Eicosanoid metabolism in cholesterol-enriched arterial smooth muscle cells: reduced arachidonate release with concomitant decrease in cyclooxygenase products

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Abstract A biochemical correlation between vascular cholesterol metabolism and eicosanoid biosynthesis has not been fully elucidated. To assess the effects of cholesteryl ester (CE) accretion on eicosanoid synthesis, we studied eicosanoid metabolism in cultured rabbit aortic smooth muscle cells (SMC) following lipid-enrichment by incubation with cationized LDL (cLDL). SMC exposed to cLDL synthesized 50% less immunoreactive 6-keto-$PGF_{1\alpha}$ than untreated cells when exposed to the calcium ionophore, A-23187. In addition, cLDL-treatment reduced arachidonate acid (AA)-induced prostacyclin (PGI_2) production sevenfold. Components of cLDL decreased eicosanoid biosynthesis in the following rank-order: linoleate > cholesterol > apo-cLDL. Lipid-enriched cells incorporated amounts of [1-^{14}C]AA into phosphatidylcholine and phosphatidylethanolamine equal to control cells, but subsequent exposure to ionophore released significantly less radioactivity as free arachidonate (AA), with proportionally less conversion to eicosanoids. Ionophore released equivalent amounts of AA from all phospholipids, suggesting specificity for uptake, but not release of AA by cellular phospholipases. Cells enriched in CE had an eightfold decrease in percentage of phospholipid-derived AA relative to linoleate as compared to controls. Taken together, our data demonstrate that SMC metabolism of cLDL leads to cholesterol and CE accretion concomitant with diminished production of eicosanoids. Potential mechanisms for this effect include competitive inhibition of eiconsanoid production by linoleate derived from LDL, direct inhibition of phospholipase A_2 activity by cholesterol, and decrease in cyclooxygenase activity. These findings may have pathophysiological significance in that a reduction in PGI_2 synthetic capacity of arterial SMC may exacerbate CE deposition since PGI_2 promotes intracellular CE hydrolysis. —**Pomerantz, K. B., and D. P. Hajjar.** Eicosanoid metabolism in cholesterol-enriched arterial smooth muscle cells: reduced arachidonate release with concomitant decrease in cyclooxygenase products. *J. Lipid Res.* 1989. **30:** 1219–1231.

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Relationships between alterations in vascular eicosanoid biosynthesis and cholesteryl ester (CE) metabolism in atherosclerosis remain undefined. Such metabolic interactions are suggested by the observation that aspirin contributes to the reduction in the incidence and severity of a second myocardial infarction in those who have suffered previous myocardial damage (1). A reduction of vascular prostacyclin (PGI_2) production may play a role in these events. Synthesis of PGI_2, the major eicosanoid produced by arterial tissue, is reduced in human and rabbit atherosclerotic blood vessels (2–5). Moreover, cultured smooth muscle cells (SMC) and endothelial cells derived from human atherosclerotic aorta demonstrate reduced capacity for PGI_2 and PGE_2 synthesis from either exogenous or endogenous arachidonic acid (AA) (6, 7). While increased vascular PGI_2 production has also been reported in human and animal atherosclerosis (8, 9), these observed differences may be due to a time-dependent effect of hypercholesterolemia on PGI_2 production, where the onset of cholesterol-feeding initially increases, then progressively decreases, vascular PGI_2 production ex vivo (10).

The mechanisms by which atherogenic stimuli alter vascular PGI_2 production are also not understood. Eicosanoid metabolism is a function of substrate availability (11, 12) as well as activities of the arachidonoyl-CoA-lysophospholipid acyltransferases and eicosanoid synthetic enzymes (13, 14). Although lipid peroxides present in plasma LDL may inhibit PGI_2 synthetase (15), this

Abbreviations: CE, cholesteryl ester; cLDL, cationized LDL; PGI_2, prostacyclin; HETE, hydroxyeicosatetraenoic acid; SMC, smooth muscle cells; TBS-G, Tris-buffered saline-gelatin; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; ACAT, acyl-CoA:cholesterol acyltransferase.

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mechanism may not be the only mechanism by which LDL inhibits eicosanoid generation since SMC cultured from atherosclerotic vessels retain their deficit in PGI_2 synthetic capacity (7). This hypothesis is supported by ex vivo studies in which hypercholesterolemia resulted in retarded endothelial cell regeneration of PGI_2 synthetic capacity after injury (16).

In this report, we have assessed the metabolic effects of intracellular CE accumulation on eicosanoid metabolism of arterial SMC. Cells were initially exposed to cationized LDL which increased the level of intracellular CE 10- to 12-fold more than SMC exposed to media containing normolipemic serum or media containing unmodified, native LDL (17–19). Such CE-enriched cells morphologically (17) and biochemically (18, 19) resembled foam cells characteristic of atherosclerotic lesions. Mechanistic studies reported herein describe the effects of CE enrichment on eicosanoid synthesis in arterial SMC.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM), penicillin–streptomycin, and Fungizone (amphotericin B) were from Gibco (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT). N,N-dimethyl-1,3-propanediamine was from Kodak Chemicals (Rochester, NY); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl was from Aldrich Chemicals (Milwaukee, WI). [1-^{14}C]Arachidonic acid (AA) (40–60 mCi/mmol), [^{3}H]AA ([5,6,8,9,11,12,14,15-^{3}H(N)-], 87.4 Ci/mmol), [^{3}H]6-keto-$PGF_{1\alpha}$ (115 Ci/mmol), [^{3}H]PGE_2 (169.5 Ci/mmol), [^{3}H]$PGF_{2\alpha}$(195 Ci/mmol), [^{3}H]15-HETE (183.4 Ci/mmol), [^{3}H]12-HETE (225 Ci/mmol), [^{3}H]5-HETE (152.9 Ci/mmol), [^{3}H]LTB_4 (32 Ci/mmol), [1-^{14}C]oleic acid (56 mCi/mol), and [^{3}H]cholesterol (72 Ci/mmol) were from New England Nuclear (Boston, MA). Albumin was from Sigma Chemicals (St. Louis, MO). Phospholipid standards were obtained from Avanti Polar Lipids (Birmingham, AL). Neutral lipids and AA were obtained from Nu-Chek-Prep (Elysian, MN). Eicosanoid standards were from Cayman Chemicals (Kalamazoo, MI). All organic solvents (HPLC grade) and other biochemicals (reagent grade) were obtained from Fisher Scientific (Springfield, NJ).

Preparation of LDL, cLDL, and apocLDL

Plasma low density lipoproteins (LDL) were isolated by preparative ultracentrifugation of pooled donor plasma in a Beckman 55.2 rotor at densities between 1.019 and 1.063 g/ml using NaBr to adjust density (20). Cationized LDL (cLDL) was prepared from LDL by covalent attachment of N,N-dimethyl-1,3-propanediamine to aspartate and glutamate residues of native LDL using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl as catalyst (17) at pH 6.5. Demonstration of successful derivitization of LDL to cLDL was accomplished by differential migration of native and cLDL at pH 8.6 by agarose gel electrophoresis. Lipoproteins were dialyzed against 12 l of 0.154 M NaCl, 0.05 M EDTA, 10 mM HEPES (pH 7.4) for 48 h prior to concentration against polyethylene glycol or Aquacide. To prepare apocLDL, cLDL was lyophilized prior to lipid extraction with ethanol–diethyl ether 3:1 at 4°C. ApocLDL was solubilized in 5.0 M guanidine HCl followed by dialysis against phosphate-buffered saline. Lipoproteins were added to tissue culture media just prior to initiation of experiments.

Isolation and culture of rabbit aortic smooth muscle cells

SMC were isolated and cultured as previously described (21). All experiments were performed on cells between passages 3 and 7. To induce CE accumulation in SMC, confluent cultures in 25-cm^2 flasks were incubated with cLDL (100 μg/ml) in 2 ml of DMEM containing 10% fetal bovine serum for 7 days. Fresh media containing cLDL was added every 3 days. In selected control experiments, cells were also exposed to free cholesterol (22) and Na linoleate (23).

Morphological studies

For determination of overall localization of lipid in SMC cultures, cells were stained with Oil Red O (24). For ultrastructural studies, cells were washed in PBS, fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer. Cells were then dehydrated using ethanol, and embedded in situ using Epon 812 containing dodecenyl succinic anhydride, nadic methyl anhydride, and DMP-30 at 60°C for 48 h. Thin (50 nm) sections were prepared, mounted on 100-mesh grids, stained with 7.5% uranyl acetate, and post-stained with Reynold's lead citrate. Sections were then examined using a JEM-100CX transmission electron microscope (25).

Quantitative lipid assays

The major stable hydrolytic product of PGI_2, 6-keto-$PGF_{1\alpha}$, was quantitated by radioimmunoassay (21). The antibody was used in a 1:8000 dilution in order to achieve approximately 40–50% binding in the absence of unlabeled ligand. Cross-reactivity of the antibody with other eicosanoids was less than 0.1% for PGE_1, PGE_2, PGA_1, $PGF_{1\alpha}$, 13,14-dihydro-15-keto-PGE_1, 6-keto-PGE_1, thromboxane A_2, and AA. Each sample (0.1 ml in duplicate without extraction) received 0.1 ml of Tris-buffered saline–gelatin (TBS-G, 0.01 M Tris, 0.154 M NaCl, 0.1% gelatin, pH 7.4), containing [^{3}H]6-keto-$PGF_{1\alpha}$ (6000

cpm/tube), and 0.1 ml TBS-G containing anti-6-keto-$PGF_{1\alpha}$ antibody. After overnight incubation at 4°C, the bound radioactivity was separated from free ligand by addition of 1.0 ml of TBS-G containing dextran-coated charcoal (25 mg Dextran T-70 per 250 mg Norit A activated charcoal per 100 ml TBS-G), and allowed to stand for 20 min at 4°C, followed by centrifugation at 4°C. The radioactivity in the supernatant (bound ligand) was then quantified. Binding of the unknowns was compared to concurrently derived standard curves calculated as a function of log cpm toward known amounts of 6-keto-$PGF_{1\alpha}$(6.2–1000 pg/0.1 ml). The data were expressed as ng/ml or ng/mg cell protein.

For studies of eicosanoid synthesis in SMC, cells were labeled with 2.0 μCi of [1-^{14}C]AA (21). Post-culture media from these incubations were subjected to acidic ethyl acetate extraction followed by development on Silica Gel G thin-layer chromatography (TLC) plates in the organic phase of ethyl acetate–isooctane–acetic acid–water 110:50:20:100 (v/v/v/v) after 30 min of humidification. Samples were also measured by high performance liquid chromatography (HPLC). The eicosanoid extraction procedure in these studies was that of Powell (26). Post-culture media of arterial SMC prelabeled with [^{3}H]AA and subsequently exposed to agonists was diluted to 10% methanol, and acidified to pH 3.5. [^{3}H]LTB_4 was used to monitor recovery since preliminary experiments revealed that SMC did not synthesize detectable quantities of this eicosanoid or other di-HETEs. Samples were then subjected to solid phase extraction employing octadecylsilane-derivitized silica (Analtech, Newark, DE). After application of the sample, the columns were sequentially washed with 10% methanol, water, and petroleum ether. HETEs and AA were then eluted using petroleum ether–chloroform 65:35 (v/v); remaining eicosanoids were eluted with 100% methanol. Samples were evaporated under nitrogen, reconstituted in acetonitrile, and stored at −20°C. Immediately prior to analysis, samples were reconstituted into methanol–water 30:70 (v/v). Recovery of prostaglandins and HETEs was typically greater than 95%, while recovery of AA was 75–80%.

The HPLC chromatographic separation of eicosanoids was performed as described by Henke, Louzan, and Eling (27), with a Waters HPLC System. Eicosanoids were separated using a 3.5 × 250 mm analytical column (C-18 microsorb reverse-phase silica, 50 μm particle size, Rainin Instrument Co., Woburn, MA) maintained at 25°C. The column remained equilibrated in 55% methanol between sample runs. A step gradient to separate eicosanoids was utilized as follows: 55% methanol to 28 min, 66% methanol to 54 min, 77% methanol to 72 min, and 100% methanol to 100 min at a solvent flow rate of 1.0 ml/min; radioactivity of the column effluent was assessed using a Radiomatic Flow-One Model HS in-line liquid scintillation spectrometer using Flow-Scint II; data were corrected for recovery, and expressed as dpm of each eicosanoid/mg of cell protein.

Lipoprotein lipids were extracted by the method of Bligh and Dyer (28). Lipids from SMC were extracted by the method of Hara and Radin (29). The cholesterol and CE content of LDL and SMC were measured by gas–liquid chromatography (GLC), 3% OV-17 on 100–120 mesh WHP, Supelco) using β-sitosterol as an internal standard (30). Lipoprotein and cellular lipid classes were separated by TLC on silica gel 60 (E. Merck, Co.) by sequential development in diethyl ether–acetic acid–petroleum ether 100:3:97 (v/v/v), and diethyl ether–petroleum ether 3:97 (v/v) (31). For fatty acid analyses, relevant lipid species were treated with 14% BF_3 in methanol to form fatty acid methyl esters. The methyl esters were separated and quantitated by GLC using 10% Silar CP on Gas-Chrom-Q (100–120 mesh, Supelco). Diheineicosanoyl phosphatidylcholine was used as an internal standard (21). Phospholipids were separated into their individual components by TLC (preadsorbent high-efficiency silica gel type HLF [Analtech]) by development of an aliquot of the total cell lipid extract in denatured alcohol–chloroform–ammonium hydroxide 50:15:6 (v/v/v) (32).

Acyl CoA:cholesterol acyltransferase (ACAT) activity. Cholesteryl ester synthetic activity was performed as described by Hajjar et al. (33) and Fabricant et al. (34). Cellular homogenates were assayed by measuring the synthesis of CE from [1-^{14}C]oleoyl CoA using exogenous free cholesterol incorporated into unilamellar liposomes containing egg phosphatidylcholine. The ACAT assay consisted of enzyme (150 μg protein of cell homogenate), 12.5 μM oleoyl CoA, 12.5 μM exogenous cholesterol, 1.0 mM egg phosphatidylcholine in 74 mM Tris HCl buffer (pH 7.4), 5 mM mercaptoethanol, and 0.025% bovine serum albumin (33, 34). Isotonic sucrose buffer with substrate but without homogenate was used as control. After 60 min at 37°C, the incubation was stopped with 5.0 ml of chloroform–methanol 2:1 (v/v) containing 50 μg of unlabeled cholesteryl oleate as a carrier. The lipids were extracted by the method of Folch, Lees, and Sloane Stanley (35), separated by thin-layer chromatography, and quantitated by liquid scintillation counting. Data were expressed as pmol CE formed/h per mg protein.

Esterification of [^{3}H]oleic acid into cellular CE. Confluent SMC cultures in 35-mm wells were allowed to incorporate a [^{3}H]oleic acid–albumin mixture (final concentration of 100 μM oleate–20 μM albumin in the absence of serum) for 24 h at 37°C (36). Cells were lipid-extracted (29), and SMC lipids were subsequently analyzed for distribution of radioactivity in cellular lipids by TLC (24).

Miscellaneous assays

Lipoprotein- and SMC-protein content were determined by the method of Lowry et al. (37). Cellular DNA was quantitated by modifications of the methods of

Cesarone, Bolognesi, and Santi (38). Cell viability was assessed by in situ staining with trypan blue. Cell number was determined either by hemocytometer or by automated counting using a Coulter counter.

Statistical analyses

Results of these experiments were analyzed by either Student's *t* test or by analysis of variance. Differences between treatments were tested using the Newman-Keuls test.

RESULTS

Characterization of CE-enriched cells

Effect of CE enrichment on morphology and fine structure. Early passage aortic SMC exposed to cLDL accumulate a variable but significant quantity of Oil Red O-positive material (**Fig. 1**, panel A). The distribution of Oil Red O positivity was localized to the cytoplasm. Importantly, there was no Oil Red O staining of the cell surface or the exposed plastic. Cells also lost their stellate configuration, and developed a more rounded appearance (Fig. 1, panel A). Untreated SMC did not stain with Oil Red O, and maintained their typical hill-and-valley configuration (Fig. 1, panel B). Transmission electron microscopic analysis confirmed the intracellular localization of the cell-associated lipid. SMC exposed to cLDL demonstrated an expanded lysosomal compartment containing numerous lipid inclusions. While most of the lipid inclusions in lipid-loaded SMC were membrane-delimited, nonmembrane bound intracellular lipid inclusions were also present. No plasma membrane-associated lipid was observed (panel C). These ultrastructural alterations were not apparent in untreated SMC (panel D).

Effect of CE enrichment on cellular lipid metabolism. Cell lipid analysis of SMC exposed to lipoprotein-deficient serum (LPDS) or LPDS containing native LDL revealed that LPDS and LDL did not alter either the cholesterol or CE content relative to cells maintained in DME containing 10% fetal bovine serum. However, SMC exposed to medium containing cLDL resulted in a nearly twofold increase of free cholesterol, and a sevenfold increase in CE (**Fig. 2**). The fatty acid profile of CE and phospholipids (**Table 1**) in normal and CE-enriched SMC revealed that the fatty acid compositions in CE-enriched SMC resembled those of the cLDL particle and not that of the normal SMC. The principal change observed in cellular CE was a dramatic increase in linoleic acid at the expense of stearic and palmitic acids. The percentage of arachidonic acid in cellular CE was unchanged as a result of CE enrichment. This shift is due principally to the increase in the mass of total cellular CE-fatty acids (13.3 μg/mg protein in normal SMC vs. 106.7 μg/mg protein in CE-enriched cells). The profile of cellular and lipoprotein phospholipid fatty acids shows that cells exposed to cLDL underwent a significant increase in percentage of linoleate relative to controls and a corresponding decrease in the percentage of oleate. The percentage of other fatty acids, including AA, was unaltered in CE-enriched SMC (Table 1B).

The influence of cholesterol enrichment on the synthesis of nascent CE was evaluated by quantitating the esterification of isotopic oleic acid into CE by cells exposed to cLDL or LDL. In addition, the intrinsic activity of acyl CoA:cholesterol acyltransferase (ACAT, the enzyme that catalyzes the esterification of fatty acids to cholesterol) was also evaluated. CE-enriched cells demonstrated over a fourfold ($P<0.05$) increase in the synthesis of CE (**Table 2**) relative to control cells. Native LDL did not increase the esterification of oleate into CE over that synthesized by control cells (data not shown). However, the intrinsic activity of ACAT was not altered relative to control cells (Table 2).

Effect of CE enrichment on cell viability and proliferation. Cells grown in 35-mm wells were quantified at confluent density, and then exposed to DME containing 10% FBS in the presence or absence of cLDL (100 μg/ml) for 4 days. After this time untreated cells continued to proliferate ($6.8 \pm 0.3 \times 10^5$ cells/well on day 4 vs. $4.6 \pm 0.5 \times 10^5$/well on day 0, mean ± SEM, $P<0.05$). Exposure of SMC to cLDL did not alter cell number after the 4-day interval ($6.4 \pm 0.4 \times 10^5$/well, P = ns). Greater than 96% of control SMC and SMC exposed to cLDL after 4 days remained viable (data not shown). The effects of cLDL on cellular protein and DNA content were also evaluated. After 7 days, the protein and DNA content of cells exposed to cLDL was compared to content in untreated cells. Exposure of SMC to cLDL increased cellular protein (38.8 ± 3.0 μg/cm² vs. 26.8 ± 2.3 μg/cm², n = 3, $P<0.05$), and DNA (1.25 ± 0.01 μg/cm² vs. 0.96 ± 0.01 μg/cm², n = 3, $P<0.05$) content relative to normal cells. However, CE enrichment did not alter the cellular protein/DNA ratio (31.0 ± 2.7 vs. 29.9 ± 2.5, n = 3, P = ns).

Effect of CE enrichment on eicosanoid metabolism

Effect of cLDL on eicosanoid biosynthesis. To determine whether CE enrichment altered PGI_2 synthetic capacity, normal and CE-enriched SMC were exposed to A-23187 (a nonspecific agonist that releases endogenous AA from cell membrane phospholipids via activation of cellular phospholipases). Supernatants were assayed for immunoreactive 6-keto-$PGF_{1\alpha}$. CE enrichment significantly reduced production of PGI_2 by SMC following exposure to A-23187. The basal production of 6-keto-$PGF_{1\alpha}$ of CE-enriched cells was not significantly different from untreated cells (**Table 3**).

Effect of cLDL constituents on eicosanoid biosynthesis. To determine which moiety of cLDL reduced eicosanoid syn-

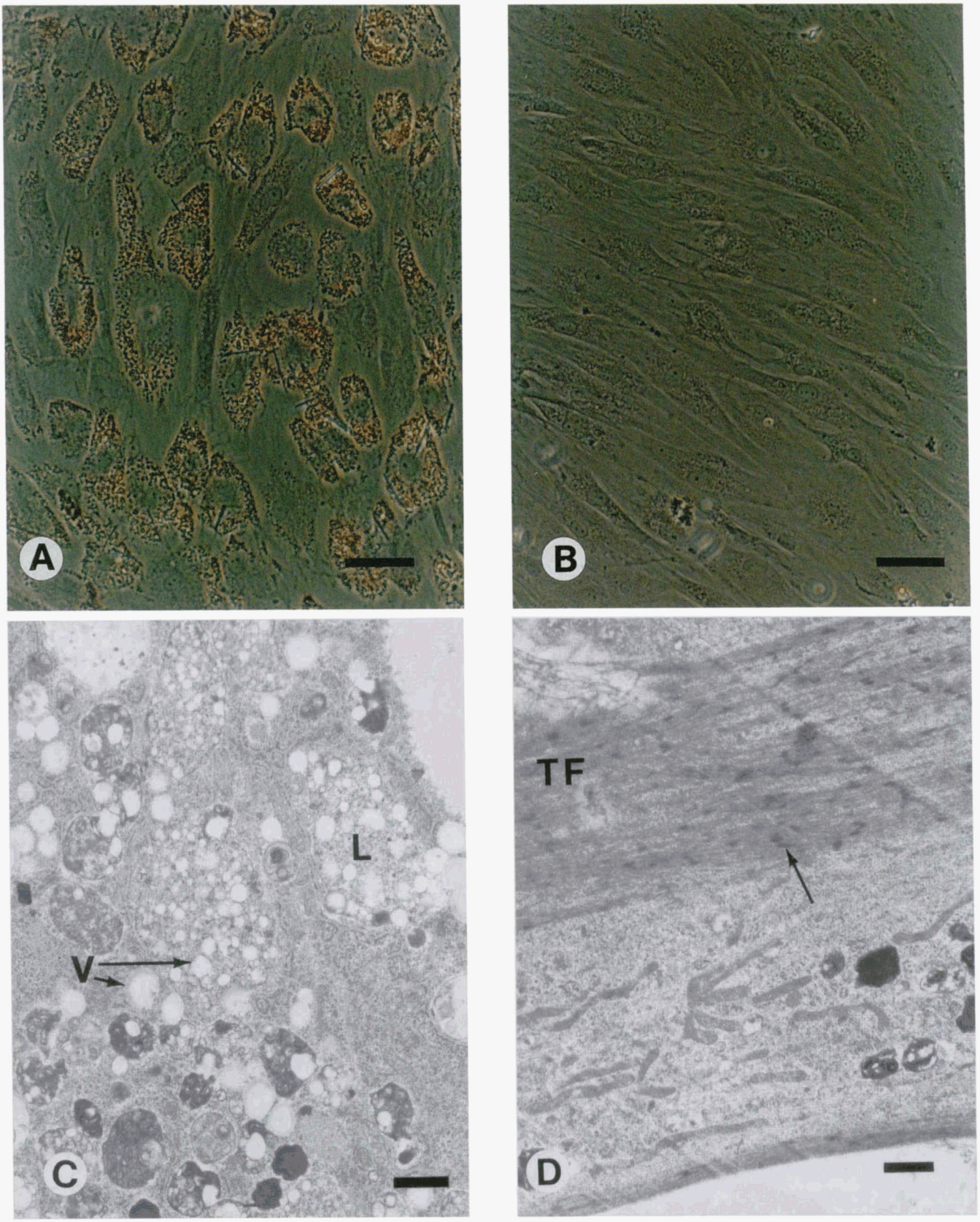

Fig. 1. Effect of cLDI on cell morphology. Phase-contrast photomicrographs of cells stained with Oil Red O: panel A, cells exposed to cationized LDL, 100μg protein/ml for 7 days; panel B, untreated SMC (260 ×, bar = 50μm). Transmission electron micrographs demonstrating ultrastructural localization and morphology of CE: panel C, CE-enriched SMC (10,100 ×); panel D, normal cells (9,6000 ×). Vacuoles (V) are restricted to the intracellular compartment, and are localized in both lysosomes (L) and cytoplasm. Thick and thin filaments (TF) as well as dense bodies (arrow) are also present; bar = 1 μm.

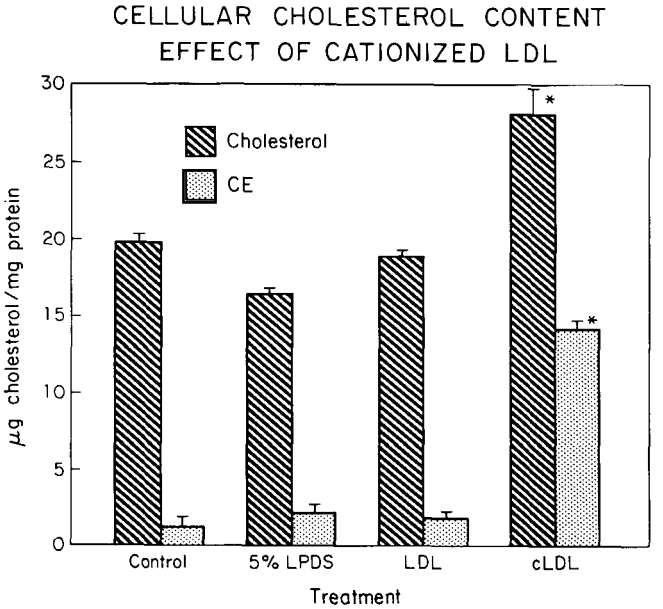

Fig. 2. *Effect of native and cLDL on cholesterol and CE content of SMC.* Cells were exposed to DMEM containing 10% fetal bovine serum. Lipoprotein-deficient serum (LPDS), or LPDS containing LDL or cLDL for 48 h. Cellular cholesterol and CE (μg/mg cell protein) were then determined in duplicate for each group (mean ± SEM*, $P<0.05$). Free and esterified cholesterol accumulated after exposure to cLDL, but not in the cells exposed to media containing fetal bovine serum, LPDS alone, or LPDS in the presence of native LDL.

thetic capacity, cells were exposed for 1 week to media alone or media containing cLDL, apo-cLDL (the delipidated cLDL apoproteins), free cholesterol, or Na linoleate (the principal unsaturated fatty acid in LDL phospholipids and CE). Prior to exposure to A-23187, parallel cultures from each treatment group were labeled with [^{3}H]AA. Supernatants from unlabeled cells exposed to A-23187 were examined for immunoreactive 6-keto-$PGF_{1\alpha}$, while eicosanoids in the supernatants from labeled cells exposed to A-23187 were quantitated by TLC. Cells exposed to cLDL and Na linoleate demonstrated reduced capacity to synthesize eicosanoids; the apo-cLDL and cholesterol did not alter ionophore-induced immunoreactive 6-keto-$PGF_{1\alpha}$ (**Table 4**). All treatments reduced eicosanoid release relative to control cells by SMC labeled with [^{3}H]AA.

Mechanisms of reduced eicosanoid biosynthesis by CE-enriched SMC

Effect of CE enrichment on AA incorporation and distribution into cell lipids and eicosanoids. Using [1-^{14}C]AA, we evaluated the influence of CE enrichment on cellular AA metabolism. Normal and CE-enriched SMC labeled with [1-^{14}C]AA were exposed to media in the absence or presence of A-23187. Normal and CE-enriched cells incorporated similar quantities of [1-^{14}C]AA into total cellular lipids

TABLE 1. Effect of cLDL on CE and phospholipid fatty acid profile of smooth muscle cells

Fatty Acid	Controls	CE-Enriched Cells	cLDL
CE fatty acids			
16:0	27.8	17.9[a]	15.2
16:1	0.9	2.4	2.5
18:0	18.9	3.2[a]	2.0
18:1	20.9	21.6	22.0
18:2	27.7	48.7[a]	51.7
20:4	3.6	4.3	5.8
Phospholipid fatty acids			
16:0	28.6	30.5	34.8
16:1	2.4	1.2	2.5
18:0	16.7	17.7	19.3
18:1	30.9	18.9[a]	14.5
18:2	1.7	14.9[a]	22.7
20:4	17.9	15.6	8.0

SMC were exposed to media ± cLDL (100 μg/ml) for 1 week. Cells were then extracted in situ. CE and phospholipid fatty acids were determined by GLC (see Methods). Data are expressed as the mean percentage of total fatty acid methyl ester, n = 2. Each sample was measured in duplicate.

[a] $P < 0.05$.

(87.4 ± 6.8% vs. 84.5 ± 7.5% of total dpm, n = 5). Examination of the distribution of the isotope in cell lipid classes showed that AA was incorporated into cellular phospholipids and triglycerides with similar efficiency in both normal and lipid-enriched SMC (**Table 5**). In addition, there was a significant increment of [1-^{14}C]AA in CE in lipid-enriched cells relative to normal cells. When SMC were subsequently exposed to A-23187, there was a reduction in radioactivity in phospholipids with a concomitant increase of radioactivity in free fatty acid. However, the magnitude of these changes was significantly less in the CE-enriched cells (Table 5). The content of AA and cyclooxygenase products in the supernatants of these cells was evaluated by TLC. Control and CE-enriched SMC released equal basal quantities of AA, PGI_2, and PGE_2 into the media (**Table 6**). However, after exposure to A-23187, normal cells produced significantly

TABLE 2. Effect of lipid enrichment on the rate of CE synthesis and activity of acyl-CoA:cholesterol acyltransferase

Treatment	CE Synthesis[a]	ACAT[b]
Control	0.39 ± 0.02[c]	1.1 ± 0.4
cLDL	1.67 ± 0.38[d]	1.1 ± 0.2

SMC were exposed to media ± cLDL (100 μg/ml) for 1 week. CE synthesis was determined by the rate of incorporation of [^{3}H]oleate-albumin into cellular CE. The activity of ACAT was measured in sucrose homogenates as described in Methods.

[a] Data are expressed as nmol of cholesteryl oleate formed per mg cell protein per 24 h. This is representative of two experiments.

[b] Data are expressed as pmol of cholesteryl oleate formed per mg cell protein per h. This is representative of four experiments.

[c] Mean ± SEM of triplicate samples.

[d] $P < 0.05$.

TABLE 3. Effect of CE enrichment on PGI_2 release from aortic smooth muscle cells: effect of A-23187

Treatment	Control	A-23187
Normal cells	0.2 ± 0.1[a]	32.4 ± 4.0[b]
CE-enriched cells	2.3 ± 1.8	6.4 ± 0.5[b,c]

SMC exposed to media ± cLDL (100 μg/ml) for 1 week were then exposed to media with or without A-23187 (5.0 μM, 30 min). 6-Keto-$PGF_{1\alpha}$ was measured in supernatants by radioimmunoassay. These data are representative of three different experiments.

[a]Mean ± SEM of triplicate samples; ng/mg protein.

[b]$P < 0.01$ control versus A-23187.

[c]$P < 0.05$ normal versus CE-enriched cells.

more [^{14}C]AA, [^{14}C]6-keto-$PGF_{1\alpha}$, and [^{14}C]PGE_2 into the media than did CE-enriched SMC. To address the possibility of differential HETE production induced by CE enrichment, eicosanoids in the supernatants from normal and CE-enriched SMC prelabeled with [^{3}H]AA and subsequently exposed to A-23187 were also analyzed by HPLC. SMC synthesized 6-keto-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$, and 17-hydroxyheptatrienoic acid, and released residual unmetabolized AA in response to A-23187 (**Fig. 3, Table 7**). CE-enriched cells released significantly less AA and synthesized significantly less cyclooxygenase products than did normal cells (Fig. 3B, Table 7). Lipoxygenase products were not detected by normal or CE-enriched cells following ionophore stimulation.

Effect of CE-enrichment on phospholipase A_2 activity. To determine whether CE-enrichment altered phospholipase A_2 activity, normal and CE-enriched cells radiolabeled with [1-^{14}C]AA as described above were exposed to A-23187 in the presence of fatty acid-free bovine serum albumin (39, 40). In the presence of fatty acid-free BSA, significantly greater amounts of AA were liberated from control cells in response to A-23187 than by CE-enriched cells, with concomitant inhibition of the conversion of AA to 6-keto-$PGF_{1\alpha}$ (**Table 8**). Furthermore, it was possible to examine the specificity of incorporation and release in the presence and absence of CE-enrichment. To this end, the cell lipid extract was fractionated by high efficiency thin-layer chromatography to separate and quantify the relative mass and radioactivity in individual phospholipids. The percentage mass distribution of each phospholipid in SMC was as follows: sphingomyelin (SM), 17.9; phosphatidylcholine (PC), 62.6; phosphatidylinositol (PI) and phosphatidylserine (PS), 3.6; and phosphatidylethanolamine (PE), 2.3. The percent distribution of radioactivity in individual phospholipids of control cells was as follows: SM, 1.2 ± 0.2; PC, 52.0 ± 4.5; PI + PS, 11.2 ± 4.7; and PE, 35.5 ± 2.6. The distribution of [1-^{14}C]AA in cellular phospholipids was unchanged with respect to ionophore stimulation or in the presence or absence of CE accumulation (data not shown). The data also demonstrate a disproportionate in corporation of AA into PI + PS and PE, relative to SM and PC.

Effect of CE enrichment on cyclooxygenase activity. To determine whether reduction in eicosanoid synthetic capacity was due to alterations in cyclooxygenase activity, normal and CE-enriched SMC were exposed to exogenous AA (10 μM, for 30 min), which bypasses phospholipase A_2 activation. Supernatants were then assayed for immunoreactive 6-keto-$PGF_{1\alpha}$. After CE enrichment, AA-induced PGI_2 synthesis was reduced over sevenfold (10.8 ± 0.7 ng/mg protein synthesized by control cells vs. 1.5 ± 0.7 ng/mg protein synthesized by CE-enriched cells, $P<0.05$, n = 4). Fatty acid compositional analyses of these cells revealed that these CE-enriched cells had an altered phospholipid fatty acid composition relative to normal cells as described in Table 2 (data not shown).

TABLE 4. Effect of cationized LDL constituents on eicosanoid biosynthesis

Treatment	6-Keto-$PGF_{1\alpha}$	Eicosanoid			
		6-Keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	AA
	ng/mg protein	*dpm × 10^3/mg protein*			
Control	1.38 ± 0.08[a]	57.6 ± 7.4	16.6 ± 2.2	130.4 ± 27.7	353.2 ± 68.2
cLDL	0.63 ± 0.11[b]	12.5 ± 2.7[b]	4.2 ± 0.7[b]	48.6 ± 7.6[b]	60.3 ± 5.3[b]
18:2	0.51 ± 0.06[b]	16.1 ± 5.2[b]	5.6 ± 0.6[b]	21.5 ± 4.6[b]	83.2 ± 13.5[b]
Chol	1.41 ± 0.10	35.1 ± 6.3[b]	11.6 ± 1.8	72.7 ± 11.6[b]	218.3 ± 23.4[b]
Apo-cLDL	1.07 ± 0.02	38.0 ± 2.3[b]	11.2 ± 0.7	77.3 ± 8.5[b]	207.8 ± 27.9[b]

SMC were exposed to media alone (control) or media containing cationized LDL (100 μg/ml), Na linoleate (18:2) (150 μM), cholesterol (Chol) (100 μM), or apo-cLDL (100 μg/ml), for 1 week with one media change. Parallel cultures were labeled with [^{3}H]AA prior to exposure to A-23187 (5.0 μM, 30 min). 6-Keto-$PGF_{1\alpha}$ was quantified by radioimmunoassay on supernatants from unlabeled cells (column 1), while eicosanoids in the supernatants from labeled cells were quantified by TLC. These data are representative of two such experiments.

[a]Mean ± SEM of three replicates per treatment group.

[b]$P < 0.05$ compared to control cells.

TABLE 5. Effect of CE enrichment on [1-^{14}C]AA metabolism: effect of A-23187 on the distribution of [1-^{14}C]AA in cell lipid classes

Treatment	PL ($\times 10^6$)	FFA ($\times 10^3$)	TG ($\times 10^5$)	CE
Control	2.42 ± 0.09[a] (78.2)[b]	9.21 ± 0.54 (0.29)	6.31 ± 0.19 (20.14)	6047 ± 466 (0.18)
A-23187	1.86 ± 0.16[c] (71.5)	89.72 ± 9.06[c] (3.43)	5.63 ± 0.62 (21.54)	3757 ± 702 (0.14)
cLDL	2.25 ± 0.03 (78.3)	9.96 ± 0.62 (0.34)	5.27 ± 0.01 (18.36)	65236 ± 2051[d] (2.27)
cLDL + A-23187	1.91 ± 0.4 (73.6)	40.15 ± 7.46[c,d] (1.55)	5.59 ± 0.29 (21.48)	60598 ± 2865[d] (2.29)

SMC were exposed to media ± cLDL (100 μg/ml) for 7 days. Cells were then exposed to (1-^{14}C]AA for 24 h prior to exposure to media with or without A-23187 (5.0 μM, 30 min). Cells were lipid-extracted and the radioactivity in cellular lipid classes (PL, phospholipid; FFA, free fatty acids; TG, triglycerides; CE, cholesteryl esters) was determined by TLC. These data do not add up to 100% because of minor incorporation into 1,2- and 1,3-diglycerides. This experiment is one of five such experiments.

[a]Values given as dpm/mg cellular protein; mean ± SEM of five replicates per treatment group.

[b]Data in parentheses indicate % of total radioactivity.

[c]$P < 0.05$, A-23187 versus control.

[d]$P < 0.05$, normal cells versus CE-enriched cells.

DISCUSSION

The concept that eicosanoids are implicated in the pathophysiology of atherosclerotic disease is based, in part, on observations that eicosanoid biosynthesis is reduced in this disease (2-5). To identify and characterize mechanisms of altered vascular eicosanoid metabolism during cellular lipid accumulation, we evaluated the effects of CE enrichment on eicosanoid synthetic capacity by arterial SMC in vitro by exposure to cLDL. In preliminary studies, we demonstrated that exposure of SMC to cLDL produces intense CE accumulation as demonstrated by morphologic and biochemical criteria used by others (17). Oil Red O staining and transmission electron microscopic analysis revealed cytoplasmic vacuoles associated with lysosomes and non-membrane-bound components (Fig. 1, panels A and C). Biochemical data revealed that there was a 1.4-fold increase in free cholesterol, an 8-fold increase in CE (whose fatty acid profile of cellular CE was similar to that of cLDL [Fig. 2, Table 1]), and a 4-fold increase in the CE synthesis over controls (Table 2). Taken together, these results demonstrate that SMC incorporated LDL-CE into lysosomes; the CE was partially hydrolyzed and re-esterified to form cytoplasmic CE. In addition to the above findings, Stein, Halperin, and Stein (41) demonstrated that a significant portion of cLDL remained extracellular and bound to the cell membrane after 3 days of exposure to cationized LDL. In our experimental design, we avoided this potential artifact by extending the exposure time from 3 days to 1 week, which provided sufficient time for the cells to internalize the cell membrane-associated cLDL.

We demonstrate that CE-enriched arterial SMC synthesized less eicosanoids than normal SMC. These alterations were not due to changes in cell number or viability. The reduction in eicosanoid synthetic capacity occurred independently of the stimulus as documented using TLC (Tables 4, 6A), HPLC (Table 6B), and immunoassay (Tables 3, 4), and has specificity since the esterification of AA into cellular triglycerides remained unaltered (Table 5). The mechanism by which CE enrichment reduced eicosanoid metabolism was therefore evaluated.

TABLE 6. Effect of CE enrichment on ionophore-induced eicosanoid biosynthesis and release of arachidonic acid

Treatment	6-Keto-$PGF_{1\alpha}$	PGE_2	AA
	dpm/mg cell protein, mean ± SEM		
Control cells	94 ± 1	126 ± 10	1287 ± 226
+ A-23187	4534 ± 444[a,c]	1043 ± 137[a,c]	67678 ± 5669[a,c]
cLDL cells	115 ± 5[b]	120 ± 25[b]	1500 ± 235[b]
+ A-23187	775 ± 397[b,c]	337 ± 50[b,c]	16472 ± 3543[b,c]

Supernatants from experiments depicted in Table 5 were assayed for [1-^{14}C]eicosanoids and [^{14}C]AA by TLC. This experiment is one of five such experiments. In each column, $P < 0.05$ for similar letters; five replicates per treatment.

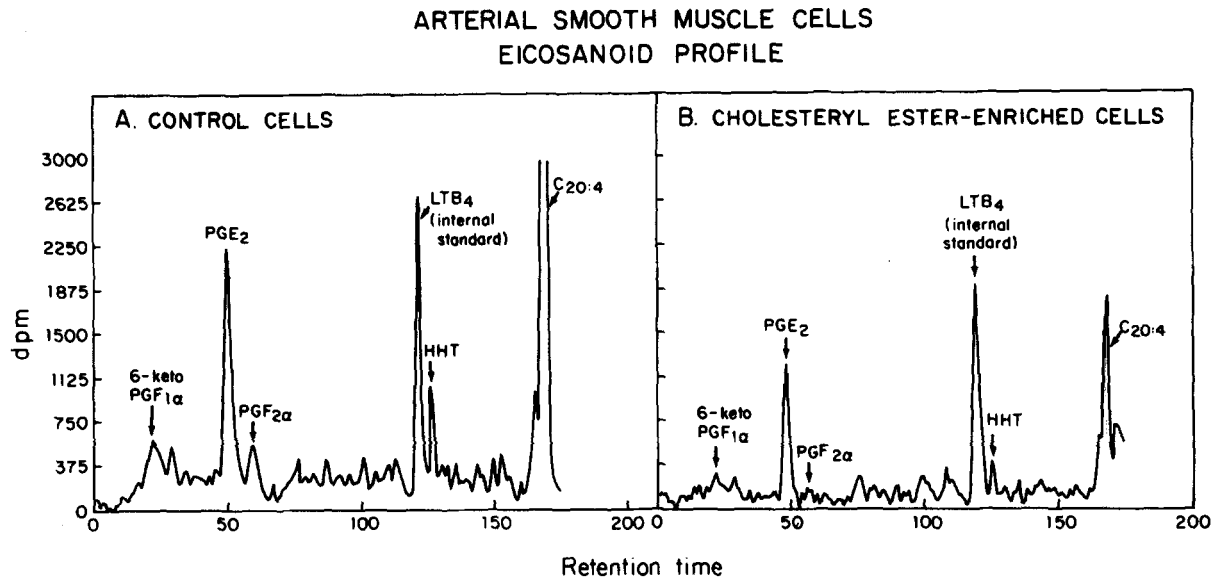

Fig. 3. Effect of CE enrichment on eicosanoid synthetic capacity; representative HPLC profiles of eicosanoid products synthesized from normal (panel A) and CE-enriched (panel B) aortic SMC incubated with [^{3}H]AA prior to washing and exposure to 5.0 μM A-23187. After addition of [^{3}H]leukotriene B_4 to monitor recovery, supernatants were diluted to 10% methanol, acidified to pH 3.5, and extracted on reverse-phase C-18 columns. Eicosanoids were eluted from the column with petroleum ether–chloroform 65:35 and methanol. Samples reconstituted into 30% methanol were analyzed by HPLC as described in Methods. Identity of each eicosanoid was determined by comparing retention times to those of authentic eicosanoid standards. Radioactivity of each eicosanoid was corrected for recovery of added [^{3}H]leukotriene B_4 and normalized to cell protein. Data from two such experiments, each performed in quintuplicate, are summarized in Table 7.

First, AA uptake and distribution into total and individual cellular phospholipids (principally PC and PE) were unimpaired by CE enrichment (Table 5). In addition, A-23187 did not alter the percent distribution of AA in cellular phospholipids. These data indicate that there was preferential incorporation of AA into cellular lipids, but no specificity of AA release. These data also demonstrate that CE enrichment does not selectively alter a specific phospholipid pool that is phospholipase-sensitive and accessible to cyclooxygenase. Secondly, when control and CE-enriched cells that had been prelabeled with [^{14}C]AA were exposed to ionophore, there was reduced [^{14}C]AA release and conversion to PGI_2, PGE_2, $PGF_{2\alpha}$, and HHT (all of which are products of normal SMC (7, 42, 43)) by the CE-enriched cells (Table 7). This was also reflected in a mass decrease of 6-keto-$PGF_{1\alpha}$ after A-23187 stimulation (Tables 3 and 4). Thus, CE enrichment impaired release and subsequent metabolism of arachidonic acid to oxygenated eicosanoids.

To determine whether phospholipase A_2 activity was altered by CE enrichment, we examined the effects of A-23187 on hydrolysis of [^{14}C]AA-labeled cellular phospholipid in the presence of fatty acid-free bovine serum albumin, which acts as a sink for released free fatty acids, thus preventing reincorporation of released AA into cel-

TABLE 7. Effect of CE enrichment on eicosanoid release

Eicosanoid	Control	Cationized LDL[a]
	dpm/mg cell protein, mean ± SEM	
6-Keto-$PGF_{1\alpha}$	10137 ± 639	4169 ± 570
PGE_2	14885 ± 1394	5543 ± 1144
$PGF_{2\alpha}$	4632 ± 348	1553 ± 252
HHT	4517 ± 446	1605 ± 215
AA (× 10^4)	8.37 ± 0.6	0.995 ± 0.008

Normal and CE-enriched SMC incubated in media containing [^{3}H]AA were exposed to A-23187 (5.0 μM, 30 min). Supernatants from cells used in experiments depicted in Fig. 3 were analyzed for eicosanoid content by HPLC. Data are normalized for recovery of [^{3}H]leukotriene B_4 and normalized to cell protein. CE enrichment resulted in a generalized decrease in arachidonate release and conversion to other eicosanoids after exposure to A-23187. Data are from two experiments, each performed in quintuplicate.

[a] $P < 0.05$ for all values for cLDL compared to corresponding control values.

TABLE 8. Effect of CE enrichment on release of arachidonic acid

Treatment	6-Keto-$PGF_{1\alpha}$	AA (× 10^3)
	dpm/mg protein, mean ± SEM	
A-23187 (control)	652 ± 75	22.7 ± 1.5
cLDL + A-23187	453 ± 54	12.1 ± 1.4[a]

SMC, prepared as described in Table 5, were exposed to A-23187 (10 μM) in the presence of fatty acid-free bovine serum albumin (0.2%) for 30 min at 37°C. Eicosanoids were separated by TLC. This is representative of two such experiments, each performed in quintuplicate.

[a] $P < 0.05$ cLDL versus control group.

lular phospholipids as well as blocking conversion into eicosanoids (39, 40). Hydrolysis of labeled phospholipid and, thus, release of [^{14}C]AA induced by the calcium ionophore was significantly reduced by CE-enriched cells (Table 8). These data strongly suggest that inhibition of phospholipase A_2 is one potential mechanism by which CE enrichment reduces eicosanoid synthetic capacity.

Next, the mechanisms by which CE enrichment reduced phospholipase A_2 were evaluated. First, linoleic acid (the principal unsaturated fatty acid in LDL-CE) potently inhibited AA release and eicosanoid biosynthesis (Table 3). Phospholipids of CE-enriched cells were also increased in their percentage of linoleate, at the expense of oleate, with no alterations in AA or other fatty acids, including palmitate and stearate (Table 1), demonstrating specificity of the alterations in fatty acid composition after exposure to cLDL. This reduction in the oleic acid/linoleic acid ratio with no alteration in other fatty acids has been reported in other experimental systems evaluating the effects of dietary (or exogenous) linoleate on cellular phospholipid fatty acid composition (23, 44, 45). These observations are important since linoleic acid is typically esterified in the *sn*-2 position of membrane phospholipids, as is AA (46, 47), and can inhibit eicosanoid generation (Table 4) at the level of cyclooxygenase by competing with arachidonate for conversion to PGH_2 following phospholipase A_2 activation (23, 45, 48). Thus, LDL, which is the principal carrier of linoleic acid in plasma, may be proatherogenic not only by providing cholesterol to cells, but also by providing significant quantities of linoleate. This observation may also have pathophysiologic relevance in vivo since cholesteryl linoleate accumulates in developing atherosclerotic lesions (49, 50). Secondly, cholesterol inhibited eicosanoid production in cells metabolically labeled with [^{3}H]AA (Table 3) to a significantly lesser extent than linoleate. These data are consistent with an earlier report demonstrating that cholesterol can inhibit phospholipase A_2 activity in murine fibroblasts (51) presumably by decreasing enzyme-substrate interactions (52). Thirdly, the apo-cLDL also partially inhibited eicosanoid generation by labeled (but not unlabeled) cells. Although the mechanism of this observation remains speculative, such an effect may be related to the documented ability of apo-cLDL to interact nonspecifically with cell membranes (41), and thereby prevent the interaction of the eicosanoid agonist (A-23187) with its receptor, calmodulin (53). The apparent difference in response to labeled and unlabeled cells to A-23187 by cholesterol- and apo-cLDL-treated SMC may be due to redistribution of [^{3}H]AA into cellular phospholipid compartments that are not accessible to phospholipases (54).

PGI_2 release by CE-enriched cells was also reduced following exposure to exogenous AA, which bypasses phospholipase A_2 activation. These observations suggest that cyclooxygenase activity itself may be reduced following CE enrichment. These data are reinforced by recent preliminary experiments demonstrating that CE enrichment reduces the levels of cyclooxygenase enzyme, as measured by ELISA (data not shown). The mechanism by which cyclooxygenase activity is reduced by CE enrichment is currently under investigation.

In summary, we have proposed that CE enrichment reduces eicosanoid metabolism by two separate mechanisms. First, CE enrichment inhibits AA release from phospholipids (presumably by phospholipase A_2) by competitive inhibition between arachidonate-containing phospholipids and phospholipids containing linoleate (derived from LDL-CE); and noncompetitive inhibition by cholesterol, also derived from LDL-CE (Fig. 3). Secondly, CE enrichment reduces cyclooxygenase activity, possibly by reduction in the synthesis of this enzyme. However, other potential mechanisms may be operative to inhibit PGI_2 generation. One possibility includes the presence of lipid peroxides, which are a constituent of oxidized LDL (55) and hypercholesterolemic (but not normal) plasma (56). These peroxides, which inhibit PGI_2 formation (4), are found in atherosclerotic plaques but not in adjacent normal areas of the blood vessel wall or normal intima (56, 57). It is also possible that CE enrichment may reduce calcium flux (and thus inhibit phospholipase A_2 activity). However, arterial calcium transport is, in fact, elevated in atherosclerotic tissue (58, 59), and calcium channel blockers may have antiatherogenic potential (60–62).

Finally, it has been documented that atherosclerotic vessels displayed significantly reduced capacity for synthesizing PGI_2 (2–7). We believe that these observations are significant since PGI_2 and other eicosanoids have recently been implicated in control of cellular cholesterol content by both autocrine and paracrine mechanisms (63–65). Therefore, reduced PGI_2 synthetic capacity following injury (16, 66, 67) may exacerbate CE deposition owing to the fact that this eicosanoid can normally stimulate cytoplasmic CE hydrolysis in arterial SMC via cyclic AMP-dependent protein kinase (63, 68, 69). Since other studies have shown that the stable PGI_2 analog, carbacyclin, and HDL can reduce triglyceride, cholesterol, and CE content in intimal SMC derived from plaque areas (70), eicosanoid-mediated cholesterol efflux from CE-enriched cells may therefore provide a cellular mechanism by which these biologically active lipids play an active role in regulating intracellular cholesterol content.

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