Metabolic activity of cholesteryl esters in aortic smooth muscle cells is altered by prostaglandins I_2 and E_2

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Abstract Among the biochemical processes associated with the atherogenic process are increased aortic cholesteryl ester (CE) accumulation and altered prostaglandin (PG) production. The precise physiological role of PG, particularly prostacyclin (PGI_2), in the control of CE metabolism in intact aortic smooth muscle cells remains to be fully elucidated. We report here that cytosolic neutral cholesteryl ester hydrolytic activity (NCEH) in intact cultured aortic smooth muscle cells is significantly increased by 75–250 nM PGI_2 at the end of a 2-hr incubation period. This effect was mediated by increased intracellular cAMP levels since the effect of PGI_2 on NCEH activity was abolished in the presence of an inhibitor of adenylate cyclase activity, viz., dideoxyadenosine (DDA). Although the addition of 20–100 μM dibutyryl cAMP (Bt_2cAMP) and 50–100 μM sodium arachidonate also increased NCEH activity twofold, 6-keto $PGF_{1\alpha}$, PGE_1, and PGE_2 did not increase the activity of this enzyme. In contrast to these findings, 75–250 nM PGE_2 significantly inhibited CE synthetic activity (ACAT) approximately 60%. Arachidonate or Bt_2cAMP did not affect ACAT activity. This decrease in ACAT activity induced by PGE_2 does not appear to be mediated by cAMP. Taken together, these findings suggest that PGI_2, a well known potent vasodilator and inhibitor of platelet aggregation, and PGE_2 may have an important regulatory role in aortic CE metabolism.—**Hajjar, D. P., and B. B. Weksler.** Metabolic activity of cholesteryl esters in aortic smooth muscle cells is altered by prostaglandins I_2 and E_2. *J. Lipid Res.* 1983. **24:** 1176–1185.

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Accretion of cholesterol and cholesteryl ester (CE) is considered the hallmark of the pathogenesis of human arteriosclerosis (1). Aside from their deposition in the extracellular matrix of the aortic smooth muscle cell, these sterols also accumulate to a significant extent in lysosomal and cytosolic compartments of these cells (2). Biochemical processes associated with CE accumulation in the arterial wall during atherogenesis include altered activity of the major enzymes responsible for CE hydrolysis and synthesis. For this reason, considerable research has been aimed at the investigation of those factors that modulate the activity of the enzymes responsible for the regulation of cholesterol/CE balance in the cell. Hydrolysis of CE via lysosomal (acid) and cytosolic (neutral) CE hydrolase activity (ACEH, NCEH; EC 3.1.1.13) and esterification of cholesterol via microsomal acyl CoA: cholesterol-O-acyltransferase (ACAT; EC 2.3.1.26) have been identified as major mechanisms by which these sterols are metabolized (2). Activity of sterol-metabolizing enzymes may be affected in cell homogenates by prostaglandins (3–6), other hormones (7–10), and lipoproteins (11–13). Prostaglandins, in particular, are produced by arterial cells but their production by atherosclerotic vessels may be different from normal arteries (14–18).

Although the physiological role of prostacyclin (PGI_2) in hemostasis and thrombosis has been studied in detail (19), little is known about the physiological role of PGI_2 in the control of lipid metabolism in *intact* aortic smooth muscle cells. Recently, we tested the hypothesis that PGI_2 and other prostaglandins may affect lysosomal ACEH activity and alter sterol accumulation in intact cultured aortic smooth muscle cells (20). Physiological levels of PGI_2 significantly increased lysosomal ACEH activity fourfold and decreased cholesterol accumulation in the cells. This effect was mediated via increased intracellular levels of cAMP. PGE_1 and PGE_2 had no effect on ACEH activity or on sterol accumulation. The effects of these prostaglandins on cytosolic CE metabolism (NCEH and ACAT activity) were not previously

Abbreviations: ACAT, acyl CoA:cholesterol O-acyltransferase; ASA, aspirin; CE, cholesteryl ester; cAMP, cyclic adenosine 3′,5′-monophosphate; Bt_2cAMP, dibutyryl cAMP: DDA, dideoxyadenosine; FFA, free fatty acids; MIX, 1-methyl-3-isobutylxanthine, MEM, minimal essential medium; NCEH, neutral cholesteryl ester hydrolase; PGI_2, prostaglandin I_2 = prostacyclin; ACEH, acid cholesteryl ester hydrolase; RIA, radioimmunoassay.

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tested when lysosomal ACEH activity was examined since a quantitative, reproducible assay for optimal NCEH activity in aortic cells has only recently been developed (21).

Accordingly, in the present study, the effects of several prostaglandins, including PGI_2 and PGE_2, on cytosolic CE hydrolytic (NCEH) and synthetic (ACAT) activity were investigated in intact cultured aortic smooth muscle cells. Taken together, the results of our recently published experiments (20) and the studies reported here indicate the potential role of PGI_2 and PGE_2 in the control of aortic CE metabolism.

EXPERIMENTAL

[1-^{14}C]Cholesteryl oleate (sp act 55 mCi/mmol), [1,2-^{3}H]cholesterol (sp act 40 Ci/mmol), [1-^{14}C]oleoyl CoA (sp act 49–53 mCi/mmol), ^{125}I-cAMP radioimmunoassay (RIA) kits, and Aquasol-2 liquid scintillation cocktail were purchased from New England Nuclear Corp., Boston, MA. Cholesterol and cholesteryl oleate were purchased from Applied Science Laboratories, Inc., State College, PA. Egg phosphatidylcholine (lecithin) and sodium eicosatrienoate were obtained from Supelco, Inc., Bellafonte, PA. Acetylsalicylic acid (ASA), N^6, O^2-dibutyryl adenosine 3′-5′-cyclic monophosphate, essentially fatty acid-free bovine albumin (Fraction V), 1-methyl-3-isobutylxanthine (MIX), 4-methylumbelliferyl-α-D glucopyranoside, neutral alumina (WN-3), sodium butyrate, and sodium taurocholate were purchased from Sigma Chemical Co., St. Louis, MO. 2′,5′-Dideoxyadenosine was obtained from P. L. Biochemicals, Milwaukee, WI. Sodium arachidonate was purchased from Nu-Chek, Elysian, MN. Thin-layer silica gel chromatoplates (K5; 250-μ thick) were purchased from Whatman Corp., Clinton, NJ, platinum oxide from ICN Nutritional Biochemical Co., Cleveland, OH, and highly purified organic solvents (nanograde quality) from Malinckrodt, St. Louis, MO. Prostacyclin (PGI_2), 6-keto $PGF_{1\alpha}$, PGE_1, and PGE_2 were generous gifts 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) and Dulbecco's phosphate buffered saline were purchased from Flow Laboratories, Inc., McLean, VA. Eagle's modified medium (1 × MEM) supplemented with nonessential amino acids (0.01 ml/ml media) and Fungizone (25 μg/ml) were purchased from Flow Laboratories, Inc., McLean, VA. L-Glutamine (200 mM), penicillin (50 units/ml), streptomycin (5 μg/ml), and fetal bovine serum (heat-inactivated) were purchased from Grand Island Biological Company (GIBCO), 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 smooth muscle cells. Rabbits were fed commercial rabbit ration (Purina Rabbit Chow) and water ad libitum.

Tissue culture and microscopy

Adventitial tissue was removed from the isolated descending thoracic rabbit aorta with sterile precautions. Aortic explants were prepared for culturing smooth muscle cells according to the general procedures of Ross (22).

Cells grown from aortic tissue explants were confirmed to be smooth muscle cells by their growth patterns as observed by phase contrast microscopy and ultrastructural characteristics by transmission electron microscopy (22). Cultured smooth muscle cells grew in the typical "hill and valley" formation characteristic of smooth muscle cells, and did not stain with antibody to Factor VIII (von Willebrand Factor).

Electron microscopy of the cultured cells was done by the methods previously described (20). The presence of microfibrils, dense-bodies, or fusiform condensations scattered throughout the myofilament bundles and basement-like membrane material was observed in these cells.

Biochemical methods

In experiments designed to assess the response of CE metabolizing enzymes or neutral-α-glucosidase activity to PG, Bt_2cAMP, free fatty acids (FFA), or sodium butyrate, smooth muscle cells 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 24 hr. The cell cultures were next washed three times with Dulbecco's phosphate-buffered saline (37°C); the cells were then incubated at 37°C with the factors under investigation in Eagle's MEM containing 1.0 mM MIX (a cAMP phosphodiesterase inhibitor) for 120 min before harvesting and assay of cAMP levels and enzyme activities. Appropriate buffer controls were used containing MIX. Cells from explants used in these experiments were subpassaged twice. In those experiments in which we inhibited endogenous synthesis of PGI_2, cells were incubated with 1.0 mM ASA for 20 min before the addition of PG, FFA, or Bt_2cAMP. Optimal results were observed by treating these cultured cells for this length of time and concentration of ASA (20). To inhibit production of cAMP, 0.5 mM dideoxyadenosine (DDA), a specific inhibitor of adenylate cyclase activity (23, 24), was added to specific wells 20 min before the initiation of the ex-

periment. Optimal results were obtained with this concentration of DDA and time of pre-incubation (20). In addition to the buffer controls for Bt_2cAMP, sodium butyrate was used as a control since butyrate itself may influence cellular metabolism.

Several experiments were also conducted to determine if the activity of a microsomal marker enzyme would be affected by the addition of PG in the range of concentrations used in the CE metabolism studies. Neutral-α-glucosidase was chosen as a microsomal marker enzyme (25). The experimental design for the assay of α-glucosidase activity was similar to the protocol previously described for NCEH and ACAT activities.

Concentration ranges of PG, Bt_2cAMP, FFA, and sodium butyrate were as follows: 12.5–250 nM PGI_2 or 25–250 nM 6-keto $PGF_{1\alpha}$ were prepared in 10 mM Na_2CO_3; for 10–100 μM sodium arachidonate and 20–100 μM Bt_2cAMP, the materials were each dissolved in 10 μl of absolute ethanol and diluted with normal saline (pH 7.0). Sodium eicosatrienoate was dissolved initially in 10 μl of 10 mM Na_2Co_3 and then diluted in normal saline (pH 7.0) to 10–100 μM; sodium butyrate was dissolved in HEPES-buffered saline to 500 nM (pH 7.0). To assess the effects of PG other than those related to PGI_2 metabolism on NCEH and ACAT activities, we tested 25–250 nM PGE_1 and PGE_2. PGE_1 and PGE_2 were initially dissolved in 10 μl of absolute ethanol and then diluted with normal saline (final pH 7.0). Their effects on NCEH and ACAT or neutral-α-glucosidase activities were evaluated in a similar fashion as the other PG. When PG, FFA, sodium butyrate, Bt_2cAMP, or inhibitors were tested, the appropriate ethanol and/or buffer controls were used. The final pH of the reaction medium was 7.2.

Finally, to measure PGI_2 produced by these cells after challenge with sodium arachidonate, the culture 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 (26).

Assay of cAMP

Following removal of the supernatant from the incubations for the assay of 6-keto $PGF_{1\alpha}$ or extracellular cAMP levels, cell cultures designated for intracellular cAMP assays were placed in an ice bath and 2.0 ml of ice-cold (0–4°C) isotonic buffer (250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.2) was added to the wells containing cells. Cells were harvested with a rubber policeman (ca. 2.5×10^5 cells/well) and this suspension was divided into two 1.0-ml aliquots; one aliquot was used for the assay of protein, NCEH, ACAT, and neutral-α-glucosidase activities (see below), while the remaining aliquot was used for the assay of cAMP(20).

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 of ice-cold isotonic sucrose buffer. Cell suspensions (ca. 2.5×10^5 cells) were subsequently transferred to a Duall glass homogenizer. An additional 1.0 ml of isotonic sucrose buffer was added, and cells 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 enzyme activity and protein. Assays of NCEH and ACAT activities were performed on the day of preparations of the homogenate since there is a loss of activity upon freezing. Assays of neutral-α-glucosidase activity and protein were done on samples of cellular homogenates frozen at −70°C. These samples were not stored for longer than 2 weeks.

Assay of neutral CE hydrolase (NCEH) activity

Cholesteryl [1-^{14}C]oleate served as a substrate for the optimal assay of NCEH activity (21). It was prepared as a mixed micelle consisting of cholesteryl oleate, phosphatidylcholine, and sodium taurocholate according to the general procedure of Vahouny, Weersing, and Treadwell (27). This substrate provided highest enzyme activity in cultured aortic smooth muscle cells as compared to other substrates tested (21). Briefly, this substrate was made as follows: 10 μCi of cholesteryl [1-^{14}C]oleate (sp act 55 mCi/mmol), freed of contaminating fatty acids by the extraction procedure of Belfrage and Vaughan (28), was added to a chloroform solution containing 3.8 μmol of phosphatidylcholine and 0.80 μmol of unlabeled cholesteryl oleate. The solvent was removed with a stream of N_2 (g) in a 37°C water bath, and the lipids were resuspended in 8.0 ml of 100 mM potassium phosphate buffer, pH 7.0, containing 2 μmol of sodium taurocholate. The suspension was transferred to a water-jacketed glass vessel maintained at 46°C, and sonicated with a Branson Sonifier/Cell Disruptor, model W-350 (0.5-inch horn) at an output setting of 4 (about 100 watts). The clear solution was centrifuged at 3000 rpm for 10 min to remove any metal fragments released from the sonicator horn. The substrate was stored at 4°C for up to 4 weeks under N_2 (g). A fatty acid standard was prepared similarly, except that [1-^{14}C]oleic acid was substituted for the radioactive cholesteryl oleate. This standard was used with each set of assays to measure the efficiency of extraction of liberated fatty acids following enzymatic hydrolysis.

For the standard assay of NCEH activity, an incubation mixture was prepared by the addition of 50 μl of micellar cholesteryl [1-^{14}C]oleate substrate to 800

μl of 100 mM potassium phosphate buffer, pH 7.0, containing 0.05% bovine serum albumin. The reaction was begun by addition of 150 μl of cell homogenate diluted in homogenization medium (ca. 150 μg protein). The incubation was carried out in stoppered tubes at 37°C for 60 min. Substrate blanks were run under identical conditions with homogenization medium added in place of the enzyme. The principal components of the final reaction mixture were enzyme, 6.0 μM cholesteryl oleate, 23.7 μM phosphatidylcholine, 12.5 μM sodium taurocholate, 0.04% bovine serum albumin, and 85 mM potassium phosphate buffer, pH 7.0. The reaction was stopped, and the unhydrolyzed substrate was removed by addition of 16.3 ml of methanol–choloroform–heptane 1.4:1.3:1.0 (v/v/v), followed by the addition of 5.3 ml of 50 mM potassium carbonate–50 mM potassium borate buffer, pH 10.0. The mixture was vortexed for 5 min, shaken for 30 min, and then centrifuged for 10 min at 2500 rpm to clear the phases. The amount of liberated [1-^{14}C]oleate in the upper aqueous phase was determined by the addition of a 1.0-ml aliquot to 15 ml of Aquasol-2 liquid scintillation cocktail for counting. Quenching was corrected by automatic external standardization. Extraction of the fatty acid standard by the same procedure routinely gave 85–90% of the [1-^{14}C]oleate in the upper phase.

Assay of acyl CoA:cholesterol O-acyltransferase (ACAT) activity

Activity of ACAT was assayed by measuring the synthesis of cholesteryl oleate from radioactive oleoyl CoA employing exogenous free cholesterol which was incorporated into egg lecithin liposomes as described in detail by Hajjar et al. (29). Optimum assay conditions used with cultured aortic smooth muscle cells gave results similar to those findings obtained from aortic tissue homogenates previously described by Hajjar et al. (29).

The ACAT assay was initiated by the addition of 250 μl of diluted cell homogenate (ca. 200 μg protein) to a solution containing 250 μl of the liposomal preparation and 500 μl of 150 mM Tris-HCl buffer, pH 7.4, containing 10 mM mercaptoethanol and 0.05% defatted bovine serum albumin. The final reaction mixture contained enzyme, 8.32 nmol of oleoyl CoA, 10.4 nmol of cholesterol, 0.02 nmol of oleic acid, and 1.1 μmol of egg lecithin in 75 mM Tris-HCl buffer, pH 7.4, containing 5 mM mercaptoethanol and 0.025% bovine serum albumin. The incubation was carried out at 37°C in a Dubnoff shaking water bath for 60 min. Incubation medium with substrate but without homogenate was used as a blank. After incubation, the reaction was stopped with 5 ml of chloroform–methanol 2:1 (v/v) containing 50 μg of unlabeled cholesteryl oleate as a carrier, and the lipid was extracted by the procedures of Folch, Lees, and Sloane Stanley (30). More than 95% of the oleoyl CoA remained in the aqueous phase. The extracted lipids from the organic phase were separated by thin-layer chromatography of silica gel chromatoplates (31). Areas of the plate corresponding to the position of cholesteryl esters were scraped into vials containing 15 ml of Aquasol-2–water 10:1 (v/v) (New England Nuclear) and counted. Quenching was corrected by automatic external standardization.

Assay of neutral-α-glucosidase activity

To examine the effects of PGI_2, 6-keto $PGF_{1\alpha}$, PGE_1, PGE_2, arachidonate, and Bt_2cAMP on a microsomal marker enzyme not related to CE metabolism, neutral-α-glucosidase activity was assayed using the methods described extensively by Peters, Muller, and de Duve (32).

Units of activity

For NCEH, 1 unit of activity corresponds to the hydrolysis of 1 μmol of cholesteryl oleate per min. One unit of ACAT activity corresponds to the esterification of 1 μmol of cholesterol to oleoyl CoA per min. For neutral-α-glucosidase, 1 unit of activity corresponds to the hydrolysis of 1 μmol of 4-methyl umbelliferyl-α-D-glucopyranoside per min. Activities of NCEH and ACAT are expressed as microunits per mg protein. Neutral-α-glucosidase activity is expressed as milliunits per mg protein.

Analysis of protein

Protein content was determined by the method of Lowry et al. (33) with bovine serum albumin as a standard.

Statistical analyses

Mean NCEH and ACAT activities after incubation with increasing concentrations of PGI_2, 6-keto $PGF_{1\alpha}$, PGE_1, PGE_2, FFA, and Bt_2cAMP were compared with the use of a single factor analysis of variance (34). Subsequent pairwise comparisons were performed with the use of Duncan's multiple range test (34). Mean intracellular cAMP concentrations after incubation of cells with PG and MIX were compared also with the use of a single factor analysis of variance.

RESULTS

Incubation of intact cultured aortic cells with 75–250 nM PGI_2 resulted in a significant ($P < 0.05$) twofold increase in NCEH activity by 60 min (**Figs. 1A** and **2A**).

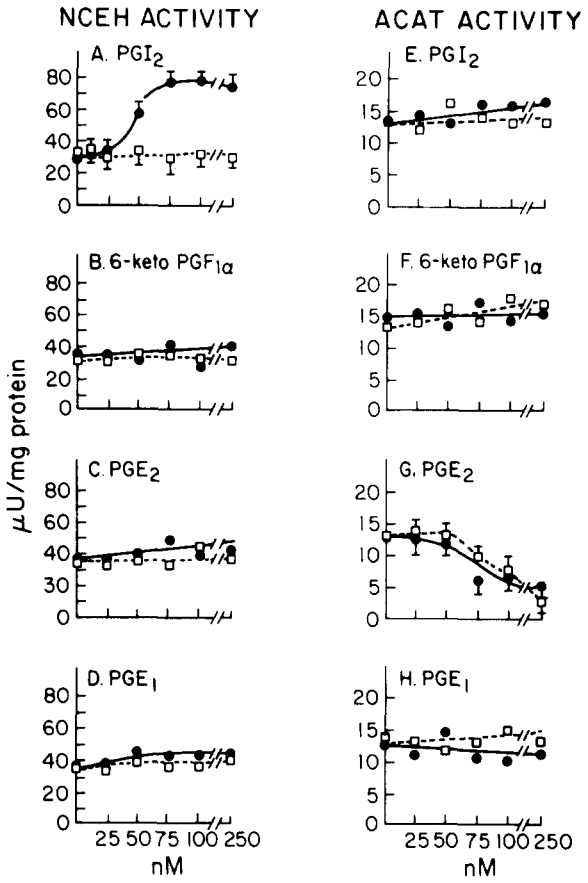

Fig. 1. Effects of PG on NCEH and ACAT activity in cells treated (□ - - - □) and not treated (● —— ●) with 0.5 mM DDA. Each point represents the mean ± SEM from six separate analyses. Cells were incubated with the various concentrations of PG and 1 mM MIX for 2 hr prior to assay of enzyme activities. In those cells treated with DDA, this inhibitor was added to the cultures 20 min prior to the start of the experiment; after which, it remained in the wells with the PG and MIX during the 2-hr experimental period. A significant increase ($P < 0.05$) in NCEH activity was observed following the addition of 75–250 nM PGI_2 (1A); this effect was abolished by treatment with DDA. No effect on NCEH was observed with 6-keto $PGF_{1\alpha}$. In contrast to these findings, ACAT activity was inhibited 60% by 75–250 nM PGE_2 in both DDA-treated and untreated cells (1G) but not by PGI_2 (1E), 6-keto $PGF_{1\alpha}$ (1F), or PGE_1 (1H).

This response was maximal at 90 min and did not change up to 120 min of incubation (Fig. 2A). Neither 6-keto $PGF_{1\alpha}$, PGE_2, nor PGE_1 significantly increased cytosolic CE hydrolytic activity in the range of PG concentrations tested (Fig. 1B–D). In contrast to these findings, only PGE_2 of all the PG tested affected CE synthetic (ACAT) activity (Fig. 1E–H). As shown in Fig. 1G and Fig. 2B, ACAT activity was inhibited approximately 60% after incubation of cells for 120 min with 250 nM PGE_2. Although PGI_2 and PGE_2 affected NCEH and ACAT activities, respectively, these PG did not affect the activity of the microsomal enzyme, neutral-α-glucosidase. The following results were obtained for neutral-α-glucosidase activity (mean ± SE in mU/mg protein): untreated cells, 1.1 ± 0.2; 250 nM PGI_2, 1.6 ± 0.3; 250 nM PGE_2, 1.5 ± 0.2; 250 nM PGE_1, 1.3 ± 0.2; and, 250 nM 6-keto $PGF_{1\alpha}$, 0.9 ± 0.1.

As shown in Fig. 1A, the increase in NCEH activity resulting from the addition of PGI_2 appears to be mediated by increased cAMP levels since pre-incubation of cells with 0.5 mM DDA, an inhibitor of adenylate cyclase activity, abolished the enhancement of NCEH activity by PGI_2. A similar trend was not observed for ACAT activity when DDA was pre-incubated with the cells, since they responded similarly to increasing concentrations of PGE_2 in the presence or absence of DDA (Fig. 1G). Activity of NCEH or ACAT was not altered by DDA alone (Figs. 1A and 1G).

Unlike 6-keto $PGF_{1\alpha}$, PGE_1, and PGE_2, 250 nM PGI_2 significantly ($P < 0.05$) increased the intracellular levels of cAMP in these SMC (Table 1), particularly in the presence of 1.0 mM MIX, a cAMP phosphodiesterase inhibitor. Dose-response analysis indicated that 1.0 mM MIX was the optimal concentration in order to provide maximal levels of intracellular cAMP (**Fig. 3A**). Finally, results in Table 1 show that, in the presence of 0.5 mM DDA, 250 nM PGI_2 did not raise the intracellular cAMP level in these cultured cells which instead maintained the basal level of cAMP. Most of the cyclic AMP remained intracellular for at least 60 min after the initial challenge of the intact cultured cells with 250 nM PGI_2 (Fig. 3B). In this experiment, 1.0 mM MIX was included to enhance detection of elevated cAMP because without it there was only a small increase in cAMP in response to PGI_2 as compared to cells pre-incubated with MIX.

The direct effects of Bt_2cAMP on NCEH activity were examined. Twenty to 100 μM Bt_2cAMP produces a significant ($P < 0.05$) twofold increase in NCEH activity (**Fig. 4A**) following incubation times of 30–120

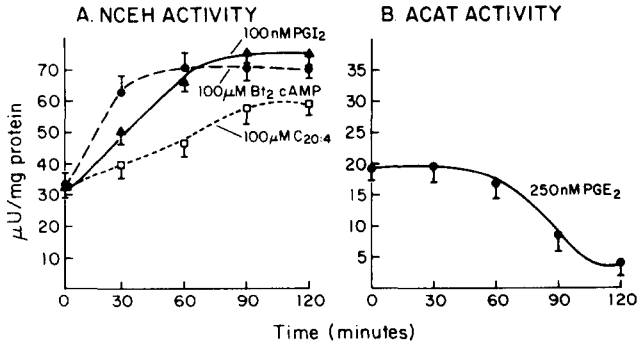

Fig. 2. A. Time course of the stimulatory effect of 100 nM PGI_2 (▲ —— ▲), 100 μM Bt_2cAMP (● - - - ●), and 100 μM sodium arachidonate, $C_{20:4}$ (□ - - - □) on NCEH activity. Routinely, 120-min incubation times were used. B. Time course of the inhibitory effect of 250 nM PGE_2 on ACAT activity. Routinely, 120-min incubation times were used. Each point in panels A and B represents the mean value ± SEM from six separate analyses.

TABLE 1. Effects of PGI_2, 6-keto $PGF_{1\alpha}$, PGE_1, and PGE_2 on intracellular cAMP levels in aortic smooth muscle cells

PG	cAMP Content (pmol/10^5 cells)		
	No Additive	MIX	DDA
PGI_2	14.7 ± 1.4^a	26.3 ± 3.9^{ab}	2.4 ± 0.3
6-Keto $PGF_{1\alpha}$	3.1 ± 0.5	5.8 ± 0.9	2.7 ± 0.4
PGE_1	5.9 ± 0.8	5.4 ± 0.9	3.0 ± 0.4
PGE_2	4.7 ± 0.7	5.3 ± 1.7	3.1 ± 0.8
Basal levels (controls)	3.6 ± 0.5^{ac}	5.8 ± 0.4^{bc}	2.0 ± 0.3

Data represent mean ± SE for four separate analyses. MIX (1.0 mM) or DDA (0.5 mM) were preincubated with cells for 20 min prior to addition of 250 nM PG. Cells were then incubated at 37°C with the inhibitors and PG for 10 min; after which, intracellular cAMP levels were assessed according to methods described in the text. See Fig. 1 for enzyme activities in those cells treated with either MIX and PG together or MIX, DDA, and PG added alone.

[a] Values with the same corresponding letter are significantly ($P < 0.05$) different.

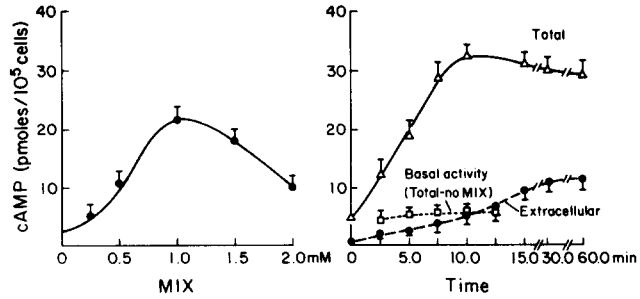

Fig. 3. Left panel: dose response curve for MIX. Optimal concentration of MIX routinely used to inhibit cAMP phosphodiesterase activity was 1.0 mM. Incubations of MIX with the cells were carried out for 2 hr at 37°C. Right panel: time course of total and extracellular cAMP levels produced from aortic smooth muscle cells. Cells were stimulated with 250 nM PGI_2 for 2.5–60 min. Total (intracellular and extracellular; (△ —— △) and extracellular levels (● - - - ●) of cAMP were measured in the presence of 1.0 mM MIX. Basal levels (□ - - - □) of cAMP represent the amounts of cAMP present without the addition of MIX or PGI_2. Data are presented as mean values ± SEM for six separate analyses.

min (Fig. 2A). Increasing concentrations of cAMP alone, in contrast to Bt_2cAMP, did not significantly increase NCEH activity, presumably since cAMP is not transported across the *intact* cell membrane into the cells. Addition of Bt_2cAMP did not significantly affect the activities of ACAT (Fig. 4C) and neutral-α-glucosidase (data not shown) in the range of concentrations used.

To assess the contribution of endogenous PG production to the increase in NCEH activity produced by Bt_2cAMP, cells were treated with 1.0 mM ASA for 20 min prior to the addition of increasing concentrations of Bt_2cAMP (Fig. 4A). Preliminary results had shown that incubation of cultured aortic smooth muscle cells with 1.0 mM ASA for 20 min inhibited PG production for at least 24 hr following the removal of the ASA.[2] Activity of NCEH in ASA-treated cells was less responsive to 12.5–60.0 μM Bt_2cAMP as compared to untreated cells. It is likely that endogenous PGI_2 acting on adenylate cyclase enhances the effect of Bt_2cAMP in augmenting cAMP sufficiently to result in increased NCEH activity. However, the activities were similarly increased in both groups of cells following the addition of 80–100 μM Bt_2cAMP (Fig. 4A).

Because the effects of Bt_2cAMP were modest, sodium butyrate was tested as a control, particularly since butyrate may in itself have profound influences on cellular metabolism. A significant effect of sodium butyrate on NCEH activity could not be demonstrated even in the presence of 1.0 mM MIX to reduce cAMP breakdown. The following results were obtained (mean ± SE in μU NCEH activity/mg protein): untreated cells, 32 ± 4; 500 nM sodium butyrate, 34 ± 5; MIX-treated cells, 33 ± 3; and 500 nM sodium butyrate + MIX, 30 ± 4.

To determine the effects of endogenous PG on CE

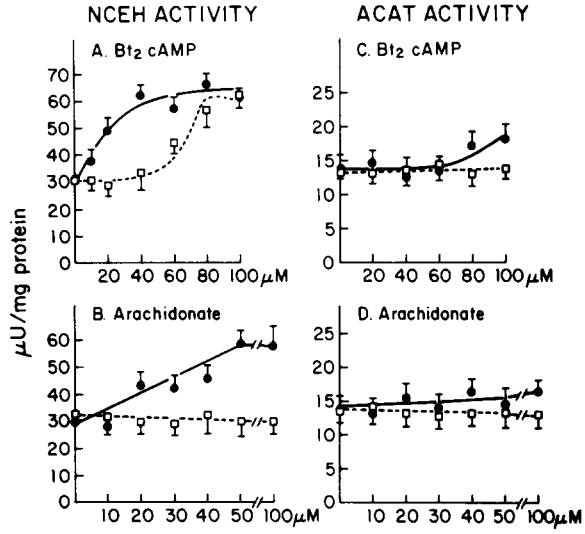

Fig. 4. A. Dose-response relationship for Bt_2cAMP stimulation of NCEH activity in the absence (● —— ●) and presence (□ - - - □) of 1.0 mM ASA after 2 hr of incubation. Activity of NCEH was significantly increased in the presence of 20–100 μM Bt_2cAMP in non-ASA-treated cells; in the presence of ASA, 80–100 μM Bt_2cAMP significantly ($P < 0.05$) increased NCEH activity. Cells were treated with ASA 20 min prior to the addition of Bt_2cAMP. B. Sodium arachidonate dose-response curve. Activity of NCEH was significantly ($P < 0.05$) increased by 50–100 μM sodium arachidonate (● —— ●) but not by sodium eicosatrienoate (□ - - - □) after 2 hr of incubation. C. Addition of Bt_2cAMP in the presence (□ - - - □) or absence (● —— ●) of ASA did not affect ACAT activity. Incubation conditions were the same as in panel A. D. Addition of sodium arachidonate (● —— ●) or sodium eicosatrienoate (□ - - - □) did not affect ACAT activity. Incubation conditions were the same as in panel B. Data presented in all four panels are mean values ± SEM for six separate analyses.

[2] Hajjar, D. P., and B. B. Weksler. Unpublished observations.

metabolism, sodium arachidonate was added to cultured cells (Figs. 4B and 4D). Sodium eicosatrienoate, a substrate for cyclooxygenase (the enzyme that converts arachidonate to PG endoperoxides) but one that cannot be later converted to PGI_2 or 6-keto $PGF_{1\alpha}$, served as a fatty acid control in this experiment (20). Sodium arachidonate (50–100 μM) significantly ($P < 0.05$) enhanced NCEH activity (Fig. 4B) but did not enhance ACAT activity (Fig. 4D) or neutral-α-glucosidase activity (data not shown). The maximal response of NCEH activity to 100 μM arachidonate occurred within a 120-min incubation period (Fig. 2A). No effect was seen on NCEH or ACAT activities upon the addition of sodium eicosatrienoate.

As anticipated, ASA-treated cells significantly decreased PGI_2 production following addition of 50 μM sodium arachidonate (4.1 nM 6-keto $PGF_{1\alpha}$ in control cultures (2×10^5 cells) as compared to 0.2 nM 6-keto $PGF_{1\alpha}$ in ASA-treated cultures) and had significantly decreased NCEH activity: (59.4 ± 4.0 to 29.9 ± 3.6 μU/mg protein). These results suggest that PG must be formed from arachidonate for enhancement of NCEH activity to occur. Although 100 μM exogenous sodium arachidonate did not inhibit ACAT activity (Fig. 4D), 75–250 nM *exogenous* PGE_2 significantly ($P < 0.05$) reduced ACAT activity (Fig. 1G) presumably because exogenous arachidonate may be converted to several PG in rabbit aortic smooth muscle cells.[2]

Similar to the results reported recently from this laboratory (20), the concentrations of PGI_2, PGE_2, and Bt_2cAMP used in this study did not affect cell proliferation (data not shown). In addition, PGI_2, PGE_2, or Bt_2cAMP did not alter the cell number or viability as determined by cell counts and trypan blue exclusion.

DISCUSSION

This study has shown for the first time that PGI_2 and PGE_2 can alter CE metabolism in *intact* aortic smooth muscle cells by affecting NCEH and ACAT activities, respectively.

The ability of PGI_2 to increase NCEH activity appears to be mediated by cAMP. The following data support these conclusions. *1*) Increasing concentrations of PGI_2 produced greater than a twofold increase in NCEH activity. *2*) Sodium arachidonate, at a concentration sufficient to stimulate endogenous PGI_2 production, significantly enhanced CE hydrolytic activity in nonaspirinated smooth muscle cells but not in aspirin-treated cells. *3*) Pretreatment of cells with an inhibitor of adenylate cyclase activity (DDA) abolished the enhancement of NCEH activity produced by exogenous PGI_2. *4*) Addition of Bt_2cAMP to either aspirin-treated or untreated cells also resulted in a twofold increase in NCEH activity.

With respect to CE synthesis, PGE_2 does not appear to inhibit ACAT activity through altering intracellular cyclic AMP levels since we observed that: *1*) this PG inhibited ACAT activity approximately 60% in the presence and absence of DDA (Fig. 1G), and *2*) PGE_2, even at a rather high concentration (0.25 μM), did not alter intracellular levels of cAMP in these aortic cells (Table 1). Alternative mechanism(s) by which PGE_2 affects ACAT activity are currently under investigation.

Previous studies in this laboratory showed that 10–20 nM PGI_2 can increase the activity of the *lysosomal* CE hydrolase by raising intracellular cAMP in intact aortic cells (20). In these studies of similar design, results provided here show that PGI_2 (and arachidonate) can alter *cytosolic* (neutral) CE hydrolytic activity in intact aortic cells. This effect was observed, though, at much higher concentrations of PGI_2 than were required to stimulate CEH activity in lysosomes. We speculate that NCEH may require high levels of endogenous cAMP to be stimulated via protein phosphorylation in intact aortic cells. These findings support the observations of others who have shown that arachidonic acid or PGI_2 can stimulate cAMP production in aortic smooth muscle cells (35), endothelial cells (36), or fibroblasts (37). Moreover, it has been reported that Bt_2cAMP can increase the activity of the neutral CE hydrolase in homogenates of ovarian and adrenal tissue (38–40), and that Bt_2cAMP can reduce the intracellular levels of CE in cultured intimal cells of atherosclerotic human aorta (41). The direct effect of Bt_2cAMP on NCEH activity in intact aortic smooth muscle cells has not been previously described.

Based on the recent findings of Tertov et al. (41), Hajjar et al. (20), and the results reported here, it is reasonable to suggest that the loss of CE from cultured smooth muscle cells of normal or atherosclerotic aortas by the addition of PGI_2 or Bt_2cAMP may be due to increased CEH activity. Interest in the role of cAMP in atherogenesis has spanned several years. For example, Numano and associates (42, 43) have demonstrated that induction of atherosclerosis in rabbits fed a 1% cholesterol diet was associated with reduced aortic intimal concentration of cAMP after 3–4 months on the diet. This atherosclerosis was characterized by the presence of foam cells, significant amounts of connective tissue matrix materials, fatty degeneration, and fibrous lesions. Taken together these findings suggest that as atherosclerosis progresses intracellular pools of cAMP decrease, resulting in less stimulation of CEH activities in the arterial smooth muscle cells and thereby predisposing to increased intracellular lipid (sterol) accumulation.

Other investigations have demonstrated an effect of E or F series PG on CE hydrolytic and synthetic activity in aorta or other types of tissue; however, a mechanism of PG action on enzyme activity was not identified. Using pigeon aorta, Subbiah and Dicke (4) have shown that PGE_1 can inhibit both CE synthesis and hydrolase activity while $PGF_{1\alpha}$ stimulates CE hydrolase activity. Likewise, Berberian, Ziboh, and Hsia (3) demonstrated a dose-dependent inhibition of cholesterol-esterifying activity (presumably ACAT activity) in rabbit aorta by PGE_2, while Subbiah (5) reported no effect of PGE_2 on CE synthetase or microsomal CE hydrolase in pigeon aorta homogenates. Several other tissues have been used to examine the effects of these PG on CE metabolism, including adrenocortical tissue (44), testes (6), and ovarian tissue (45). However, it is noteworthy that, in these experiments, homogenates were prepared prior to the addition of nonphysiological (micromolar) concentrations of PG. Thus, membrane-associated enzyme systems and phosphorylation cascade sequences may not have been intact in the preparations used. Further, the concentrations of PG used were 4–8 times greater than the amounts used in this investigation. In our preliminary experiments, prostaglandins were also added directly to homogenates prior to assay of enzyme activities to determine if nanomolar quantities of PG could affect NCEH and ACAT activities in homogenates as compared with intact cells. No demonstrable effect on these enzyme activities resulted when nanomolar concentrations of PG were added to cell homogenates.

Since PGI_2 increased NCEH activity and PGE_2 decreased ACAT activity, it was also determined if these prostaglandins specifically affected only enzymes involved in CE metabolism. Accordingly, the effects of these PG on a microsomal enzyme not related to CE metabolism were evaluated. Neutral-α-glucosidase activity was not altered by the PG used in this study, indicating that specific enzyme activation can be produced in intact cells by nanomolar concentrations of PG. Other investigators have shown that E and F series PG or elevated cAMP levels can stimulate marker enzyme activities in homogenized tissue (46). However, the PG concentrations used in those studies were much greater (10- to 100-fold differences) than the levels used in this investigation.

The precise physiological significance of PGI_2-mediated elevation of cAMP levels, which in turn leads to altered lipid metabolism, cannot be established by this study. However, from data previously reported on the effects of PGI_2 on lysosomal CE hydrolysis (20) and from those results stated here, PGI_2 and PGE_2 may have an important role in regulation of the intracellular cholesterol/CE balance in rabbit arterial tissue. We conclude that PGI_2 can increase both lysosomal and cytoplasmic CE hydrolytic activity via cAMP but not CE synthetic activity. This may result in increased cholesterol availability as substrate for esterification by ACAT in the cytoplasm. Accordingly, PGE_2, if produced in sufficient quantities, may assist in the regulation of cholesterol esterification by inhibiting ACAT activity.

Several studies indicate that PGI_2 production is decreased in atherosclerotic vessels (15, 16). This may diminish both lysosomal and cytoplasmic CE hydrolytic activities resulting in decreased turnover of CE in vascular tissue. This would favor CE accumulation, which could promote further development of atherosclerotic vascular changes. Similarly, decreased PGE_2 production could promote enhanced cholesterol esterification with the same end result. Clarification of these possible metabolic alterations during experimental atherosclerosis awaits further analysis. Clearly, a more detailed investigation of the role of PG in the control of sterol metabolism, particularly in the presence of increased concentrations of intracellular cholesterol and CE, may provide valuable information about the process of lipid accumulation during diseases such as atherosclerosis.

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