COX-2 gene expression as well as the enzyme activity in C6 cells, this natural product is suggested to be valuable for drug development for the treatment of these brain diseases and inflammations.

In conclusion, we for the first time demonstrated that γ-mangostin, a tetraoxygenated diprenylated xanthone isolated from mangosteen, suppresses IKK activity to inhibit LPS-induced NF-κB activation without affecting cell viability in C6 glioma cells and thereby decreases COX-2 induction.

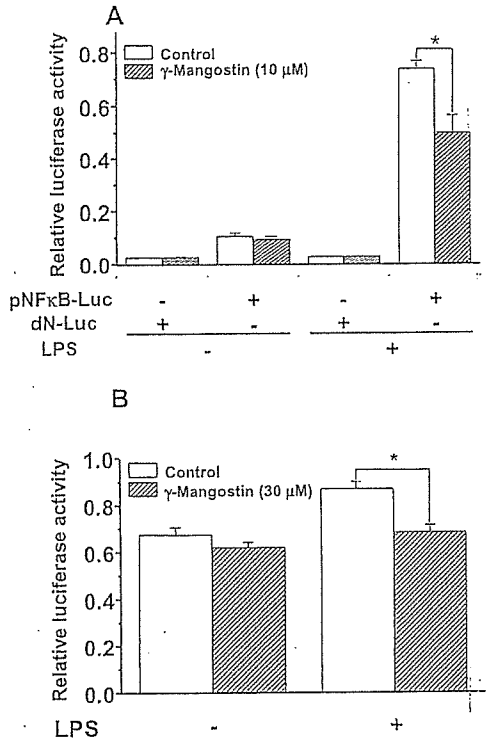

Fig. 7. Inhibitory effect of γ-mangostin on LPS-induced enhancement of luciferase activity of pNFκB-Luc (A) or phPES2(−327/+59)-Luc (B) in C6 cells. Cells were transfected with 0.475 μg/well pNFκB-Luc or dN-Luc (the NF-κB–responsive element-deficient pNFκB-Luc), or 0.4 μg/well phPES2(−327/+59)-Luc, and 0.025 μg/well of pRG-TK plasmid. After transfection, cells were preincubated with the indicated concentration of γ-mangostin or without this compound for 3 h and then incubated in the absence or presence of 1 μg/ml LPS for 8 h. Cells were harvested, and thereafter the luciferase activity was determined as described under *Materials and Methods*. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to *R. reniformis* luciferase activity. Each column represents the mean ± S.E.M. ($n = 3$). *, $P < 0.05$ compared with the value for cotransfected cells with pNFκB-Luc (A), or phPES2(−327/+59)-Luc (B) and pRG-TK plasmid, which were treated with LPS alone (0.1% DMSO).

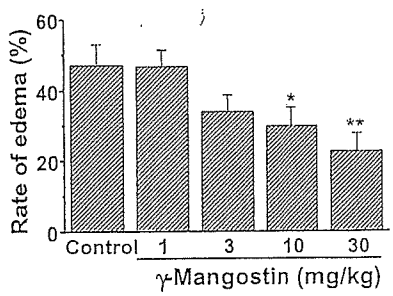

Fig. 8. Concentration-dependent inhibition of rat carrageenan-induced paw edema by γ-mangostin. Rats were injected i.p. with the indicated doses of γ-mangostin 30 min before carrageenan injection. Each column represents the mean ± S.E.M. ($n = 6$). *, $P < 0.05$ and **, $P < 0.01$ compared with the value for the vehicle (DMSO).

This study also demonstrated that γ-mangostin had an anti-inflammatory activity in vivo. These anti-inflammatory properties of this natural compound revealed by our present study using the combined methods of biochemistry and molecular biology sufficiently account for the anti-inflammatory action of the fruit hull of mangosteen. The chemical structure of this natural product is totally different from those of the NSAIDs reported so far, including aspirin and sodium salicylate. These findings thus suggest that γ-mangostin serves not only as a new attractive pharmacological tool for studying the molecular mechanism underlying inflammation but also as a new lead compound for drug development for the prevention and/or treatment of inflammation and brain diseases, including amyotrophic lateral sclerosis and brain tumors.

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Involvement of the 3′-untranslated region of cyclooxygenase-2 gene in its post-transcriptional regulation through the glucocorticoid receptor

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Abstract

Functional roles of the 3′-untranslated region (3′-UTR) of the human Cyclooxygenase-2 (COX-2) gene were evaluated by transient transfection using luciferase (Luc) reporter vectors into bovine arterial endothelial cells (BAEC). Insertion of the 3′-UTR into the downstream of a Luc coding region resulted in decreased reporter activity (23%), although insertion into the upstream was no effect. The reporter activity of the downstream insertion but not the upstream insertion was induced by bacterial lipopolysaccharide (LPS). Moreover, LPS selectively stabilized COX-2 mRNA. Next, to evaluate the role of the 3′-UTR together with glucocorticoid receptor (GR), a GR-expression vector was cotransfected with the reporter vector of the downstream insertion of the 3′-UTR. As a result, the LPS-induced reporter activity was suppressed by dexamethasone in a dose-dependent manner. These data suggest that the 3′-UTR of the COX-2 gene is involved in not only the induction by LPS but also the suppression by DEX of COX-2 expression at the post-transcriptional level.

Keywords: Cyclooxygenase; mRNA stability; AUUUA motif; Post-transcriptional regulation; Dexamethasone

Introduction

Prostaglandin (PG) endoperoxide synthase (EC1.14.99.1) known as cyclooxygenase (COX) is the rate-limiting enzyme in the biosynthesis of prostaglandins and thromboxane (Smith et al., 1996;

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Herschman, 1996). There exist two distinct isozymes, COX-1 and COX-2 (Kosaka et al., 1994; Appleby et al., 1994). While COX-1 is constitutively expressed in most cells, the expression of COX-2 is low under basal condition and induced by inflammatory mediators such as bacterial lipopolysaccharide (LPS) and cytokines, suggesting that COX-2 plays a critical role in inflammation. However, growing lines of evidence indicate that expression of COX-2 is differently and strictly regulated in various cell types and plays key roles in tumorigenesis, development, and circulatory homeostasis (Vane et al., 1998; DuBois et al., 1998; Oshima et al., 1996; Lim et al., 1997). Importantly, nuclear receptors such as glucocorticoid receptor (GR) and PPARγ modulate COX-2 expression mediated through both their ligands and expression patterns (Inoue et al., 1999, 2000). Dexamethasone (DEX), a synthetic glucocorticoid with a potent anti-inflammatory property, suppresses COX-2 expression in macrophage-like differentiated U937 cells, but not in vascular endothelial cells. This cell type-specific regulation may be physiologically important because thromboxane A_2 produced by macrophages has the opposite effect of prostacyclin produced by vascular endothelial cells. We have reported that this different effect of DEX is due to the modulation of COX-2 promoter activity by GR (Inoue et al., 1999). However, involvement of GR in the post-transcriptional regulation of COX-2 gene remains to be elucidated.

Transcriptional regulation of the COX-1 and COX-2 genes has been reported in numerous cell types using luciferase reporter vectors containing the 5′-flanking region of these genes. In bovine arterial endothelial cells (BAEC), the human COX-2 gene ($-1432/+59$) showed promoter activity induced by LPS and TPA, whereas the human COX-1 gene ($-1010/+69$) showed constitutive promoter activity (Inoue et al., 1995). However, the promoter activity of the human COX-2 gene ($-1432/+59$) was 15-fold higher than that of the COX-1 gene ($-1010/+69$) in BAEC, which was in contrast to the result that the amount of intrinsic COX-1 mRNA was greater than that of COX-2 mRNA (Inoue et al., 1995). Interestingly, the entire 3′-UTR of the human COX-2 gene encoded by exon 10 contains 17 copies of Shaw-Kamen sequence (AUUUA), which is found in many immediate-early genes and have been shown to enhance mRNA degradation (Kosaka et al., 1994; Appleby et al., 1994). In fact, recent reports showed that the 3′-UTR is involved in regulated stabilization of COX-2 mRNA especially by inducers of COX-2 expression (Newton et al., 1998; Gou et al., 1998, Dean et al., 1999; Dixon et al., 2000). However, there is no direct evidence that 3′-UTR is involved in the DEX-mediated suppression of COX-2 gene. On the other hand, we have found that the COX-2 promoter region acquires its suppressive response to DEX by transfection of expression vector for the glucocorticoid receptor (GR) into BAEC since BAEC express no detectable levels of GR (Inoue et al., 1999). In this study, we investigated the relationship between the 3′-UTR of the COX-2 gene and the GR. We indicated that the 3′-UTR is involved in suppression of COX-2 expression by DEX mediated through GR at the post-transcriptional level.

Materials and methods

Cell culture and reagents

BAEC were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland), 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate in a humidified atmosphere of 5% CO2 in air. DEX and LPS (from

Escherichia coli serotype 055, B5) were obtained from Sigma (St. Louis, MO) and used at concentrations of 100 nM and 1 μg/ml, respectively.

RNA analysis

Total RNA was isolated using the acid guanidinium thiocyanate procedure. RNAs were then subjected to electrophoresis. The cDNA probes for COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously (Inoue et al., 1999). The levels of mRNA were calculated on the basis of hybridization signals by imaging analyzers Fujix BAS 2500.

Plasmid construction

pGV-C (pGL2-Control Vector), the luciferase vector under control of the constitutive SV40 promoter/enhancer and pRShGRα, an expression vector for the human GR (Giguíre et al., 1986) were described previously (Inoue et al., 1999). Fig. 1 shows pGV-C and its derived reporter vectors containing the 3′-UTR of human COX-2 gene. This fragment contains a part of the coding region (57 bp) and the full-length of the 3′-UTR, which contains 17 copies of the ATTTA motif followed by 3 copies of the polyadenylation signal (AATAAA), and was obtained from the human COX-2 genomic clone λhPESII95 (kindly supplied by Dr. T. Tanabe) as described previously (Inoue et al., 2002). A reporter vector pG-3UCOX2 will express luciferase mRNA under controls of the SV40 enhancer/promoter and of the 3′-UTR (Inoue et al., 2002). This same fragment was ligated into *Sma* I site of pGV-C, which is located in the upstream of the coding region, in a sense (pG-5F3UCOX-2) and anti-sense orientations (pG-5R3UCOX-2), respectively. These clones are used as controls to evaluate the role of the 3′-UTR as enhancer/silencer for the SV40 promoter. DNA sequencing of relevant regions confirmed all of the constructs.

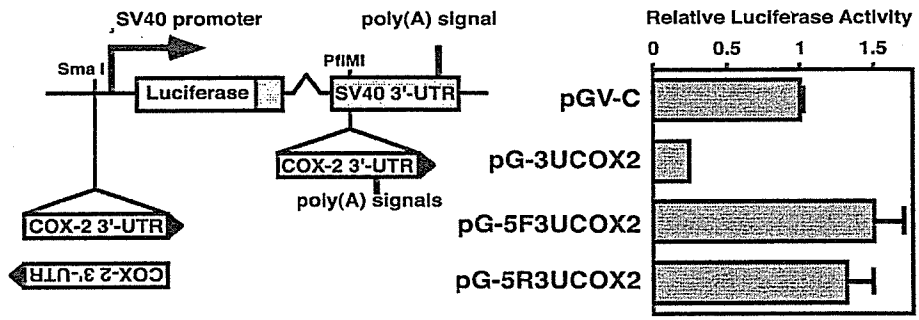

Fig. 1. Effect of the 3′-UTR of the human COX-2 on luciferase expression in a heterologous reporter system driven by SV40 promoter/enhancer. A 3′-UTR of the human COX-2 gene, which contains 17 copies of the AUUUA mRNA instability sequence and several polyadenylation signals, was inserted into pGV-C, a luciferase vector under control of the SV40 promoter/enhancer. A pG-3UCOX2 was constructed by insertion of the 3′-UTR into the downstream of a luciferase coding region in a sense orientation. pG-5F3UCOX2 and pG-5R3UCOX2 were constructed by insertion of the 3′-UTR into the upstream in a sense and anti-sense orientations, respectively. BAEC were transfected with each luciferase reporter together with pCMV-β-galactosidase control vector. Forty-eight hours after transfection, the cells were harvested, lysed and assayed for both luciferase and β-galactosidase activities. The results are presented as luciferase activities relative to the normalized luciferase activity of pGV-C. The data are presented as means ± standard deviations of three separate wells.

DNA transfections

Transfection of the reporter plasmids into BAEC was carried out as described previously (Inoue et al., 1999). Forty-eight hours after transfection using Trans IT™-LT-1 (Mirus), the cells were treated with LPS and/or DEX for 5 h. The cells were harvested, and their luciferase and β – galactosidase activities were determined by a luminometer (Berthold) and a method using chlorophenol red β-D-galactopyranoside as a substrate, respectively.

Results and discussion

The 3′-UTR of COX-2 destabilizes its mRNA, but does not suppress the transcriptional activity

The expression of COX-2 is tightly regulated at the levels of both post-transcription and transcription. To determine if the 3′-UTR of COX-2 mediates post-transcriptional regulation by changing mRNA stability and modulates the transcriptional activity, we examined the decay of chimeric luciferase cDNA constructs containing the 3′-UTR of COX-2 in three different positions. As shown in Fig. 1, transfection of pG-3UCOX2, a reporter vector containing the 3′-UTR at the downstream region of a luciferase coding region resulted in 23% reporter activity relative to that of pGV-C control vector in BAEC. On the other hand, pG-5F3UCOX2 and pG-5R3UCOX2, reporter vectors containing the 3′-UTR at the upstream region in a sense and anti-sense orientation, respectively, resulted in similar reporter activities with that of pGV-C. These results suggest that the 3′-UTR of COX-2 destabilizes its mRNA, but does not suppress the transcriptional activity. This destabilization of COX-2 mRNA by its 3′-UTR will explain the differences between higher COX-2 promoter activity and lower amount of COX-2 mRNA compared with those of COX-1 in BAEC (Inoue et al., 1995).

Transient stabilization of mRNA by LPS mediated through the 3′-UTR in BAEC

We have shown that LPS induces the COX-2 promoter activity in BAEC using luciferase reporter vectors of the human COX-2 gene (Inoue et al., 1995). Therefore, we next examined the involvement of the 3′-UTR of COX-2 in the induction of COX-2 mRNA by LPS. To eliminate the effect of the SV40 promoter activity of pG-3UCOX2, the luciferase activity of pG-3UCOX-2 was normalized by that of pGV-C since both reporter vectors used an identical SV40 promoter. As shown in Fig. 2A, normalized luciferase activity of pG-3UCOX2 maximally induced in 2.7 fold at 5 h and then returned in 1.8 fold at 7.5 h and 1.5 fold at 10 h after LPS treatment, respectively. On the other hand, treatment of LPS for 5 h did not induce the luciferase activity of pG-5F3UCOX2 or pG-5R3UCOX2 (data not shown). Next, to examine whether LPS attenuates the COX-2 mRNA instability, we chased the decay of COX-2 mRNA after the addition of actinomycin D, an inhibitor of transcription. As shown in Fig. 3, induced COX-2 mRNA by LPS for 4 h was almost completely degraded 2 h after the addition of actinomycin D in the absence of LPS. However, when the decay of COX-2 mRNA was significantly delayed in the presence of LPS. Taken together, these results indicates that LPS attenuates the mRNA instability mediated through the 3′-UTR of COX-2. Interestingly, the COX-2 promoter activity (– 327/ + 59) was maximally induced not at 5 h but at 7.5 h after LPS treatment (Fig. 2B), suggesting that the post-transcriptional regulation of COX-2 mRNA will has a different time-dependency of the transcriptional regulation of COX-2 gene.

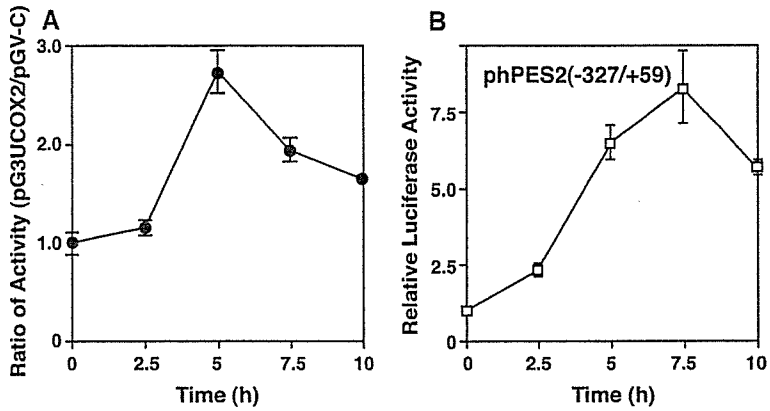

Fig. 2. Effect of LPS on the post-transcriptional control of the 3′-UTR of the human COX-2. BAEC were transfected with pGV-C, pG-3UCOX2 or phPES2(−327/+59) together with pCMV-β-gal as an internal control for the transfection. Forty-eight hours after transfection, the cells were treated with or without LPS. At the indicated times, cells were harvested, lysed and assayed for both luciferase and β-galactosidase activities. The results are presented as ratio of luciferase activities of pG-3UCOX2 relative to that of pGV-C (A) or the luciferase activity of phPES2(−327/+59) (B). The data are presented as means ± standard deviations of three separate wells. phPES2(−327/+59) is a luciferase reporter vector driven by human COX-2 promoter region between −327 and +59 bp.

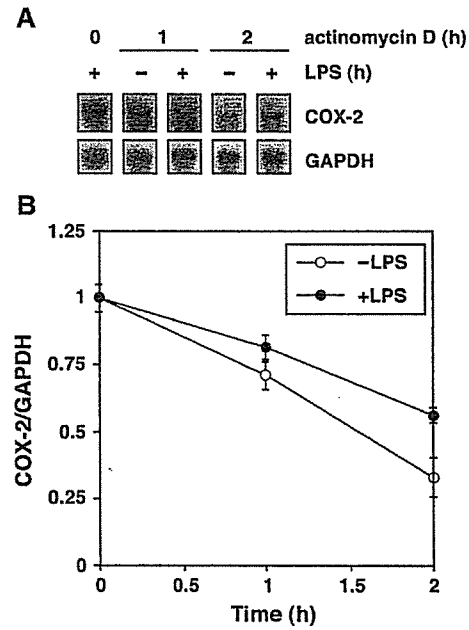

Fig. 3. Effect of LPS on COX-2 mRNA stability. BAEC were exposed to LPS (1 μg/ml) for 4 hours and then treated with actinomycin D (5 μg/ml) for a further 1 and 2 hours in the presence or absence of LPS. Extracted RNAs (10 μg/lane) were analyzed by RNA blot analysis (A). Expression levels of COX-2 mRNAs were normalized with amounts of GAPDH mRNA, and standardized to the value obtained at 4 hours of LPS-treatment. The data are presented as means ± standard deviations of three separate wells (B).

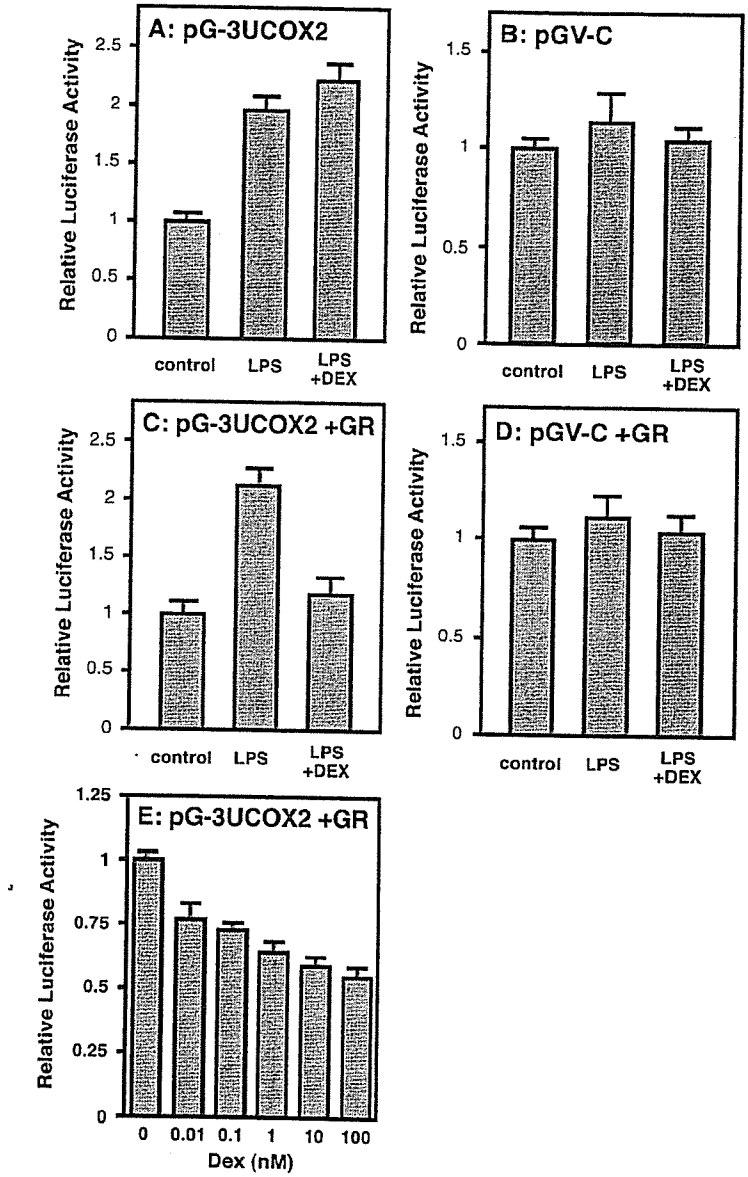

Fig. 4. Effect of DEX on the post-transcriptional control of the 3′-UTR of the human COX-2 in the presence or absence of GR. pG-3UCOX2 (0.3 μg) was transfected into BAEC without (A) or with pRShGRα (0.3 μg) (C), an expression vector for the GR, and with pCMV-βgal (0.05 μg) as an internal control for the transfection. For control experiments, pGV-C was used as a reporter vector instead of pG-3UCOX2 without (B) or with (D) pRShGRα. pGV-B, a promoterless reporter vector, was used instead of pRShGRα as a control and to maintain the total amount of plasmid equal (0.65 μg/well) for each transfection. Forty-eight hours after transfection, the cells were incubated for 5 h with or without 1 μg/ml LPS in the absence or presence of 100 nM DEX (A–D) or the indicated concentrations of DEX (E). The cells were then harvested, lysed and assayed for both luciferase and β-galactosidase activities. The results are presented as luciferase activities relative to the normalized luciferase activity in each control. The data are presented as means ± standard deviations of three separate wells.

DEX destabilizes the LPS-induced stabilization of mRNA by the 3′-UTR of COX-2 in the presence of the GR

To examine the effect of DEX on the 3′-UTR of COX-2, a reporter assay was performed using pG-3UCOX2 and pGV-C (Fig. 4A and B). In both cases, DEX did not suppress the reporter activity. This result is consistent with those reports by two groups (Newton et al., 1998; Gou et al., 1998), who concluded that the 3′-UTR of COX-2 is involved in the IL-1-induced stabilization of COX-2 mRNA, but not the DEX-induced down-regulation. However, we have found that DEX-mediated suppression of the promoter activity of the human COX-2 gene (−327/+59) is modulated by expression of the GR in BAEC (Inoue et al., 1999). Therefore, to examine the effect of DEX on the 3′-UTR in the presence of the GR, cotransfection of pRShGRα with the reporter vector pG-3UCOX2 into BAEC was performed. As shown in Fig. 4C, DEX suppressed the LPS-induced reporter activity in BAEC. On the other hand, even in the presence of the GR, DEX did not suppress the reporter activity in pGV-C (Fig. 4D), pG-5F3UCOX2 or pG-5R3UCOX-2 (data not shown). Moreover, the suppressive effect of DEX on the luciferase activity of pG-3UCOX-2 was dose-dependent (Fig. 4E). This dose-dependency is similar to that of the COX-2 promoter activity (−327/+59) in the presence of the GR (Inoue et al., 1999), indicating that the GR modulates the COX-2 expression at both the transcriptional and post-transcriptional levels.

DEX-mediated inhibition of COX-2 expression has previously been reported by several laboratories (Kujubu and Herschman, 1992; Xie et al., 1993; DeWitt and Meade, 1993; Masferrer et al., 1994; Crofford et al., 1994; Smith et al., 1996). This regulation occurs at both transcriptional and post-transcriptional levels. Concerning the transcriptional control, the NF-κB site (−223/−214) of the human COX-2 promoter region is involved in both the LPS-induced expression and its suppression by DEX in macrophage-like differentiated U937 cells (Inoue and Tanabe, 1998), and other cis-acting elements are also involved in BAEC transfected with the GR (Inoue et al., 1999). As for the post-transcriptional control, there are several reports indicating that DEX-mediated suppression of COX-2 mRNA occurs at the post-transcriptional level due to its destabilization (Newton et al., 1998; Xie et al., 1993; Ristimäki et al., 1996). Especially, the AUUUA sequence of the 3′-UTR was reported to be involved in the control of mRNA instability (Newton et al., 1998; Gou et al., 1998; Dean et al., 1999; Dixon et al., 2000). However, there has been no reports that the 3′-UTR is involved in the DEX-mediated suppression since DEX did not suppress the luciferase activity of pG-3UCOX2 (Fig. 4A) or similar constructs (Newton et al., 1998; Gou et al., 1998). This study demonstrates, for the first time, that the 3′-UTR is involved in the DEX-mediated suppression because of sufficient supply of the GR to the reporter system (Fig. 3C). Thus, this assay system will provide a suitable tool to analyze the molecular mechanism by which DEX interacts with the 3′-UTR to control the mRNA stability.

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HDL_3 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. HDL 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—HDL_3 (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 HDL_3 is involved in COX-2 mRNA transcription and stabilization. As a consequence of COX-2-induction by HDL_3 prostacyclin production increased, incubation with a COX-2 selective inhibitor blocked this effect. Moreover, HDL_3 increased caveolin-1 phosphorylation, thus promoting PGI-synthase shuttling from the membrane to the perinuclear area.

Conclusion—We conclude that in endothelial cells, HDL modulate 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;24:1-8.**)

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 posses several anti-atherosclerotic effects,[2] including the induction of prostacyclin (PGI_2), a strong vasorelaxant[3] that acts also as an inhibitor of platelet and leukocyte activation.[4] The stimulatory effect on PGI_2 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 PGI_2 synthesis in endothelial cells,[8,9] whereas COX-1 is mainly involved in TXA_2 synthesis by platelets.[8,9] Whether COX-2 inhibition is useful as related to an increase of cardiovascular risk is uncertain.[10] HDL induces COX-2 expression in rabbit smooth muscle cells[11] 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 HDL_3 on COX-2 expression and eicosanoid production in cultured human endothelial cells.

Methods

HDL_3 (dose 1.125 to 1.21 g/mL) was isolated from human plasma and protein content was determined as described.[22] HDL_3 was used within 6 hours from isolation. No LPS contamination was detected as assessed by the endotoxin kit (Sigma, Italy).

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HUVECs were isolated and cultured as described.[23] In all experiments, cells were preincubated with serum-free medium for 6 hours,[22-24] then HDL_3 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;[23] 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,[25] was used at 0.1 μmol/L.

Immunocytochemistry

Cells were cultured on coverslips in 24-well plates. Fixed cells[23] 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′, then propidium iodide (2,5 μg/mL) was added for 30′. For the studies of COX-2 co-localization with PGI-S, mPGES-1 and phospho-caveolin-1 fixed cells were incubated overnight with the antibody, followed by incubation with anti IgG FITC-conjugated (30′), anti-COX-2 phycoerythrin-labeled for 1 hour, and TOPRO 3 (Molecular Probes) (1:500) for 15′. 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.[22,24] 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 melting peaks of the amplicons were as expected (not shown). The primers used, the amplicon size, and the melting temperature are indicated in online Table I (available online at http://atvb.ahajournals.org). Each sample was analyzed in duplicate using the IQ™ Cycler (Biorad). The PCR amplification was related to a standard curve ranging from 10^{-11} M to 10^{-14} M.

Transcription Assay

The construction of various reporter vectors for the human COX-2 gene has been described previously.[20,21] 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,[26-28] we performed transfection experiments in CHO cells, a cell line widely used for studies involving the effects of HDL in vitro.[29,30] 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.[21]

Detection of Prostaglandin Release by Competitive Enzyme Immunoassay

Competitive enzyme immunoassay kits for 6-keto $PGF_1\alpha$, TXB_2, and PGE_2 were from Cayman. HUVECs were exposed to HDL_3 (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

HDL_3 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 HDL_3 (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 HDL_3. The induction was maximal after 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, HDL_3 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 HDL_3 without changes of COX-1 expression (Figure 1b). On three-dimensional reconstruction, COX-2 localized in the perinuclear area and in the cytoplasm[31] (Figure 1c).

Effects of HDL_3 on Intracellular Kinase Pathways and on COX-2 Promoter Activity

HDL_3 activated ERK1/2 and p38 MAPK, with a the peak of phosphorylation reached after 5 to 10 minutes of incubation (Figure 2). Several transcription factors are activated through MAPK-dependent pathways.[17-19] HDL_3 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 HDL_3 (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 HDL_3 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 HDL_3 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 HDL_3-treated cells and 1.26±0.16-fold in LPS treated cells (Figure 3; P=NS versus control).

Involvement of p38-MAPK in HDL_3-Induced COX-2 Protein and mRNA Expression and Stabilization

Cells were preincubated with the MEK1 inhibitor U 0126 (25 μmol/L) or the p38 MAPK inhibitor SB 203580 (1 μmol/L) for 1 hour. HDL_3 (30 μg/mL) were added for 2 and 4 hours to evaluate COX-2 mRNA and protein expression. U0126 did not affect HDL_3-induced COX-2 expression.

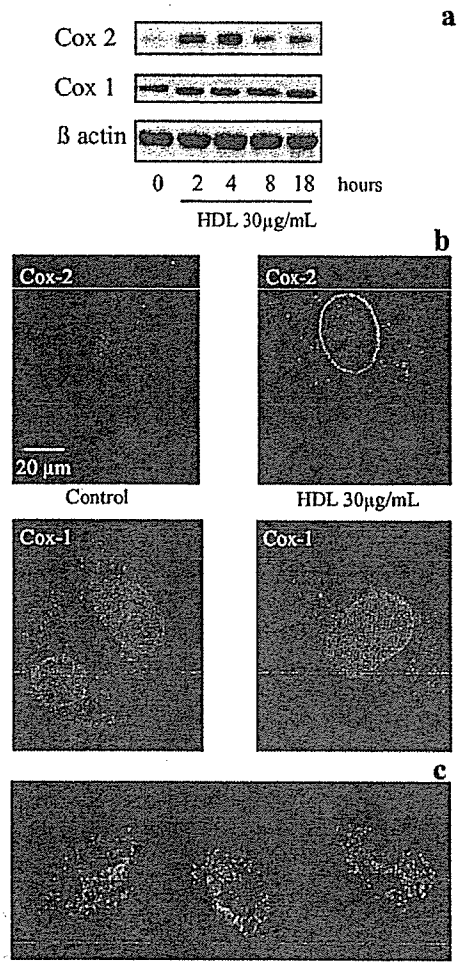

Figure 1. Time dependency of COX-1 and COX-2 expression as detected by Western immunoblotting (a) and indirect immunofluorescence (b) in HUVEC incubated with HDL_3 for 4 hours (30 μg/mL). (The green signal represents COX-1 or COX-2, whereas the red one is the nuclear staining with propidium iodide). A three-dimensional reconstruction showing that COX-2 localizes mainly in the perinuclear cytoplasm is shown (c). Three different projections are shown. The results are representative of 4 separate experiments.

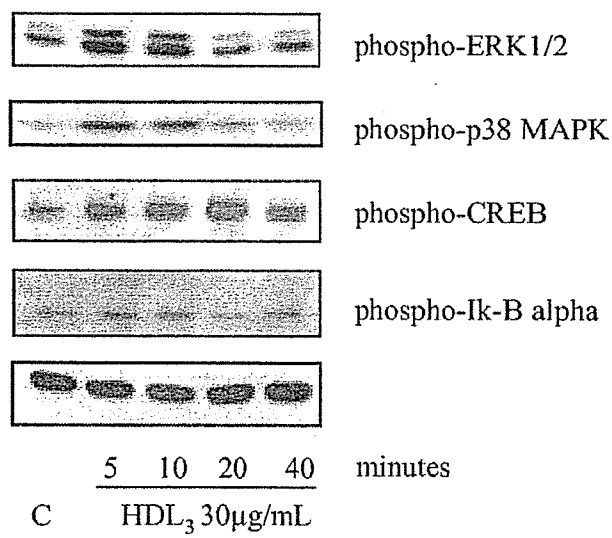

Figure 2. Time-dependent phosphorylation of ERK1/2, P38 MAPK, CREB, and Ik-B alpha after incubation of endothelial cells with HDL_3. HUVEC were incubated from 5 minutes up to 40 minutes with HDL_3 (30 μg/mL). The results are representative of 4 experiments.

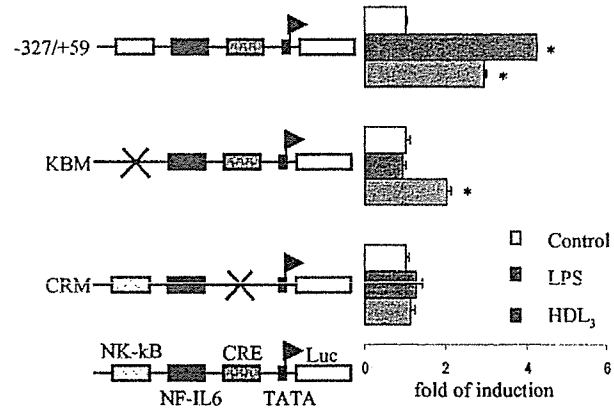

Figure 3. Identification of the regions responsible for HDL_3-induced promoter activity of the human COX-2 gene. The 5′-flanking region of the human COX-2 gene with site-specific mutations are represented schematically on the left. After transfection, CHO cells were incubated for 6 hours with LPS (1 μg/mL), used as positive control, and with HDL_3 (30 μg/mL). The results are presented as relative luciferase activity normalized to cellular protein content. Each experiment was performed in triplicate. *$P<0.01$ versus control.

SB203580 strongly inhibited HDL_3-mediated COX-2 mRNA and protein expression (Figure 4). Because p38 MAPK stabilizes COX-2 mRNA,[32] we investigated whether HDL_3 possesses this effect. To asses the stability of COX-2 mRNA in HUVEC, actinomycin D (2 μg/mL) was added to cells after 2 hours of HDL_3 incubation and COX-2 mRNA levels were measured up to 60 minutes (Figure 4). Simultaneous addition of SB203580 (1 μmol/L) and actinomycin D to the cells after a 2-hour stimulation with HDL_3 resulted in a more rapid decrease in COX-2 mRNA levels, suggesting mRNA stabilizing effect by p38 MAPK activity.

Effects of HDL_3 on Eicosanoid Production

The effects of HDL_3 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 $PGF_1\alpha$ (PGI_2 main metabolite) was 73.14±6.79 pg/mg of

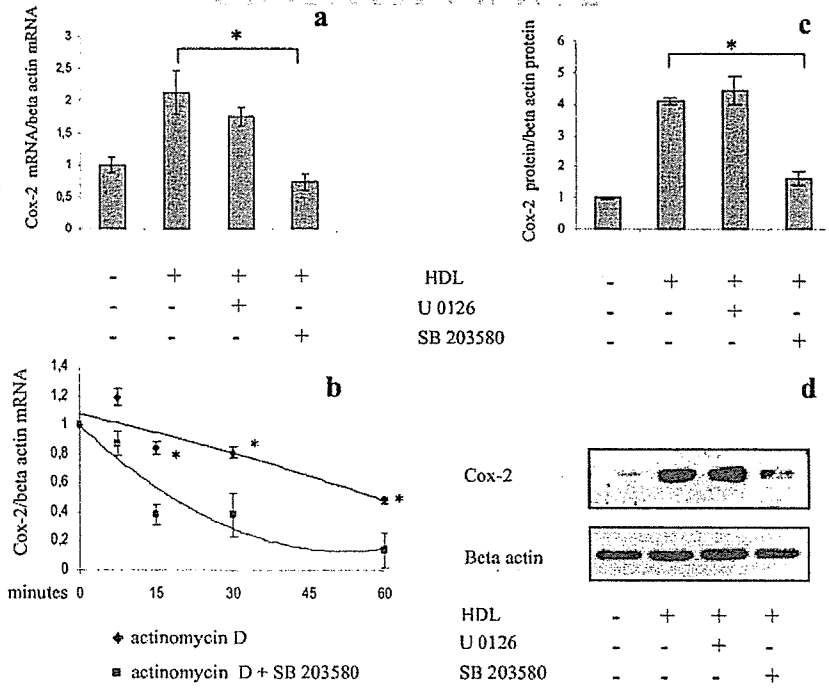

Figure 4. Involvement of p38 MAPK pathway in COX-2 mRNA (a and b) and protein expression induced by HDL_3 (c and d). HUVECs were incubated for 2 hours (for mRNA detection) and 4 hours (for protein detection) with HDL_3 (30 μg/mL) alone or in the presence of U0126 (25 μmol/L) or of SB203580 (1 μmol/L). a, COX-2 mRNA expression was assessed with real-time quantitative PCR; the target sequence was normalized to the beta actin content. b, Inhibition of p38 MAPK destabilizes COX-2 mRNA expression induced by HDL_3. HUVECs were incubated for 2 hours with HDL_3 (30 μg/mL), then actinomycin D alone or actinomycin D plus SB203580 were added (time 0). COX-2 mRNA levels were measured using real-time quantitative PCR from time 0 minutes up to 60 minutes. Data are mean±SD of 4 separate experiments. *$P<0.01$. c and d, COX-2 protein expression was assessed by immunoblotting. c, The results are mean±SD of 4 separate experiments and are normalized for beta actin content. *$P<0.01$.

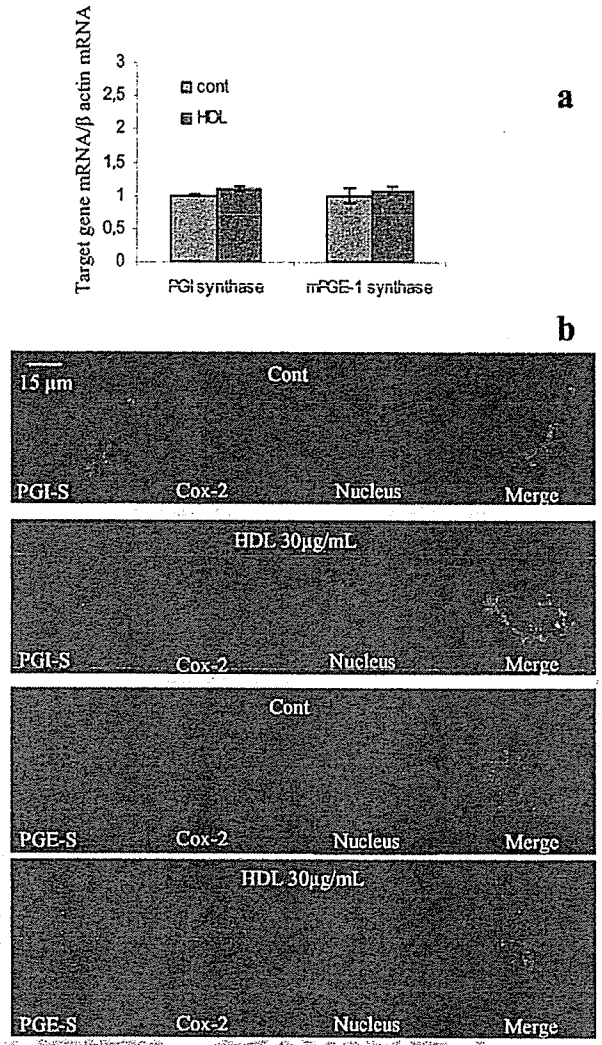

Figure 5. Effects of HDL_3 on PGI-S and PGE-S mRNA expression and cellular localization. a, HUVECs were incubated for 2 hours with HDL_3 (30 μg/mL) and COX-2 mRNA levels were measured using quantitative real-time PCR. Data are mean±SD of 4 separate experiments. b, cellular localization of COX-2 and PGI-S or PGE-S. The green signal is for PGI-S or PGE-S, the red signal is for COX-2, and the blue signal is the staining for the nucleus. The results are representative of 4 experiments.

cellular protein. Incubation of endothelial cells with HDL_3 increased 6-keto $PGF_1\alpha$ 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] HDL_3-induced 6-keto $PGF_1\alpha$ production was reduced to 77.95±10.19 pg/mg of cellular protein and PGE_2 resulted in 74.10±3.45 pg/mg of cellular protein and was not affected by HDL_3 incubation.

Effects of HDL_3 on PGI Synthase Expression and Cellular Localization

As HDL_3 induces COX-2 expression and increases PGI_2 release, we investigated whether HDL_3 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.[33] Caveolin-1 is the main protein of caveolae, and when phosphorylated[34] it moves into the cytoplasm,[34,35] shuttling PGI-S in the perinuclear area where it couples to COX-2,[35] 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 HDL_3 induces COX-2 expression and PGI_2 release in human endothelial cells via p38 MAPK activation. The activation of this signaling pathway promotes COX-2 mRNA transcription and stabilization.

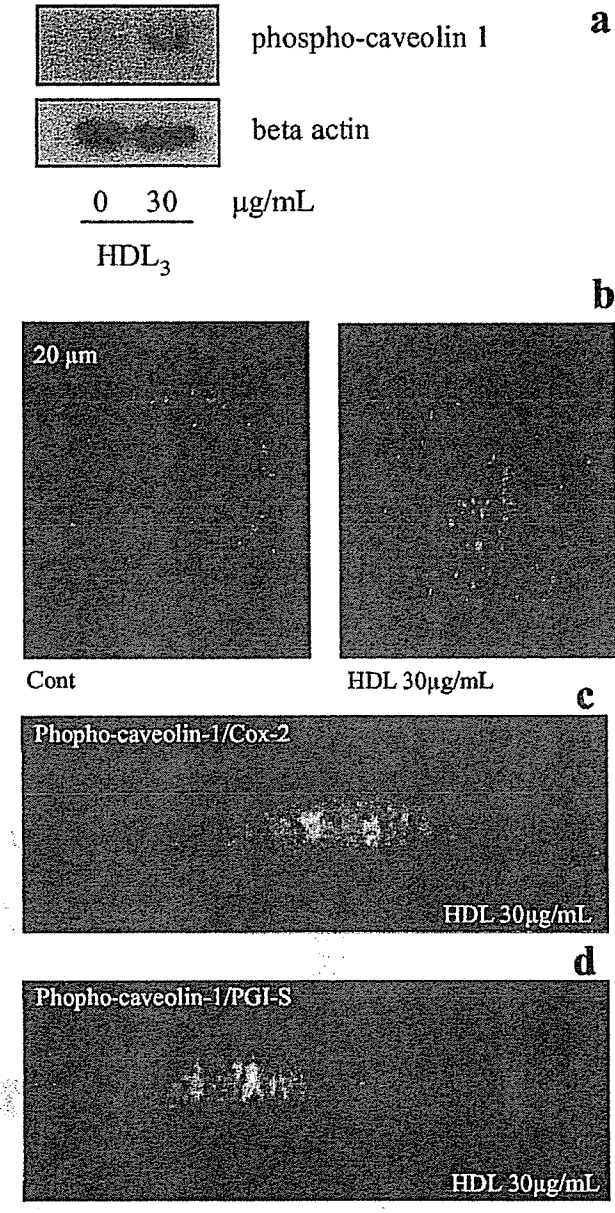

Figure 6. Effects of HDL_3 on caveolin-1 phosphorylation and cellular localization. a, HUVECs were incubated for 4 hours with HDL_3 (30 µg/mL) and phospho-caveolin-1 levels were determined by Western blotting. b, cellular localization of phospho-caveolin-1. The green signal is for phospho-caveolin-1, the blue is staining for the nucleus. c, Cellular localization of phospho-caveolin-1 and COX-2 (transversal projection). The green signal is for phospho-caveolin-1, the red signal is for COX-2, and the blue signal is the nucleus. d, Cellular localization of phospho-caveolin-1 and PGI-S (transversal projection). The green signal is for phospho-caveolin-1, the red signal is for PGI-S, and the blue signal is the nucleus. The results are representative of 4 experiments.

On incubation of cells with HDL_3, 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.[35,36] This effect is specific for COX-2; in fact, COX-1 was mainly localized in the cytoplasm and was not modulated by HDL_3; moreover, PGI_2 synthesis was downregulated by a specific COX-2 inhibitor.

The molecular mechanisms by which HDL_3 induces COX-2 are unknown. Here we show that HDL_3 activates 2 of the major kinases pathways involved in COX-2 gene transcription: ERK1/2 and p38 MAPK.[13,14] HDL can activate ERK1/2 via cell surface S1P receptor in astroglial cells.[37] However, the possibility that MAPK activation results from plasma membrane cholesterol depletion cannot be excluded.[38] In support of this hypothesis, Smith et al[39] showed that increasing concentration of LDL or free cholesterol decreases COX-2 expression and PGI_2 synthesis. As HDL triggers the release of cholesterol from cells,[2] our observation suggests that cellular cholesterol balance plays an important role in determining COX-2 levels.

HDL_3 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,[40] thus suggesting that CRE plays a relevant role in COX-2 induction by a number of stimuli.[40] Using transient transfection experiments, we demonstrated that mutation in CRE abrogated the luciferase activity induced by HDL_3, confirming the role of CRE in HDL_3-induced COX-2 gene transcription.

NF-kB has also been suggested to be involved in determining COX-2 gene transcription.[20,21] We show that a mutation in the NF-kB response element abrogates luciferase activity induced by LPS, used as a positive control, while it slightly decreases HDL_3-induced luciferase activity, suggesting a minor role of this pathway in COX-2 induction by HDL_3.

As transcriptional regulation of the COX-2 gene occurs via activation of MAPKs,[13,14] we investigated whether inhibition of ERK1/2 or p38 MAPK pathway affected HDL_3-induced COX-2 mRNA and protein expression. We show that the p38 MAPK pathway is responsible for the induction of COX-2 by HDL_3.

P38 MAPK plays a housekeeping role in maintaining COX-2 mRNA stability[32] via the recognition of the AUUUA motifs present in the 3′ untranslated region of COX-2.[41] We therefore studied COX-2 mRNA stability in cells stimulated with HDL_3. 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.[9] Eicosanoids are involved in a variety of physiological processes in atherosclerosis and thrombosis, including leukocyte–endothelial cell adhesion, vasorelaxation, and platelet aggregation.[9] The dominant prostaglandin produced by endothelial cells is PGI_2.[4] PGI_2 is believed to play a protective role in atherothrombosis.[4] COX-2 contributes significantly to systemic PGI_2 synthesis in humans;[42] 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_3 that increases PGI_2 release mediated by AA in endothelial cells. This effect is dependent mainly on COX-2 as indomethacin eptyl ester, a specific COX-2 inhibitor, abolished PGI_2 release induced by HDL_3. This observation may also be relevant to the recent observation that COX-2 inhibitors may increase CHD risk.[10] In vitro 30 μg/mL of HDL_3 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 as 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_3 does not increase PGE_2, a proatherogenic eicosanoid, synthesized mainly via COX-2,[12] 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_3-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[33] and is activated when shuttled from the plasma membrane in the perinuclear area;[35] moreover, disruption of caveolae organization downregulates prostacyclin production and impairs angiogenesis.[43,44] Here we demonstrate that HDL_3 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.[33,35] Nitric oxide is responsible for several beneficial effects of HDL on endothelial cells,[45] such as helping to maintain endothelial integrity, facilitating vascular relaxation, inhibiting cell adhesion to vascular endothelium, decreasing radical oxygen production, and inhibiting apoptosis.[45] 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[46]

In summary, our data suggest that in human endothelial cells, HDL can modulate COX-2 expression via p38 MAPK-dependent COX-2 mRNA transcription and stabilization. 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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Native LDL and Oxidized LDL modulate cyclooxygenase-2 expression in HUVECs through a p38-MAPK, NF-κB, CRE dependent pathway and affect PGE_2 synthesis

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Abstract. Native low density lipoproteins (n-LDL) and oxidized low density lipoproteins (Ox-LDL) play a central role in atherogenesis and possess a wide variety of biological properties. We investigated whether n-LDL or Ox-LDL modulate cyclooxygenase-1 and -2 (Cox-1 and Cox-2) expression and prostaglandins release in human endothelial cells via an MAPK-dependent pathway. HUVECs were incubated in the presence of n-LDL or Ox-LDL (30 μg/ml for both) for 2-15 h. Real-time PCR, western blotting and immunocytochemistry were used to investigate Cox-1 and Cox-2 expression. N-LDL and Ox-LDL induced Cox-2 expression in a time- and dose-dependent manner. The Cox-2 protein was strongly induced 2 h after exposure to n-LDL or Ox-LDL, the induction was maximal after 4 h and sustained for at least 8 h. The effect was specific for Cox-2, as Cox-1 expression was not modulated either by n-LDL or by Ox-LDL. The induction of Cox-2 expression was mainly dependent on the activation of p38 MAPK. Transient transfection analysis using a Cox-2 promoter showed that n-LDL and Ox-LDL exert their effects at the transcriptional level via NF-κB and CREB activation. N-LDL and Ox-LDL increased PGE_2 release in a Cox-2-dependent manner while TXA_2 and PGI_2 release were not affected either by n-LDL or Ox-LDL. The finding that n-LDL and Ox-LDL induces Cox-2 in human endothelial cells through a p38 MAPK, NF-κB, CREB dependent pathway thus modulating PGE_2 release, suggests a new mechanism by which these lipoproteins induce endothelial dysfunction, sustaining inflammatory processes in the arterial wall.

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Introduction

The expression of cyclooxygenase-2 (Cox-2), a rate-limiting enzyme of PG synthesis which catalyses the conversion of arachidonic acid (AA) to PGG_2 and further to PGH_2 (1), is increased within human atherosclerotic lesion, particularly in macrophages and endothelial cells (2,3), supporting the hypothesis of the inflammatory nature of the atheroma (4). Native LDL and oxidized LDL influence several processes involved in atherosclerosis (5,6), including leukocyte adhesion and attachment to endothelial cells, chemotaxis, cytotoxicity, stress response and expression of cytokines, growth factors or prostaglandins (PGs) (7-13). PGs, synthesized by the cyclooxygenase enzyme (Cox) (1), are essential mediators in the regulation of cardiovascular function and are closely related to atherogenesis (14-16). Two major forms of Cox, Cox-1 and Cox-2, have been identified (17,18). While Cox-1 is constitutively expressed in most cell types, Cox-2 is induced after stimulation by various growth factors and cytokines (19,20). Cox-2 expression is modulated by growth factors and cytokines via mitogen-activated protein kinase (MAPKs) cascade (21-23). Once activated, the MAPKs could modulate several transcription factors such as CREB, NF-IL6, AP-1 or NF-κB, (24,25) all of which are involved in Cox-2 gene transcription (26,27).

In the present study we investigated whether n-LDL or Ox-LDL modulate Cox-1 or Cox-2 expression in human endothelial cells via an MAPKs pathway. Moreover, as Cox-2 derived prostanoids exert pro-atherogenic or anti-atherogenic roles (28), the effects of n-LDL or Ox-LDL on PGI_2, TXA_2 and PGE_2 production were studied.

Materials and methods

Lipoproteins. LDL (1.019-1.063 g/ml) were obtained from freshly isolated human plasma by preparative ultracentrifugation (29) and dialysed in PBS containing 0.01% EDTA. The protein content was determined by the Lowry method (30). LDL (0.2 mg protein/ml) were oxidized with 20 μM $CuSO_4$ for 24 h at 37°C, as previously described (9). The oxidation was blocked by the addition of 40 μM butylated