Prostacyclin Modulates Cholesteryl Ester Hydrolytic Activity by Its Effect on Cyclic Adenosine Monophosphate in Rabbit Aortic Smooth Muscle Cells

DAVID P. HAJJAR, BABETTE B. WEKSLER, DOMENICK J. FALCONE, JOHN M. HEFTON, KAREN TACK-GOLDMAN, and C. RICHARD MINICK,
Departments of Biochemistry, Pathology, and Medicine, New York Hospital-Cornell Medical Center, New York 10021

ABSTRACT We tested the hypothesis that prostacyclin (PGI_2), 6-keto-prostaglandin$F_{1\alpha}$(6-keto-$PGF_{1\alpha}$), and several E series prostaglandins (PG) may affect the activity of cholesteryl ester (CE) hydrolase since our previous experiments indicated that smooth muscle cells (SMC) in neointima of injured rabbit aorta (*a*) acquire the capacity to produce PGI_2 and (*b*) have increased lysosomal CE hydrolytic (acid cholesteryl ester hydrolase [ACEH])activity. Using cultured SMC from rabbit thoracic aorta, we demonstrated that PGI_2, 6-keto-$PGF_{1\alpha}$, and 6-keto-PGE_1 enhanced ACEH activity fourfold. No significant effects on ACEH activity were observed with PGE_1 or PGE_2. Preincubation of SMC with an inhibitor of adenylate cyclase activity (dideoxyadenosine) abolished the effect of these PG on CE hydrolytic activity. Addition of dibutyryl cAMP to these SMC significantly increased ACEH activity. Although concentrations of PGI_2 used significantly increased cAMP levels, proliferation of these SMC was not observed.

In related experiments, we determined if the addition of PGI_2, 6-keto-$PGF_{1\alpha}$, or 6-keto-PGE_1 to cultured aortic SMC would enhance the egress of unesterified cholesterol and CE from these SMC. A significant loss of total cholesterol from PG-treated SMC was observed at the end of 14 d. Results suggest that increased synthesis of PGI_2 by neointimal SMC in the injured aortic wall may, at least in part, explain the changes in CE catabolism and accumulation following injury. These PG may also be important in CE metabolism and accumulation in human arteries.

INTRODUCTION

Intimal thickening resulting from proliferation of smooth muscle cells (SMC)[1] with accumulation of free and esterified cholesterol in the vascular wall is a characteristic feature of atherosclerosis. The mechanism(s) leading to the accumulation of cholesterol (CH) and cholesteryl ester (CE) have not been fully defined. Hydrolysis of CE via lysosomal (acid) CE hydrolase (ACEH; EC 3.1.1.13) is a major mechanism by which CE is catabolized (1–3). Activity of enzymes responsible for CE metabolism may be modulated by prostaglandins (PG) (4–7), other hormones (8–11), and lipoproteins (12–14).

Although the role of prostacyclin (PGI_2) in hemostasis and thrombosis has been extensively investigated (15), little is known concerning the effect of PGI_2 on lipid metabolism or proliferation of aortic SMC. Recently, we have shown that following deendothelialization of rabbit aorta, neointimal SMC acquire the capacity to produce PGI_2 in an amount similar to that produced by endothelial cells of the adjacent uninjured

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[1] *Abbreviations used in this paper*: ACEH, acid cholesteryl ester hydrolase; ASA, acetylsalicylic acid; CE, cholesteryl ester; CH, cholesterol; DDA, 2′,5′ dideoxyadenosine; MEM, minimal essential medium; MIX, 1-methyl-3-isobutylxanthine; NAGase, *N*-acetyl-β-glucosaminidase; PG, prostaglandin(s); PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; PGI_2, prostacyclin; RIA, radioimmunoassay; SMC, smooth muscle cells.

artery (16). Injured aortas have also been shown to exhibit increased CE hydrolytic activity (17). Therefore, we examined the possible direct effects of PGI_2 and several other PG on CE hydrolytic activity and sterol accumulation in intact cultured rabbit aortic SMC. Results indicate that (*a*) treatment with PGI_2, 6-keto-PGE_1, 6-keto-$PGF_{1\alpha}$, but not PGE_1 or PGE_2, significantly increased CE hydrolytic activity in intact SMC; (*b*) this increase is mediated at least in part by cAMP; and (*c*) these PG lead to a decrease in cellular accumulation of CH.

METHODS

[1-^{14}C]Cholesteryl oleate (55 mCi/mmol sp act), [1-^{14}C]oleic acid (40 mCi/mmol sp act), [6-^{3}H]thymidine (25 Ci/mmol sp act), ^{125}I-cAMP radioimmunoassay (RIA) kits, and Aquasol-2 liquid scintillation cocktail were purchased from New England Nuclear, Boston, MA. CH, cholesteryl oleate, and sodium palmitate were obtained from Applied Science Laboratories, Inc., State College, PA. Egg lecithin and sodium eicosatrienoate were purchased from Supelco, Inc., Bellafonte, PA. Acetylsalicyclic acid (ASA), N^6, O^2-dibutyryl adenosine 3′5′-cyclic monophosphate, calf thymus DNA, essentially fatty acid free bovine albumin (Fraction V), β-glycerol phosphate, 1-methyl-3-isobutylxanthine (MIX), neutral alumina (WN-3), 4-methylumbelliferyl-2 acetamido-2-deoxy-β-D-glucopyranoside, sodium butyrate, sodium taurocholate, and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, MO. 2′5′ Dideoxyadenosine (DDA) was obtained from P. L. Biochemicals, Inc., Milwaukee, WI. 3′5′-Diaminobenzoic acid dihydrochloride and 2-methyoxyethanol were purchased from Aldrich Chemical Co. Inc., Milwaukee, WI. Sodium arachidonate was obtained from NuChek, Elysian, MN. Thin-layer silica gel chromatoplates (K6; 250 μm thick) were obtained from Whatman, Inc., Chemical Separation Div., Clifton, NJ, platinum oxide from ICN Nutritional Biochemicals, Cleveland, OH, and highly purified organic solvents (nanograde quality) from Mallinckrodt, Inc., Science Products Div., St. Louis, MO.

PGI_2, 6-keto-$PGF_{1\alpha}$, 6-keto-PGE_1, PGE_1, and PGE_2 were a generous gift from Dr. John Pike of the Upjohn Co., Kalamazoo, MI.

Disposable tissue culture materials were purchased from Corning Glass Works, Corning, NY. Tissue culture plates (Linbro) were purchased from Flow Laboratories, Inc., Rockville, MD. Eagle's modified medium (1× Minimal Essential Medium [MEM]) supplemented with nonessential amino acids (0.05 ml/ml) and fungizone (250 mg/ml) was purchased from Flow Laboratories, Inc. L-Glutamine (200 mM), penicillin (5,000 U/ml), streptomycin (5 mg/ml) and fetal bovine serum (heat inactivated) were purchased from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY.

Experimental animals. Young New Zealand White female rabbits weighing 2.5–3.0 kg were used as a source for cultured thoracic aortic SMC. Rabbits were fed commercial rabbit ration (Purina rabbit chow, Ralston Purina Co., St. Louis, MO.) and water ad lib.

Tissue culture and microscopy. Adventitial tissue was removed from the isolated descending thoracic rabbit aorta with sterile precautions. Aortic explants were prepared for culturing SMC according to a modification of the general procedures of Ross (18).

Cultured SMC grew in typical "hill and valley" formation. For electron microscopy, cells cultured in Corning plastic flasks were fixed *in situ* with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) at room temperature, washed with buffer, and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) for 1 h (19). Details of the dehydration and embedding procedures have been described (19). Sections were cut, stained with uranyl acetate and lead citrate, and examined with a Philips EM-301 electron microscope. The presence of microfibrils, dense bodies, or fusiform condensations scattered throughout the myofilament bundles and basement membranelike material were observed in these cells.

Cells grown from aortic explants were confirmed to be SMC by their growth pattern as observed by phase contrast microscopy and ultrastructural characteristics by transmission electron microscopy (18).

Biochemical methods. In experiments designed to measure the response of CE hydrolase activity to the PG, dibutyryl cAMP, FFA, or sodium butyrate, SMC were initially plated in wells (2-cm^2 surface area; 24 wells/plate) at a density of ~2.0×10^5 cells/well in Eagle's MEM with 10% fetal calf serum and allowed to adhere for 48 h. Cells were then washed three times with Eagle's MEM without serum. The factors under investigation were then added to SMC and cells were incubated at 37°C in Eagle's MEM without serum for 2 h before harvesting and assay of CE hydrolase activity. Cells from explants used in these experiments were subpassaged two times. Initial experiments showed that the maximum effect of the PG on ACEH activity could be obtained by incubating the intact SMC with PGI_2 for 2 h. To inhibit endogenous synthesis of PGI_2, SMC were incubated with 1.0 mM ASA for 20 min before the addition of PG, FFA, or dibutyryl cAMP. Optimal results were obtained by treating these cultured SMC for this length of time and concentration of ASA. To inhibit production of cAMP, 0.5 mM DDA, a specific inhibitor of adenylate cyclase activity (20, 21), was added to specific microwells 20 min before the initiation of the experiment. Optimal results were obtained with this concentration of DDA and time of preincubation. In addition to the buffer controls for dibutyryl cAMP experiments, sodium butyrate was also used, since butyrate itself may influence cellular metabolism.

Several experiments were also conducted to determine if alterations in cell marker enzyme activity were affected by the addition of PGI_2, 6-keto-PGE_1, or 6-keto-$PGF_{1\alpha}$ in the range of PG concentrations used in the CE metabolism studies. Acid phosphatase and *N*-acetyl-β-glucosaminidase (NAGase) were chosen as marker enzymes for SMC lysosomes (22). The experimental design was similar to the protocol previously described for ACEH activity.

Concentration range of PG, dibutyryl cAMP, FFA, and sodium butyrate were as follows: 2–20 nM PGI_2 or 11–55 nM 6-keto-$PGF_{1\alpha}$ were prepared in 10 mM Na_2CO_3; 11–55 nM 6-keto-PGE_1, 10–40 μM sodium arachidonate, 8–40 μM sodium palmitate, and 5–50 μM dibutyryl cAMP, were each dissolved in 10 μl absolute ethanol and diluted with normal saline (final pH 7.0); sodium eicosatrienoate was dissolved initially in 10 μl 0.01 M Na_2CO_3 and diluted in normal saline (final pH 7.0) to 8–40 μM; and sodium butyrate was dissolved in Hepes-buffered saline to 50–500 nM (pH 7.0). To assess the effects of PG other than those related to PGI_2 metabolism on ACEH activity, 100–1,000 nM PGE_1 and PGE_2 were tested. PGE_1 or PGE_2 was initially dissolved in 10 μl absolute ethanol, and diluted in normal saline (final pH 7.0) and their effects on ACEH activity were evaluated in a similar fashion as the other PG. When PG, FFA, sodium butyrate, dibutyryl

cAMP, or inhibitors were tested, the appropriate ethanol and/or buffer controls were used. The final pH of the reaction media was 7.3.

Finally, to obtain the concentration of PGI_2 produced by these cells after challenge with sodium arachidonate, sodium eicosatrienoate, or sodium palmitate, the cell medium was removed and frozen at −70°C. The quantity of PGI_2 released by the cells was determined by RIA for 6-keto-$PGF_{1\alpha}$. Details of this RIA have been published (23). Cross-reactivity with 6-keto-PGE_1 is <1%.

Assay of cAMP. Following removal of the supernatant from the incubations for the assay of 6-keto-$PGF_{1\alpha}$, the wells containing SMC cultures designated for cAMP assays were placed in an ice bath and 1.0 ml of ice-cold (0–4°C) isotonic buffer (250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.3) was added. Cells were harvested with a rubber policeman (~2.5×10^5 cells/well) and this suspension was divided into two 0.5-ml aliquots: one aliquot was used for the assay of ACEH activity, DNA, and protein (see below), while the remaining aliquot was used for the assay of cAMP (24). The aliquot designated for cAMP assay was treated with 0.1 vol of 50% TCA to a final concentration of 5% TCA. This preparation was vortexed and stored frozen at −70°C. After thawing, the TCA-precipitable cell debris was removed by centrifugation at 2,230 *g* for 15 min at 4°C. Tracer [^{3}H]cAMP (100 fmol) was added to each sample for recovery determinations. Samples were subsequently applied to Pasteur-pipette columns containing 500 mg of neutral alumina (type WN-3). Columns were allowed to run dry and then rinsed with 2.0 ml 0.5 M TCA followed by 2.5 ml distilled water, and 0.5 ml 0.2 M sodium acetate buffer (pH 6.3). The cAMP was subsequently eluted from the column with 1.0 ml 0.2 M sodium acetate buffer, pH 6.3, and measured with a cAMP RIA kit using the acetylation modification. Recoveries averaged 70–80%.

In those experiments in which cAMP was not measured, cells were also harvested, over ice, with a rubber policeman, after the addition of 1.0 ml ice-cold isotonic sucrose buffer. Cell suspensions (~2.0×10^5 cells) were subsequently transferred to a Duall glass homogenizer (Kontes Co., Vineland, NJ). An additional 1.0 ml of isotonic sucrose buffer was added, and SMC were homogenized to a uniform suspension at 0–4°C for a total of 4 min. Separate aliquots of this homogenate were then used for the assay of the enzyme activity, DNA, and protein. Assays of ACEH and NAGase activities were performed on the day of preparation of the homogenate, since there is a loss of activity upon freezing (17, 25). Assays of acid phosphatase activity, DNA, and protein were done on samples of cellular homogenates frozen at −70°C. These samples were not stored for longer than 2 wk.

Lysosomal ACEH activity. Activity of lysosomal ACEH was assayed at pH 3.9 by the method of Haley et al. (25). We used 12.7 μM cholesteryl [1-^{14}C]oleate (10 μCi) as substrate suspended in an egg lecithin-digitonide dispersion. It has been demonstrated by subcellular fractionation that ACEH is localized primarily in the lysosomes of SMC (25). After incubation of the substrate with the cell homogenate (~50 μg protein) for 60 min in a Dubnoff shaking water bath at 37°C, released oleic acid (~2,300 dpm/assay tube) was separated by organic solvent extraction using 3.25 ml methanol/chloroform/heptane (1.4:1.3:1, vol/vol/vol) and 1.05 ml 50 mM K_2CO_3-H_3BO_3 buffer, pH 10 (25). Radioactivity was assayed by a liquid scintillation counter. Quenching was corrected by automatic external standardization. In all experiments, efficiency of fatty acid extraction was determined to be ≥90%. For preliminary studies, different methods of substrate preparation and CE of varying fatty acid composition were compared. Optimum assay conditions for ACEH activity using cultured aortic SMC were similar to those findings obtained from aortic tissue homogenates previously described by Hajjar et al. (17).

Assay of lysosomal marker enzyme activities. To examine the effects of PGI_2, 6-keto-PGE_1, and 6-keto-$PGF_{1\alpha}$ on other lysosomal enzymes in addition to ACEH, acid phosphatase and NAGase activities were assayed. Acid phosphatase activity was assessed by a chemical modification of the cytochemical methods of Gomori (26) and Barka and Anderson (27). Briefly, the incubation medium contained 0.05% Triton X-100, 800 μl 50 mM sodium acetate buffer (pH 5.0) and 10 μl 1.25% sodium-β-glycerol phosphate (wt/vol), pH adjusted to 4.8 with 1 N HCl. To this medium, 300 μl cell homogenate (~50 μg protein) was added and incubated for 30 min at 37°C in a Dubnoff shaking water bath. The reaction was stopped with the addition of 500 μl 10% $HClO_4$ (wt/vol). Endogenous inorganic phosphate in each cell homogenate was determined separately before the addition of β-glycerol phosphate (28). NAGase activity was determined by the fluorometric method outlined by Peters et al. (29). Briefly, 50 μl of a fluorogenic substrate solution consisting of 10 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside dissolved in methoxyethanol was added to a reaction mixture consisting of 500 μl 0.2 M sodium citrate buffer (pH 4.8), 10 ml 0.02% Triton X-100, and 440 μl cell homogenate (pH 4.8; ~75 μg protein). Reaction was allowed to proceed at 37°C for 60 min, the reaction was then stopped with 50 mM glycine-5 mM EDTA (pH 10.4), and the fluorescence determined in a Perkin-Elmer fluorescence spectrophotometer (model 204, Perkin-Elmer Corp., Eden Prairie, MN).

Units of activity. For all hydrolases, 1 U of activity corresponds to the hydrolysis of 1 μmol substrate/min. For acid phosphatase activity, 1 mU of activity equals 1 ng Pi released/min. All activities are expressed as milliunits per milligram DNA.

Addition of PG to cell cultures: effects on CH and CE accumulation. The effects of PGI_2, 6-keto $PGF_{1\alpha}$, and 6-keto-PGE_1 on CH and CE accumulation were assessed in cultured aortic SMC.

To lipid-load aortic SMC, were fed media containing 25% fetal bovine serum every other day for 12 d before initiation of the experiment. After first subpassage from arterial explants, cells were grown to confluence (day 0) in 60 × 15-mm wells/plate. On 0, 2, 6, and 10 d of the 14 d experimental period, medium was changed and 100 nM of either PGI_2, 6-keto-$PGF_{1\alpha}$, or 6-keto-PGE_1 was added to the cell cultures. Cells were harvested at 0, 4, 8, 12, and 14 d to assess their CH and CE content. Approximately 1–2 × 10^6 cells/well were present during the sampling period. No changes in the viability of the SMC were observed based on trypan blue exclusion in PG-treated cells during the experimental period. A control group of cultured cells were also maintained throughout the experimental period without the addition of any PG. No major changes in the ultrastructural characteristics of the cells were observed as a result of this treatment.

On the designated day of sampling, cells were harvested and centrifuged at 1,500 *g* for 10 min and subsequently washed two times with serum-free medium. They were suspended in 2 ml normal saline for the analysis of CH and CE. CH and CE content of the postculture media was also assessed at 14 d and compared with fresh culture media to determine if PG-treated cells had an increased capacity to excrete free and esterified CH.

Analysis of CH and CE. Of the 2.0-ml cell suspension prepared for lipid and DNA analysis, 1.5 ml was used for the assay of free and esterified CH by microfluorometric analysis. The remaining 0.5-ml aliquot was used for the assay of DNA.

Lipid was extracted from the 1.5-ml aliquot of the cell suspension or 2.0 ml of the fresh culture or postculture media by homogenization of these aliquots in 15.0 ml chloroform/methanol (2:1, vol/vol). The lipid was subsequently extracted according to Folch et al. (30) method. The organic phase, which contained the cellular lipid, was evaporated under N_2 (gas). Any trace of water from the upper phase rinse of the Folch extract was removed by adding 5 ml benzene/methanol (1:1, vol/vol) and by evaporating the benzene-water azeotrope under N_2 (gas).

The lipid extract was then completely hydrogenated with the use of platinum oxide as a catalyst in methanol/dichloromethane (9:1, vol/vol). The extent of hydrogenation was determined by gas-liquid chromatography (31). Hydrogenation was necessary to avoid interference by unsaturated bonds (29) in the fluorometric quantitation of lipids.

The hydrogenated extract and a standard containing free and esterified CH at a concentration of 1 $\mu g/\mu l$ were then fractionated by thin-layer chromatography on Whatman K6 silica gel analytical thin-layer chromatography plates (20 × 20 cm, 250 μm; Whatman, Inc., Chemical Separation Div., Clifton, NJ) with the use of a two-solvent system as described by Hojnacki and Smith (33) and modified by Hajjar et al. (31). CH and CE were subsequently quantitated by microfluorometric analysis (32).

Thymidine incorporation into SMC. In these experiments, confluent monolayers of aortic SMC grown in T-25 Corning flasks were trypsinized as follows: culture medium was removed, primary cell cultures were washed with phosphate-buffered saline (pH 7.2) and then incubated in 4 ml 0.25% trypsin-0.02% EDTA for 5 min at 37°C. Fetal bovine serum was added to approximately one-fifth the volume to stop tryptic activity, after which SMC were collected by centrifugation at 500 *g* for 10 min at room temperature. The cells were resuspended in culture medium, and cell number and viability were determined by trypan blue (0.25%) exclusion in a hemocytometer. Cells were seeded at 1×10^5 cells/well in 24-well tissue culture plates. The plates were incubated at 37°C in water-saturated air containing 5% CO_2 for 1 wk.

At this time, cells were confluent and the culture medium was not replaced for the next 48 h. PGI_2, dissolved in 10 mM Na_2CO_3 was added to separate culture wells at a final concentration of 50, 150, 250, and 300 nM. Dibutyryl cAMP, dissolved in phosphate-buffered saline, was added to separate culture wells in concentration of 5, 10, 50, 150, and 250 μM. Control wells received equivalent volumes of MEM. Culture plates were then incubated at 37°C in water-saturated air containing 5% CO_2 for an additional 24 h.

3 h after the addition of PGI_2 or dibutyryl cAMP, 1 μCi [6-^{3}H]thymidine in 1 μl of culture medium was added to each well. 24 h later, the culture medium was aspirated from the wells. Cells were washed three times with phosphate-buffered saline and trypsinized as previously described. Dissociated cells from each well were collected on glass fiber filter paper. After the filter papers were dry and placed in glass vials, 2.0 ml Aquasol-2 liquid scintillation cocktail was added. Samples were counted in a liquid scintillation counter, and quenching was corrected by automatic external standardization.

Analysis of DNA and protein. The content of DNA in the SMC was assessed by a microfluorometric method using 3'5'-diaminobenzoic acid (34) and calf thymus DNA as standard. Protein was determined by the method of Lowry et al. (35) with bovine serum albumin as standard.

Statistical analysis. Mean ACEH activities after incubation with increasing concentrations of PGI_2, 6-keto-PGE_1, 6-keto-$PGF_{1\alpha}$, dibutyryl cAMP, and FFA were compared with the use of single-factor analysis of variance (36). Subsequent pairwise comparisons were performed with the use of Duncan's multiple range test (36). The mean concentration of CH and CE at 0, 4, 8, 12, and 14 d in control cells and cells treated with PGI_2, 6-keto-$PGF_{1\alpha}$, or 6-keto-PGE_1 were compared with the use of 4 × 5 factorial analysis of variance (36). The analysis revealed significant interaction between factors (PG) used and time; therefore, simple-effects analysis was also performed. Mean concentration of CH and CE in the preculture and postculture media were compared at 14 d with the use of the single-factor analysis of variance.

RESULTS

Findings of these experiments revealed that lysosomal ACEH activity in cultured aortic SMC is increased approximately fourfold ($P < 0.05$) following the addition of 8–20 nM PGI_2, 44–55 nM 6-keto-PGE_1, or 44–55 nM 6-keto-$PGF_{1\alpha}$ (Fig. 1). Activity of ACEH

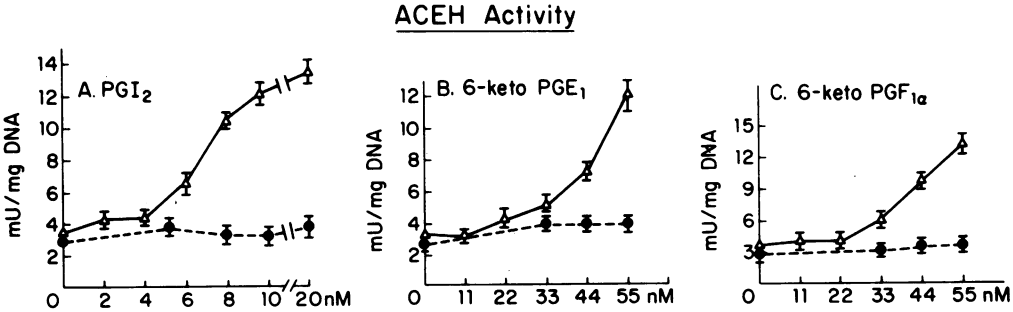

FIGURE 1 Effects of PG on ACEH activity in SMC treated (●- - -●) and not treated (Δ——Δ) with 0.5 mM DDA. Each point represents the mean±SEM for eight separate analyses. Significant increase ($P < 0.05$) in ACEH activity was observed following the addition of 8–20 nM PGI_2 (1A) and 44–55 nM 6-keto-PGE_1, or 44–55 nM 6-keto-$PGF_{1\alpha}$ (1B and 1C). These increases were abolished by treatment with DDA.

TABLE I
Effect of PGI_2 on ACEH Activity and cAMP Levels in the Presence of MIX or DDA

	Specific activity ACEH	cAMP content
	mU/mg DNA	*pmol/10^5 cells*
10n M PGI_2	12.1±0.6^{aa}	6.8±0.3^{ee}
20n M PGI_2	13.4±0.9^{bc}	8.9±0.4^{fg}
20n M PGI_2 + MIX	13.2±0.8^{d}	16.9±0.8^{gh}
20n M PGI_2 + DDA	4.0±0.3^{c}	2.2±0.4^{g}
Control 1 (No additives)	3.4±0.6^{ab}	3.0±0.3^{efi}
Control 2 (MIX)	4.1±0.2^{d}	5.2±0.4^{hi}
Control 3 (DDA)	2.8±0.2	1.8±0.2

* Data represent mean±SE of four separate analyses. MIX (1.0 mM) or DDA (0.5mM) were added to SMC 20 min before the addition of PGI_2; the SMC were then incubated at 37°C with the inhibitors for 2 h. Values with corresponding letters are significantly different ($P < 0.05$).

was equally enhanced from base-line levels by PGI_2, regardless of the presence or absence of MIX, a phosphodiesterase inhibitor (Table I). These PG specifically stimulated lysosomal ACEH activity, without any significant effect on the activity of two other lysosomal enzymes, acid phosphatase and NAGase. Results of these experiments for acid phosphatase activity are as follows (mean±SE): untreated cells: 208±26 mU/mg DNA; 100 nM PGI_2: 141±27 mU/mg DNA; 55 nM 6-keto-$PGF_{1\alpha}$: 259±30 mU/mg DNA; and, 55 nM 6-keto-PGE_1: 231±45 mU/mg DNA. For NAGase activity, the following results were obtained: untreated cells: 120±4 mU/mg DNA; 100 nM PGI_2: 159±27 mU/mg DNA; 55 nM 6-keto-$PGF_{1\alpha}$: 121±3 mU/mg DNA; and 55 nM 6-keto-PGE_1: 113±7 mU/mg DNA.

As shown in Fig. 1, increases in ACEH activity resulting from the addition of these PG appear to be mediated by increased cAMP since 0.5 mM DDA, an inhibitor of adenylate cyclase activity, abolished the enhancement of ACEH activity produced by each PG tested. Enzyme activity was not altered by DDA alone (Table I). PGI_2 (20 nM) significantly increased ($P < 0.05$) the intracellular levels of cAMP in these SMC from 3.0±0.3 pmol/10^5 cells to 8.9±0.4 pmol/10^5 cells (Table I), particularly if SMC were treated with 1.0 mM MIX (16.9±0.8 pmol/10^5 cells) as compared with MIX controls (5.2±0.4 pmol/10^5 cells). In the presence of 20 nM PGI_2 and 0.5 mM DDA, the intracellular levels of cAMP were not increased but rather maintained at 2.2±0.4 pmol/10^5 cells as compared with DDA controls: 1.8±0.2 pmol/10^5 cells.

Further studies were designed to investigate the direct effects of dibutyryl cAMP on ACEH activity (Fig. 2A). Our results demonstrate that 20 μM dibutyryl cAMP produces a significant twofold ($P < 0.05$) increase in ACEH activity. In contrast, lysosomal marker enzyme activities were not altered by dibutyryl cAMP in the range of concentrations used in this investigation (data not shown). Because the effects of dibutyryl cAMP were rather modest, sodium butyrate was tested as a control particularly since butyrate may in itself have profound influences on cellular metabolism. We could not demonstrate a significant effect of sodium butyrate on ACEH activity (untreated cells: 3.4±0.6 mU/mg DNA; 500 nM sodium butyrate: 3.8±0.7 mU/mg DNA), even in the presence of MIX

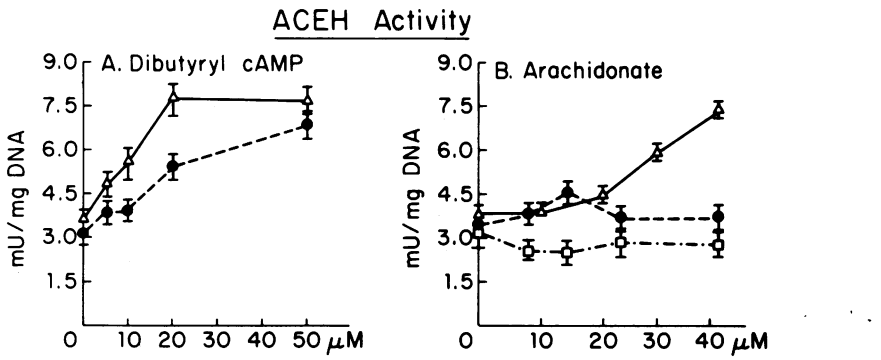

FIGURE 2 Effects of dibutyryl cAMP (2A) and sodium arachidonate (2B) on ACEH activity. Activity of ACEH was significantly stimulated ($P < 0.05$) by 20 and 50 μM dibutyryl cAMP in SMC untreated (△——△) or pretreated (●- - -●) with ASA. In Fig. 2B, the effects of sodium arachidonate (△——△), sodium palmitate (●- - -●), and sodium eicosatrienoate (□-·-·-□) on ACEH activity are depicted. Fig. 2B shows a significant enhancement ($P < 0.05$) of ACEH activity in SMC by the addition of 30–40 μM sodium arachidonate but not by sodium palmitate, or sodium eicosatrienoate. Each point in Figs. 2A and 2B represents the mean±SEM for four separate analyses.

that would reduce cAMP catabolism (MIX-treated control cells: 3.7±0.3 mU/mg DNA; 500 nM sodium butyrate plus MIX, 2.8±0.5 mU/mg DNA). We also determined the contribution of endogenous PG production to the increase in ACEH activity produced by dibutyryl cAMP. Activity of ACEH in ASA-treated SMC appears less sensitive to 20 μM dibutyryl cAMP than ACEH activity in untreated SMC. The activities were similar after the addition of 50 μM dibutyryl cAMP (Fig. 2A).

To determine the effects of endogenous PG on CE catabolism, sodium arachidonate was added to cultured SMC (Fig. 2B). Sodium palmitate and sodium eicosatrienoate were used as controls. 30–40 μM sodium arachidonate significantly enhanced ($P < 0.05$) both PG production and ACEH activity. When SMC were challenged with 10, 20, 30, and 40 μM sodium arachidonate, they produced 3.7, 4.1, 4.8, and 5.8 nM 6-keto-$PGF_{1\alpha}$, respectively. These cells contained 3.7±0.4, 4.4±0.2, 5.9±0.2, and 6.8±0.1 mU/mg DNA ACEH activity, respectively. The quantities of endogenous PGI_2, which significantly enhanced ACEH activity, were comparable to those effects observed following addition of exogenous PGI_2 (Fig. 1A). Neither sodium palmitate, a fatty acid unrelated to PG production, nor sodium eicosatrienoate (a substrate for cyclooxygenase but one that cannot be later converted to PGI_2, 6-keto-$PGF_{1\alpha}$, or 6-keto-PGE_1) affected ACEH activity (Fig. 2B) or the base-line production of 6-keto-$PGF_{1\alpha}$ (data not shown). As anticipated, ASA-treated SMC significantly decreased PGI_2 production by SMC following addition of 40 μM sodium arachidonate (2.4 nM as compared with 0.2 nM) and led to a significant decrease in ACEH activity (6.8±0.1 to 3.9±0.1 mU/mg DNA). These results suggest PG must be formed from sodium arachidonate for enhancement of ACEH activity to occur.

In experiments designed to ascertain the effects, if any, of other PG on ACEH activity, PGE_1 and PGE_2 were tested. We also tested PGE_1 and PGE_2 in SMC pretreated with 1 mM ASA and 1 mM MIX to block the contribution of endogenous arachidonic acid and reduce the catabolism of cAMP. Neither PGE_1 nor PGE_2 significantly enhanced ACEH activity in the presence or absence of ASA and MIX (untreated cells: 3.4±0.6 mU/mg DNA; 1 μM PGE_1: 4.3±1.6 mU/mg DNA; 1 μM PGE_2: 5.3±1.0 mU/mg DNA; SMC treated with ASA±MIX: 3.7±0.2 mU/mg DNA; 1 μM PGE_1 + ASA + MIX: 3.2±0.2 mU/mg DNA; 1 μM PGE_2 + ASA + MIX: 3.2±0.4 mU/mg DNA) (mean±SE).

Since PGI_2, 6-keto-$PGF_{1\alpha}$, and 6-keto-PGE_1 affected hydrolytic activity of CE in these SMC, their overall effects on CH and CE accumulation were assessed over a 2-wk period. A significant decrease ($P < 0.05$) in CH and CE content was observed at 14 d following the addition of PGI_2, 6-keto-$PGF_{1\alpha}$, or 6-keto-PGE_1 at 0, 2, 6, and 10 d (Fig. 3). This decrease in CH and CE content appears to result from excretion of these sterols into the medium. However, it is noteworthy that CE accounted for ~15% of the total sterol content (CH and CE) in the medium, regardless of treatment of SMC. At 14 d, the postculture media from PGI_2-treated cells contained 244±24 μg CH/ml and 48±4 μg CE/ml (mean±SE), significantly greater ($P < 0.05$) than the total amount of CH and CE in the medium fed to the cells (187±20 μg CH/ml and 28±4 μg CE/ml) or in the postculture media from the untreated cells (196±10 μg CH/ml and 30±3 μg CE/ml). Postculture media from the 6-keto-$PGF_{1\alpha}$- and 6-keto-PGE_1-treated SMC contained 242±18 μg CH/ml and 44±6 μg CE/ml, and 253±17 μg CH/ml and 48±5 μg CE/ml, re-

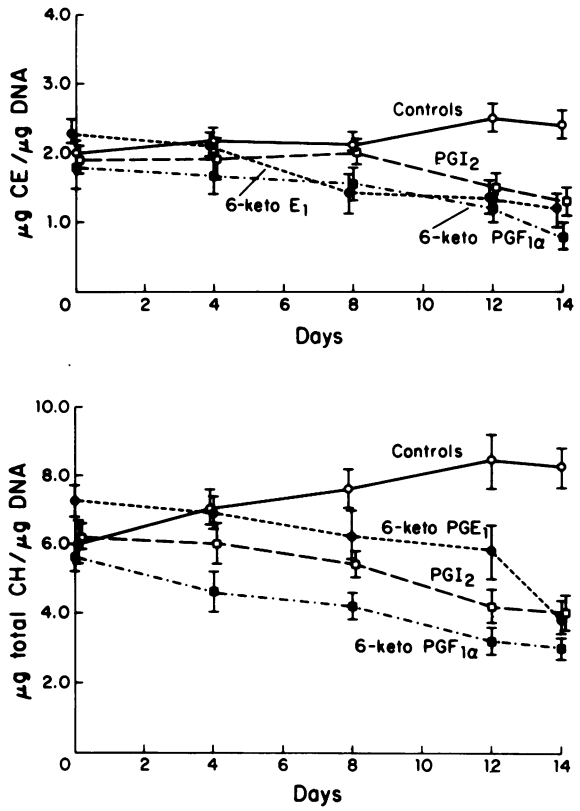

FIGURE 3 Effects of PGI_2 (□ - - - □), 6-keto-PGE_1 (● - - - ●), and 6-keto-$PGF_{1\alpha}$ (■ - · - · - ■) on CH and CE accumulation in cultured SMC as compared with controls (○ —— ○). Cells were challenged separately with 100 nM PGI_2, 6-keto-PGE_1, or 6-keto-$PGF_{1\alpha}$ at 0, 2, 6, and 10 d. Five separate analyses of CH and CE concentration were done at 0, 4, 8, 12, and 14 d. Each point represents the mean±SEM. At day 0, there were no significant differences in total CH or CE content in the four groups of SMC. However, there was a significant decrease in total CH and CE accumulation ($P < 0.05$) by 14 d in SMC treated with PG as compared with untreated SMC.

spectively. These values were also significantly greater ($P < 0.05$) than the amount of sterol present in the media fed to the cells or in the postculture media of untreated cells.

To determine if the effect of PGI_2 or dibutyryl cAMP on CE hydrolytic activity was, at least in part, related to proliferation of SMC, we measured the incorporation of [^{3}H]thymidine into these cells in the presence of increasing concentrations of PGI_2 or dibutyryl cAMP (Fig. 4). PGI_2 and dibutyryl cAMP did not alter the incorporation of [^{3}H]thymidine into these cells. In addition, PGI_2 or dibutyryl cAMP did not significantly alter the cell number or viability as determined by cell counts and trypan blue exclusion.

DISCUSSION

We have demonstrated that PGI_2, 6-keto-$PGF_{1\alpha}$, and 6-keto-PGE_1 increase CE hydrolytic activity in cultured aortic SMC. This effect appears to be mediated by cAMP. The following data support these conclusions: (*a*) increasing concentrations of PGI_2, 6-keto-$PGF_{1\alpha}$, and 6-keto-PGE_1 produced a fourfold increase in CE hydrolytic activity (ACEH). (*b*) Sodium arachidonate, at a concentration sufficient to stimulate endogenous PGI_2 production, significantly enhanced CE hydrolytic activity in untreated SMC but not in ASA-treated cells. (*c*) Pretreatment of SMC with an inhibitor of adenylate cyclase activity abolished the enhancement of CE hydrolytic activity produced by exogenous PG. (*d*) Addition of dibutyryl cAMP to either ASA-treated or untreated SMC resulted in a twofold increase in CE hydrolytic activity. This is the first evidence that we know of to indicate that PGI_2, 6-keto-$PGF_{1\alpha}$, or 6-keto-PGE_1 have an effect on CE hydrolytic activity in intact cells and that these effects are mediated via stimulation of intracellular cAMP levels. These findings in aortic SMC support the observations of others who have shown that arachidonic acid or PGI_2 can stimulate cAMP formation in fibroblasts (37) or endothelial cells (38). Further, others have shown that dibutyryl cAMP may increase the activity of a neutral cholesteryl ester hydrolase in ovarian (39) and adrenal (40, 41) tissue. To the best of our knowledge this is the first report that has described an effect of dibutyryl cAMP on lysosomal ACEH activity.

Other investigators have demonstrated an effect of E and F series PG on unspecified CE hydrolytic activity in aortic or cell homogenates (5–7). However, in their experiments homogenates were prepared before the addition of nonphysiologic concentrations of PG. Thus, membrane-associated enzyme systems and phosphorylation cascade sequences that mediate cyclic nucleotide action may not be intact. Further, they used quantities of PG 6–10 times greater than the amounts used in this investigation. In other experiments, we added PG directly to homogenates before the assay of enzyme activities, as others have done (5–7), to determine if nanomolar quantities of PG could stimulate lysosomal enzyme activity and compare them with our observations on intact cells. We could not demonstrate an effect on enzyme activity when nanomolar concentrations of PG were added to aortic homogenates.

Since our results indicated that PGI_2, 6-keto-PGE_1, and 6-keto-$PGF_{1\alpha}$ significantly enhanced lysosomal ACEH activity, we attempted to determine if the effects of these PG on CE catabolism were specific for certain lysosomal enzymes. The activities of two lysosomal enzymes, acid phosphatase and NAGase were unaffected by addition of PGI_2, 6-keto-$PGF_{1\alpha}$, or 6-keto-PGE_1, indicating that specific enzyme activation

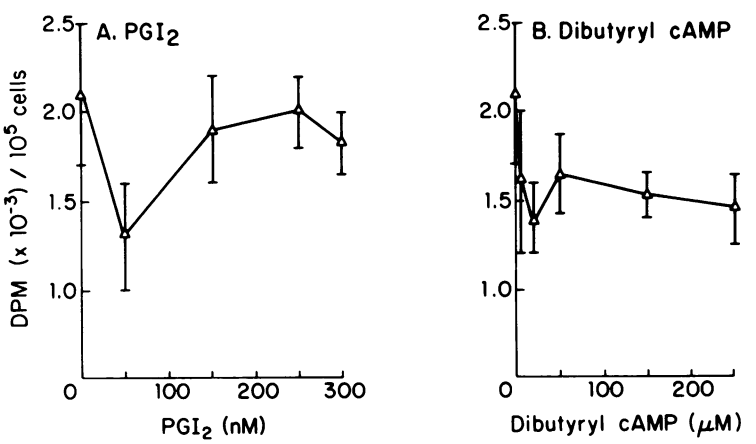

FIGURE 4 Incorporation of [6-^{3}H]thymidine into cultured SMC following addition of (A) PGI_2 and (B) dibutyryl cAMP. Each point represents the mean±SEM for four separate analyses. No effects were observed on incorporation of [^{3}H]thymidine by PGI_2 or dibutyryl cAMP.

can be produced by nanomolar concentrations of PG in intact SMC. Other investigators have shown that E and F series PG or elevation in cAMP can destroy lysosomal latency and thereby stimulate activity in homogenized tissues (42, 43). However, the PG concentrations used in those studies were much greater than the levels used in this investigation.

We found that PGE_1 and PGE_2 did not significantly affect ACEH activity in intact, cultured aortic SMC in a concentration range of 100–1,000 nM. In contrast to these findings, it has been demonstrated that PGE_1 and PGE_2 can affect CE metabolism in cell homogenates by inhibiting CE hydrolytic activity in micromolar amounts, at a neutral pH (4–6). However, due to the differences in experimental design, it is difficult to compare our results with these findings.

That the stable hydrolysis product of PGI_2, i.e., 6-keto-$PGF_{1\alpha}$, affected ACEH activity via cAMP was unexpected, since this PG is generally considered to be biologically inactive (44). Recently, Wong et al. (45, 46) have suggested that 6-keto-$PGF_{1\alpha}$ may be converted by 9-hydroxyprostaglandin dehydrogenase to 6-keto-PGE_1, which stimulates adenylate cyclase activity (47), albeit in some systems it is less active than PGI_2 (48). Conversion of 6-keto-$PGF_{1\alpha}$ to 6-keto-PGE_1 by the SMC would be a possible explanation of the results we obtained.

We observed that PGI_2, 6-keto-PGE_1, and 6-keto-$PGF_{1\alpha}$ significantly enhanced CE hydrolytic activity in treated SMC as compared with untreated SMC (Fig. 1). Increased lysosomal hydrolysis of CE has been demonstrated to release free CH from lysosomes and this CH is either reesterified in the cytoplasm by acyl CoA cholesteryl *O*-acyltransferase to form CE droplets (49, 50), or is transported by sterol carrier proteins to the cell surface for excretion (51). The cytoplasmic CE can, in turn, be hydrolyzed by a nonlysosomal CE hydrolase to form CH (49, 50), which can also be transported by sterol carrier proteins. In the experiments reported here, we observed a significant decrease of ~35% in free and esterified CH in SMC treated with PGI_2, 6-keto-$PGF_{1\alpha}$, or 6-keto-PGE_1 over a 2-wk observation period. Possible mechanisms leading to increased egress of CH may involve increased transport of CH to the cell surface by sterol carrier proteins (51), where it can then bind to serum proteins such as high density lipoproteins. We did find a significant increase of CH in the medium at the end of the 2-wk experimental period. Also, there was a small increase in CE in the medium that may have resulted from residual CH esterifying activity (lecithin cholesterol acyl transferase) in the serum used in these experiments (data not shown). It is interesting that the proportion of total sterol present in the medium as CE was similar in both control and PG-treated cultures.

One possible interpretation of the results of our experiments can be summarized as follows: PGI_2 may stimulate adenylate cyclase activity producing increased intracellular levels of cAMP. Cyclic AMP, in turn, may subsequently enhance CE catabolism (52, 53) and CH egress from the cell, as it has been recently shown in adipocytes that dibutyryl cAMP can significantly reduce lipid accumulation (54).

While PGI_2 is well known as a vasodilator and inhibitor of platelet aggregation (55–58), our experiments demonstrated that PGI_2, 6-keto-$PGF_{1\alpha}$, and 6-keto-PGE_1 have appreciable effects on CE catabolism and accumulation in cultured aortic SMC and suggest the possibility that PGI_2 may have similar effects in vivo. In hypercholesterolemic rabbits, aortic PGI_2 production measured at the surface of previously deendothelialized aortas was significantly reduced as compared to similar areas in normocholesterolemic rabbits (59). In experiments of similar design, ACEH activity in deendothelialized aortas was significantly reduced in hypercholesterolemic rabbits as compared with normocholesterolemic rabbits (17). Thus, PGI_2 may influence the pathogenesis of degenerative arterial disease both through its effects on platelets and its effect on CE accumulation.

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REFERENCES

1. St. Clair, R. W. 1976. Metabolism of the arterial wall and atherosclerosis. *In* Atherosclerosis Reviews. R. Paoletti and A. M. Gotto, Jr., editors. Raven Press, New York. **1**: 61–117.
2. Takano, T., W. J. Black, T. J. Peters, and C. deDuve. 1974. Assay, kinetics, and lysosomal localization of an acid cholesteryl esterase in rabbit aortic smooth muscle cells. *J. Biol. Chem.* **249**: 6732–6737.
3. Brecher, P., H. Y. Pynn, and A. V. Chobanian. 1977. Effect of atherosclerosis on lysosomal cholesteryl esterase activity in rabbit aorta. *J. Lipid Res.* **18**: 154–163.
4. Berberian, P. A., V. A. Ziboh, and S. L. Hsia. 1977. Inhibition of cholesterol esterification in rabbit aorta by prostaglandin E_2. *Atherosclerosis.* **27**: 213–220.
5. Subbiah, M. T. R., and B. Dicke. 1977. Effect of prostaglandins E_1 and $F_{1\alpha}$ on the activities of cholesteryl ester synthetase and cholesteryl ester hydrolase of pigeon aorta in vitro. *Atherosclerosis.* **27**: 107–111.

6. Subbiah, M. T. R. 1978. Prostaglandin E_2 biosynthesis and effect in pigeon aorta: possible role in atherogenesis. *Atherosclerosis.* **29:** 487–495.
7. Takatori, T., and A. Yamaoka. 1978. Effects of prostaglandins on the metabolism of cholesteryl ester in rat testes: changes in the synthesis and hydrolysis of cholesteryl ester and the activity of cholesterol side-chain cleavage enzyme. *J. Endocrinol.* **79:** 41–48.
8. Lakshmanan, M. R., C. M. Nepokroeff, G. C. Ness, R. E. Dugan, and J. W. Porter. 1973. Stimulation by insulin of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol-synthesizing activities. *Biochem. Biophys. Res. Commun.* **50:** 704–710.
9. Nepokroeff, C. M., M. R. Lakshmanan, G. C. Ness, R. E. Dugan, and J. W. Porter. 1974. Regulation of the diurnal rhythm of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase activity by insulin, glucagon, cyclic AMP, and hydrocortisone. *Arch. Biochem. Biophys.* **160:** 387–393.
10. Vahouny, G. V., R. Chanderbhan, R. Hinds, V. A. Hodges, and C. R. Treadwell. 1978. ACTH-induced hydrolysis of cholesteryl esters in rat adrenal cells. *J. Lipid Res.* **19:** 570–577.
11. Edwards, P. A., D. Lemongello, and A. M. Fogelman. 1979. The effect of glucagon, norepinephrine, and dibutyryl cyclic AMP on cholesterol efflux and on the activity of 3-hydroxy-3-methylglutaryl CoA reductase in rat hepatocytes. *J. Lipid Res.* **20:** 2–7.
12. Bates, S. R., and G. H. Rothblat. 1974. Regulation of cellular sterol flux and synthesis by human serum lipoproteins. *Biochim. Biophys. Acta.* **360:** 38–55.
13. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* **46:** 897–930.
14. Balasubramanian, S., J. L. Goldstein, J. R. Faust, G. Y. Brunshede, and M. S. Brown. 1977. Lipoprotein-mediated regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and cholesteryl ester metabolism in the adrenal gland of the rat. *J. Biol. Chem.* **252:** 1771–1779.
15. Vane, J. R., and S. Bergstrom, editors. 1979. Prostacyclin. Raven Press, New York.
16. Eldor, A., D. J. Falcone, D. P. Hajjar, C. R. Minick, and B. B. Weksler. 1981. Recovery of prostacyclin production by de-endothelialized rabbit aorta. *J. Clin. Invest.* **67:** 735–741.
17. Hajjar, D. P., D. J. Falcone, S. Fowler, and C. R. Minick. 1981. Endothelium modifies the altered metabolism of the injured aortic wall. *Am. J. Pathol.* **102:** 28–39.
18. Ross, R. 1971. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell Biol.* **50:** 172–186.
19. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52:** 2745–2756.
20. Haslam, R. J., M. M. L. Davidson, and J. V. Desjardins. 1978. Inhibition of adenylate cyclase by adenosine analogues in preparations of broken and intact human platelets. *Biochem. J.* **176:** 83–95.
21. Haslam, R. J., M. M. L. Davidson, J. E. B. Fox, and J. A. Lynham. 1978. Cyclic nucleotides in platelet function. *Thromb. Haemostasis.* **40:** 232–240.
22. Shio, H., N. J. Haley, and S. Fowler. 1978. Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. II. Morphometric analysis of lipid-filled lysosomes and lipid droplets in aortic cell population. *Lab. Invest.* **39:** 390–397.
23. Levin, R. I., E. A. Jaffe, B. B. Weksler, and K. Tack-Goldman. 1981. Nitroglycerin stimulates synthesis of prostacyclin by cultured endothelial cells. *J. Clin. Invest.* **67:** 762–769.
24. Coffey, R. G., E. M. Hadden, and J. W. Hadden. 1981. Phytohemagglutinin stimulation of guanylate cyclase in human lymphocytes. *J. Biol. Chem.* **256:** 4418–4424.
25. Haley, N. J., S. Fowler, and C. deDuve. 1980. Lysosomal acid cholesteryl esterase activity in normal and lipid-laden aortic cells. *J. Lipid. Res.* **21:** 961–969.
26. Gomori, G. 1950. An improved histochemical technique for acid phosphatase. *Stain Technol.* **25:** 81–85.
27. Barka, T., and P. J. Anderson. 1962. Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler. *J. Histochem. Cytochem.* **10:** 741–753.
28. Itaya, K., and M. Ui. 1966. A new micromethod for the colorimetric determination of inorganic phosphate. *Clin. Chim. Acta.* **14:** 361–366.
29. Peters, T. J., M. Muller, and C. deDuve. 1972. Lysosomes of the arterial wall. I. Isolation and subcellular fractionation of cells from normal rabbit aorta. *J. Exp. Med.* **136:** 1117–1139.
30. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
31. Hajjar, D. P., T. N. Wight, and S. C. Smith. 1980. Lipid accumulation and ultrastructural change within the aortic wall during early spontaneous atherogenesis. *Am. J. Pathol.* **100:** 683–706.
32. Nicolosi, R. J., S. C. Smith, and R. F. Santerre. 1971. Simultaneous fluorometric analysis of five lipid classes on thin-layer chromatograms. *J. Chromatogr.* **60:** 111–117.
33. Hojnacki, J. L., and S. C. Smith. 1974. Separation of six lipid classes on one thin-layer chromatogram. *J. Chromatogr.* **90:** 365–367.
34. Kissane, J. M., and E. Robins. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.* **233:** 184–188.
35. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein determination with the Folin phenol reagent. *J. Biol Chem.* **193:** 265–275.
36. Winer, B. J. 1962. Statistical Principles in Experimental Design. McGraw-Hill Book Co., New York.
37. Claesson, H. E. 1980. Prostaglandin I_2 synthesis and elevation of cyclic AMP levels in 3T3 fibroblasts. *Biochim. Biophys. Acta.* **618:** 399–406.
38. Hopkins, N. K., and R. R. Gorman. 1981. Regulation of endothelial cell cyclic nucleotide metabolism by prostacyclin. *J. Clin. Invest.* **67:** 540–546.
39. Williams, M. T., M. R. Clark, W. Y. Ling, W. J. LeMaire, M. G. Caron, and J. M. Marsh. 1978. Role of cyclic AMP in the actions of luteinizing hormone on steroidogenesis in the corpus luteum. *In* Advances in Cyclic Nucleotide Research. W. J. George and L. J. Ignarro, editors. Raven Press, New York. **9:** 573–582.
40. Naghshinek, S., C. R. Treadwell, L. Gallo, and G. V. Vahaouny. 1974. Activation of adrenal sterol ester hydrolase by dibutyryl cAMP and protein kinase. *Biochem. Biophys. Res. Commun.* **61:** 1076–1082.
41. Trzeciak, W. H., and G. S. Boyd. 1974. Activation of cholesterol esterase in bovine adrenal cortex. *Eur. J. Biochem.* **46:** 201–207.

42. Szego, C. M. 1972. The role of cyclic AMP in lysosome mobilization and their nucleotropic translocation in steroid hormonal target cells. *In* Advances in Cyclic Nucleotide Research. P. Greengard and G. A. Robinson, editors. Raven Press, New York. **1:** 541–564.
43. Ignarro, L. J. 1975. Regulation of lysosomal enzyme release by prostaglandins, autonomic neurohormones, and cyclic nucleotides. *In* Lysosomes in Biology and Pathology. J. T. Dingle and R. T. Dean, editors. Elsevier North-Holland, Inc., New York. **4:** 481–523.
44. Tateson, J. E., S. Moncada, and J. R. Vane. 1977. Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. *Prostaglandins.* **13:** 389–397.
45. Wong, P. Y-K., K. U. Malik, D. M. Desiderio, J. C. McGiff, and F. F. Sun. 1980. Hepatic metabolism of prostacyclin (PGI_2) in the rabbit: formation of a potent novel inhibitor of platelet aggregation. *Biochem. Biophys. Res. Commun.* **93:** 486–494.
46. Wong, P. Y-K., W. H. Lee, C. P. Quilley, and J. C. McGiff. 1981. Metabolism of prostacyclin: formation of an active metabolite in the liver. *Fed. Proc.* **40:** 2001–2004.
47. Rapp, N. S., T. V. Zenser, and B. B. Davis. 1981. 6-Keto-prostaglandin E_1 stimulation of rat and rabbit renal adenylate cyclase-cyclic AMP systems. *Biochim. Biophys. Acta.* **673:** 163–169.
48. Miller, O. V., J. W. Aiken, R. J. Shebuski, and R. R. Gorman. 1980. 6-Keto-prostaglandin E_1 is not equipotent to prostacyclin (PGI_2) as an antiaggregatory agent. *Prostaglandins.* **20:** 391–400.
49. Brown, M. S, Y. K. Ho, and J. L. Goldstein. 1980. The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters. *J. Biol. Chem.* **255:** 9344–9352.
50. Goldstein, J. L., and M. S. Brown. 1977. The low density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* **46:** 897–930.
51. Marsh, J. M. 1976. The role of cyclic AMP in gonadal steroidogenesis. *Biol. Reprod.* **14:** 30–53.
52. Kuehl, F. A. 1974. Prostaglandins, cyclic nucleotides, and cell function. *Prostaglandins.* **5:** 325–340.
53. Kuehl, F. A., V. J. Cirillo, and H. G. Oien. 1976. Prostaglandin-cyclic nucleotide interactions in mammalian tissues. *In* Prostaglandins: Chemical and Biochemical Aspects. S. M. M. Karim, editor. University Park Press, Baltimore, MD. **5:** 191–225.
54. Spiegelman, B. M., and H. Gren. 1981. Cyclic AMP-mediated control of lipogenic enzyme synthesis during adipose differentiation of 3T3 cells. *Cell.* **24:** 503–510.
55. Bunting, S., R. Gryglewski, S. Moncada, and J. R. Vane. 1976. Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac arteries and inhibits platelet aggregation. *Prostaglandins.* **12:** 897–913.
56. Gryglewski, R. J., S. Bunting, S. Moncada, R. J. Flower, and J. R. Vane. 1976. Arterial walls are protected against deposition of platelet thrombi by a substance (prostaglandin X) which they make from prostaglandin endoperoxide. *Prostaglandins.* **12:** 685–713.
57. Moncada, S., R. J. Gryglewski, S. Bunting, and J. R. Vane. 1976. An enzyme isolated from arteries transforms prostaglandin endoperoxide to an unstable substance that inhibits platelet aggregation. *Nature (Lond.).* **263:** 663–665.
58. Dusting, G. J., S. Moncada, and J. R. Vane. 1977. Prostacyclin (PGI_2) is the endogenous metabolite of arachidonic acid which relaxes coronary arteries. *Prostaglandins.* **13:** 3–15.
59. Eldor, A., D. J. Falcone, D. P. Hajjar, C. R. Minick, and B. B. Weksler. 1982. Diet-induced hypercholesterolemia inhibits the recovery of prostacyclin by the injured rabbit aorta. *Am. J. Pathol.* **107:** 186–190.