Eicosanoid Metabolism in Cholesterol-Enriched Arterial Smooth Muscle Cells. Evidence for Reduced Posttranscriptional Processing of Cyclooxygenase I and Reduced Cyclooxygenase II Gene Expression†

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ABSTRACT: Eicosanoid biosynthetic activity by the cyclooxygenase pathway is reduced in smooth muscle cell-derived foam cells [Pomerantz, K. B., & Hajjar, D. P. (1989) *J. Lipid Res. 30*, 1219–1231; Pomerantz, K. B., & Hajjar, D. P. (1990) *Biochemistry 29*, 1892–1899]. The present study identifies those mechanisms which contribute to reduced production of cyclooxygenase products following cholesterol enrichment of arterial smooth muscle cells. Cyclooxygenase activity, as assessed by the conversion of exogenous arachidonate to 6-keto-$PGF_{1\alpha}$, was reduced approximately 8-fold in intact lipid-laden cells relative to untreated cells. Microsomes from cholesterol-enriched cells also converted less [^{3}H]arachidonic acid to 6-keto-$PGF_{1\alpha}$ and PGE_2 relative to microsomes from untreated cells. The reduction in cyclooxygenase activity paralleled the reduced mass of the constitutive form of cyclooxygenase (COX-1) and PGI_2 synthase by approximately 80% and 33%, respectively. Northern blot hybridization analyses of COX-1 mRNA steady-state levels revealed no differences between normal and cholesterol-enriched cells under basal conditions, indicating that cholesterol enrichment did not alter COX-1 gene expression. Furthermore, cholesterol enrichment did not alter the relative levels of COX-1 mRNA expression over time following exposure of the cells to actinomycin D, indicating that cholesterol enrichment did not significantly alter the rate of COX-1 mRNA degradation. Recovery of PGI_2 biosynthesis in untreated cells exposed to serum following the inactivation of COX occurred within 12 h, while the recovery of COX activity in lipid-enriched cells did not return to levels observed in untreated cells even after up to 48 h, suggesting that the induction of COX-2 (inducible form of cyclooxygenase) synthesis by growth factors or cytokines is impaired. Indeed, cholesterol enrichment attenuated IL-1β-, PDGF-, and TNFα-induced PGI_2 synthesis relative to controls and was consistent with the results of *in vitro* labeling experiments demonstrating that cholesterol enrichment reduced the incorporation of [^{35}S]methionine into immunoprecipitable COX-1 and COX-2 following induction by PDGF. Cholesterol enrichment also reduced the induction of COX-2 mRNA steady-state levels following exposure to PDGF. Taken together, these data demonstrate that reduced eicosanoid synthesis in smooth muscle-derived foam cells is due, in part, to impaired transcription of mRNA for COX-1 and COX-2 as well as fatty acid remodeling in membrane phospholipids. These findings support the hypothesis that cholesterol enrichment alters posttranscriptional processing of COX-1 expression, as well as altering COX-2 gene expression.

Reduced eicosanoid biosynthesis has been observed following alterations in lipoprotein and lipid metabolism in numerous *in vivo* and *in vitro* studies. First, hypercholesterolemia has been associated with reduced prostacyclin (PGI_2)[1] synthetic activity (Serneri et al., 1986; Gryglewski et al., 1988; Fogelberg et al., 1990). Second, hypercholesterolemia inhibited the regeneration of PGI_2 synthesis after endothelial cell injury (Eldor et al., 1982). Third, smooth muscle cells derived from atherosclerotic rabbit aorta retain the deficit in eicosanoid synthetic capacity, even after repeated subcultures (Larrue et al., 1981). These findings suggest that lipoproteins or the products of lipoprotein catabolism down-regulate eicosanoid biosynthesis long after their removal from a hypercholesterolemic environment. To this end, numerous studies have been performed to evaluate the influence of plasma lipoproteins or cholesterol enrichment following exposure to modified forms of LDL on eicosanoid biosynthesis by isolated vascular cells.

All purified lipoproteins studied, including high-density lipoprotein (HDL) (Pomerantz et al., 1984, 1985; Spector et al., 1985; Fleisher et al., 1982; Van Sickle et al., 1986), low-density lipoprotein (LDL) (Pomerantz et al., 1984, 1985; Spector et al., 1985; Van Sickle et al., 1986; Habenicht et al., 1990), acetylated LDL (Hartung et al., 1986; Diez et al., 1989), and oxidized LDL (Yokode et al., 1988; Zhang et al., 1990), acutely stimulate vascular cell eicosanoid biosynthesis by either mobilizing endogenous arachidonate from membrane phospholipids through the stimulation of phospholipases (Pomerantz et al., 1984, 1985), donating substrate derived from lipoprotein cholesteryl esters (Pomerantz et al., 1984, 1985; Spector et al., 1985; Van Sickle et al., 1986; Habenicht et al., 1990), or activating cyclooxygenase (Zhang et al., 1990).

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[1] Abbreviations: PG, prostaglandin; ELISA, enzyme-linked immunosorbent assay; PGI_2, prostacyclin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; cLDL, cationized LDL; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DMEM, Dulbecco's Modified Eagle's medium; FBS, fetal bovine serum; GAP, glyceraldehyde phosphate dehydrogenase; COX-1, cyclooxygenase, constitutive form; COX-2, cyclooxygenase, mitogen-inducible form; mRNA, messenger RNA; PDGF, platelet-derived growth factor; IL-1, interleukin-1; TNF, tumor necrosis factor.

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However, the effect of cholesterol enrichment (using modified forms of LDL) on vascular eicosanoid biosynthesis clearly is different from that observed by acute stimulation by isolated lipoproteins. We previously demonstrated that smooth muscle cell-derived foam cells displayed a diminished eicosanoid synthetic capacity that was due to competitive inhibition of arachidonate metabolism by linoleate derived from LDL cholesteryl ester and a noncompetitive inhibition of phospholipase A_2 activity by free cholesterol (Pomerantz & Hajjar, 1989). The reduction in eicosanoid synthetic activity was associated with coincident reductions in eicosanoid-sensitive cholesteryl ester hydrolase activities (Pomerantz & Hajjar, 1990). In addition, we also demonstrated that arachidonic acid-induced PGI_2 production was inhibited following cholesterol enrichment (Pomerantz & Hajjar, 1989), suggesting the possibility that COX and/or PGI_2 synthase activities may be reduced as a consequence of cholesterol accumulation.

Since COX is essential for prostaglandin (and thromboxane A_2) synthesis from arachidonic acid, the regulation of COX has been the subject of intense study (Raz et al., 1989a; Fu et al., 1990; Maier et al., 1990). COX is present as two gene products consisting of constitutive and inducible forms known as COX-1 and COX-2, respectively. COX-2 is up-regulated by phorbol esters (Kujubu & Herschman, 1992; Kujubu et al., 1993; Kujubu et al., 1991), IL-1β (Raz et al., 1988; Breviairio et al., 1990; O'Banion et al., 1992), PDGF (Goerig et al., 1988; Habenicht et al. 1985), and EGF (Pash & Bailey, 1988; Bailey et al., 1985). In contrast, COX activity may be down-regulated by dexamethasone (O'Banion et al., 1991; Phillips et al., 1993; Kujubu et al., 1993) and lipocortin, a dexamethasone-inducible protein (Fu et al., 1990). Since the influence of modified LDL on COX gene regulation has been unexplored, we tested the hypothesis that cholesterol enrichment of arterial smooth muscle cells may alter the activity and levels of COX-1, COX-2, and PGI_2 synthase.

EXPERIMENTAL PROCEDURES

Materials. Dulbecco's Modified Eagle's medium (DMEM), Hanks' balanced salt solution (HBSS), medium M-199, penicillin, streptomycin, and amphotericin B (fungizone) were from Gibco (Grand Island, NY). Fetal bovine and horse serum were purchased from Hyclone (Logan, UT). *N,N*-Dimethyl-1,3-propanediamine was from Kodak Chemicals (Rochester, NY); 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride was purchased from Aldrich Chemicals (Milwaukee, WI). Eicosanoid standards, a rabbit polyclonal antibody generated against ram seminal vesicle COX-1 but which recognizes COX-1 and COX-2 (henceforth called pan-COX antibody), and a rabbit polyclonal antibody generated against murine COX-2 were obtained from Cayman Biochemicals (Ann Arbor, MI). [^{14}C]Arachidonate, [^{3}H]arachidonate, [^{3}H]-6-keto-$PGF_{1\alpha}$, and [^{125}I]protein A were purchased from New England Nuclear (Boston, MA). Neutral lipids and arachidonate were obtained from Nu-Chek Prep (Elysian, MN). Purified COX-1, a murine monoclonal antibody directed against COX-1 [cyo-1, which shows no cross-reactivity against COX-2 (Dr. Sankaran, Oxford Biomedical Research, Inc., personal communication)], and a murine cDNA probe for COX-2 (a 1.9-kb piece of murine cDNA hybridizing to a single 4.2-kb message without cross-reacting with the 2.7-kb COX-1 mRNA) were purchased from Oxford Biomedical Labs (Oxford, MI). Purified PGI_2 synthase, a rabbit polyclonal antibody directed against PGI_2 synthase, cDNA probes for COX-1 (a 1.8-kb *Eco*RI piece of murine cDNA inserted into SP65 vector, which hybridizes to a single 2.7-kb mRNA and does not cross-react with COX-2 under high-stringency conditions), and glyceraldehyde phosphate dehydrogenase (GAP; a 1.2-kb *Pst*I piece of murine cDNA inserted into pBR322 which hybridizes a single 1.3-kb mRNA) were generously provided by Dr. David DeWitt (Department of Biochemistry, Michigan State University). A cDNA probe for 18S was kindly provided by Dr. Paul Szabo (Department of Medicine, Cornell University). HEPES and Tris were obtained from Sigma Corporation (St. Louis, MO). Interleukin-1β (IL-1β) was obtained from Cistron (Pine Brook, NJ). Platelet-derived growth factor (PDGF) was purchased from R&D Systems (Minneapolis, MN). Acrylamide, bisacrylamide, TEMED, and ammonium persulfate were from National Diagnostics (Manville, NJ). Nitrocellulose and Immobilon membranes were obtained from Millipore Corporation (Bedford, MA). All organic solvents (HPLC grade) and biochemicals were obtained from Fisher Scientific (Springfield, NJ).

Preparation of Plasma LDL and Cationized LDL (cLDL). LDL (1.019–1.063 g/mL) was isolated by the preparative ultracentrifugation of pooled donor human plasma (Havel et al., 1955). LDL was cationized using *N,N*-dimethyl-1,3-propanediamine as adduct and 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride as catalyst at pH 6.5 (Goldstein et al., 1977; Pomerantz & Hajjar, 1989). Lipoproteins were dialyzed against 0.154 M NaCl, 0.3 mM EDTA, and 0.1 M $NaHPO_4$ (pH 7.4) for 48 h prior to concentration by ultrafiltration and added to tissue culture media just prior to initiation of experiments. Native and cLDL preparations routinely contained less than 2 nmol of thiobarbituric acid-reactive substances/mg of protein, as compared to Cu^{2+}-oxidized LDL which possessed >15.0 nmol/mg of protein.

Isolation and Culture of Aortic Smooth Muscle Cells. Smooth muscle cells were propagated from explants of rabbit and rat thoracic aorta and cultured as previously described (Pomerantz et al., 1984). Cells were identified as smooth muscle by their characteristic hill-and-valley morphology. Rabbit cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/fungizone (v/v/v). Rat cells were maintained in M-199 including the same supplements. All cells were incubated at 37 °C in 5% CO_2 in air.

Preparation of Cholesterol-Enriched Smooth Muscle Cells. Confluent cultures were incubated with cLDL (100 μg of protein/mL) in DMEM containing 10% fetal bovine serum for 7 days. Fresh medium containing cLDL was added on day 4 (Pomerantz & Hajjar, 1989). We have previously shown that cholesterol-enriched smooth muscle cells excluded trypan blue to the same extent as untreated smooth muscle cells (Pomerantz & Hajjar, 1989). In addition, spontaneous LDH release into PBS was similar between control and cholesterol-enriched smooth muscle cells, respectively (10.4% [control] vs 6.0% [cholesterol-enriched smooth muscle cells] of total cellular LDH). Normal and cholesterol-enriched cells also contained similar amounts of LDH enzyme (10.5×10^3 B-B units/35-mm plate). Cells were rendered quiescent by incubation in DMEM containing 1% insulin/transferrin/selenium (ITS).

COX Activity—Intact Smooth Muscle Cells. COX activity was estimated by the conversion of exogenous sodium arachidonate (10 μM) to 6-keto-$PGF_{1\alpha}$. Sodium arachidonate was prepared as an ethanolic solution, with vehicle (ethanol, 0.075%) used as the control. Cells were exposed to medium alone (control) or medium containing sodium arachidonate for 30 min at 37 °C. The amount of 6-keto-$PGF_{1\alpha}$ in the

supernatants was estimated by radio-immunoassay (Pomerantz et al., 1984).

Microsomal COX Activity. Untreated and cholesterol-enriched smooth muscle cells grown in 75-cm^2 flasks were washed, scraped into PBS/10 mM EDTA, pelleted by centrifugation, and resuspended in 1.0 mL of PBS/EDTA buffer. After sonication over ice in PBS/EDTA, unbroken cells and nuclei were removed by centrifugation at 7500*g*. Microsomes were then pelleted by centrifugation of the 7500*g* supernatant at 100000*g* for 60 min. Microsomes were resuspended in 1.0 mL of assay buffer (0.2 M Na_2PO_4, 10 mM EDTA, and 100 μg of norepinephrine); 10-μL aliquots were assayed for protein. The reaction was started by adding approximately 90 μg of microsomal protein to 0.5 μCi of [^{3}H]-arachidonate and then incubated at 37 °C for 15 min and terminated by extraction into 800 μL of ethyl acetate/methanol/0.4 M citric acid (15:2:1, v/v/v). Eicosanoid standards (6-keto-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$, TxB_2, PGD_2, and arachidonate, 5 μg each) were added to the organic phase and then developed by thin-layer chromatography (Pomerantz et al., 1984). COX activity was expressed as the percent conversion of [^{3}H]arachidonate to COX products/μg of protein.

ELISA for COX-1. In situ ELISA was used to quantify COX-1 in intact cells and was performed as previously described (Weksler, 1990). Briefly, normal and cholesterol-enriched smooth muscle cells plated into 96-well cluster plates were washed in 0.5 mM HEPES containing calciuma nd magnesium and fixed in HEPES containing 0.02% glutaraldehyde for 20 min at room temperature. After washing, the cells were passivated and made permeable using HEPES buffer containing 0.05% Tween-20 and 1% normal horse serum. After they were washed in HEPES/Tween, cells were exposed to cyo-1 antibody (1:50) overnight at 4 °C. After they were washed again in HEPES/Tween, cells were exposed to biotinylated horse anti-mouse antibody (1:200 in HEPES/Tween containing 1% horse serum) and visualized using avidin–alkaline phosphatase complex using 2 mM *p*-nitrophenyl phosphate (Calbiochem) in 10% diethanolamine. Plates were scanned on a Titertech multiscanner. DNA was then estimated using Hoechst reagent (McCaffrey et al., 1988). The absorbance of COX-1 (OD_{492}) was then normalized to the absorbance of DNA (Weksler, 1990). Data are expressed as relative absorbance units/unit of DNA (mean ± SEM) and are the difference between specific (cyo-1) and nonspecific binding (horse serum).

Metabolic Labeling of Cells/Immunoprecipitation. Cells were first incubated in methionine-free DMEM containing 1% ITS. Cells were then exposed to media containing Tran^{35}S label (150 μCi/mL) for 2 h. Cells were washed in ice-cold PBS, followed by a 30-min incubation in 350 μL of lysis buffer (25 mM Tris (pH 8.0), 144 mM NaCl, 1.0 mM PMSF, 2.0 mM EDTA, 1% NP-40, 20 μg/mL aprotinin, and 0.2 unit/mL α_2 macroglobulin). Cells were then scraped into ultracentrifuge tubes and incubated for an additional 45 min. Following a 30-min centrifugation at 30000*g*, the supernatant was collected, and an aliquot was taken for the determination of TCA-precipitable counts; 7.5×10^6 TCA-precipitable counts were diluted into a final volume of 500 μL of buffer A (42 mM Tris (pH 7.0), 188 mM NaCl, 6 mM EDTA, 2.5% Triton X-100, 20 μg/mL aprotinin, and 1 mM PMSF) containing myoglobin (0.1 mg/ml) and normal rabbit serum (1:50 dilution), to which was added 50 μL of protein A agarose beads first equilibrated with 50 μL of buffer B (47 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, and 20 μg/mL aprotinin). Each sample was cleared twice with protein A Agarose for 30 min. Following addition of 3.0 μL of pan-COX antibody (Tai & Tai, 1984), COX-2 antibody, or normal rabbit serum, the lysates were incubated for 4 h, followed by transfer to fresh tubes containing protein A agarose beads equilibrated in buffer B. After a 2-h incubation, beads were centrifuged at 15000*g* for 15 min and washed three times in buffer I (24 mM Tris (pH 8.0), 148 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, and 20 μg/mL aprotinin) and buffer B. Agarose beads were then overlaid with 40 μL of Laemmli sample buffer containing dithiothreitol, heated to 95 °C for 4 min, centrifuged at 12000*g* for 4 min, and run out on 7.5% PAGE gels (Laemmli, 1970). The PAGE gels were fixed, stained with Coomassie blue, incubated with Enhance, dried, and then subjected to autoradiography using Kodak X-OMAT film with intensifying screens at –80 °C. Autoradiographs were quantified using the ADOBE Printshop for MacIntosh and quantitated using NIH Image 1.41 programming.

Northern Blot Hybridizations. Total RNA was prepared (Chirgwin et al., 1979) from untreated and cholesterol-enriched smooth muscle cells grown in 75-cm^2 flasks. Cells were washed once in PBS, solubilized in 4.0 M guanidinium isothiocyanate buffer containing β-mercaptoethanol, and homogenized by trituration through a 22-gauge needle. After separation of the RNA by ultracentrifugation through 5.7 M cesium chloride (35 000 rpm in an SW-50.2 rotor, using a Beckman ultracentrifuge), RNA was precipitated using sodium acetate and ethanol. The purity (OD_{260}/OD_{280}) and quantity (OD_{260}) of the RNA were determined by UV spectrophotometry. RNA (20 μg) was electrophoresed through denaturing agarose gels containing 1.0% formaldehyde and ethidium bromide in MOPS buffer and blotted onto Zetaprobe membranes by capillary action in 10× SSC. Blots were then rinsed in 10× SSC, UV-cross-linked, washed (0.5% SDS and 0.1× SSC at 65 °C), prehybridized (0.25 M Na_2HPO_4, 0.25 M NaCl, 1 mM EDTA, and 7% SDS containing formamide at 43 °C for 5 min), and then hybridized (overnight at 43 °C) to cDNA probes (COX-1, COX-2, GAP, and 18S) prepared with a Random Primed DNA Labeling Kit (Boehringer-Mannheim). After they were washed under high-stringency conditions (2× to 0.1× SSC containing 0.1% SDS at 65 °C, 15 min each), the blots were subjected to autoradiography as described above. Data are expressed as absorbance ratios of COX-1 or COX-2 to GAP. Initial experiments were performed to determine whether cholesterol enrichment altered the expression of constitutively expressed genes, namely, glyceraldehyde phosphate dehydrogenase (GAP) (Xie et al., 1991) or 18S, expressed as the GAP/18S ratio. Cholesterol enrichment did not alter the expression of GAP/18S mRNA steady-state levels relative to controls (4.13 ± 0.19 vs 3.5 ± 0.36, $n = 3$, $p > 0.10$). Therefore, for most Northern analyses reported herein, *COX-1* and *COX-2* mRNA steady-state levels are normalized to the expression of GAP, except for experiments employing actinomycin D, where the data are normalized to the expression of 18S.

Western Analysis for PGI_2 Synthase. To detect and quantify PGI_2 synthase, normal and cholesterol-enriched smooth muscle cells in 150-cm^2 flasks were scraped into PBS, pelleted, and then extracted in 500 μL of 20 mM Tris buffer containing 1.0 mM EDTA and 20 mM CHAPS. After brief centrifugation to remove particulate matter, the supernatant was subjected to centrifugation at 12000*g* for 15 min. Total cell protein (100 μg) was diluted into loading buffer (0.25 M Tris, 20% SDS, 50% glycerol, and 2% dithiothreitol), boiled

for 5 min, and then subjected to electrophoresis (Laemmli, 1970) through 7.5% polyacrylamide. Proteins were transferred to Immobilon-P (Millipore) in a buffer containing 38 mM glycine, 48 mM Tris, and 0.035% SDS (Towbin et al., 1979). After overnight blocking in 20 mM Tris and 0.154 M NaCl (pH 7.4) containing 0.05% Tween-20 and 5% nonfat dry milk at 4 °C, blots were exposed to primary antibodies (anti-PGI_2 synthase, and anti-β-galactosidase, 1:50) for 3 h at 25 °C. The anti-PGI_2 synthase antibody cross-reacts 100% with antigen derived from rabbit and human tissue (DeWitt et al., 1983) and does not react with other cellular proteins, including actin (data not shown). Blots were then washed in TBS/Tween (3 × 150 mL) and TBS (1 × 150 mL). For visualization, blots were exposed to [^{125}I]protein A (10^6 cpm) for 30 min, followed by autoradiography. The developed peaks were scanned using an LKB 2202 Ultrascan laser densitometer and quantified by peak area ratios.

Miscellaneous Assays. Cellular and lipoprotein protein contents were measured by the method of Lowry et al. (1951), using bovine serum albumin as standard. Thiobarbituric acid-reactive substances (TBARS) were measured as an index of oxidation in native and cLDL preparations (Marnett et al., 1979). Lactate dehydrogenase (LDH) was measured in cells and in conditioned media using a colorimetric assay kit (Sigma, St. Louis, MO).

Statistical Analysis. Data are expressed as mean ± SEM and analyzed by the student's *t*-test or analysis of variance, followed by the Newman Keuls test when more than two factors were simultaneously analyzed. Differences with $p < 0.05$ were considered significant.

RESULTS

Initial experiments were performed to determine whether cholesterol enrichment reduced the COX-1 activity of intact quiescent rabbit smooth muscle cells. Arachidonic acid-induced 6-keto-$PGF_{1\alpha}$ synthesis by cholesterol-enriched cells following exposure to arachidonate was nearly 8-fold less than untreated cells (Figure 1). However, cholesterol enrichment did not alter basal 6-keto-$PGF_{1\alpha}$ production to untreated controls. When expressed as the percent conversion of exogenously added arachidonic acid (10 μM or 20 nmol/well) to 6-keto-$PGF_{1\alpha}$, intact normal smooth muscle cells converted 0.18 ± 004% of the exogenous arachidonic acid to 6-keto-$PGF_{1\alpha}$ (36 ± 0.001 pmol/well). In contrast, cholesterol-enriched cells converted 0.031 ± 0.001% of exogenous arachidonic acid to 6-keto-$PGF_{1\alpha}$ (20 nmol of arachidonic acid to 6 ± 2 pmol of 6-keto-$PGF_{1\alpha}$). Since arachidonic acid-induced stimulation of eicosanoid biosynthesis bypasses phospholipase A_2 and C activation, and PGI_2 synthase is present in 20-fold higher levels than COX activity in arterial smooth muscle cells (DeWitt et al., 1983), these results demonstrate that cholesterol enrichment can reduce COX activity.

To determine the temporal relationship between exposure to cLDL and reduction in COX-1 activity, confluent cultures of rabbit smooth muscle cells were exposed to medium in the presence or absence of cLDL for 7 days with one medium change. At discrete time intervals, cells were exposed to 10 μM arachidonic acid (30 min at 37 °C), followed by radio-immunoassay of 6-keto-$PGF_{1\alpha}$ in the supernatant (Figure 2). COX-1 activity by control cells declined gradually as cells continued past confluence. However, COX-1 activity by cells exposed to cLDL continued to become further depressed over the remainder of the 7-day time course. The time course by which cLDL reduces COX-1 activity is parallel to the rate at which cells accumulate lipid (Pomerantz & Hajjar, 1989).

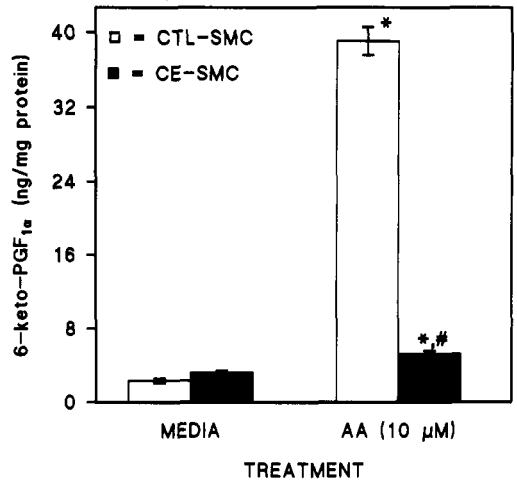

FIGURE 1: Cholesterol enrichment reduces COX activity in rabbit smooth muscle cells. Smooth muscle cells (passage 4) at confluent density were exposed to media ± cLDL (100 μg/mL) for 1 week. Cells were then washed in PBS and exposed to serum-free DMEM ± arachidonate (AA, 10 μM) for 30 min at 37 °C. 6-Keto-$PGF_{1\alpha}$ was measured in supernatants by radio-immunoassay. These data are representative of six experiments, using 12 replicates per treatment group. Data are expressed as ng of 6-keto-$PGF_{1\alpha}$/mg of protein (mean ± SEM, * = $p < 0.01$ control vs AA, * = $p < 0.01$, control vs cholesterol-enriched smooth muscle cells, # = $p < 0.05$, control/AA vs CE-enriched/AA).

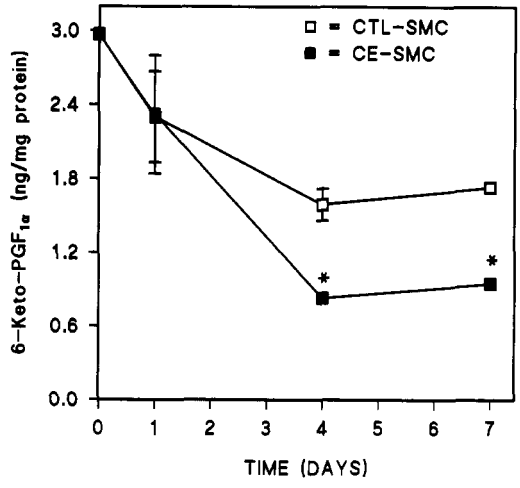

FIGURE 2: Effect of cholesterol accumulation on COX activity—time course. Confluent rabbit smooth muscle cell cultures (passage 9) grown in 24-well cluster plates were exposed to DMEM containing 10% fetal bovine serum in the absence (box) and presence (■) of cLDL (100 μg/mL). At specific time intervals, cells were exposed to serum-free media containing arachidonic acid (10 μM, for 30 min at 37 °C). Data are expressed as ng of 6-keto-$PGF_{1\alpha}$/mg of protein (mean ± SEM, * = $p < 0.05$, control vs cLDL). This is representative of six separate experiments, with each treatment perfrmed in triplicate.

To determine the dose–response relationship between exposure to cLDL and reduction in COX-1 activity, rabbit smooth muscle cells were exposed to increasing concentrations of cLDL (0–75 μg/mL) for 1 week. Cells were then exposed to arachidonic acid (10 μM for 30 min), followed by radio-immunoassay of 6-keto-$PGF_{1\alpha}$ in the conditioned media. cLDL at concentrations of less than 50 μg/mL did not inhibit COX-1 activity, while concentrations of cLDL greater than 50 μg/mL inhibited 6-keto-$PGF_{1\alpha}$ release in a dose-dependent manner (Figure 3). This dose–response parallels the dose–response by which cells accumulate oil red O-positive lipid.

Since COX-1 is a microsomal protein, experiments were performed to determine whether cholesterol enrichment

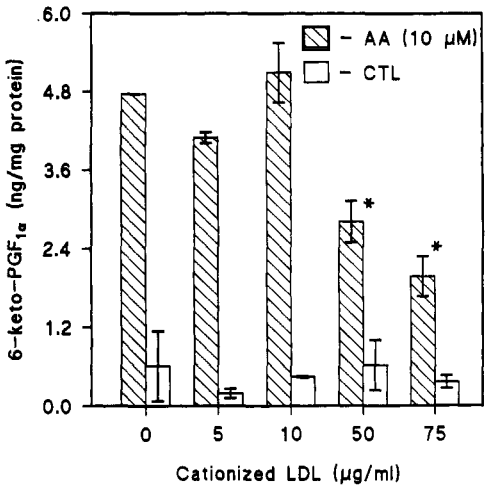

FIGURE 3: Effect of cholesterol enrichment on COX activity—dose–response relationship. Confluent rabbit smooth muscle cells (passage 9) grown in 24-well cluster plates were exposed to DMEM containing 10% fetal bovine serum and increasing concentrations of cLDL (0–75 μg of protein/mL) for 7 days with one medium change. Cells were then exposed to serum-free media alone (white bars) or media mcontaining arachidonic acid (hatched bars, 10 μM, for 30 min at 37 °C). Data are expressed as ng of 6-keto-$PGF_{1\alpha}$/mg of protein (mean ± SEM, * = $p < 0.05$, cLDL vs control).

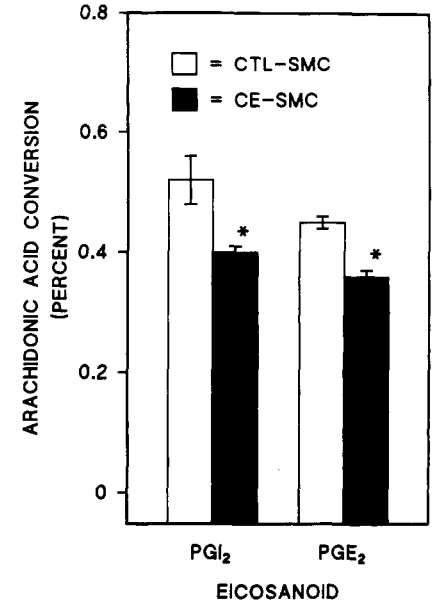

FIGURE 4: Cholesterol enrichment reduces microsomal COX activity. Microsomes from normal and cholesterol-enriched rabbit smooth muscle cells (passage 7) were prepared as described in Experimental Procedures. COX activity of equal amounts of microsomes (90 μg/sample) was determined by evaluating the conversion of [^{3}H]-arachidonate (0.5 μCi) to COX products. Samples were extracted into ethyl acetate and developed by thin-layer chromatography as described in Experimental Procedures. The data are expressed as the percent of total radioactivity converted to 6-keto-$PGF_{1\alpha}$ and PGE_2. An equal percent of arachidonate was associated with unmetabolized substrate by each treatment group (74.4 ± 1.32 for control cells vs 76.2 ± 1.04 for cholesterol-enriched cells, p = ns). This is representative of two such experiments, with triplicate samples for each treatment group (mean ± SEM, * = $p < 0.05$).

reduced the rate of conversion of [^{3}H]arachidonate to COX products, as assessed by TLC (Figure 4). Microsomes from untreated rabbit cells converted 1.0% of total radioactivity to PGI_2 and PGE_2, while microsomes derived from cholesterol-enriched smooth muscle cells converted only 0.7% of total radioactivity to these eicosanoids ($n = 3$, $p < 0.01$). Other COX products ($PGF_{2\alpha}$, PGD_2, and TxB_2, PGB_2), lipoxygenase products, or epoxyeicosatetraenoic acids were undetectable. Thus, cholesterol enrichment reduced microsomal COX-1 activity by approximately 22%, suggesting the possibility that cholesterol enrichment reduced COX-1 mass.

To directly determine whether cholesterol enrichment reduced COX-1 activity by reducing COX-1 mass, the relative abundance of this protein was measured by *in situ* ELISA employing a highly specific monoclonal antibody (cyo-1). In two separate experiments with eight replicates per treatment group, cholesterol enrichment reduced COX-1 immunoreactivity by approximately 78% (2.23 ± 0.01 OD_{492}/unit of DNA vs 0.51 ± 0.01, * = $p < 0.01$) and 30% (0.81 ± 0.01 OD_{492}/unit of DNA vs 0.57 ± 0.01, * = $p < 0.01$), respectively. There was no difference in nonspecific binding between control and cholesterol-enriched smooth muscle cells (data not shown). The decrease in COX-1 content occurred notwithstanding an increase in total cell protein following cholesterol enrichment (Pomerantz & Hajjar, 1989). These data suggest that a potential mechanism by which cholesterol-enrichment-reduced COX-1 activity may be due to decreased COX-1 mass.

Experiments were then performed to determine whether cholesterol enrichment altered the expression of mitogen-inducing COX, namely, COX-2. To address this possibility, we took advantage of two important characteristics of COX-2. First, COX-1 and COX-2 are inactivated by free radical-mediated peroxidation of the enzyme during catalytic conversion of arachidonic acid to PGH_2 (Egan et al., 1976). Second, COX-2 activity is augmented by serum by induction of the transcription and translation of COX-2 mRNA (Raz et al., 1989a,b; O'Banion et al., 1991). To determine whether cholesterol enrichment alters COX-2 synthesis, the rate of the recovery of COX-2 activity in normal and cholesterol-enriched cells in response to serum stimulation was examined. Normal and cholesterol-enriched smooth muscle cells were first exposed to 10 μM arachidonic acid (30 min at 37 °C). Cells were then postincubated in fresh medium containing 10% fetal bovine serum for up to 48 h. At discrete time intervals, COX-2 activity was quantified by re-exposure to 10 μM arachidonate in serum-free medium; the conditioned medium was then assayed for 6-keto-$PGF_{1\alpha}$ (Figure 5). As depicted in panel A, COX activity was reduced in cholesterol-enriched smooth muscle cells relative to untreated cells. Panel B depicts the recovery of COX-2 activity induced by serum and its modulation in cholesterol-enriched cells. In untreated cells, COX was rapidly inactivated by arachidonate (89% after 30 min). COX-2 activity demonstrated a well-characterized "rebound effect" after approximately 12 h, which gradually declined over time (Pash & Bailey, 1988; Bailey et al., 1985). However, the profile of the recovery of COX-2 activity (and, hence, content) by cholesterol-enriched cells was markedly different. Cholesterol-enriched cells contained reduced COX-2 activity (panel A) and demonstrated nearly undetectable COX activity 30 min after arachidonate-induced inactivation (panel B). In addition, the recovery of COX-2 activity in cholesterol-enriched cells was approximately linear over time. When compared to its original COX activity there is a rebound effect, albeit not nearly as marked as the rebound effect observed by untreated cells exposed to arachidonic acid. In addition, COX-2 activity did not return to the levels observed inuntreated cells. Smooth muscle cells remained cholesterol-enriched during the entire 48-h period of COX-2 recovery (data not shown). Thus, cholesterol enrichment not only reduced COX-2 activity but it also impaired the ability of smooth muscle cells to regenerate COX-2 in response to serum.

Elements present in serum, such as PDGF (Habenicht et al., 1985; Goerig et al., 1988; Hajjar et al., 1987) and IL-1β

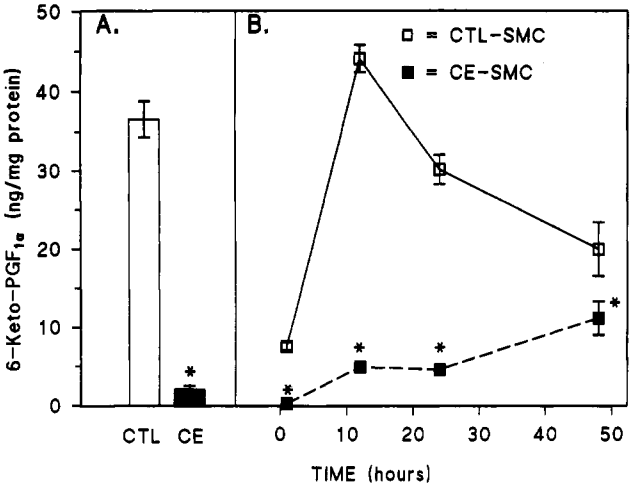

FIGURE 5: Cholesterol enrichment attenuates serum-induced COX activity. Normal and cholesterol-enriched rabbit smooth muscle cells (passage 4) were first exposed to DMEM ± 10 μM arachidonate (30 min at 37 °C) to inactivate COX. Supernatants were assayed for 6-keto-$PGF_{1\alpha}$ by radio-immunoassay (A). Cells were then incubated for up to 48 h in DMEM containing 10% fetal bovine serum in the absence of further cholesterol enrichment. At 0.5-, 12-, 24-, and 48-h intervals, cells were washed and reexposed to arachidonate (10 μM). Supernatants were assayed for 6-keto-$PGF_{1\alpha}$ by radio-immunoassay (B). This experiment is representative of four such experiments, with three replicates per treatment group. Data are expressed as ng of 6-keto-$PGF_{1\alpha}$/mg of protein (mean ± SEM, * = $p < 0.05$ from control smooth muscle cells). (The 12- and 24-h time points of cholesterol-enriched smooth muscle cells are not statistically different from each other, but both points are statistically significantly different from time 0 and 48 h post-arachidonate challenge.)

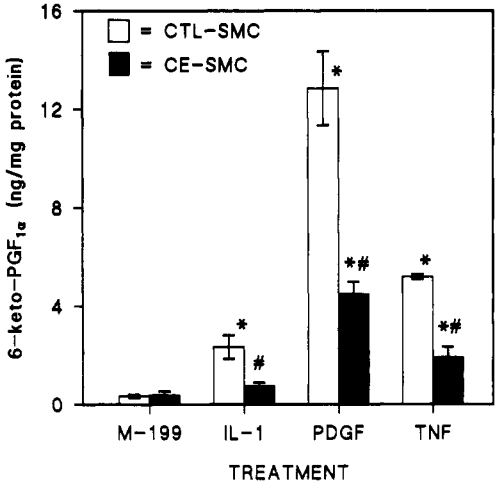

FIGURE 6: Cholesterol enrichment inhibits IL-1β-, PDGF-, and TNFα-induced PGI_2 production. Normal and cholesterol-enriched rat smooth muscle cells in 24-well cluster plates were exposed to M-199/1% ITS alone (control) or in media containing IL-1β (1.0 unit/mL), PDGF (10 ng/mL), or TNFα (50 ng/mL) for 24 h at 37 °C. 6-Keto-$PGF_{1\alpha}$ was measured in conditioned media by radio-immunoassay. Data are expressed as ng of 6-keto-$PGF_{1\alpha}$/mg of protein (mean ± SEM, * = $p < 0.05$ from agonist vs control, # = $p < 0.05$ normal vs cholesterol-enriched smooth muscle cells). This is a representative of three separate experiments, with each treatment performed in triplicate.

(Raz et al., 1988; Breviairio et al., 1990; Maier et al., 1990), stimulate PGI_2 production by activation of cellular phospholipases and by induction of the rate of transcription and translation of COX-2. To determine whether cholesterol enrichment interferes with growth factor/cytokine induction of smooth muscle cell PGI_2 generation, normal and cholesterol-enriched smooth muscle cells were exposed to M-199/1% ITS

Table I: Cholesterol Enrichment Reduces IL-1β-Induced PGI_2 Synthesis: Dose–Response Relationship[a]

IL-1β (units/mL)	control SMC	CE-enriched SMC
0	1.56 ± 0.28	1.42 ± 0.14
0.1	2.63 ± 0.08*	1.95 ± 0.14*,#
1.0	2.94 ± 0.08*	1.75 ± 0.19*,#
10.0	4.35 ± 0.30*	3.08 ± 0.23*,#

[a] Rat smooth muscle cells (SMC) prepared as described in Figure 6 were exposed to M-199/1% ITS containing increasing concentrations of IL-1β for 24 h at 37 °C. Supernatants were assayed for 6-keto-$PGF_{1\alpha}$. Data are expressed as ng of 6-keto-$PGF_{1\alpha}$/mg of cell protein (mean ± SEM, with * = $p < 0.05$, IL-1β vs media alone, # = $p < 0.05$ control vs cholesterol-enriched smooth muscle cells). This is representative of three experiments, with each treatment performed in triplicate.

Table II: Cholesterol Enrichment Inhibits IL-1β-Induced PGI_2 Synthesis: Time Course[a]

time (h)	control SMC	CE SMC	IL-1β	CE SMC IL-1β
0.5	0.7 ± 0.2	0.5 ± 0.1	2.3 ± 0.2*	0.5 ± 0.1*
1.0	0.5 ± 0.1	0.4 ± 0.1	1.7 ± 0.1*	0.4 ± 0.1*
4.0	1.2 ± 0.2	0.7 ± 0.1	5.8 ± 0.5*	0.8 ± 0.1*
24.0	2.0 ± 0.4	2.3 ± 0.1	7.9 ± 0.7*	2.1 ± 0.3*

[a] Rat smooth muscle cells grown and cholesterol-enriched as described in Figure 6 were exposed to M-199/1% ITS alone (control) or these media containing IL-1β (1.0 unit/mL, 1.0 mL/well) for up to 24 h at 37 °C. At 0.5, 1, 4, and 24 h, supernatants were assayed for 6-keto-$PGF_{1\alpha}$; data are expressed as ng of 6-keto-$PGF_{1\alpha}$/mg of cell protein (mean ± SEM, with * = $p < 0.05$). This is representative of two experiments, with each treatment performed in triplicate.

(control) or these media containing IL-1β (1.0 unit/mL), PDGF (10 ng/mL), or TNF (50 ng/mL). Medium conditioned for 24 h was assayed for 6-keto-$PGF_{1\alpha}$ (Figure 6). Each of these agonists stimulated 6-keto-$PGF_{1\alpha}$ production by rat smooth muscle cells with the following decreasing rank order of potency: PDGF > TNF > IL-1β. Importantly, cholesterol enrichment significantly reduced 6-keto-$PGF_{1\alpha}$ production in response to each of these agonists.

These data show that cholesterol enrichment reduced eicosanoid biosynthesis by inhibiting the activity of common pathways leading to eicosanoid generation. These pathways include cellular phospholipase activation and *de novo* synthesis of COX. To investigate these processes more closely with respect to cholesterol enrichment, we first evaluated the dose–response relationship of IL-1β on PGI_2 generation. IL-1β increased 6-keto-$PGF_{1\alpha}$ production in 24-h-conditioned media from control cells in a dose-dependent manner, with maximal effects seen at 10 units/mL; cholesterol enrichment reduced IL-1β induction of 6-keto-$PGF_{1\alpha}$ at all dose points (Table I).

Next, normal and cholesterol-enriched smooth muscle cells were exposed to IL-1β (5.0 units/mL) for up to 24 h; 6-keto-$PGF_{1\alpha}$ was then measured at 30 and 60 min (to assess the effects of cholesterol enrichment on phospholipase activation and COX-1 activity) and at 4–24 h (to determine the effects of cholesterol enrichment on the induction of COX-2). Cholesterol enrichment reduced the acute (30–60 min) chronic (4–24 h) stimulation of 6-keto-$PGF_{1\alpha}$ release following IL-1β (Table II), demonstrating that cholesterol enrichment reduces eicosanoid generation by inhibiting phospholipase activity, as well as inhibiting COX-1 and COX-2 expression.

The above data suggest that cholesterol-enrichment reduced cytokine/growth factor-induced PGI_2 synthesis by inhibiting mitogen induction of the steady-state levels of COX-1 and COX-2 mRNAs. To test this hypothesis, total RNA from normal and cholesterol-enriched smooth muscle cells exposed to PDGF (10 ng/mL) (Figure 7) for discrete time intervals up to 24 h was examined by Northern analysis using cDNA

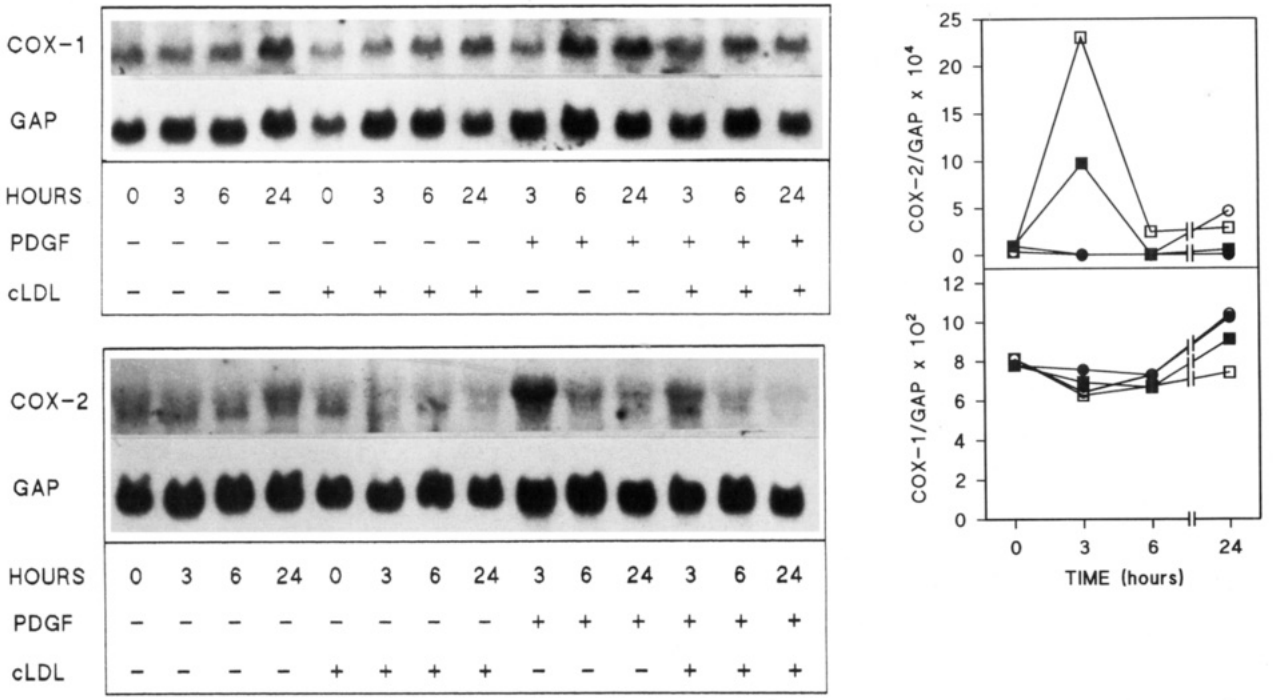

FIGURE 7: Influence of cholesterol enrichment on PDGF induction of COX-1 and COX-2 mRNA steady-state levels—kinetic analysis. Normal and cholesterol-enriched rat smooth muscle cells grown in 75-cm² flasks were exposed to M-199/1% ITS ± PDGF (10 ng/mL) for up to 24 h at 37 °C. Extracted RNA (20 μg) was subject to Northern analysis, using probes hybridizing to COX-1 (A, top left) COX-2 (B, bottom left), and GAP. Data are normalized to the expression of GAP and expressed as arbitrary absorbance units. Densitometric analysis of COX-1 and COX-2 Northern blots was performed using a Molecular Dynamics Phosphoimager using Image-Quant programming and is depicted in C (right). Treatment groups are as follows: ○, control smooth muscle cells, M-199 alone; ●, cholesterol-enriched smooth muscle cells, M-199 alone; □, control smooth muscle cells, exposed to PDGF; ■, cholesterol-enriched smooth muscle cells, exposed to PDGF.

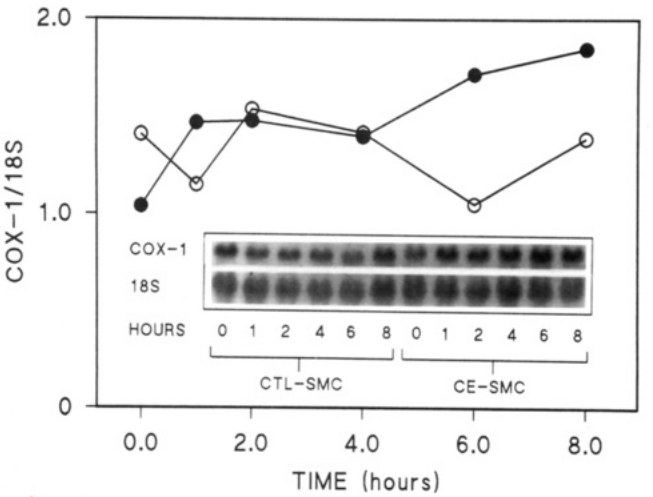

FIGURE 8: Effect of cholesterol enrichment on COX-1 mRNA stability. Normal and cholesterol-enriched smooth muscle cells were exposed to actinomycin D (5.0 μg/mL) for up to 8 h. At discrete intervals, total RNA was extracted; 20 μg of RNA was subjected to Northern analysis (top) using probes directed against COX-1 and 18S and normalized to the expression of 18S. Densitometric analysis of the above plot is expressed as arbitrary absorbance units. This is representative of two such experiments. Treatment groups are as follows: ○, control smooth muscle cells; ●, cholesterol-enriched smooth muscle cells.

probes for COX-1 (panel A) and COX-2 (panel B) and normalized to the expression of GAP. Quantitative analysis for COX-1 and COX-2 Northern blots is summarized in panel C. Under unstimulated conditions, COX-1 is constitutively expressed and was not affected by either cholesterol enrichment or exposure to PDGF (panels A and C). In contrast, COX-2 steady-state mRNA levels in unstimulated normal cells are essentially undetectable; PDGF increased COX-2 mRNA steady-state levels by over 20-fold after 3 h and returned to low, but detectable, levels after 6–24 h (panels B and C). Cholesterol enrichment inhibited the PDGF induction of COX-2 mRNA steady-state levels by approximately 40% after an 8-h exposure to PDGF, which returned to nearly undetectable levels after 24 h (panels B and C).

We next examined the effect of actinomycin D on COX-1 mRNA steady-state levels to determine whether cholesterol enrichment inhibited basal COX expression by decreasing COX-1 mRNA stability. Normal and cholesterol-enriched smooth muscle cells were exposed to actinomycin D (5.0 μg/mL) for up to 8 hours. At discrete intervals, total RNA was extracted, and COX-1 mRNA levels were determined by Northern analysis and normalized to the expression of 18S (Figure 8). Actinomycin D did not alter expression of COX-1 mRNA up to 8 h, indicating that reduced COX expression by cholesterol-enriched smooth muscle cells is not due to reduced COX-1 stability.

The above data demonstrate that cholesterol enrichment inhibited PGI_2 and PGE_2 synthesis by smooth muscle cells commensurate with reducing COX-2 mRNA steady-state levels without altering basal COX-1 mRNA steady-state levels. These observations support the hypothesis that cholesterol enrichment decreased eicosanoid synthesis by inhibiting COX protein synthesis. To test this hypothesis, quiescent normal and cholesterol-enriched smooth muscle cells were placed in media in the absence and presence of PDGF for 3 h; Tran^{35}S label was added to methionine-free medium for the final 2 h. COX-1 and COX-2 were immunoprecipitated from aliquots of cell lysates containing equal amounts of TCA-precipitable radioactivity using pan-COX antibody or preimmune serum (Figure 9). Pan-COX antibody immunoprecipitated one prominent 70-kDa protein from quiescent smooth muscle cells and a minor band at slightly higher molecular mass (ca. 71 kDa); cholesterol enrichment reduced COX-1 protein by approximately 25%. Exposure of smooth muscle cells to PDGF

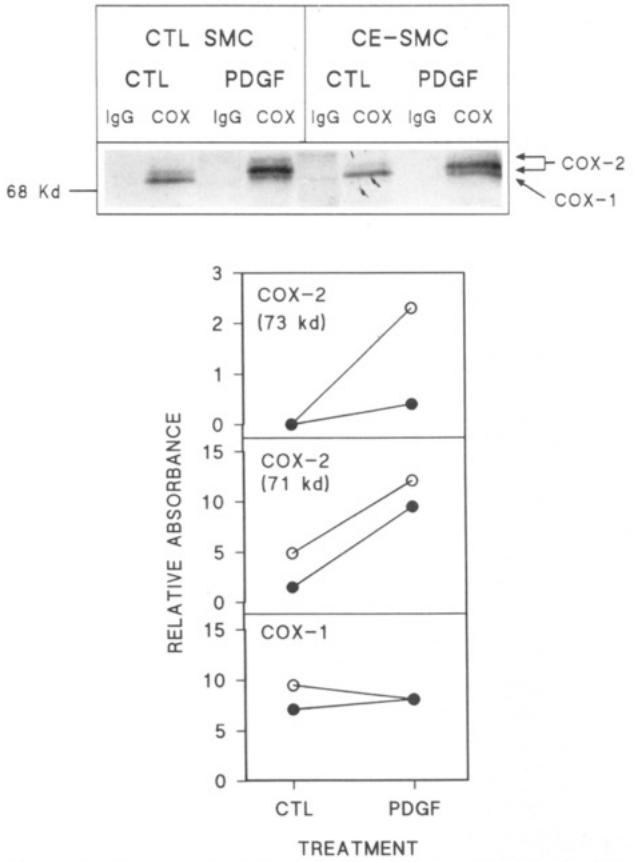

FIGURE 9: Cholesterol enrichment reduces [^{35}S]methionine/cysteine incorporation into COX protein(s). (A) PAGE autoradiograph. Normal (lanes A–D) and cholesterol–enriched (lanes E–H) rat smooth muscle cells grown in 25-cm^2 flasks were preincubated in methionine-free M-199/1% ITS alone or these media containing PDGF for 1 h, followed by coincubation of PDGF with Tran^{35}S label (150 μCi/mL) for an additional 2 h as described in Experimental Procedures. Normal smooth muscle cells incorporated significantly more [^{35}S]-methionine/cysteine into TCA-precipitable counts than cholesterol-enriched smooth muscle cells (8.26 ± 0.92 vs 5.16 ± 0.56%, $p <$ 0.02). Equal amounts of TCA-precipitable radioactivity (7.0×10^6 dpm) were immunoprecipitated using COX antibody or nonimmune serum, followed by SDS–PAGE and autoradiography. (B) Densitometric analysis of the PAGE autoradiograph. Data were obtained using a laser densitometer and are expressed as arbitrary absorbance units. Each value represents the mean of two scans for each lane. This is representative of two experiments, with each treatment performed in duplicate. Treatment groups are as follows: ○, control smooth muscle cells; ●, cholesterol-enriched smooth muscle cells.

reduced the amount of radioactivity in COX-1 (ca. 15%) and significantly increased expression of a COX-2 71/73-kDa doublet, which has been shown by others to be COX-2 (Kujubu & Herschman, 1992; Han et al., 1990; Phillips et al., 1993; Kujubu et al., 1993). Cholesterol-enriched cells, when exposed to PDGF, contained 22% less COX-2 (71 kDa) and 82% less COX-2 (73 kDa) than control cells exposed to PDGF, but contained similar amounts of COX-1. Neither COX-1 nor COX-2 was immunoprecipitated using normal rabbit serum. Overall, these data reinforce the hypothesis that cholesterol enrichment reduces the synthesis of nascent COX proteins.

Experiments were then performed to determine the kinetics of COX-1 and COX-2 protein expression in normal and cholesterol-enriched smooth muscle cells. Rat smooth muscle cells were first incubated in serum-free M-199/1% ITS overnight and then exposed to these media in the absence and presence of PDGF for 1, 4, and 22 h. Cells were then incubated in methionine-free medium containing Tran^{35}S label for an additional 2 h. Equal amounts of TCA-precipitable radioactivity were used for the immunoprecipitation (Figure 10) using pan-COX antibody (panel A) and COX-2 antibody (panel B). Densitometric analyses for COX-1 and COX-2 immunoprecipitations are depicted in panel C. In control smooth muscle cells, radioactivity declines slowly over time, consistent with COX-1 turnover over the 24-h time period. Cholesterol enrichment reduced radioactivity in COX-1, but the rate of decline of COX-1 radioactivity was similar between normal and cholesterol-enriched smooth muscle cells. PDGF did not significantly alter the kinetics of COX-1 turnover in either normal or cholesterol-enriched smooth muscle cells. PDGF significantly increased the COX-2 expression after 3 h in both the 71- and 73-kDa COX-2 forms (2-fold). The rate of COX-2 (71 kDa) turnover was slower than that for the 73-kDa COX-2 form; COX-2 (73 kDa) returned to basal levels after 6 h. The COX-2 (71 kDa) isoform returned to base line after 24 h. Cholesterol enrichment reduced PDGF-induced COX-2 (73 kDa) by 23% and COX-2 (71 kDa) by approximately 10%. Rates of turnover of both COX-2 isoforms were similar between normal and cholesterol-enriched cells in the absence or presence of PDGF, suggesting that synthesis and not degradation regulates COX-2 expression.

Finally, to evaluate the possibility that reduced PGI_2 synthesis following cholesterol enrichment was due not only to reductions in COX but also to alterations in PGI_2 synthase, the content of PGI_2 synthase was measured by Western analysis. Cholesterol enrichment reduced the PGI_2 synthase content by approximately 30%/100 μg of total cell extract ($p < 0.05$) (Figure 11).

DISCUSSION

We have previously reported that smooth muscle cell-derived foam cells are deficient in eicosanoid synthetic capacity, principally due to inhibition of arachidonic acid release and conversion to eicosanoids by linoleic acid and cholesterol derived from the hydrolysis of LDL cholesteryl esters (Pomerantz & Hajjar, 1989). At that time, we also found that cholesterol enrichment inhibited arachidonic acid-induced PGI_2 synthesis by smooth muscle cells, suggesting that COX and/or PGI_2 synthase may be affected by cholesterol enrichment. We now provide mechanistic data demonstrating that intact cholesterol-enriched arterial smooth muscle cells display reduced eicosanoid synthetic capacity due to their inability to generate COX-1 and COX-2 proteins, commensurate with either no change or a decrease in COX-1 and COX-2 mRNA steady-state levels.

We first explored the influence of cholesterol enrichment on the regulation of COX-1 activity. Using arachidonic acid-induced PGI_2 release as an indirect measurement of COX-1 activity, our data revealed that cholesterol enrichment significantly reduced COX-1 activity (Figure 1). Time course studies revealed that reduction in COX-1 activity occurs after 4 days of exposure to cLDL or when the cells begin to accumulate intracellular lipid (Figure 2). Prior to this time, most of the lipoprotein is extracellular and surface-bound (Pomerantz & Hajjar, 1989; Stein et al., 1979). Since PGI_2 production by cholesterol-enriched cells did not change during the last 3 days of exposure to cLDL, significant cholesterol accumulation may not be a prerequisite for reduction in COX-1 activity. However, the effects of lipid enrichment on PGI_2 are also dose-dependent, since reductions in PGI_2 synthesis occur only at concentrations of cLDL (>50 μg of protein/mL) that promote significant lipid accumulation (Figure 3). These results support the hypothesis that reduced COX-1 activity may be due to the metabolism of cholesteryl esters, rather than to the accumulation of cholesteryl esters *per se*.

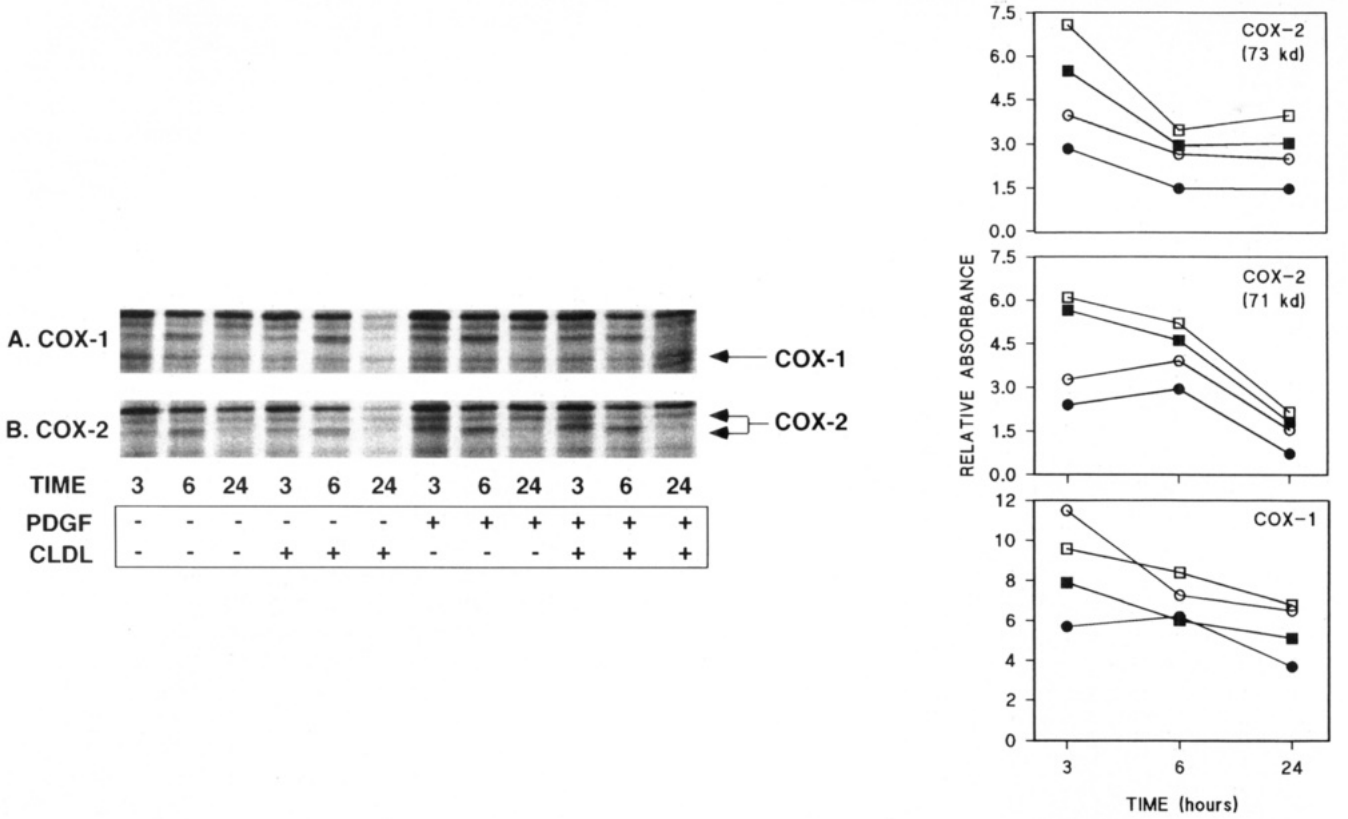

FIGURE 10: Cholesterol enrichment reduces [^{35}S]methionine/cysteine incorporation into COX proteins—kinetic analysis. Normal and cholesterol-enriched rat smooth muscle cells in 75-cm^2 flasks were first exposed to serum-free M-199/1% ITS overnight. Cells were then exposed to M-199 ± PDGF for the indicated times minus 2 h and then incubated in methionine-free M-199 containing Tran^{35}S label (150 μCi/mL) for an additional 2 h. Normal cells incorporated equal amounts of [^{35}S]methionine/cysteine into TCA-precipitable counts as cholesterol-enriched smooth muscle cells (8.6 ± 0.92 vs 8.4 ± 0.56%, p = ns). Equal amounts of TCA-precipitable radioactivity (7.0 × 10^6 dpm) were then immunoprecipitated using pan-COX antibody (A) or COX-2 antibody (B) followed by SDS–PAGE. Radioactivity comigrating with COX-1 and COX-2 (C, right) was quantified directly by phosphorimage analysis (Molecular Dynamics using Image-Quant software) and is expressed in arbitrary absorbance units; imaging was performed using Photoshop (MacIntosh). Treatment groups are as follows: ○, control smooth muscle cells, M-199 alone; ●, cholesterol-enriched smooth muscle cells, M-199 alone; □, control smooth muscle cells, exposed to PDGF; ■, cholesterol-enriched smooth muscle cells, exposed to PDGF.

Since COX-1 is a microsomal protein, we evaluated the influence of cholesterol enrichment on microsomal COX-1 activity. Our results demonstrate that, when compared to intact cells, microsomes derived from normal cells converted 3-fold more arachidonic acid to 6-keto-PGF$_{1\alpha}$, consistent with previously published reports on the degree of enrichment of COX activity in microsomes vs whole cells (Van Der Ouderaa et al., 1977). We also demonstrated that cholesterol enrichment reduced microsomal COX-1 activity (Figure 4). The results of these experiments suggest that cholesterol enrichment reduces COX-1 mass. In fact, COX-1 mass, as measured *in situ* by ELISA, was also reduced (see Results), suggesting that the reduction in COX-1 activity as a result of cholesterol enrichment was due to a reduction in COX-1 content. The magnitude of reduction of COX-1 activity by intact cells (84%) was greater than the magnitude of reduction of COX-1 activity by microsomes (22%). While the mechanism of this effect is not clear, it may be due to differences in substrate accessibility to COX-1; the cell membrane of cholesterol-enriched cells may represent a greater barrier to arachidonate penetration than cell membranes from normal cells.

To explore potential mechanisms by which cholesterol enrichment modulates COX-1 expression, we evaluated the influence of cholesterol enrichment on transcriptional and posttranscriptional processes leading to the expression of COX-1. COX-1 is a 70-kDa protein whose message is constitutively expressed (DeWitt & Smith, 1988). Since the 5′-flanking region of the COX-1 gene has no CAAT or TATA boxes, but does have two SP-1 and three AP-1 sites, one of which is adjacent to a negative glucocorticoid regulatory element (Kraemer et al., 1992), it may be subject to transcriptional regulation. Our data demonstrate that COX-1 mRNA is constitutively expressed in smooth muscle cells and is not up-regulated by PDGF or affected by cholesterol enrichment (Figure 7). Second, actinomycin D treatment of normal and cholesterol-enriched cells revealed that COX-1 mRNA is very stable ($t_{1/2} \geq 8$ h) and also is unaffected by cholesterol enrichment (Figure 8). However, COX-1 protein was reduced, whether measured by ELISA, by immunoprecipitation following metabolic labeling (Figure 10), or indirectly by estimation of its activity (Figures 1–3). Thus, our data suggest that cholesterol enrichment may inhibit posttranscriptional processes leading to COX-1 expression, on the basis of observations that cholesterol enrichment reduced COX-1 protein synthesis and the corresponding COX activity without significantly altering COX-1 mRNA levels (Figure 7) or degrading the COX-1 protein (Figure 10). The precise mechanism by which cholesterol enrichment reduces post-transcriptional processing of COX-1 mRNA is not known, but it may due to the inhibition of translation of COX-1 mRNA transcripts.

To gain further insight into the mechanisms by which cholesterol enrichment inhibited eicosanoid synthesis, we explored the possibility that cholesterol enrichment might modify the induction of COX-2. In contrast to COX-1, COX-2 is not constitutively expressed. Its 8.9-kb cDNA (Xie

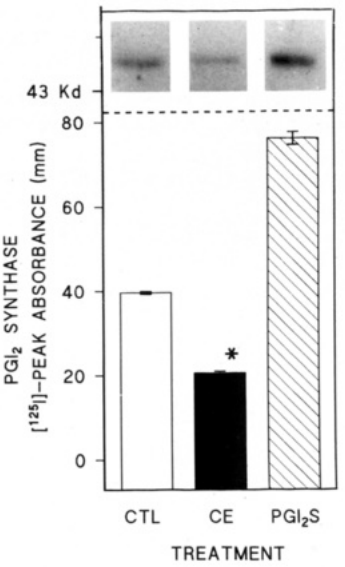

FIGURE 11: Cholesterol enrichment reduces PGI_2 synthase. Normal and cholesterol-enriched smooth muscle cells were extracted into Tris buffer containing CHAPS, electrophoresed through SDS–PAGE, electroblotted onto Immobilon, and immunoblotted as described in Experimental Procedures. Visualization was performed using [^{125}I]-protein A. Top: autoradiogram of control (lane 1) and cholesterol-enriched smooth muscle cells (lane 2) and authentic PGI_2 synthase (1.5 μg, lane 3). Bottom: densitometric analysis of scans in the top panel. These data are representative of three such experiments.

et al., 1991) encodes a 4.1-kb mRNA, which is translated into a 71-/73-kDa protein doublet (O'Banion et al., 1992; Phillips et al., 1993; Kujubu et al., 1993). The promoter region of COX-2 contains a TATA box, a serum-responsive element, an AP-1 site, an NF-κB site, and several SP-1 and SP-2 sites (Xie et al., 1993). Thus, COX-2 is inducible by serum (O'Banion et al., 1991, 1992; Kujubu et al., 1993), IL-1β (O'Banion et al., 1992), lipopolysaccharide (Phillips et al., 1993), and phorbol esters (Kujubu & Herschman, 1992; Kujubu et al., 1991). These agents stimulate eicosanoid production acutely (within 1.0 h) by activating cellular phospholipases and by up-regulating transcription and translation of COX-2 over a 5–24-h period (Habenicht et al., 1985; Goerig et al., 1988; Breviairio et al., 1990). COX-2 mRNA is also rapidly degraded due to the presence of multiple AUUUA repeats in its 3′-untranslated end (O'Banion et al., 1992). Conversely, COX-2 expression is down-regulated by glucocorticoids, but the mechanism of this effect is obscure and may involve transcriptional (O'Banion et al., 1991) or posttranscriptional mechanisms (Xie et al., 1993).

In an initial series of experiments, we first examined the effects of cholesterol enrichment on serum-induced generation of COX-2 activity, following peroxide-mediated inactivation of existing COX-2 (Egan et al., 1976). Cholesterol-enriched cells not only possessed less COX activity than untreated cells (Figure 5A) but were also hyporesponsive to the inductive effects of serum on COX-2 synthesis (Figure 5B), supporting the hypothesis that cholesterol enrichment inhibited serum-induced synthesis of COX-2 enzyme. In addition, cholesterol enrichment inhibited IL-1β-, PDGF-, and TNF-α-induced PGI_2 generation (Figure 6 and Tables I and II). Time course studies revealed that cholesterol enrichment reduced both the acute and delayed elevation of PGI_2 synthesis in response to IL-1β (Table II), providing further supporting evidence that cholesterol enrichment inhibited the induction of COX-2 protein synthesis in addition to the inhibition of phospholipase A_2 and COX-1.

Importantly, COX-2 mRNA steady-state levels (Figure 7) and protein levels (especially the 73-kDa form) (Figures 9 and 10) are also decreased in cholesterol-enriched cells following exposure to PDGF. The significance of the latter observation is unclear. The 73-kDa COX-2 isoform is believed to be glycosylated, with the 71-kDa form believed to be a deglycosylated enzyme. Our data suggest that cholesterol enrichment preferentially reduces the synthesis of the 73-kDa form. Since cholesterol enrichment reduces cytokine/growth factor-induced eicosanoid production (Figure 6 and Tables I and II) commensurate with reducing PDGF-induced COX-2 mRNA steady-state levels (Figure 7) and protein synthesis (Figures 9 and 10), these data support the concept that cholesterol enrichment interferes with COX-2 gene expression. The mechanism(s) by which cholesterol enrichment interferes with COX-2 expression is(are) not known.

We also demonstrated that the mass of PGI_2 synthase is reduced following cholesterol enrichment (Figure 11). This observation has two important implications from the point of view of altered eicosanoid generation. First, these data suggest that cholesterol enrichment has coordinate effects in inhibiting the enzymes responsible for PGI_2 generation. Secondly, we may be overestimating the degree of COX reduction by using a radio-immunoassay of 6-keto-$PGF_{1\alpha}$ as an index of COX activity. However, conversion of arachidonic acid to PGE_2 and PGI_2 was reduced to a similar degree in microsomes, suggesting the quantitative importance of COX vs PGI_2 synthase in the global reduction in eicosanoid synthetic capacity following cholesterol enrichment. In addition, PGI_2 synthase is present in significantly higher amounts that COX in smooth muscle cells and may not be a limiting step in generating PGI_2.

The effects of cholesterol enrichment on eicosanoid generation are variable. In six separate experiments, the range of inhibition of arachidonate-induced PGI_2 release is from approximately 50% to nearly 90%. This variability was also noted in our ELISA measurements and is due to the following: (i) differences in the cell populations used, as cells were derived from different animals (rabbit and rat smooth muscle cells were used in this study, and rat smooth muscle cells generally elaborate more COX products than rabbit smooth muscle cells); (ii) differences in the age of the cultures [PGI_2 synthesis decreases with age [this paper and Ager et al. (1982)], and cells from each species were used until passage 9 (rabbit) or passage 18 (rat) since smooth muscle cells undergo senescence at these approximate passages]; (iii) intrinsic differences in each cLDL preparation, which were derived from pooled anonymous donor plasma; (iv) differences in the degree of cholesterol enrichment between experiments, which ranged from 6 to 30-fold; and (v) differences in the ratio of COX product formation. While smooth muscle cells elaborate mainly PGI_2 and PGE_2, the PGI_2/PGE_2 ratio is variable. *However, cholesterol enrichment reduced eicosanoid release independent of these variables.*

It is also unlikely that oxidation mediates cLDL-induced reduction in eicosanoid release for the following reasons. (i) oxLDL does not promote cholesterol enrichment (Kraemer et al., 1993) due to its toxicity (Hessler et al., 1979; Morel et al., 1983) or lack of internalization through a scavenger receptor (Parthasarathy et al., 1986). (ii) Although LDL autooxidizes under serum-free conditions (Esterbauer et al., 1987; Parthasarathy, 1987), it is protected from oxidation by apo-A1 or apo-A1-containing serum (Parthasarathy et al., 1990; Ohta et al., 1989), which are the conditions we use to cholesterol-enrich cells. (iii) Native LDL in medium plus

fetal bovine serum over a 7-day period *did not* promote cholesterol enrichment or alter PGI_2 generation (Pomerantz & Hajjar, 1989).

Finally, the effect of cholesterol enrichment on the reduction of COX function is specific since we have previously shown that cholesterol enrichment stimulated the inducible form of nitric oxide synthase (Pomerantz et al., 1993) but did not alter lysosomal β-galactosidase (Hajjar et al., 1989).

In summary, the results of our experiments suggest that the defect in PGI_2 synthetic capacity is due in part to the inhibition of the expression of COX and PGI_2 synthase proteins, as well as to the documented reduction in substrate availability (due to linoleic acid competition) and inhibition of phospholipase A_2 activity by cholesterol (Pomerantz & Hajjar, 1989). Alterations in these processes may explain the reduced eicosanoid synthetic capacity in atherosclerotic vessels and provide evidence to support the role for PGI_2 in mediating intracellular cholesterol trafficking (Hajjar et al., 1987; Pomerantz & Hajjar, 1990).

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