7The sterol response element binding protein regulates cyclooxygenase-2 gene expression in endothelial cells

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Abstract We previously demonstrated that cholesterol deprivation increases endothelial cyclooxygenase-2 (COX-2)-dependent prostacyclin [prostaglandin I_2 (PGI_2)] production in vitro. Cholesterol directly regulates gene transcription through the sterol response element binding protein (SREBP). In this work, we demonstrate that SREBP directly regulates COX-2 expression. Cholesterol reduces human COX-2 promoter-luciferase reporter construct activity in transiently transfected endothelial cells. Conversely, cotransfection with a constitutively active mutant SREBP increases COX-2 promoter activity. SREBP-1a and -2 specifically bind a putative sterol response element (SRE) sequence in the COX-2 promoter. This sequence competes for SREBP binding to a low density lipoprotein receptor consensus sequence in an electromobility-shift assay. These data indicate that endothelial COX-2 is regulated by cholesterol via the SREBP pathway. The present study identifies COX-2 as the first vascular gene without a clear role in lipid metabolism transactivated by SREBP, and suggests that enhanced production of PGI_2 through this pathway may be an additional benefit of cholesterol-lowering therapies.—Smith, L. H., M. S. Petrie, J. D. Morrow, J. A. Oates, and D. E. Vaughan. **The sterol response element binding protein regulates cyclooxygenase-2 gene expression in endothelial cells. *J. Lipid Res.* 2005.** 46: **862–871.**

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Elevated levels of low density lipoprotein (LDL) are a well-established risk factor for cardiovascular disease (1) and are associated with an impairment of endothelial-dependent vasodilator capacity. Endothelial-dependent vasodilation is mediated by the combined effects of nitric oxide, prostacyclin [prostaglandin I_2 (PGI_2)], and endothelial-derived hyperpolarizing factor (2). In the vasculature, PGI_2 is synthesized by the inducible form of the cyclooxygenase enzyme, cyclooxygenase-2 (COX-2) (3, 4). PGI_2 plays important roles in vascular homeostasis, vasodilation, and platelet quiescence (5–8).

Extensive analysis of the effects of cholesterol and fatty acids on hepatic gene expression resulted in the discovery of the sterol response element binding protein (SREBP) (9) and established the role of SREBP as a lipid-dependent transcription factor (10). Although numerous transcriptional targets of SREBP have been identified (11), little is known about the effects of SREBP on gene expression in extra-hepatic tissues, or upon genes not directly associated with lipid homeostasis. Intracellular cholesterol concentrations are maintained through two distinct yet ultimately convergent pathways: LDL receptor (LDLR)-mediated endocytosis of plasma lipoprotein (12) and de novo synthesis via the mevalonate pathway (13). Under conditions of cholesterol deprivation, SREBPs increase the expression of the LDLR and the enzymes of the mevalonate pathway (14). SREBPs are transcription factors that are activated by a posttranscriptional two-step cleavage process. Inactive, precursor SREBP is sequestered in the endoplasmic reticulum (ER), where it resides complexed with the SREBP cleavage-activating protein (SCAP) (15). Under low-cholesterol conditions, the SCAP transports SREBP to the Golgi apparatus, where it is cleaved by two specific proteases. The site 1 protease (S1P) and site 2 protease (S2P) release the transcriptionally active N-terminal domain of SREBP (16). This bHLH-zip portion migrates to the nucleus and transactivates gene expression by binding sterol response elements (SREs) in target genes (10). The three forms of SREBP are SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1a is a powerful activator of all SREBP-responsive genes (17). SREBP-1c, a splice variant of SREBP-1a, has a short transactivation domain and is less potent than SREBP-1a. Although they differ in potency, SREBP-1a and

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Abbreviations: COX, cyclooxygenase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; LDL, low density lipoprotein; PGE_2, prostaglandin E_2; PGI_2, prostaglandin I_2; SREBP, sterol response element binding protein.

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-1c activate genes involved in the synthesis of fatty acids and the formation of triglycerides and phospholipids. SREBP-2 is encoded by a separate gene, and preferentially activates genes involved in cholesterol homeostasis (17). Cholesterol and fatty acids exert a negative feedback mechanism on their own synthesis by inhibiting the translocation of SREBP to the Golgi, and thus prevent SREBP activation (18).

We recently demonstrated that LDL-cholesterol decreases PGI_2 production in a dose-dependent manner. In that study, we observed that modulating both LDL and free cholesterol altered endothelial COX-2 gene expression. Total cholesterol deprivation increased COX-2 mRNA and protein, whereas high concentrations of cholesterol had the opposite effect. COX-1 gene expression was unaffected by changes in ambient cholesterol levels (19). To further define the mechanism by which cholesterol regulated COX-2, we examined the role of the cholesterol-sensitive transcription factor, SREBP. In this report, we demonstrate that endothelial cells express functional SREBP, and provide evidence that endothelial COX-2 is directly regulated by SREBP at the transcriptional level. We further show that SREBP binds to an SRE located at −422 bp from the transcription start site of COX-2 and that this site is necessary for SREBP-mediated activation of the human COX-2 promoter.

EXPERIMENTAL PROCEDURES

Matched lots of delipidated and normal fetal bovine serum (FBS) were purchased from Calicobiologicals (Reamstown, PA). All other cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO). 25-Hydroxycholesterol was purchased from Steraloids (Newport, RI). Custom oligonucleotides were synthesized by Sigma-Genosys Biotechnologies (The Woodlands, TX). Human SREBP antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Precast NuPage (10% Bis-Tris MES) gels, buffers, polyvinylidene difluoride (PVDF) membrane, Seeblue Plus2 molecular weight marker, and sample buffer were purchased from Invitrogen (Carlsbad, CA). The luciferase promoterless vector, pGL-2 basic, and dual-luciferase assay reagents were purchased from Promega (Madison, WI). Organic solvents (HPLC grade) and biochemicals were purchased from Sigma-Aldrich. Lovastatin was a gift of Merck and Co. (Rahway, NJ).

Plasmids

CMV-SREBP1a-460, CMV-SREBP2-468, and SRE-Luc were provided by Dr. Joseph Goldstein (20). Human COX-2 promoter constructs were provided by Dr. Steve Prescott and Dr. Hyroishi Inoue (21, 22).

Cell culture

Confluent primary cultures of human umbilical vein endothelial cells (HUVECs) were isolated as previously described (23) and cultured in supplemented medium 199 (15% FBS, endothelial mitogen, penicillin, streptomycin, and heparin), on gelatin-coated plates. Primary cultures of bovine aortic endothelial cells (BAECs) were obtained from fresh bovine aortas and cultured as described previously (24, 25). Cells were serum starved overnight and then treated for 24 h with cholesterol-rich or cholesterol-deficient medium. Cholesterol-rich medium contained 1 μg/ml 25-hydroxycholesterol + 10 μg/ml cholesterol in 5% lipid-depleted serum; cholesterol-deficient medium contained no cholesterol in 5% lipid-depleted serum. All experiments used IL-1α (1 ng/ml) as a positive control for the induction of COX-2.

Immunoblot analysis

Total cellular protein was isolated from 100 mm culture dishes (52.0 cm^2) containing approximately 3.0×10^6 cells, using 0.5 ml protein lysis buffer as previously described (26). Proteins were separated using a 10% acrylamide Bis-Tris, MES SDS-PAGE. Proteins were then transferred to PVDF membrane and blocked overnight with 5% nonfat dry milk at 4°C. Blots were exposed to primary antibody (1:1,000 dilution for SREBP monoclonal antibody) for 2 hrs at 25°C. Secondary antibody (1:5,000) was added after four washings with Tris-buffered saline/Tween-20, and incubated for 1 h at 25°C. The blots were visualized using ECL-Plus (Pharmacia; Uppsala, Sweden) and exposure to Kodak X-OMAT blue autoradiographic film.

Reverse transcription-polymerase chain reaction

Total cellular RNA from HUVEC was isolated 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 using the Advantage One-Step RT-PCR kit (Clontech; Palo Alto, CA). Primers used for amplification are listed in **Table 1**. Amplified PCR products were cloned and sequenced to confirm target specificity. Sequenced clones were used as PCR positive controls.

Real-time quantitative PCR (qPCR) was performed on cDNA synthesized from 1.0 μg of total RNA using I-Script cDNA synthesis kit (Bio-Rad; Hercules, CA). HUVECs were treated with increasing doses of lovastatin (0.1, 1.0, 10.0 μM) for 48 h, and total RNA was isolated as described above. IQ-SYBR Supermix was used with an I-cycler instrument (Bio-Rad) to amplify COX-2 and 18s cDNAs. All primers used for qPCR were designed using Beacon Designer II (PREMIER Biosoft; Palo Alto, CA), and are listed in Table 1. PCR thermocycling steps were 95°C for 3.0 min, then 40 cycles of 95°C for 15 s and 61.3°C for 30 s, followed by melting curve data collection with 201 cycles of 0.2°C/8 s increments and analysis. Standard curves were created by making serial dilutions of purified COX-2 and 18s amplicons (ranged from 0.0 ng/ml to 10,000 ng/ml) and run in parallel with sample cDNAs on a single PCR plate in triplicate. Data analyses were performed using the relative standard curve method and are presented as fold induction of COX-2 mRNA over control samples.

Transfections and luciferase assay

BAECs were cultured in 38 mm 12-well plates at a density of 1×10^5 cells/well. At 70–80% confluence, they were transfected using lipofectamine PLUS (Invitrogen) with 0.6 μg of the COX-2 pGL-2 construct and 0.2 μg of the plasmid pCMV-RLuc, which contains the renilla luciferase gene under control of the CMV promoter. COX-2 promoter constructs were co-transfected with increasing concentrations (5.0–500.0 ng) of pCMV-SREBP1a-460 or pCMV-SREBP2-468. Firefly-luciferase activity was corrected for renilla-luciferase activity and is presented as relative light units (RLUs). RLUs reported reflect a value normalized to the activity of the empty vector.

Immunofluorescence

HUVECs were grown to confluence in 2-well glass chamber slides. Cells were treated with cholesterol-rich or cholesterol-deficient media for 48 h. The chambers were rinsed and the cells fixed in 4% formaldehyde. Nonspecific sites were blocked using 10% normal goat serum in PBS for 1 h at 25°C. Cells were incubated with anti-SREBP-1 and -2 polyclonal antibodies or SCAP

TABLE 1. Oligonucleotides used in PCR and EMSA

Oligonucleotide	Sequence	Reference
S1p Sn	TGCTCCCACCTGACTTTGAAG	(52)
S1p As	GCTGTGAAGTATCCGTTGAAAGC	(52)
S2p Sn	CGGATGCTGGGGCTGTAAGG	(52)
S2p As	CGGTCAGGTAGACGACAGTCC	(52)
SCAP Sn	ATTTGCTCACCGTGGAGATGTT	(52)
SCAP As	GAAGTCATCCAGGCCACTACTAATG	(52)
BP1a Sn	GGCCGAGATGTGCGAACT	(53)
BP1a As	TTGTTGATGAGCTGGAGCATGT	(53)
BP1c Sn	GGAGCCATGGATTGCACATT	(53)
BP1c As	GGCCCGGGAAGTCACTGT	(52)
BP2 Sn	GCGTTCTGGAGACCATGGA	(52)
BP2 As	ACAAAGTTGCTCTGAAAACAAATCA	(52)
COX-2 Sn	AACCAGAGAGAAATGAGTTTTGACG	(52)
COX-2 As	TGCATTGGAAACATCGACAGTG	(52)
GAPDH Sn	TGGAATTACCGCGGCTGC	(52)
GAPDH As	CGGCTACCACATCCAAGGAAGG	(52)
LDLR SRE	AAATCACCCCACTGCAAAATCACCCCACTGCAAAATCACCCCACTGCA	(54)
COX-2 SRE	GCGATCAGTCCACCTGAAAATCAGTCCACCTGAAAATCAGTCCACGCG	this work
SRE loss	CCCCGGTATCCCATCCAAGGCGAT**A**AGTCC**T**GAA**C**TGGCTCTCGGAA	this work
SRE gain	GGTATCCCATCCAAGGCGATCA**CC**CCA**C**AACTGGCTCTCG	this work

COX, cyclooxygenase; EMSA, electromobility shift assay; LDLR, LDL receptor; S1p, site 1 protease; S2p, site 2 protease; SCAP, SREBP cleavage-activating protein; SRE, sterol response element. Underscoring indicates the response element region; bolded nucleotides represent the mutated bases.

monoclonal antibody for 1 h at 25°C (1:500 dilution). Secondary antibodies conjugated to Alexa488 goat anti-rabbit or Alexa594 rabbit anti-mouse (1:1,000) were added after a washing with PBS and incubated for 0.5 h at 25°C. Cells were then washed again with PBS and coverslipped using aqueous mounting media containing the nuclear stain 4′,6-diamidino-2-phenylindole (Molecular Probes; Eugene, OR). The ER was labeled using SelectFX Alexa488 ER labeling Kit (Molecular Probes) according to the manufacturer's protocol. Epifluorescence was visualized and captured as photomicrographs with an Optronics digital camera affixed to an Olympus ×30 microscope. Digital image analysis was performed using ImagePro Plus 4.0 imaging software (Media Cybernetics; San Diego, CA).

Electromobility shift assay

The interaction of SREBP and the SRE in the COX-2 promoter was analyzed by electromobility shift assay (EMSA) as described (27). Recombinant SREBP-1 and -2 (10 ng), purified from a bacterial expression vector, was a gift of Dr. Michael Waterman (28). Protein samples were preincubated for 5 min on ice with 1.0 μg of poly(dI/dC) (Pharmacia Biotech) and 1 μg of single-stranded nonspecific oligonucleotide in a reaction buffer containing 10 mM Tris, pH 7.4, 100 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.3 μg BSA, 0.1% Triton-X 100, and 5 mM $MgCl_2$ before the addition of the radiolabeled probe. The unlabeled competitor oligonucleotides were added during preincubation. LDLR SRE consensus oligonucleotides with canonical SRE motifs (29), and COX-2 SRE oligonucleotides (Table 1) were end-labeled with [^{32}P]dATP through the use of T4 polynucleotide kinase, adjusted to 50,000 cpm/0.25 ng/μl, and 1 μl/19 μl sample was added. After additional incubation for 5 min at room temperature, the samples were directly loaded on a prerun 6.0% polyacrylamide gel with 5% glycerol and 5% Ficoll (MW 400,000; Sigma) in 0.5× TBE (0.5×0.045 mol/l Tris-borate, 0.002 ml EDTA) and run at 20 miliamps (mA) for 2 h. The gel was vacuum dried and exposed to Kodak XO-Mat Blue X-ray film.

Mass spectroscopy

COX activity was determined by the conversion of exogenous arachidonic acid into primary prostaglandin metabolites as previously described (19). PGI_2 and PGH_2 rearrange in aqueous solution to the stable metabolites 6-keto-$PGF_{1\alpha}$, and prostaglandin E_2 (PGE_2)/prostaglandin D2, respectively (30). These metabolites were analyzed and quantified by stable isotope dilution techniques GC/negative-ion chemical ionization MS as O-methyloxime, pentafluorobenzyl ester, and trimethylsilyl ether derivatives. Compounds were detected by MS based on the M-$CH_2C_6F_5$ ions *m/z* 524 for PGE_2/D_2, *m/z* 614 for 6-keto-$PGF_{1\alpha}$ and TxB_2, and *m/z* 528 for [$^2H_4$]PGE_2 and 618 for [$^2H_4$]6-keto-$PGF_{1\alpha}$ and TxB_2, were included as internal standards (31).

PCR-based site-directed mutagenesis

For PCR-based site-directed mutagenesis of the SRE located at −429 bp of the COX-2 promoter construct, the following primer sets were generated (bolded nucleotides represent the mutated bases and underscoring indicates the response element region): loss-of-function SRE mutant primer, sense strand (5′-CCCCGGTATCCCATCCAAGGCGAT**A**AGTCC**T**GAACTGGCTCTCGGAAGCGCTCGG-3′); and gain-of-function SRE mutant primer strand (5′-GGTATCCCATCCAAGGCGATCA**CC**CCA**C**AACTGGCTCTCG-3′). Gain-of-function mutant primer was designed to convert the putative COX-2 SRE into the canonical LDLR SRE. PCR-based site-directed mutagenesis was performed as described (32). The sequence integrity of the mutated promoter regions was confirmed by automated sequencing and restriction endonuclease analysis.

Statistical analysis

Statistical analyses of data were performed using GraphPad Prism 4 software. All experiments were performed three times in triplicate, unless otherwise specified. Reported data were analyzed by ANOVA using the Bonferroni or Dunnett's correction for multiple comparisons.

RESULTS

HUVECs express functional SREBP

To test the hypothesis that COX-2 gene expression is effected by SREBP, it was first necessary to determine whether vascular endothelial cells express the SREBP, and its acti-

vating proteins. HUVECs were cultured on glass 2-well chamber slides in cholesterol-rich or cholesterol-deficient media for 48 h. The presence of SREBP was determined using a polyclonal antibody against the DNA binding domain of human SREBP. This antibody does not discriminate between different isoforms of SREBP, because the DNA binding domain is highly conserved among the SREBPs. Immunoreactive precursor (inactive) SREBP (red fluorescence) was colocalized to the ER in cholesterol-loaded endothelial cells using antibodies against the ER-specific protein PDI (protein disulfide isomerase, green fluorescence), as indicated by the yellow fluorescence (**Fig. 1A**). Conversely, HUVECs incubated under conditions of total cholesterol deprivation showed marked SREBP staining within the nucleus (blue fluorescence), as indicated by the strong magenta fluorescence (Fig. 1B). In addition to SREBP, we determined that SCAP was also expressed and colocalized with the ER in HUVECs (Fig. 1C). This staining pattern is consistent with previously reported immunolocalization studies of SREBP and SCAP in hepatocytes (33). Western blotting (Fig. 1D) showed that precursor SREBP (125 kDa) was present in HUVECs. Incubation of these cells in cholesterol-deficient media activates SREBP, as indicated by the appearance of the smaller, nuclear SREBP (64 kDa). RT-PCR analysis further confirmed the expression of SREBP-1, SCAP, S1P, and S2P mRNA in HUVECs (Fig. 1E). Interestingly, we did not detect a significant amount of SREBP-2 mRNA in HUVECs.

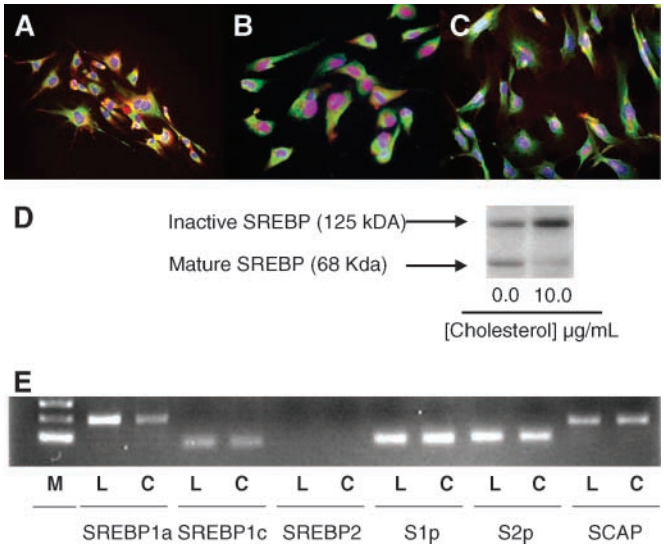

Fig. 1. Human endothelial cells express sterol response element binding protein (SREBP). Vascular endothelial SREBP is activated by cholesterol deprivation. Human endothelial cells stained for SREBP (red) and PDI (green) show that SREBP is colocalized to the ER in the presence of excess cholesterol, as indicated by the yellow fluorescence (A), and is transported into the nucleus in the absence of cholesterol, as indicated by the magenta fluorescence (B). Total cellular protein from human umbilical vein endothelial cells (HUVECs) was separated by NuPAGE gel, followed by immunoblotting with an anti-SREBP1 monoclonal antibody. Precursor SREBP is the inactive form of the protein. Mature SREBP is the cleaved activated form, and is thus smaller in molecular mass (C). SREBP cleavage activating protein (SCAP; red) is localized to the perinuclear region of the ER (PDI; green) under cholesterol replete as indicated by the yellow fluorescence. In some cells SCAP is not associated with the ER. (D). E: RT-PCR using the specified primers (Table 1) amplified SREBP, SREBP cleavage-activating protein (SCAP), site 1 protease (S1p), and site 2 protease (S2p) mRNA. L, cholesterol-depleted conditions (0.0 µg/ml); C, cholesterol-enriched media (10.0 µg/ml).

Analysis and identification of sequences that confer cholesterol regulation on the COX-2 promoter

In our previous study, we observed that cholesterol regulated COX-2 gene expression at the level of transcription. To characterize the effect of cholesterol on the COX-2 promoter, we obtained a luciferase reporter plasmid that contains 7.2 Kbp of the human COX-2 promoter (22). The activity of the -7.2COX-2Luc reporter construct promoter was greatest when no cholesterol was present in the media, and this activity was decreased 73% by the addition of 10 µg/ml exogenous free cholesterol (17.8 ± 1.6 to 4.8 ± 0.4 RLU; $P = 0.02$) (**Fig. 2A**). When the COX-2 promoter construct was cotransfected with CMV-SREBP-1a-460, which encodes only the DNA binding and transactivation domains of SREBP-1, the constitutively active SREBP increased luciferase activity 5-fold, compared with those cells transfected with empty vector (Fig. 2B). COX-2 gene expression, therefore, is regulated by cholesterol through the promoter, and the cholesterol-sensitive transcription factor SREBP is sufficient to increase promoter activity.

Next, we analyzed the 5′-upstream regulatory sequences of human COX-2 for the presence of an SRE. A computer analysis of sequence motifs using Vector NTI (Informax, Inc., Bethesda, MD) identified a 10 bp sequence (ATCAGTCCAC, at −422 bp) that is highly similar to the SRE-1 in the human squalene synthase (HSS) gene (ATCACGCCAG) (34). When compared with the HSS SRE-1, the only mismatched base (6th, T instead of G) is located in a position that is not conserved in different SREBP target genes (Table 1).

To localize the regulatory elements that mediate the effects of SREBP, we obtained a series of 5′ progressive deletions of the human COX-2 promoter starting from −1.4 Kbp, and their activities were measured in transfection experiments, either alone (with and without cholesterol) or in combination with cotransfected CMV-SREBP-1a460 (**Table 2**). Figure 2C, D show the relative luciferase activities of these constructs in BAECs. Deletion up to −459 bp did not decrease the sensitivity of these constructs to cholesterol. However, deletion up to −327 bp, which removes the SRE motif located at −422 bp, resulted in a 60% reduction in luciferase activity. Similar results were obtained when cells were cultured in cholesterol-rich media and cotransfected with the constitutively active SREBP. Taken together, these findings localize the predominant sterol regulatory domain to a region from −459 bp to −327 bp of the COX-2 promoter.

We further localized the SRE in the human COX-2 promoter to this sequence by PCR site-directed mutagenesis (Table 1). Two mutant plasmids were successfully generated, and the mutations were confirmed by sequencing and restriction endonuclease analysis (data not shown). The first mutant, designated pSREloss-COX-2Luc, changed three key residues previously determined to be essential to

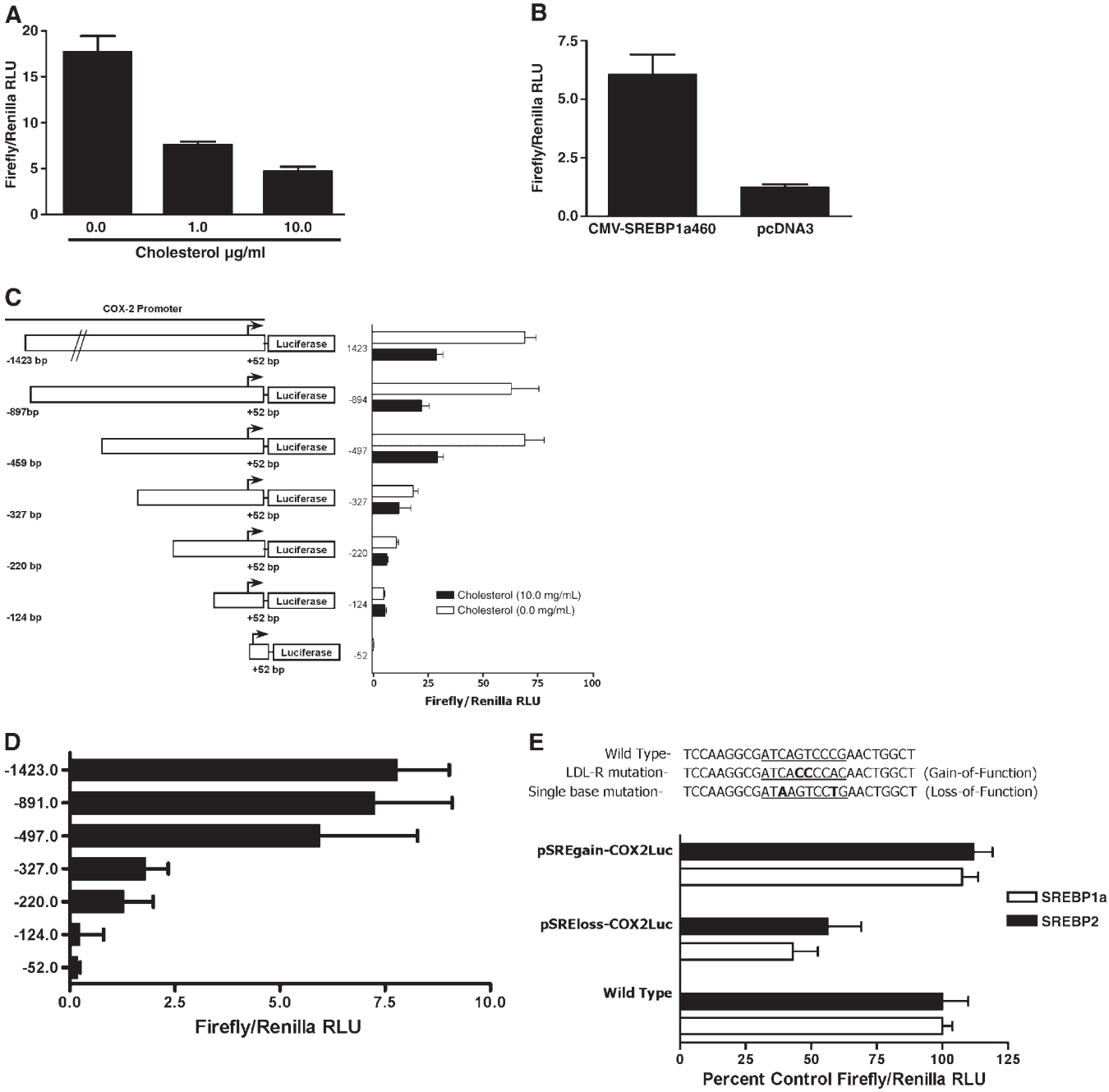

Fig. 2. The effects of cholesterol on the activity of human cyclooxygenase-2 (COX-2) promoter-luciferase constructs. A: Increasing concentrations of cholesterol reduced the activity of the human COX-2 promoter-luciferase construct in a dose-dependent fashion. Results are the mean ± SEM relative luciferase units from a representative experiment performed in triplicate that was repeated four times. B: Constitutively active SREBP encoded by pCMV-SREBP-1a 460 increases luciferase activity in the presence of exogenous cholesterol, compared with empty vector. Results are the mean ± SEM relative luciferase units from a representative experiment performed in triplicate that was repeated four times. C: Bovine aortic endothelial cells (BAECs) were transfected with deletion mutants of the human COX-2 promoter driving luciferase gene expression. After transfection, cells were grown in cholesterol-deficient media ± the addition of exogenous free cholesterol for 24 h. Results are the mean ± SEM from a representative experiment performed in triplicate that was repeated four times. D: BAECs cotransfected with constitutively active SREBP localize SREBP-responsive element in the human COX-2 promoter. BAECs transfected with a plasmid expressing only the DNA binding and transactivation domains of SREBP-1a and deletions of human COX-2 promoter-luciferase constructs localize sterol response elements (SREs) to a region between −497 and −327 bp upstream from the transcription start site. After transfection, cells were grown in cholesterol-deficient media + exogenous free cholesterol for 24 h. Results are the mean ± SEM from a representative experiment performed in triplicate that was repeated four times. E: Nucleotides essential for the binding and activation of SREBP-regulated genes were altered by PCR site-directed muatgenesis. Alignments of wild-type (unmutated) p459COX-2Luc, a loss-of-function mutant, pSREloss-COX-2luc, and the gain-of-function mutant pSREgain-COX-2luc show the mutations. Results are the means ± SEM from a representative experiment performed in triplicate that was repeated twice, and statistical significance was determined by ANOVA.

TABLE 2. SRE of selected genes aligned with the putative COX-2 SRE

Gene	SRE Promoter Sequence	Reference
LDLR	ATCACCCCAC	(29)
HMG-CoA synthase	CTCACCCCAC	(55)
Squalene synthase	ATCACGCCAG	(34)
SREBP-2	ATCACCCCAC	(56)
Gycerol-3-phosphate acyltransferase	ATCACTCCAC	(57)
COX-2	ATCAGTCCAC	this work

the binding of SREBP to its response element (14). This mutation reduced SREBP-1a-dependent luciferase activity by 48%, compared with unmutated plasmid (9.56 ± 2.13 vs. 22.26 ± 2.13 RLU; $P = 0.004$, by ANOVA). Deletion of these key nucleotides had a similar effect on SREBP-2-dependent luciferase activity. A second mutant, designated pSREgain-COX-2Luc was designed to increase the SREBP-dependent induction of the COX-2 promoter construct by converting the COX-2 SRE into the canonical LDLR SRE (35). When transfected with either pCMV-SREBP-1a460 or pCMV-SREBP2-468, the luciferase activity of pSREgain-COX-2Luc did not differ significantly from the activity observed in the unmutated construct, p459COX-2Luc (Fig. 2E).

Differential activation of COX-2 promoter constructs by SREBP-1a and SREBP-2

To further characterize the effects of SREBP induction of COX-2 promoter constructs, we transfected BAECs with p459COX-2Luc or p327COX-2Luc and increasing concentrations (5.0 ng to 500.0 ng) of pCMV-SREBP1a-460 or pCMV-SREBP2-468. We chose to focus on these COX-2 luciferase constructs because the putative SRE lies within the p459COX-2Luc, but is absent from the p327COX-2Luc. **Figure 3** shows that SREBP-1a and SREBP-2 activate the p459COX-2Luc promoter in a concentration-dependent manner, as expected for an SRE-containing promoter. In this study, SREBP-1a appears to act as a more potent activator of this promoter than SREBP-2. Although we cannot determine the relative amounts of each SREBP isoform, or their intrinsic activating potencies, this difference in potency is consistent with previous reports that the longer transactivation domain in SREBP-1a confers a greater potency than the shorter transactivation domain of SREBP-2 (10). Neither SREBP-1 nor SREBP-2 significantly activated the -327COX-2Luc reporter construct. Similar experiments were performed using the SRE-Luc construct, known to be activated by SREBP-1 and SREBP-2 (29). SRE-Luc was activated by both SREBP-1a and SREBP-2 in the same system in which we observed a significant induction of p-459COX-2Luc, and only minimal induction of p-327COX-2Luc (data not shown). Background values (pGL2-basic) were only slightly affected by the SREBP constructs. This minor induction is attributable to the binding of SREBP to E-boxes within the pGL-2 promoter (36).

SREBP-1a and -2 form specific complexes with the COX-2 SRE

To determine whether SREBP directly binds the putative COX-2 SRE site (ATCAGTCCACA at −422 bp), EMSAs

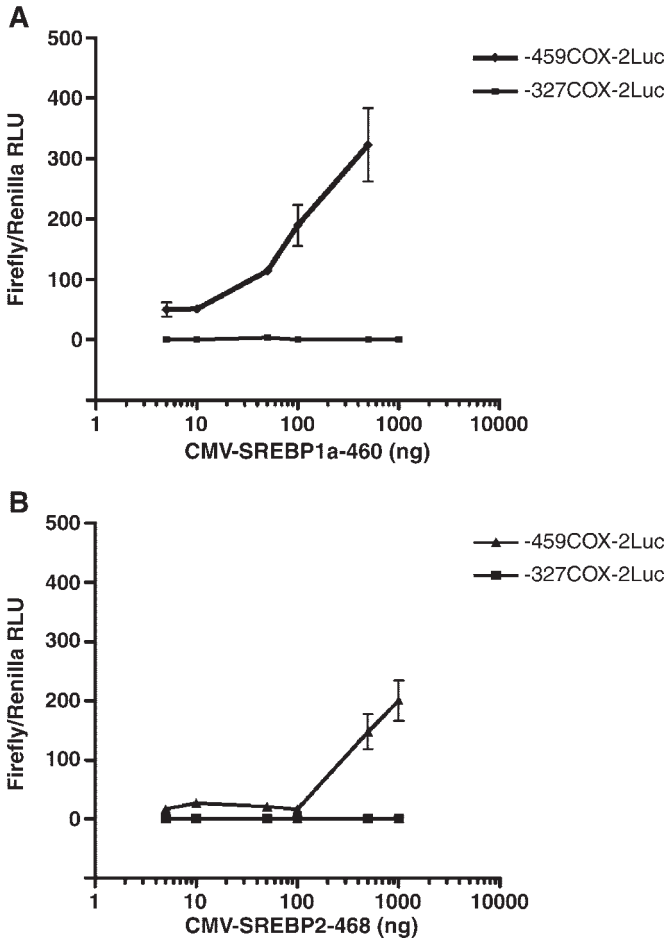

Fig. 3. SREBP-1a and -2 differentially activate COX-2 promoter constructs. A: SREBP-1a 460 induces -459COX-2Luc luciferase activity in a dose-dependent manner, but has no effect on the luciferase activity of the -329COX-2Luc construct. B: SREBP-2 468. BAECs were transfected with increasing concentrations of plasmid encoding the active form of SREBP (diamond, -459COX-2Luc; square, -327COX-2Luc). Endogenous SREBP activity was repressed by the addition of exogenous cholesterol (10.0 μg/ml). Results are the means ± SEM from a representative experiment performed in triplicate that was repeated twice ($P < 0.0001$, by ANOVA).

were performed. Recombinant SREBP-1a-460 and SREBP-2-468 were probed for their ability to interact in vitro with this sequence using a DNA probe that contained three tandem repeats of the COX-2 SRE, with each putative SRE separated by 6 bp (designated COX-2 SRE, and the DNA probe containing three repeats of this sequence, designated as COX-2 SRE 3×). SREBP-1a-460 incubated with ^{32}P-labeled COX-SRE3× resulted in the formation of three DNA–protein complexes with different mobilities (**Fig. 4A**). Complex 1 represents an interaction between COX-2 SRE3× and BSA, used in the binding buffer. This complex is also present in the negative control when no protein has been added, and a 100-fold molar excess of unlabeled COX-2SRE3× does not compete for the binding of this complex. This nonspecific interaction serves as an internal control for each binding reaction. Complex 2 probably represents an additional nonspecific DNA–protein interaction. The intensity of this band is not reduced by

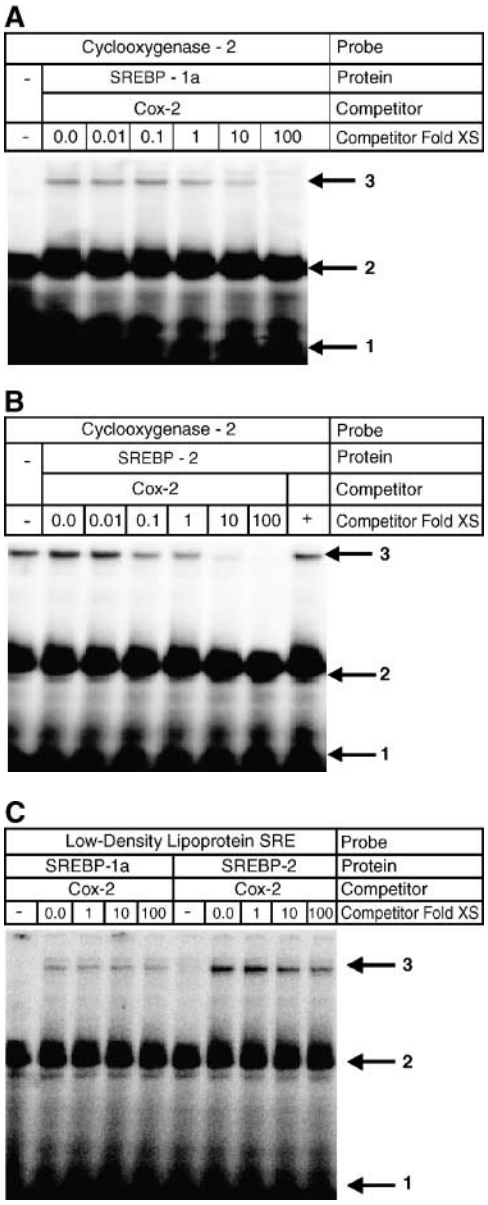

Fig. 4. Electrophoretic mobility shift analysis of the COX-2 SRE and recombinant protein.[32]P-labeled oligonucleotides of COX-2 SRE were incubated with recombinant SREBP-1a (A) or SREBP-2 (B). Free and bound probes were electrophoretically separated in a nondenaturing polyacrylamide gel. The SREBP-COX-2SRE complexes were designated 1–3 in the order of increased mobility. Complex 3 represents a specific interaction between SREBP and COX-2SRE that is competed by 100-fold molar excess of cold, unlabeled COX-2SRE. (−) Indicates the negative control reaction, in which no protein was added to the reaction mixture. (+) Indicates the positive control reaction, in which SREBP is bound with labeled LDL receptor (LDLR) SRE. C: COX-2 SRE effectively competes with LDLR SRE for SREBP complexes in electrophoretic mobility shift assay. [32]P-labeled oligonucleotides of LDLR SRE were incubated with recombinant SREBP-1a or SREBP-2. Free and bound probes were electrophoretically separated in a nondenaturing polyacrylamide gel. The SREBP-LDLR SRE complexes were designated 1–3 in the order of increased mobility. Complex 3 represents a specific interaction between SREBP and target oligos that is effectively competed by 100-fold molar excess of cold, unlabeled COX-2SRE. (−) Indicates the negative control reaction in which no protein was added to the reaction mixture.

unlabeled COX-2 SRE3×. The intensity of complex 3 was reduced in the presence of increasing concentrations of unlabeled COX-2SRE3×, indicating that complex 3 represents a specific DNA–protein interaction between SREBP and the COX-2SRE3× oligonucleotide. Recombinant SREBP-2-468 produced the same pattern of specificity (Fig. 4B).

COX-2 SRE effectively competes with LDLR SRE for SREBP complexes

SREBP binding to the COX-2 SRE was confirmed by competition with a probe containing the LDLR promoter SRE. Increasing concentrations of unlabeled COX-2 SRE reduced the intensity of SREBP/LDLR SRE complexes in a concentration-dependent manner (Fig. 4C). The LDLR probe competed more effectively than the COX-2 probe, suggesting that the COX-2 promoter has lower affinity for SREBPs than the LDLR.

Lovastatin increases endothelial PGI_2 production and induces COX-2 expression

The SREBP transcriptional pathway can also be activated by pharmacological inhibition cholesterol synthesis using HMG-CoA reductase inhibitors such as lovastatin. We therefore examined the effects of HMG-CoA reductase inhibition on endothelial PGI_2 production, COX-2 promoter activity, and COX-2 mRNA. Exposure of HUVECs to lovastatin for 48 h led to an increase in prostacyclin production (**Fig. 5A**). Cells treated with lovastatin produced 0.52 ± 0.05 ng/ml of 6-keto-PGF1α (n = 6), compared with 0.28 ± 0.01 ng/ml in vehicle-treated cells ($P = 0.0042$). Lovastatin increased the luciferase activity in BAECs transfected with the p459COX-2Luc (8.0 ± 2.2 RLU to 79.0 ± 5.8 RLU; n = 3, $P < 0.001$) in a dose-dependent manner (Fig. 5B). In contrast, cells transfected with the -327COX-2 promoter lacking the SRE were unaffected by lovastatin treatment. Lovastatin treatment also increased endothelial COX-2 mRNA production in a dose-dependent manner (1.0 ± 0.5-fold induction by vehicle treatment compared with 190.9 ± 105.5-fold induction by 10 μM lovastatin; n = 9, $P = 0.042$, by ANOVA with Dunnett's multiple comparison test; Fig. 5C). Cell viability was measured after 48 h of lovastatin treatment to exclude cell death as an artifactual cause of decreased COX-2 Luc activity. At the concentrations used in this experiment, treatment with lovastatin for 48 h did not impair endothelial viability, as assessed by examination of tryptan blue-stained cells.

DISCUSSION

The identification of SREBPs as classic transcription factors has broadened our understanding of the effects of intracellular cholesterol and fatty acids on gene expression. Studies analyzing the mechanics of cholesterol-regulated gene expression have been largely limited to cholesterologenic tissues such as the liver and intestine (10). Although many studies have identified a variety of genes regulated by SREBP, less is known about the role that

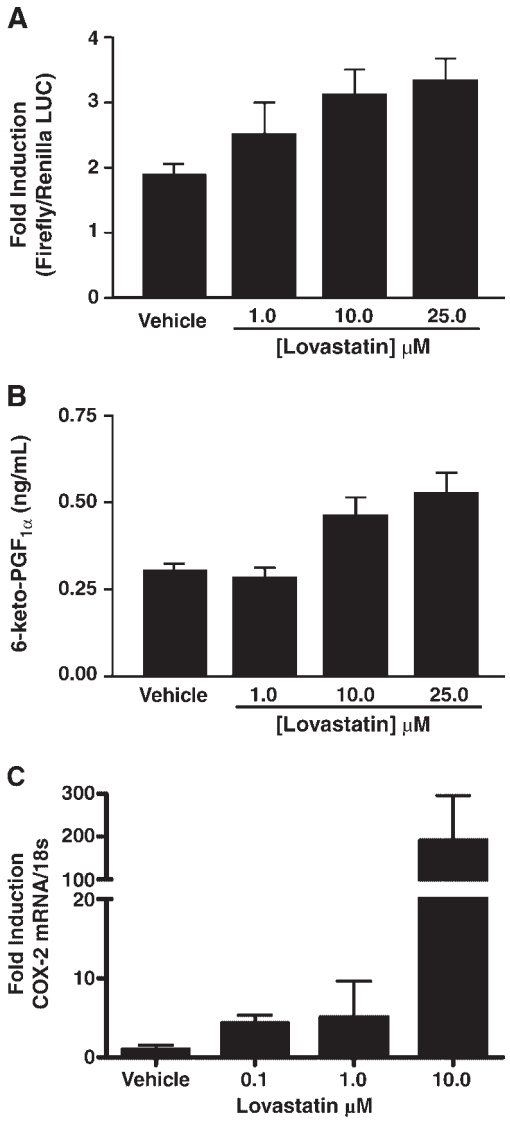

Fig. 5. The effects of lovastatin on endothelial prostaglandin I_2 production and COX-2 promoter-luciferase activity. A: BAECs were transfected with -459pCOX-2Luc, and incubated with increasing doses of lovastatin for 48 h as indicated ($P < 0.0001$, by ANOVA). B: HUVECs were subjected to increasing concentrations of lovastatin for 48 h as indicated. Production of 6-keto-$PGF_{1\alpha}$ was quantified by GC-MS as described in Experimental Procedures ($P < 0.0042$, by ANOVA). C: HUVECs were incubated with increasing concentrations of lovastatin for 48 h as indicated. COX-2 mRNA was quantified by quantitative PCR as described in Experimental Procedures. Results are the mean ± SEM from a representative experiment performed in triplicate that was repeated twice.

SREBPs play in tissues not directly associated with cholesterol, fatty acid, and lipoprotein synthesis. Although cholesterol clearly impacts endothelial cell function (37, 38), the direct effects of cholesterol on endothelial gene expression have not been investigated. We previously reported that cholesterol negatively regulates the expression of COX-2 in vascular endothelial cells (19). In that study, we observed a dose-dependent decrease in COX-2 mRNA and protein under conditions of increasing cholesterol concentrations, whereas cholesterol had no effect on the expression of COX-1 mRNA or protein. To further investigate the mechanism responsible for this cholesterol-mediated regulation, we systematically analyzed the effects of cholesterol and SREBP on the activation of the human COX-2 gene promoter. In this report, we have identified a functional *cis*-acting element within the human COX-2 promoter that specifically binds and mediates a transcriptional response to active SREBP.

There are three fundamental characteristics that identify genes as cholesterol- and SREBP-responsive (11). First, the gene in question should exhibit an inverse regulatory relationship with cholesterol. Although we previously showed that endothelial COX-2 protein and mRNA expression is induced by cholesterol depletion (19), the present study extends these observations by demonstrating that cholesterol deprivation activates the human COX-2 promoter in transiently transfected endothelial cells. In accord with this mechanism of gene regulation, we found that overexpression of either constitutively active SREBP-1a or SREBP-2 increases COX-2 promoter activity. Furthermore, targeted mutation or deletion of the COX-2 SRE decreases the ability of SREBP to increase COX-2 promoter activity. These functional data are further supported by experiments demonstrating that cholesterol deprivation increases endothelial PGI_2 production. Second, the gene in question must have sequence that corresponds to the canonical SREs as previously described (17, 35). Indeed, sequence analysis identified one highly conserved SRE at −422 bp upstream from the transcription start site in the human COX-2 promoter. This sequence is fully capable of forming specific complexes with both SREBP-1a and SREBP-2, and is an effective competitor of SREBP binding to the LDLR SRE. Third, compounds known to activate SREBP must induce the gene. The competitive inhibitor of HMG-CoA reductase, lovastatin, indirectly activates SREBP by reducing intracellular cholesterol synthesis. In the present study, lovastatin induced a dose-dependent increase in COX-2 promoter-dependent luciferase activity in cells transfected with the -459COX-2 promoter that contains the SRE. Consistent with this observation, we observed a 2-fold induction of COX-2-dependent PGI_2 production in HUVECs treated with concentrations of lovastatin. These data are fully consistent with previously reported SREBP-dependent transactivation of the LDLR promoter and other cholesterol-regulated genes (35).

We cannot exclude the possibility that cholesterol deprivation activates the COX-2 promoter by nonspecific alterations in the integrity of the ER and other membranes. Compounds that induce ER stress, such as thapsigargin and tunicamycin, have been shown to activate SREBP and other factors controlled by regulated intermembrane proteolysis (33, 39). In view of the consistent and concordant activation of the COX-2 promoter independently by cholesterol deprivation, by the HMG-CoA reductase inhibitor lovastatin and by constitutively active SREBP, we conclude that COX-2 is directly and specifically regulated by SREBP. Although this study suggests that both isoforms of SREBP are capable of inducing COX-2 expression, this associa-

tion may not fully reflect the molecular physiology of the system in vivo, inasmuch as only mRNA for SREBP-1 and not SREBP-2 was detected in HUVECs by RT-PCR. Because SREBP-1 appears to be the dominant form expressed in endothelial cells, we speculate that SREBP-1 may be the dominant endogenous regulator of COX-2 gene expression in endothelial cells. Further studies using endothelial cells derived from SREBP-1a or SREBP-2 knockout mice may be undertaken to investigate this question.

It is important to consider the current findings in the context of known regulators of COX-2 expression. Inflammatory cytokines such as IL-1 are recognized as potent inducers of COX-2 expression in a variety of tissues, including the vasculature (40). We have previously examined the combined effects of cholesterol deprivation and IL-1 on COX-2 expression, and found no evidence of synergy (19). Although the current study demonstrates that cholesterol influences COX-2 expression, it is quantitatively less potent than direct inflammatory stimuli. The regulation of COX-2 expression in atherosclerosis is even more complex. Our experimental system examines endothelial function separated from the confounding influences of smooth muscle cells, platelets, macrophages, and the inflammatory signals present in atherosclerosis. The net effects of cholesterol lowering on endothelial COX-2 expression are undoubtedly influenced by the complex calculus of other factors in vivo, including inflammatory cytokines.

The relationship between cholesterol and COX-2 has other physiological ramifications. There is evidence to suggest a reciprocal role for prostaglandins in intracellular cholesterol homeostasis. Prostacyclin has been shown to enhance neutral and acid cholesteryl ester hydrolase activity in aortic smooth muscle cells (41, 42). The cholesterol-sensitive SREBP is activated when intracellular stores of cholesterol are depleted, linking PGI_2 production and cholesterol homeostasis. As we have shown, SREBP-dependent transactivation of the COX-2 gene increases COX-2 mRNA and protein expression, which, in turn, promotes PGI_2 production. PGI_2 facilitates cholesterol ester hydrolysis, thereby increasing intracellular free cholesterol. Thus, the induction of PGI_2 production under low-cholesterol conditions provides a complementary mechanism for regulating intracellular cholesterol concentrations. Given the powerful effects of lipid-lowering therapy in reducing the risk of cardiovascular events, even in patients with normal cholesterol levels (1, 43–51), it would not be surprising to find other endothelial and vascular genes that are regulated by this important pathway. Cholesterol reduction clearly has multifaceted effects on the vasculature, including increasing the production of the vasodilator PGI_2 via an SREBP-dependent mechanism.

In summary, cholesterol regulates COX-2 gene expression through an SREBP-dependent pathway. This is the first description of a vascular gene product that is directly regulated by SREBP. These findings suggest that cholesterol-lowering therapy provides unanticipated dividends by enhancing endothelial PGI_2 production, thereby promoting vascular health, independent of effects on arterial lipid accumulation.

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