25±0.08 ng/mL). Taken together, these observations indicate that endothelial PGI₂ production is regulated by cholesterol at the transcriptional level and that cholesterol-sensitive transcriptional pathways that regulate COX-2 expression are present in vascular tissue. (Arterioscler Thromb Vasc Biol. 2002;22:983-988.)

Key Words: cyclooxygenase ■ prostacyclin ■ cholesterol ■ LDL ■ endothelial

An elevated plasma level of LDL is a major determinant of ischemic cardiovascular risk. The formation and rupture of atherosclerotic plaques, the magnitude of thrombosis, and the resultant arrhythmias are key pathophysiological processes that determine the clinical impact of ischemia resulting from hypercholesterolemia. The ultimate outcome of hypercholesterolemia manifests itself in any of these processes, culminating in myocardial infarction, stroke, and sudden cardiac death. Accordingly, therapeutic reduction of plasma LDL cholesterol levels is indicated for the primary and secondary prevention of ischemic heart disease and has been proven to reduce the incidence of ischemic cardiovascular events. Although this therapeutic benefit was anticipated, the mechanisms responsible are incompletely understood.

Prostacyclin (PGI₂) is a powerful vasodilator and is the primary eicosanoid product of vascular endothelium and smooth muscle cells. Therefore, it is poised to affect the progression of atherosclerotic disorders. PGI₂ protects against arterial thrombosis by reducing platelet–vessel wall reactivity by modulating tissue plasminogen inhibitor release, and by counteracting some of the long-term effects of hypercholesterolemia, such as inhibiting smooth muscle cell proliferation. PGI₂ synthesis results from sequential activities of phospholipase A₂, cyclooxygenase (COX), and PGI₂ synthase (PGIS). COX is the rate-limiting enzyme in the synthesis of prostaglandins from free arachidonic acid (AA). It is responsible for the cyclooxygenation and subsequent peroxidation of AA into prostaglandin H₂ (PGH₂), the substrate from which tissue-specific synthases produce individual prostaglandins. Mammalian COX exists as 2 isoforms, COX-1 and COX-2. These isoforms are produced by separate genes but are structurally homologous and possess similar kinetic profiles. There is increasing evidence demonstrating that COX-2 expression is not limited to sites of inflammation but that it is differentially regulated in various cell types and plays an important role in vascular homeostasis. Indeed, recent clinical studies have shown that vascular PGI₂ production is derived to a large extent from endothelial COX-2 activity and that inhibition of COX-2 may increase the risk of coronary thrombosis.

In vivo and in vitro studies have reported a relationship between eicosanoid biosynthesis and atherosclerosis. However, the direct effects of cholesterol on PGI₂ synthesis have not been fully investigated. In the present study, we investigate the effects of cholesterol on PGI₂ production in cultured endothelial cells. We report that PGI₂ production is negatively regulated by cholesterol via a COX-2–dependent mechanism.

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Methods

Materials

Matched lots of delipidated and normal FBS were purchased from Calico Biologicals. All other cell culture reagents were from purchased from Invitrogen. We obtained 25-hydroxycholesterol from Seraloids. Custom oligonucleotides used in reverse transcription (RT)-polymerase chain reaction (PCR) were synthesized by Sigma-Genosys Biotechnologies. Human COX-1 and PGIS antibodies were purchased from Cayman Chemical Co; secondary anti-mouse IgG–horseradish peroxidase conjugate, from Santa Cruz Biotechnologies; and purified recombinant COX-1, COX-2, and PGIS, from Oxford Biomedical Research. Human COX-2 monoclonal antibody was a generous gift from Dr Aida Habib and has previously been characterized.26 Precast NuPAGE (10% bis-tris MES) gels, buffers, PVDF membrane, See-blue Plus2 molecular weight marker, and NuPAGE sample buffer were also purchased from Invitrogen. All organic solvents (high-performance liquid chromatography grade) and biochemicals were obtained from Sigma-Aldrich.

Tissue Culture

Primary cultures of human umbilical vein endothelial cells (HUVECs) were isolated as previously described27 and cultured in medium 199 supplemented with 15% FBS, endothelial mitogen, penicillin, streptomycin, amphotericin B, and heparin. Cells between passages 2 and 4 were plated in gelatin-coated 6-well (9.6-cm2) or 24-well plates with 300,000 cells per well. Cells were washed twice with HBSS, and then 2 mL HBBS + 0.75% BSA was added to each well. Exogenous AA (10 μmol/L) was added to each well, with the exception of the negative control that received no exogenous AA. Cells were then incubated at 37°C for 30 minutes. Samples were flash-frozen in liquid nitrogen and later derivatized for gas chromatography (GC)/mass spectrometry (MS) analysis of prostaglandin metabolites. In some cases, cells were incubated twice for 20 minutes with the COX-2–selective inhibitor, SC-58236 (10 μmol/L, celecoxib), the COX-1–selective inhibitor, SC-58560 (10 μmol/L), or vehicle before the addition of exogenous AA (10 μmol/L). In experiments using acetylsalicylic acid (ASA), cells were pretreated with 300 μmol/L to abolish existing COX activity. ASA was used in this experiment as a pretreatment only and was removed by extensive washes with PBS, so as not to inhibit newly synthesized COX activity. In the present study, the use of primary cultures resulted in a batch- and passage-dependent variation in prostaglandin production. Although the absolute values that were obtained differed slightly, the ratio of product reduction remained constant. To minimize these effects, matched batches of early-passage cells were used.

Mass Spectroscopy

COX activity was determined by the conversion of exogenous AA into primary prostaglandin metabolites as previously described.29,30 PGI2 and PGH2 were rearranged in aqueous solution to the stable metabolites 6-ketoprostaglandin-F1α (6-keto-PGF1α) and prostaglandin E1 (PGE1)/prostaglandin D1 (PGD1), respectively. These metabolites were extracted by using C-18 Sep-Pak cartridges (Waters) and purified by thin-layer chromatography. Samples were chemically converted to o-methyloxime pentafluorobenzyl ester trimethylsilyl ether derivatives and quantified by stable isotope dilution techniques using GC/MS. Data were obtained with the use of a Hewlett-Packard 5890 GC/MS. The samples were measured by using internal standards [3H6]-6-keto-PGF1α and [3H6]PGE1. All eicosanoid standards were synthesized in-house. Samples were spiked with internal standard after collection but before extraction and chemical derivatization. Compounds were detected by MS based on the M-CH2C6F5 ion mass-to-charge ratio (m/z) 524 for PGE1/PGD1, m/z 614 for 6-keto-PGF1α, and thromboxane B2 (TxB2), m/z 528 for [3H6]PGE1, and m/z 618 for [3H6]-6-keto-PGF1α and TxB2, for the internal standards. Quantification of the total amounts of each prostaglandin was obtained by integrating the peak area of material in the m/z 524 or 614 channel and comparing the area of the m/z 528 or 618 channel.

Reverse Transcription–Polymerase Chain Reaction

Confluent HUVECs were serum-starved in 5% normal FBS medium for 24 hours to decrease serum-induced COX-2 expression. Dose-response experiments were performed under total lipoprotein deprivation with or without the addition of freshly isolated human LDL to create the following dose-response curve: 0.0, 15.0, 30.0, and 90.0 mg/dL. After 24 hours, total cellular RNA was isolated by using 1 mL RNAzol per 100-mm culture dish. RNA was quantified by spectrophotometry, and 2.0 μg of total RNA was used in each reaction. RT-PCR was performed by using the Advantage One-Step RT-PCR kit (Clontech). Primers used for amplification were as follows: COX-1 (sense 5′-TCTTTATTCTTCAGCAGAATGTCAACTCCC-3′/antisense 5′-AAATCCACATCTTACAGCCAGATGC-3′), COX-2 (sense 5′-TGAGGCCAAACTTTTTTTATCTCTTCG-3′/antisense 5′-AGCCATTGTTGCAAGAGCAGTTCCTC-3′), PGIS (sense 5′-TGGTGTTGGATCTCGTACA-3′/antisense 5′-CCTCACCATTACAGGGTCA-3′), and GAPDH (sense 5′-GATGACATCAAGAGGTTGTAAGGC-3′/antisense 5′-TTCTGGTGCATACCAAGGAAATTGC-3′). Target amplification was quantified by product band integrated optical density and normalized to GAPDH for each sample for each experiment. All gel images were analyzed using imaging software (UVProbe, BioRad). In addition, amplified PCR products were cloned and sequenced to confirm target specificity. Sequenced clones were used as PCR-positive controls.

Immunoblot Analysis

Dose-response experiments were performed with the use of confluent HUVEC cultures under conditions of total lipoprotein deprivation (24 hours). Freshly isolated human LDL was subsequently added. Total cellular protein was isolated from 100-mm culture dishes (52.0 cm2) containing ~3.0×105 cells with the use of 0.5 mL protein lysis buffer, as previously described.31 Supernatants were collected, and total protein content was quantified by the Bio-Rad Assay. Total cellular protein extracts (20 μg) were heated to 70°C for 10 minutes in sample buffer and separated with the use of a 10% acrylamide SDS Novex NuPAGE (bis-tris MES) gel in a Novex Xcell II module according to the manufacturer’s protocol (Invitrogen). Proteins were then transferred to charged nylon membrane by using an Xcell II transfer cassette (Invitrogen). Membranes were blocked overnight in 5% nonfat dry milk at 4°C. Blots were exposed to primary antibody (1:1000 dilution for COX-1 and 1:10,000 for monoclonal antibody COX-2) for 2 hours at 25°C. After 4 washings with Tris-buffered saline plus Tween, secondary antibody (1:1000 for COX-1 and 1:10,000 for COX-2) was added, and the blot was incubated for 2 hours at 25°C. The blots were visualized.
by using the ECL-Plus kit (Amersham Pharmacia) and exposure on Kodak-AR film. The developed bands were then quantified by densitometry with the use of computerized imaging and analysis software (UVP Labworks). The samples were quantified by comparing integrated optical density with a standard curve of recombinant protein.

Statistical Analysis
Statistical analyses of data were performed by using Prism 3 Graph-Pad software. Data from RT-PCR experiments and GC/MS quantification of prostaglandin metabolites were analyzed by ANOVA, except in Figure 3, where the data were analyzed by a 1-tailed t test, as indicated.

Results

LDL Cholesterol Decreases PGI2 Production In Vitro
The effects of purified LDL on prostaglandin production were assessed in HUVECs exposed to differing concentrations of LDL. Because PGI2 is unstable in aqueous solution and rapidly rearranges to 6-keto-PGF1α, this metabolite was quantified by GC/MS. Cells completely deprived of LDL produced >3 times the amount of 6-keto-PGF1α from exogenous AA (10 μmol/L) compared with the amount from cells treated with excess exogenous LDL (2.6±0.2 ng/mL versus 0.75±0.5 ng/mL, respectively; P<0.0001; Figure 1a). Similar effects were observed regarding the production of PGE2, and PGD2 (data not shown), implying that the observed changes in prostaglandin production resulted from an increase in COX activity and from individual prostaglandin synthases. The thromboxane metabolite, TxB2, was also measured. Under standard cell culture conditions, HUVECs produced an insignificant amount of TxB2 from 10 μmol/L exogenous AA, and removal of cholesterol with the use of LPDS had no effect (data not shown). Although the absolute quantities of 6-keto-PGF1α in these experiments varied with each cell batch, the ratio of reduction in prostaglandin synthesis was consistent throughout the present study.

Free Cholesterol (FC) Decreases PGI2 Production In Vitro
To compare the specific effect of free molecular cholesterol with that of LDL, we measured PGI2 production by cultured HUVECs in increasing concentrations of cholesterol. FC (0.1, 1.0, and 10.0 μg/mL) plus a fixed concentration of 25-OH cholesterol (1.0 μg/mL) dose-dependently reduced PGI2 production compared with a deprivation of cholesterol (0.25±0.09 ng/mL for 10.0 μg/mL FC and 0.66±0.09 ng/mL for 0.0 μg/mL FC, P<0.0001; Figure 1b). Commensurate with results obtained with increasing LDL, FC (10 μg/mL+1.0 μg/mL 25-OH cholesterol) decreased the production of PGI2 2.6-fold.

LDL Dose-Dependently Decreases COX-2 mRNA and Protein
After exposure to cholesterol-deficient medium with or without LDL for 24 hours, LDL decreased COX-2 message in a dose-dependent manner (Figure 2a). RT-PCR analysis determined that LDL had no effect on the expression of COX-1 and PGIS. In conjunction with these experiments, we examined the effects of LDL on COX-2 protein expression in HUVECs, as analyzed by Western blot. When treated with increasing doses of LDL, COX-2 protein decreased from 2.55±0.07 ng/μg total protein to 1.03±0.01 ng/μg total protein (Figure 2b). This 2.5-fold decrease in protein is comparable to the reductions in mRNA and prostaglandin synthesis. Western analysis showed that LDL did not modify the expression of COX-1 (data not shown).

Effects of Selective COX-1 and COX-2 Inhibition on PGI2 Production
The relative contribution of the COX-1 and COX-2 isoforms to the observed increase in endothelial cell PGI2 production was examined. When deprived of cholesterol, endothelial cell PGI2 production was reduced by treatment with celecoxib, a selective COX-2 inhibitor, compared with treatment with vehicle (0.404±0.022 ng/mL for celecoxib and 1.189±0.383 ng/mL for vehicle, P=0.004; Figure 3). SC-58560, a selective COX-1 inhibitor, had no effect on LPDS-induced PGI2 synthesis (0.395±0.074 ng/mL for SC-58560 and 0.427±0.009 ng/mL for vehicle). Taken together, these findings suggest that the observed increase in PGI2 synthesis in the absence of cholesterol is a COX-2–dependent phenomenon.
Effect of Transcription Inhibition on PGI₂ Production

Used only as a pretreatment, the covalent inactivation of existing COX by ASA allows PGI₂ to serve as a marker for the activity of newly synthesized COX. HUVECs were treated with ASA (300 μmol/L) or vehicle for 1.0 hours. Before cholesterol depletion or treatment with actinomycin D, ASA and preexisting 6-keto-PGF₁α were removed by extensive washes with PBS. At time zero (T₀), cells were treated with LPDS in the presence or absence of actinomycin D. To confirm the effectiveness of ASA inactivation of COX, exogenous AA was added to a group of cells at T₀. These samples were flash-frozen and later assayed with those collected at 6.5 hours (T₆.₅). An insignificant amount of 6-keto-PGF₁α was detected in these samples, confirming the inactivation of existing COX by ASA at T₀ (Figure 4, second bar). Lipoprotein deprivation induced significant PGI₂ release in aspirin-treated HUVECs by T₆.₅. In these cells, actinomycin D was found to reduce the formation of 6-keto-PGF₁α by 47.9% (Figure 4). These findings indicate that LDL depletion results in increased COX-2 message and increased 6-keto-PGF₁α production.

Discussion

In the present study, we provide evidence that LDL and FC suppress COX-2–dependent endothelial PGI₂ production. Our data demonstrate that a reduction in ambient concentrations of either LDL or FC increases COX-2 mRNA, whereas the expression of COX-1 and PGIS are unaffected. We also observed that endothelial COX-2 protein and COX-2–dependent prostaglandin production are increased under conditions of reduced cholesterol.

In multicellular tissues undergoing pathological processes in vivo, there are many potential pathways of prostaglandin synthesis with diverse influences and consequences on each cell type. LDL cholesterol was reported to increase or decrease prostaglandin production in a variety of vascular tissue preparations. In the present study, we observed a reduction of COX-2 expression in response to increased cholesterol. Although the biological mechanisms responsible for our observations and those previously reported are incompletely understood, the apparent contradiction may be simply...
explained as follows: the effects of the inflammatory response associated with atherosclerosis lead to increased COX-2 expression in complex lesions, but cholesterol alone has the opposite effect. While our manuscript was in preparation, Wong et al. reported that hypercholesterolemia reduced PGI₂ via a COX-2-independent mechanism. These experiments used ex vivo rabbit aortas bathed in AA. One likely explanation for the differences between our results and theirs is that our study used cultured endothelial cells, whereas theirs used tissue sections composed of many different cell types. Cultured HUVECs were specifically chosen as our experimental system rather than an in vivo system, because these cells provide a model of endothelial biology distinct from the complex pathophysiological and inflammatory processes of atherosclerosis. In addition, the use of primary endothelial cell cultures provides the opportunity to examine precisely the acute effects of cholesterol on cells that regulate key processes between blood and the vessel wall. These studies are limited to a biochemical assessment of cholesterol depletion on a specific cell type and may not reflect changes in COX-2 expression in vivo or in other relevant vascular beds, such as the coronary or cerebral circulation. However, it is important to note that although hypercholesterolemia is a contributing risk factor for atherosclerosis, the present study examines the acute biochemical effects of increased cholesterol in a single cell type and, therefore, must be considered separately from the long-term pathological consequences of excess plasma LDL in vivo.

The precise mechanism by which cholesterol reduces endothelial PGI₂ production may involve the sterol response element–binding protein (SREBP), the oxysterol receptor LXR, retinoids, or other undetermined pathways. There is considerable evidence to support a reciprocal role for PGI₂ in the regulation of intracellular cholesterol balance. PGI₂ has been shown to enhance neutral and acid cholesterol ester hydrolysis activity in aortic smooth muscle cells. The cholesterol-sensitive SREBP is activated when intracellular stores of cholesterol are depleted and serves as a potential link between PGI₂ production and cholesterol homeostasis. SREBP activation promotes the trans-activation of genes involved in cholesterol and fatty acid biosynthesis. We hypothesize that SREBP may also affect the transcription of COX-2 to produce PGI₂, which in turn would facilitate an increase in intracellular FC by cholesterol ester hydrolysis. Future experiments will test this hypothesis.

In summary, the present results demonstrate that in cultured endothelial cells, LDL and FC suppress COX-2 expression. This regulation is ultimately manifested by diminished production of PGI₂ by HUVECs. The dysregulation of this important vascular protective mechanism may explain the reduced capacity for eicosanoid synthesis under hyperlipidemic conditions and the enhanced endothelial function and vasoreactivity observed in patients receiving lipid-lowering therapy.

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