Cholesterol metabolism is altered by hydrolytic metabolites of prostacyclin in arterial smooth muscle cells

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Abstract Cholesteryl esters are the major lipids that accumulate in arteries during atherogenesis. The mechanisms responsible for this lipid accretion have not been completely defined. Our previous experiments have shown that prostacyclin (PGI_2) enhances cholesteryl ester catabolism by increasing cyclic AMP in cultured arterial smooth muscle cells. However, PGI_2 is rapidly degraded under physiologic conditions and endogenous levels of PGI_2 in the human circulation are extremely low. These findings suggest that it is not a circulating hormone. We tested the hypothesis that stable PGI_2 metabolites alter cholesteryl ester metabolism and cellular lipid accumulation. Ten to 100 nM dinor-6-keto $PGF_{1\alpha}$, 13,14-dihydro-6,15-diketo $PGF_{1\alpha}$, and 6,15-diketo $PGF_{1\alpha}$ increased cyclic AMP levels significantly two- to threefold with a concomitant enhancement of both lysosomal and cytoplasmic cholesteryl ester hydrolytic activities. Cholesteryl ester synthesis was unchanged by the PGI_2 metabolites. When cyclic AMP concentrations were maintained at basal levels by an adenylate cyclase inhibitor, no effect on cholesteryl ester hydrolysis was observed following addition of PGI_2 metabolites to the cells. Furthermore, addition of PGI_2 metabolites during a 1-week culture period reduced free and esterified cholesterol by 50%. These data suggest that PGI_2 metabolites: *1*) decrease intracellular cholesterol accumulation by increasing cholesteryl ester catabolism; *2*) act via enhancement of cyclic AMP; and, *3*) may represent circulating regulators of arterial cholesteryl ester metabolism. —**Etingin, O. R., B. B. Weksler, and D. P. Hajjar.** Cholesterol metabolism is altered by hydrolytic metabolites of prostacyclin in arterial smooth muscle cells. *J. Lipid Res.* 1986. **27:** 530–536.

Supplementary key words cholesteryl esters • cyclic AMP • $PGF_{1\alpha}$ • atherogenesis • cholesteryl esterase

Lysosomal and cytosolic compartmentalization of cholesteryl esters is observed in arterial smooth muscle cells during cholesteryl ester storage disease and atherogenesis (1, 2). This lipid accumulation can result from an inactive cholesteryl esterase (1, 2) and/or enhanced endocytosis of low density lipoprotein-cholesteryl esters (2).

Recently, we showed that several eicosanoids, including prostacyclin (PGI_2) and 6-keto PGE_1, but not PGE_1 or PGE_2, are involved in the regulation of cholesteryl ester hydrolysis within vascular smooth muscle cells (3, 4). These compounds increase lysosomal and cytoplasmic cholesteryl esterase activities by stimulating adenylate cyclase, raising intracellular levels of cyclic AMP (4). The net effect is to decrease intracellular cholesteryl ester accumulation by increasing cholesteryl ester hydrolysis and promoting cholesterol excretion (3). The activation of cytoplasmic cholesteryl esterase by cyclic AMP occurs by a protein kinase-dependent mechanism in adrenocortical cells (5), adipocytes (6), arterial smooth muscle cells (7), and macrophages (8).

The role of PGI_2 in diseases associated with lipid accumulation is undefined. Since PGI_2 is labile under physiologic conditions ($t_{1/2}$ = 3–5 min), and the level of PGI_2 in human blood (< 5 pg/ml) is lower than that required for its known actions (9–15), PGI_2 itself is unlikely to be a circulating regulator of arterial cholesterol metabolism. Identification of several stable metabolites of PGI_2 in human urine (16, 17) led us to postulate that PGI_2 may act in vivo through its stable metabolites to modulate cholesterol homeostasis. For this reason, we examined the effects of several PGI_2 metabolites, namely, dinor-6-keto $PGF_{1\alpha}$, 13,14-dihydro-6,15-diketo $PGF_{1\alpha}$, and 6,15-diketo $PGF_{1\alpha}$, on intracellular cyclic AMP levels, lysosomal and cytoplasmic cholesteryl esterase activities, and free and esterified cholesterol accumulation in cultured arterial smooth muscle cells.

Abbreviations: ACAT, acyl CoA:cholesterol O-acyltransferase; DDA, 2'5' dideoxyadenosine; HDL, high density lipoprotein; LDL, low density lipoprotein; PGI_2, prostacyclin.

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MATERIALS AND METHODS

Materials

[1-^{14}C]Cholesteryl oleate (sp act 55 mCi/mmol), [1-^{14}C]oleic acid (sp act 40 mCi/mmol), ^{125}I-labeled cyclic AMP radioimmunoassay (RIA) kits, and Aquasol-2 liquid scintillation fluid were obtained from New England Nuclear Corp., Boston, MA. Unlabeled cholesteryl ester and egg lecithin were obtained from Supelco Inc., Bellefonte, PA. Acetylsalicylic acid, fatty acid-free bovine serum albumin, 1-methyl-3-isobutylmethylxanthine, neutral alumina (WN-3), 4-methylumbelliferyl-β-D-galactopyranoside monohydrate, 4-methoxyethanol, sodium taurocholate, and 2'5' dideoxyadenosine were obtained from Sigma Chemical Company, St. Louis, MO. Thin-layer silica gel chromatoplates (K6; 250 μm thick) were obtained from Whatman, Inc., Clifton, NJ. Nanograde quality organic solvents were obtained from Mallinckrodt, Inc., Science Products Division, St. Louis, MO. PGI_2, 6-keto $PGF_{1\alpha}$, dinor-6-keto $PGF_{1\alpha}$, 13,14-dihydro-6,15-diketo $PGF_{1\alpha}$ and 6,15-diketo $PGF_{1\alpha}$ were gifts from Dr. John Pike of the Upjohn Co., Kalamazoo, MI.

Disposable tissue culture materials were purchased from Corning Glass Works, Corning, NY. Dulbecco's modified Eagle's medium (MEM) and Fungizone (250 μg/ml) were obtained from Flow Laboratories, Inc., McLean, VA. L-Glutamine (200 mM), penicillin (5000 IU/ml), streptomycin (5 mg/ml) and fetal bovine serum (heat-inactivated) were purchased from Gibco Laboratories, Grand Island, NY.

Tissue culture

Bovine smooth muscle cells were obtained from bovine thoracic arteries supplied by a local abattoir. Smooth muscle cells were cultured from arterial explants following the removal of adventitial tissue according to Ross (18). Cells cultured from thoracic arteries were confirmed to be smooth muscle cells by their growth pattern as observed by phase contrast microscopy and ultrastructural characteristics by transmission electron microscopy (3).

Experimental design and biochemical methods

To assess cholesteryl ester metabolic activity in response to prostaglandins, 2×10^5 smooth muscle cells were placed per well in 6-well, 35-mm diameter cluster plates in Dulbecco's MEM with 10% fetal calf serum. Cells were allowed to adhere for 24–48 hr. Cell monolayers were then washed twice with Dulbecco's MEM without serum. Aspirin was added to designated wells at a final concentration of 0.1 mM for 20 min prior to prostaglandin addition in order to inhibit endogenous PGI_2 production (3). Eicosanoids were prepared in 10 mM Na_2CO_3 after initial dilution in 10–20 μl of acetone. Control wells contained buffer plus a similar volume of acetone. Concentrations of PGI_2 metabolites (0–100 nM) were added to cells for 2 hr (the optimal time period for activation, as determined in preliminary studies) at 37°C prior to harvesting and assay of enzyme activities. This time period of 2 hr was chosen because it was within the range of optimal activation of cyclic AMP in these cells by the PGI_2 metabolites. Cells used in these experiments were subpassaged two to four times. When required, 2'5' dideoxyadenosine (DDA), an adenylate cyclase inhibitor (19), was added at a final concentration of 0.5 mM for 30 min prior to addition of prostaglandins to maintain cyclic AMP at basal levels following addition of the prostaglandins.

Isobutylmethylxanthine (MIX), a cyclic AMP phosphodiesterase inhibitor (3, 4), was also added to selected wells at a final concentration of 1.0 mM for 20 min prior to prostaglandin addition in order to prevent cyclic AMP breakdown and thus maximize intracellular cyclic AMP levels. This inhibitor was prepared in HEPES–Hanks buffer (pH 7.0), by sonication over ice for 15 min.

Experiments were also conducted to determine whether the activities of various cellular enzymes unrelated to cholesterol metabolism were also altered by the addition of prostaglandins. Activities of neutral-α-glucosidase, a microsomal marker enzyme, and β-galactosidase, a lysosomal marker enzyme, were assayed by previously published methods (20).

Assay of intracellular cyclic AMP

Intracellular levels of cyclic AMP in arterial smooth muscle cells were measured by radio immunoassay as previously described (3).

Preparation of cell homogenates

For the assay of enzyme activities, cells were harvested by scraping, as previously described, following aspiration of the incubation medium and addition of 2.0 ml of ice-cold isotonic sucrose buffer (3). Cell preparations were homogenized over ice for 30 sec and aliquots were taken for the assays of lysosomal cholesteryl esterase activity, cytoplasmic cholesteryl esterase activity, cholesteryl ester synthetic (ACAT) activity, marker enzyme activities, and protein content. Protein was determined by the method of Lowry et al. (21) with bovine serum albumin as standard.

Lysosomal (acid) cholesteryl esterase activity

Lysosomal cholesteryl esterase activity in arterial smooth muscle cells was assayed at pH 3.9 as described by Haley, Fowler, and de Duve (22).

Cytoplasmic (neutral) cholesteryl esterase activity

Cholesteryl [1-^{14}C]oleate was used as substrate at a final reaction concentration of 6.0 μM in a mixed micelle of egg lecithin/Na taurocholate/cholesteryl oleate to assay cytoplasmic cholesteryl esterase activity in arterial smooth muscle cells as previously described by Hajjar et al. (4, 7).

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Acyl CoA:cholesterol O-acyltransferase activity (ACAT)

Activity of microsomal ACAT was assayed by measuring the synthesis of cholesteryl oleate from radioactive oleoyl CoA and exogenous free cholesterol (4, 23). Oleoyl CoA and cholesterol were prepared as unilamellar liposomes as described by Hajjar et al. (23).

Units of activity

For lysosomal and cytoplasmic cholesteryl esterase and ACAT, 1 unit of activity corresponds to 1 nmole of substrate hydrolyzed or esterified per min. For marker enzymes, 1 unit of activity corresponds to 1 nmole of substrate hydrolyzed per hr. Activities were expressed as units per milligram of protein.

Effect of eicosanoids on free and esterified cholesterol accumulation

The effects of PGI_2 metabolites on total cholesterol accumulation were assessed in arterial smooth muscle cells cultured over a 1-week period (3). Cells were plated as described above. On days 0, 3, and 6 of the experiment, cells were re-fed with Dulbecco's MEM containing 10% fetal calf serum and 0.1% bovine serum albumin to which prostaglandins were then added to a final concentration of 100 nM. Cells were harvested at days 0, 4, and 7 to assess total cholesterol content. Control cultures were similarly maintained, but only the buffer used to prepare the prostaglandin stocks was added to the control cells at refeeding. Cell viability and ultrastructural characteristics were not altered by prostaglandin treatment. Before cells were harvested, they were washed twice with serum-free medium. The cellular lipids then were extracted twice in situ with hexane–isopropanol 3:2 (v/v) (24). Lipid extracts were stored at −70°C under N_2 (g) until lipid analyses were performed (within 1 week). Cells were removed from the wells following lipid extraction by the addition of 1 ml of 0.2 N NaOH for 1 hr (25). Aliquots were taken for protein determination (21).

Lipid analysis

Analysis of cholesterol and cholesteryl ester in arterial smooth muscle cells was done after evaporating samples to dryness under N_2(g). Samples were resuspended in 100–200 μl of chloroform–methanol 2:1 (v/v). The extract and a lipid standard containing cholesterol and cholesteryl ester (1 μg/μl) were fractionated separately by thin-layer chromatography (26). A two-solvent system was used to separate these sterols (26). These lipids were subsequently quantitated by scanning microfluorometry (27).

Statistical analysis

Mean values for enzyme activities following the addition of increasing concentrations of prostaglandins were compared with the use of single factor analysis of variance (28). Activities of lysosomal and cytoplasmic cholesteryl esterase in the presence of DDA and the lipid quantitation data were compared separately with the use of a two-factor analysis of variance (28).

RESULTS

The effects of increasing concentrations (0–100 nM) of three PGI_2 metabolites on cholesteryl ester metabolism in cultured bovine arterial smooth muscle cells are shown in **Fig. 1 (A–C)**. Concentrations (20–100 nM) of dinor 6-keto $PGF_{1\alpha}$, 13,14-dihydro-6,15-diketo $PGF_{1\alpha}$, or 6,15-diketo $PGF_{1\alpha}$, when added separately to smooth muscle cells, enhanced cholesteryl ester hydrolytic activities 2- to 3-fold compared to control cells treated with buffer alone ($P < 0.05$) (Fig. 1A, 1B). Cholesteryl ester synthetic (ACAT) activity was unchanged by the addition of these PGI_2 metabolites (Fig. 1C).

Activities of lysosomal and microsomal marker enzymes, β-galactosidase and neutral-α-glucosidase, respectively, were not altered by prostaglandin addition, indicating that the effect of these eicosanoids at the concentrations used appears specific for cholesteryl ester catabolic activity. β-Galactosidase in control cultures was 0.40 ± 0.08 units/mg of protein; in cultures treated with 20 nM dinor-6-keto $PGF_{1\alpha}$, 0.44 ± 0.10 units/mg of protein. Neutral-α-glucosidase in control cultures was 1.97 ± 0.14 units/mg of protein; in cultures treated with 20 nM dinor-6-keto $PGF_{1\alpha}$, 2.24 ± 0.29 units/mg of protein.

Intracellular cyclic AMP levels in these cells were doubled over baseline values by the addition of each of the PGI_2 metabolites to a final concentration of 20 nM (**Fig. 2**). As reported earlier (4), 6-keto $PGF_{1\alpha}$ did not increase cyclic AMP levels nor did it alter cytoplasmic cholesteryl ester metabolism. Lower concentrations of PGI_2 metabolites used in this study did not have a significant effect on cyclic AMP levels. Generally, higher concentrations of PGI_2 metabolites produced similar effects on cyclic AMP as did 20 nM, indicating that a final concentration of 20 nM produced a maximal response in cyclic AMP levels in these subcultured cells.

To determine whether the increase in CE hydrolytic activities by the stable metabolites of PGI_2 depended on increases in intracellular cyclic AMP, cells were exposed to 0.5 mM dideoxyadenosine (DDA), prior to the addition of the eicosanoids. Cells pretreated with DDA were assayed for cholesteryl ester hydrolytic activities after exposure to prostaglandins for 2 hr (**Table 1**). Little change from base-line levels of cholesteryl ester hydrolytic activity following addition of PGI_2 metabolites was observed ($P < 0.05$) in DDA-pretreated cells compared to control cells, indicating that enhancement of cholesteryl ester hydrolysis by these eicosanoids requires

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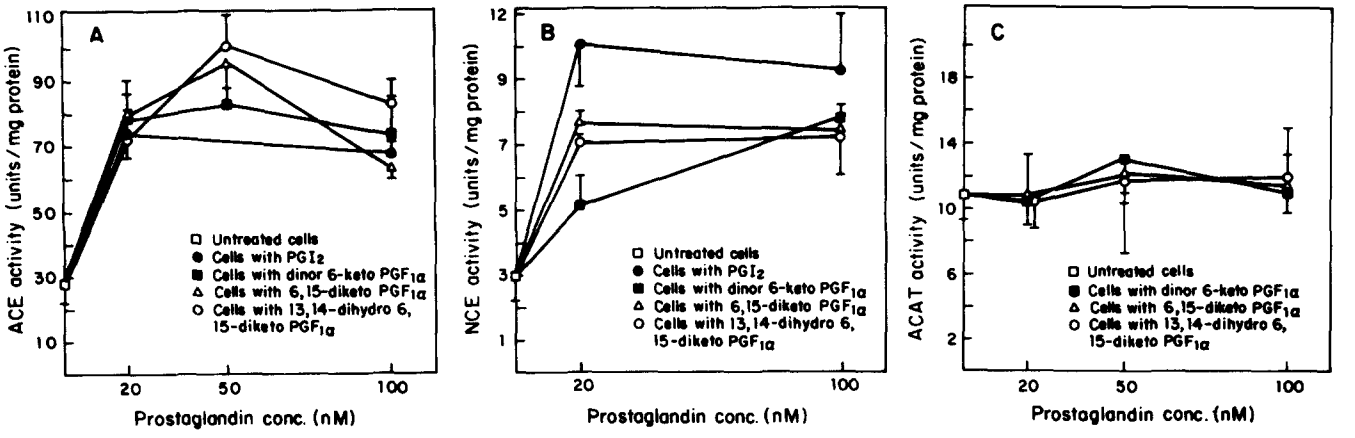

Fig. 1. Effect of PGI_2 metabolites on cholesteryl ester hydrolytic and synthetic activities in arterial smooth muscle cells. After incubation with 0.1 mM aspirin for 20 min, arterial cells were incubated with PGI_2 metabolites for 2 hr. Cells were harvested and enzyme activities were measured as outlined in Methods. Each of the PGI_2 metabolites significantly ($P < 0.05$) increased (A) lysosomal (ACE) and (B) cytoplasmic (NCE) cholesteryl esterase activities 2- to 3-fold. No effect was observed by the addition of 6-keto $PGF_{1\alpha}$ (data not shown). The PGI_2 metabolites did not significantly affect ACAT activity (C). Each point represents the mean ± SD for four separate experiments. Enzyme assays were performed in triplicate for each individual experiment.

increased levels of intracellular cyclic AMP.

To determine whether the increased cholesteryl esterase activities altered the cholesterol and cholesteryl ester content over a longer time period, smooth muscle cells were repeatedly exposed to the prostaglandin metabolites during 1 week of culture. **Fig. 3** depicts the free and esterified cholesterol content of cells following 1 week of treatment with PGI_2 metabolites added every 3 days at a final concentration of 100 nM. Although non-lipid-laden smooth muscle cells contained low levels of esterified cholesterol, prostaglandin-treated cells had a significantly ($P < 0.05$) lower cholesterol and cholesteryl ester content than untreated cells. No significant reduction in total cholesterol content was observed over 1 week in cells treated with buffer alone.

DISCUSSION

We have demonstrated for the first time that the stable metabolites of PGI_2, dinor-6-keto $PGF_{1\alpha}$ 13,14-dihydro-6,15-diketo $PGF_{1\alpha}$, and 6,15-diketo $PGF_{1\alpha}$, alter cholesterol metabolism in arterial smooth muscle cells. Each of these three metabolites increased cholesteryl esterase activity 2- to 3-fold by raising cyclic AMP (Figs. 1 and 2). The enhancement in cholesteryl esterase activity did not parallel the extent of cyclic AMP elevation; rather it appeared that a threshold effect of cyclic AMP was obtained, with a certain elevated level of cyclic AMP needed for enzyme activation, but that further increased cyclic AMP had little further augmenting effect.

The enhancement of cholesteryl ester hydrolytic activities by these PGI_2 metabolites was similar to that produced by PGI_2 itself (3, 4). Although we have shown that PGI_2 and its metabolite 6-keto PGE_1 stimulate arterial cholesteryl ester hydrolysis (3, 4), these metabolites of PGI_2 have not previously been shown to be biologically active except as weak platelet disaggregators (29, 30). When we tested this metabolite on gel-filtered platelets, concentrations of 250 nM or greater were required to inhibit platelet aggregation (data not shown).

Eicosanoid production in vascular cells is regulated by the availability of arachidonic acid, calcium, and cyclic AMP (31). Lipoproteins may also regulate PGI_2 bio-

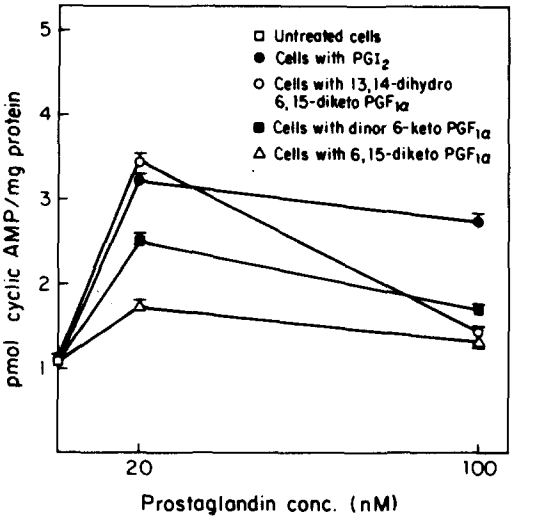

Fig. 2. Effect of PGI_2 metabolites on cyclic AMP in arterial smooth muscle cells. After incubation with 0.1 mM aspirin for 20 min, arterial cells were incubated with PGI_2 metabolites for 2 hr. Cells were harvested and intracellular cyclic AMP levels were measured. Each of the PGI_2 metabolites significantly ($P < 0.05$) increased cyclic AMP in these cells. Each point represents the mean ± SD for four separate experiments in which each assay was done in duplicate.

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TABLE 1. Effect of PGI_2 metabolites on cholesteryl ester hydrolase activity in the presence or absence of an adenylate cyclase inhibitor, DDA

Treatment	Lysosomal Cholesteryl Esterase		Neutral Cholesteryl Esterase	
	− DDA	+ DDA	− DDA	+ DDA
	units/mg protein			
Untreated cells	23.7 ± 1.4	27.0 ± 4.9	2.5 ± 0.6	2.4 ± 0.7
20 nM PGI_2	52.6 ± 3.3	30.8 ± 3.4	8.8 ± 1.2	3.9 ± 1.5
20 nM dinor-6-keto $PGF_{1\alpha}$	62.8 ± 7.5	26.2 ± 3.8	5.8 ± 0.8	3.4 ± 1.0
20 nM 6,15-diketo $PGF_{1\alpha}$	63.7 ± 3.7	34.9 ± 3.0	7.4 ± 0.9	3.1 ± 1.1
20 nM 13,14-dihydro-6,15-diketo $PGF_{1\alpha}$	73.8 ± 1.4	31.3 ± 3.6	7.0 ± 1.9	2.9 ± 1.4

Cultured cells were incubated for 2 hr in the presence or absence of 0.5 mM DDA in addition to the prostaglandins. Cholesteryl esterase activities were measured as described in the Methods section. PGI_2 and its stable metabolites significantly ($P < 0.05$) enhanced lysosomal cholesteryl esterase activity only in the absence of DDA, indicating that these eicosanoids stimulate CE hydrolysis by increasing cyclic AMP levels. Values shown represent the mean ± SD for three separate experiments in which each assay was done in triplicate.

synthesis. It has been shown that human HDL can enhance PGI_2 production in cultured endothelial cells presumably by contributing arachidonic acid (32). Conversely, human LDL-lipid peroxides can decrease PGI_2 production in aortic slices in vitro (33). We assayed PGI_2 in supernatants from cells treated with stable metabolites to ascertain whether such treatment increased PGI_2 production by these cells. PGI_2 was not different in prostaglandin-treated and non-aspirin-treated control cells (data not shown), indicating that the metabolites do not enhance cholesteryl esterase activities by augmenting endogenous PGI_2 production.

The degradation of PGI_2 in human tissue involves production of several PGI_2 metabolites (13, 34, 35) with 6-keto $PGF_{1\alpha}$ postulated to be a common intermediate (13), as demonstrated in human platelets (36), isolated perfused rabbit liver (30), and in animal studies (37). In humans, increased dinor-6-keto $PGF_{1\alpha}$ has been found in urine of patients with severe atherosclerotic disease (17). Increased eicosanoid production by atherosclerotic vessels may be a response to increased platelet activation and platelet–vessel wall interactions (17).

The level of PGI_2 circulating in human plasma is controversial, but clearly low. Estimates of measurable 6-keto $PGF_{1\alpha}$ range from 5 to 200 pg/ml plasma (< ca. 0.40 nM) (13–16). The concentrations of the stable metabolites used in this study ranged from 80 to 100 pg/ml plasma (ca. 0.20 nM) (12). In the microenvironment near the vascular wall, much higher concentrations of these substances probably are present for several reasons. *1*) 15-Hydroxyprostaglandin dehydrogenase, an enzyme that catalyzes the conversion of 6-keto $PGF_{1\alpha}$ to these metabolites, has been found within the vessel wall (38), and *2*) both endothelial and vascular intimal smooth muscle cells can produce significant amounts of PGI_2 locally, in the range of 1–10 nM (39, 40). Therefore, the circulating levels of PGI_2 and its metabolites may not accurately reflect amounts produced within arteries or at the arterial surface (40, 41).

The mechanism for enhanced cholesteryl ester hydrolysis by the other PGI_2 metabolites studied here is consistent with that previously reported, i.e., stimulation of adenylate cyclase leading to increased cyclic AMP production (3, 4). Increased levels of cyclic AMP made available to cyclic AMP-dependent protein kinases can augment phosphorylation of the cytoplasmic cholesteryl esterase, leading to increased activity (4). On the other hand, lysosomal cholesteryl esterase activity is

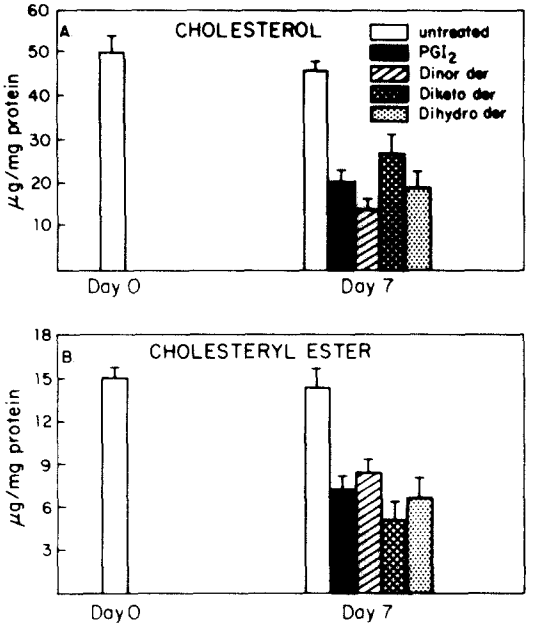

Fig. 3. Effect of PGI_2 metabolites on cholesterol and cholesteryl ester accumulation in arterial smooth muscle cells. Arterial cells were challenged three times with 100 nM of the PGI_2 metabolite (final concentration) during the 1-week experimental period. The PGI_2 metabolites used in this study were dinor-6-keto $PGF_{1\alpha}$, 6,15-diketo $PGF_{1\alpha}$, and 13, 14-dihydro-6,15-diketo $PGF_{1\alpha}$. Each of the PGI_2 metabolites significantly ($P < 0.05$) reduced the cholesterol and cholesteryl ester content of these cells. Lipid levels in control cells were unchanged during the experimental period. Range bars indicate mean ± SD for three separate experiments in which each assay was done in quadruplicate.

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directly stimulated by cyclic AMP and does not require protein kinase activation (Hajjar, D. P., et al., unpublished observations).

Cholesterol accumulation and excretion from arterial smooth muscle cells are altered in human atherogenesis (2). Decreased activities of lysosomal and cytoplasmic cholesteryl esterase may be responsible for cholesteryl ester accumulation (7, 42). In this study, we have observed that stable PGI_2 metabolites enhanced cholesteryl ester hydrolytic activities and diminished cholesterol accumulation in cultured bovine arterial smooth muscle cells. While the biologic impact of these PGI_2 metabolites in promoting cholesterol mobilization from human arteries remains to be determined, other stable PGI_2 metabolites such as carbocyclin or 6-β-PGI_1 have been reported to decrease the cholesteryl ester content from fatty streaks and plaques in human arteries (43). The data reported here suggest a potential mechanism by which PGI_2 metabolites modulate vascular lipid accumulation. This biologic activity of stable PGI_2 metabolites supports the hypothesis that these eicosanoids may have a potential role in the control of atherosclerosis.

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