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Research Article

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Increased Hydrolysis of Cholesteryl Ester with Prostacyclin Is Potentiated by High Density Lipoprotein through the Prostacyclin Stabilization

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Abstract

Prostacyclin (PGI_2) has been reported to stimulate activities of acid cholesteryl ester hydrolase (ACEH; EC 3.1.1.13) and neutral cholesteryl ester hydrolase (NCEH; EC 3.1.1.13) in the smooth muscle cells leading to a decrease in intracellular cholesteryl ester. Recently, we have found that the half-life of PGI_2 was prolonged through stabilization by HDL. HDL is known to have anti-atherogenic properties, although its precise mechanism has not been fully clarified. We therefore hypothesized that HDL can exert anti-atherogenic action by augmenting PGI_2-stimulated increases in the activities of ACEH and NCEH. After incubation with PGI_2 and HDL, a cell homogenate was made from which the activities of ACEH and NCEH were assessed. HDL significantly augmented the PGI_2-induced increase in the activities of both enzymes. This effect of HDL was abolished in the absence of PGI_2. Elevated intracellular levels of cyclic AMP were maintained for longer periods by HDL. The increase in both intracellular cyclic AMP levels and enzyme activities disappeared in the presence of an inhibitor of adenylate cyclase, 2'5'-dideoxyadenosine. Radiolabeled smooth muscle cells demonstrated a significant loss in total cholesterol and cholesteryl ester after treatment with PGI_2 and HDL, due to the increase in cholesteryl ester hydrolytic activities. These data suggest that HDL enhanced the PGI_2-stimulated hydrolysis of cholesteryl ester and augmented the PGI_2-induced reduction of cellular cholesteryl ester content by stabilizing PGI_2. (*J. Clin. Invest.* 1990. 86:1885–1891.) Key words: HDL • PGI_2

Introduction

During the progression of human atherosclerosis, cholesteryl ester (CE)[1] accumulates in arterial smooth muscle cells (1, 2). This lipid accumulation can result from inadequate CE hydrolysis and/or enhanced endocytosis of low density lipoprotein-cholesteryl esters.

There are several enzymes participating in the regulation of cholesterol/cholesteryl ester balance in cells, lysosomal (acid) and cytosolic (neutral) CE hydrolase (ACEH, NCEH; EC 3.1.1.13), which hydrolyze CE, and microsomal acyl CoA: cholesterol-*O*-acyltransferase (ACAT; EC 2.3.1.26), which esterifies cholesterol.

It has been reported that PGI_2 stimulates cholesteryl ester hydrolysis within smooth muscle cells. PGI_2 increases ACEH and NCEH activities, raising the intracellular levels of cAMP (3, 4). PGI_2 has no effect on ACAT activity (3, 4). HDL is known to be antiatherogenic (5–8). We have recently demonstrated that apolipoprotein A-I, a main constituent protein of HDL, stabilizes PGI_2 in the blood (9). PGI_2 is stabilized by HDL-associated apolipoprotein A-I, but not by free apolipoprotein A-I (10). Therefore, these findings raise the possibility that HDL enhances PGI_2-induced lysosomal and cytosolic cholesteryl ester hydrolase activation and the reduction of cellular CE content by stabilizing PGI_2. This study was performed to prove this hypothesis. We present herein data showing that PGI_2 with HDL can significantly maintain high levels of intracellular cAMP in cultured intact arterial smooth muscle cells (SMC), and that this enhancement of cAMP causes a concomitant increase in the activities of ACEH and NCEH, leading to a reduction in the cellular content of cholesteryl ester.

Methods

Materials. Cholesteryl oleate, oleic acid, 3-isobuthyl-1-methylxanthine (IBMX), 2'5'-dideoxyadenosine (DDA), and sodium taurocholate were obtained from Sigma Chemical Co. (St. Louis, MO). Eicosatetraynoic acid (ETYA) was purchased from Cayman Chemical Co. (Ann Arbor, MI). 1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride was from Pierce Co. (Rockford, IL). Cholesteryl-[1-^{14}C]oleate (2109.0 GBq/mmol), [1-^{14}C]oleate (2109.0 GBq/mmol), and [1,2,6,7-^{3}H]cholesteryl-linoleate (3330.0 GBq/mmol) were obtained from DuPont-New England Nuclear Research Products (Boston, MA). Egg lecithin, acetylsalicylic acid (ASA), and *N,N*-dimethyl-1,3-propanediamine were purchased from Wako Pure Chemical Co., (Tokyo, Japan). PGI_2 was a generous gift from ONO Pharmaceutical Co. (Osaka, Japan). All reagents were of analytical grade.

Disposable tissue culture materials were from Corning Glass Work (Corning, NY); DME was from Nissui Pharmaceutical Co. (Tokyo, Japan); FCS was from M. A. Bioproduct (Walkersville, MD).

Cell culture. Bovine smooth muscle cells were obtained from bovine thoracic aorta supplied by a local abattoir. Smooth muscle cells were cultured from arterial explants after the removal of adventitial tissue (11). 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. Bovine arterial SMC were subpassaged 2–6 times in Dulbecco's MEM with 10% fetal calf serum. Freshly isolated rabbit SMC were prepared from thoracic aorta by the method of Haley et al. (12). Rabbit SMC were subpassaged on an average of 1–2 times before use.

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1. *Abbreviations used in this paper:* ACAT, acyl CoA: cholesterol-*O*-acyltransferase; ACEH and NCEH, acid and neutral cholesteryl ester hydrolase; ASA, acetylsalicylic acid; CE, cholesteryl ester; cLDL, cationized LDL; DDA, dideoxyadenosine; ETYA, eicosatetraynoic acid; IBMX, 3-isobuthyl-1-methylxanthine; PGI_2, prostacyclin; SMC, smooth muscle cell.

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Preparation of lipoproteins and cationized LDL. HDL (d = 1.063–1.210), LDL (d = 1.019–1.063), and VLDL (d = 0.95–1006) were isolated from normal human plasma by sequential ultracentrifugation in a Beckman L8-70M ultracentrifuge (Beckman Instruments Inc., Fullerton, CA) using a 60 Ti rotor at 4°C (13). HDL and LDL were dialyzed against 200 vol of 150 mM NaCl, 0.01% EDTA, and 10 mM Tris-HCl, pH 7.4 at 4°C. The purity of HDL and LDL was controlled by SDS-PAGE under reducing conditions.

Cationized LDL (cLDL) was prepared from LDL by attachment of *N,N*-dimethyl-1,3-propanediamine to aspartate and glutamate residues of native LDL using 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride as a catalyst at pH 6.5. Cationized LDL radiolabeled with [^{3}H]cholesteryl linoleate was prepared by a reconstitution method in which the endogenous neutral lipids of cLDL were extracted with heptane and replaced with [^{3}H]cholesteryl linoleate (14).

Cell incubation with PGI_2 and HDL. To assess cholesteryl ester hydrolytic activity in response to PGI_2 with or without HDL, 2×10^5 smooth muscle cells per well were placed in 35-mm diameter plates containing the medium. Cells were allowed to adhere for 24–48 h. Cell monolayers were washed twice with Dulbecco's MEM without serum. ASA was added to designated wells at a final concentration of 0.1 mM for 20 min before PGI_2 addition in order to inhibit endogenous PGI_2 production. IBMX, a phosphodiesterase inhibitor, was added to cells at a final concentration of 1.0 mM for 20 min before PGI_2 (with or without HDL) addition to maximize intracellular cAMP levels. When required, to inhibit the production of cAMP, 0.5 mM DDA, a specific inhibitor of adenylate cyclase activity, was added to specific wells 20 min before the initiation of the experiment. PGI_2 was prepared in 10 mM sodium carbonate. Cells were incubated with various concentrations of PGI_2 (0–120 nM) and HDL (0–60 mg cholesterol/dl) for 2 h (the optimal period for activation) at 37°C before harvesting and assaying of enzyme activities.

Assay of intracellular cAMP. After the supernatant was removed, cells were harvested in 1 ml of isotonic sucrose buffer consisting of 250 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.0), and this suspension was divided into two parts of 0.5 ml aliquots: one aliquot was used for assaying of ACEH and NCEH activities and protein concentration, while the remaining aliquot was used for the cAMP assay. The aliquot for the cAMP assay was treated with 50% TCA to a final concentration of 5% TCA. This preparation was homogenized and the TCA-precipitable cell debris was removed by centrifugation at 2,230 *g* for 15 min at 4°C. Assays of intracellular levels of cAMP in the arterial SMC were done by means of a sensitive radioimmunoassay method with Yamasa cyclic AMP assay kits (Yamasa, Chiba, Japan) (15). Dioxane (0.1 ml) containing succinic anhydride and triethylamine was added to 0.1 ml of a sample in order to succinylate cyclic nucleotides in the sample. After 10 min, the mixture was diluted with 0.8 ml of 0.3 M imidazole buffer (pH 6.5). After being kept at 4°C overnight, the unbound radioactive nucleotide was absorbed by dextran-coated charcoal. The radioactivity in the supernatant containing the antibody-bound radioactive nucleotide was counted in a gamma counter (Gamma 5500; Beckman Instruments Inc.).

Preparation of cell homogenates. Cell monolayers were washed twice in 250 mM sucrose containing 1 mM EDTA and 10 mM Tris-HCl, pH 7.0. Cells were then harvested by a rubber policeman, homogenized in 0.5 ml of the same buffer, disrupted with 15 strokes in a small homogenizer (Dounce Co., Vineland, NJ), and centrifuged for 10 min at 150 *g*. The supernatant was removed with a Pasteur pipette and kept ice cold.

ACEH assay. Acid cholesteryl ester hydrolase activity was determined by the procedure of Haley et al. (12). Cholesteryl [1-^{14}C]oleate (0.37 MBq) was added to 500 μl of benzene containing 1.27 μmol of cold cholesteryl oleate and 127 μmol of egg lecithin. The solvents were removed by evaporation under a nitrogen stream, followed by lyophilization of the lipid for at least 1 h to insure all traces of the solvent had been removed. The lipids were resuspended by thorough vortex mixing into 10 ml of 100 mM KCl containing 10 mM Tris-HCl buffer, pH 7.0, and 3 mM sodium azide. This suspension was transferred to a 15-ml water-jacketed glass vessel maintained at 45°C, and sonicated for 12 min at an output of 4 (~ 100 W) (Tomy Seiko Co., Tokyo, Japan). The resulting opalescent suspension was centrifuged at 1,000 *g* for 15 min to remove any aggregated lipid and metal fragments released from the sonicator horn. The preparation was stored at 4°C and used within 2 wk.

In the standard assay, a substrate mixture was prepared by the addition of one part lecithin-cholesteryl oleate in four parts of 125 mM Na acetate buffer, pH 3.9, containing 5.0 mM Na taurocholate. The cell homogenates were diluted in sucrose buffer containing 0.1 mg digitonin/ml, and incubated for 10 min at 0°C. The reaction was started by the addition of 150 μl of the diluted enzyme to 20 μl of the substrate solution. Incubation was carried out at 37°C for 60 min. The final reaction mixture contained enzyme (200 μg protein), 1.27 mM lecithin, 12.7 μM cholesteryl oleate, 2.0 mM sodium taurocholate, 0.005% digitonin, and 50 mM sodium acetate buffer, at pH 3.9. The reaction was stopped by the addition of 3.25 ml of benzene/chloroform/methanol (1.0:0.5:1.2, vol/vol/vol) containing unlabeled oleic acid as a carrier (16). Then, 0.6 ml of 0.6 N NaOH was added. The samples, in screw-cap test tubes, were shaken together for 30 min and then centrifuged at room temperature to clear the phases. The unhydrolyzed substrate was contained in the lower phase. A 0.5-ml aliquot of the upper aqueous phase was added to 4 ml of scintillant (ACS II; Amersham Corp., Arlington Heights, IL) and counted by a liquid scintillation counter (LS-1701; Beckman Instruments Inc.). The radioactive free fatty acid standard, [1-^{14}C]oleate, was counted before and after extraction and the efficiency of extraction was determined to be ~ 90%. Data were corrected by subtracting nonspecific hydrolysis of the substrate measured in the absence of cell homogenate.

NCEH assay. Cholesteryl [1-^{14}C]oleate was also used as a substrate (17). A mixed micelle of cholesteryl oleate/egg lecithin/sodium taurocholate, prepared by a modification of the procedure described by Vahouny et al. (18), yielded the highest enzyme activity. This preparation was made as follows: 0.37 MBq of cholesteryl [1-^{14}C]oleate were added to a chloroform solution containing 3.8 μmol of egg lecithin and 0.80 μmol of unlabeled cholesteryl oleate. The solvent was removed with a stream of nitrogen gas, and the lipids were resuspended in 8.0 ml of 100 mM potassium phosphate buffer, pH 7.0, containing 2 μmol of sodium taurocholate, and sonicated as described in the ACEH assay. For the standard assay, 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 adding 150 μl of homogenate. The incubation was carried out in stoppered tubes at 37°C, for 60 min. The components of the final reaction mixture were enzyme (200 μg protein), 6.0 μM cholesteryl oleate, 23.7 μM egg lecithin, 12.5 μM sodium taurocholate, 0.04% bovine serum albumin, and 85 mM potassium phosphate buffer, pH 7.0. Released [1-^{14}C]oleic acid was separated and counted as described in the previous section.

Evaluation of PGI_2 half-life. The stability of PGI_2 was determined by a quantitative high performance liquid chromatographic method (19). Samples were injected onto a YMC A-314 C18 column (6 mm × 30 cm; Yamamura Chemical Industry, Kyoto, Japan). The mobile phase was a methanol/boric acid buffer (40 mM, pH 8.9) = 55:45 (vol/vol) with a flow rate of 1.0 ml/min. This method separates and quantitates both PGI_2 and 6-keto-$PGF_{1\alpha}$ based on their characteristic absorptions at 204 nm.

Effect of PGI_2 and HDL on the cellular cholesterol content. Reconstituted cLDL containing [^{3}H]cholesteryl linoleate was prepared. Cells were exposed to reconstituted-[^{3}H-cholesteryl-linoleate]cLDL for 7 d with one media change at 4 d. Cells were washed and then exposed to serum-free DME alone, or serum-free DME containing PGI_2 (40 nM) and/or HDL (50 mg/dl as cholesterol) in the presence of ASA and IBMX. Total cholesterol content was measured as cellular radioactivity after 24 h (20), media were removed, and cells were washed and lipids extracted into hexane/2-propanol (3:2 vol/vol). The distribution of radioactivity in SMC lipids was determined by thin layer chromatography on silica gel 60 F (21). After digestion of delipidated cells in

0.2 N NaOH, aliquots were taken for the determination of cellular protein concentration.

Determination of protein and cholesterol concentrations. Protein was determined by the method of Lowry et al. using bovine serum albumin as a standard (22). Cholesterol was determined with an analyzing system by Wako Pure Chemical Co. (Tokyo, Japan).

Statistical analysis. Values are expressed as mean±SEM. Multiple comparison test with two way analysis of variance was used for the statistical analysis. For data in Table I, Student's *t* test was used. A *P* value less than 0.05 was considered as significant.

Results

Cultured bovine smooth muscle cells were incubated with PGI_2 for the indicated times and cell homogenates were made, from which the activities of ACEH and NCEH were assessed as described in Methods. As shown in Fig. 1, the activities of ACEH and NCEH increased with the incubation time. They reached a peak at 120 min and then declined to nearly basal levels by 180 min. The addition of HDL (50 mg/dl as cholesterol) to the incubation mixture significantly ($P < 0.05$) increased the activities without changing the time of peak activity. In the following experiments, peak activities at 120 min were used.

Preincubation with PGI_2 caused a dose-dependent increase in the activity of ACEH and NCEH isolated from SMC. The activity of the enzymes was significantly enhanced with the addition of HDL in the presence of ASA (Fig. 2). This effect was also observed in the pretreatment of ETYA, a dual cyclooxygenase and lipoxygenase inhibitor (23) (data not shown).

To investigate the effects of HDL on PGI_2-stimulated enzyme activation, HDL was given simultaneously with PGI_2. As shown in Fig. 3, HDL increased the activities of both enzymes in a dose-dependent manner. The elevation of the enzyme activity was nearly maximal around 50 mg/dl of HDL. In these experiments, PGI_2 was used at the concentration where the enzyme activity increased by ~ 50% (10 and 40 nM for ACEH and NCEH, respectively). In the absence of PGI_2, HDL did not increase the activity of either enzyme (Fig. 3). Enzyme activities were determined after the incubation with PGI_2, at the concentrations indicated in Fig. 2, in the presence or absence of HDL (50 mg/dl as cholesterol). This level of HDL was chosen for the experiment because it evoked a nearly maximal response at 10 nM PGI_2 (Fig. 3). As shown in Fig. 2, HDL significantly shifted the dose-response curves upward.

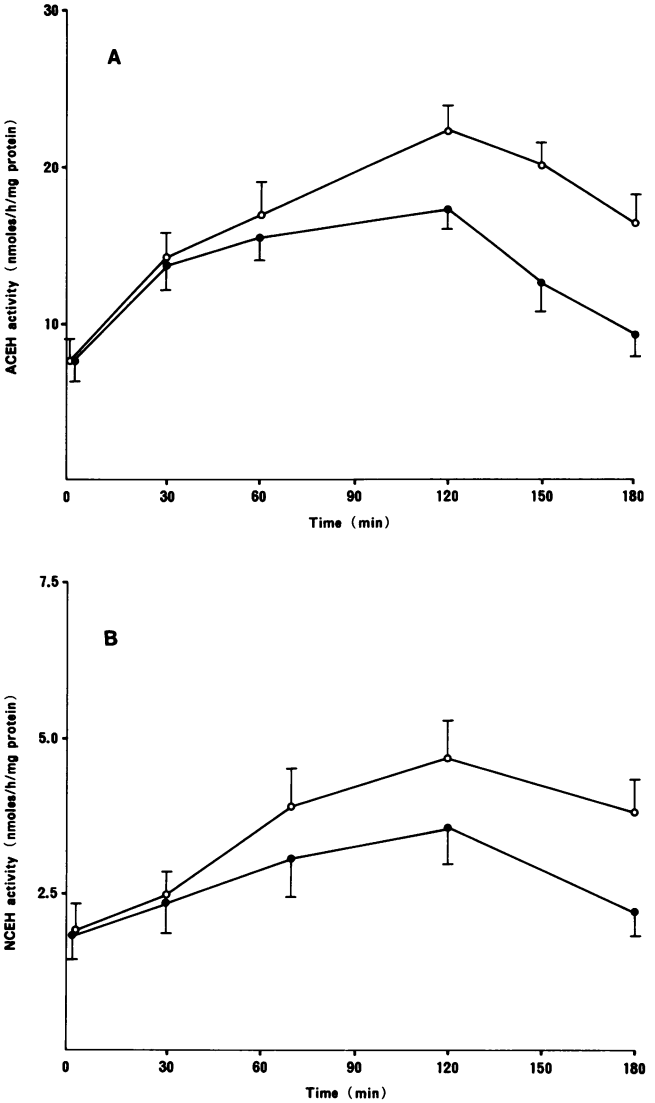

Figure 1. Time course of ACEH and NCEH activities in cultured bovine smooth muscle cells. (*A*) Cultured bovine aortic smooth muscle cells were incubated with 10 nM PGI_2 in the presence (○) or absence (●) of HDL (50 mg/dl as cholesterol) for the times indicated on the abscissa. Then, ACEH was prepared and its activity measured. Significant enhancement ($P < 0.05$) in ACEH activity was observed in the presence of HDL. (*B*) NCEH activity was determined after incubation with 40 nM PGI_2 in the presence (○) or absence (●) of HDL (50 mg/dl as cholesterol). HDL significantly ($P < 0.05$) enhanced the enzyme activity. Data are mean±SEM ($n = 3$) and are representative of two experiments performed on different cell isolates.

Table I. Effect of PGI_2 and/or HDL on Cellular Content of [^3H]Cholesteryl Ester and Total [^3H]Cholesterol

	Decrease of cellular [^{3}H]cholesteryl ester content	Decrease of cellular total [^{3}H]cholesterol content
	nmol/mg protein per 24 h	*nmol/mg protein per 24 h*
PGI_2	11.6±0.8	1.4±0.7
HDL	13.6±1.0	12.8±0.2
PGI_2 + HDL	32.2±1.6*	18.0±1.7*

Arterial SMC were incubated with reconstituted [^{3}H-cholesteryl linoleate]-cationized LDL for 7 d. Cells were washed and exposed to serum free Dulbecco's MEM alone or to the same medium containing PGI_2 (40 nM) and/or HDL (50 mg/dl as cholesterol) for 24 h. The cellular [^{3}H]cholesteryl ester and total [^{3}H]cholesterol were extracted and determined by thin layer chromatography. The cellular [^{3}H]cholesteryl ester and total [^{3}H]cholesterol contents decreased by 6.1±1.3 and 0.3±0.8 (nmol/mg cell protein per 24 h) in control cells, respectively. Data were corrected by subtracting these values and were expressed as mean±SEM of four separate analyses. Comparisons are as follow: * $P < 0.01$, PGI_2 + HDL vs. HDL or PGI_2. In the presence of both PGI_2 and HDL, the reduction in the cellular content of [^{3}H]cholesteryl ester and total [^{3}H]cholesterol was greater than the combined reduction induced by each substance (32.2±1.6 vs. 25.2±2.5, 18.0±1.7 vs. 14.2±1.0; $P < 0.05$).

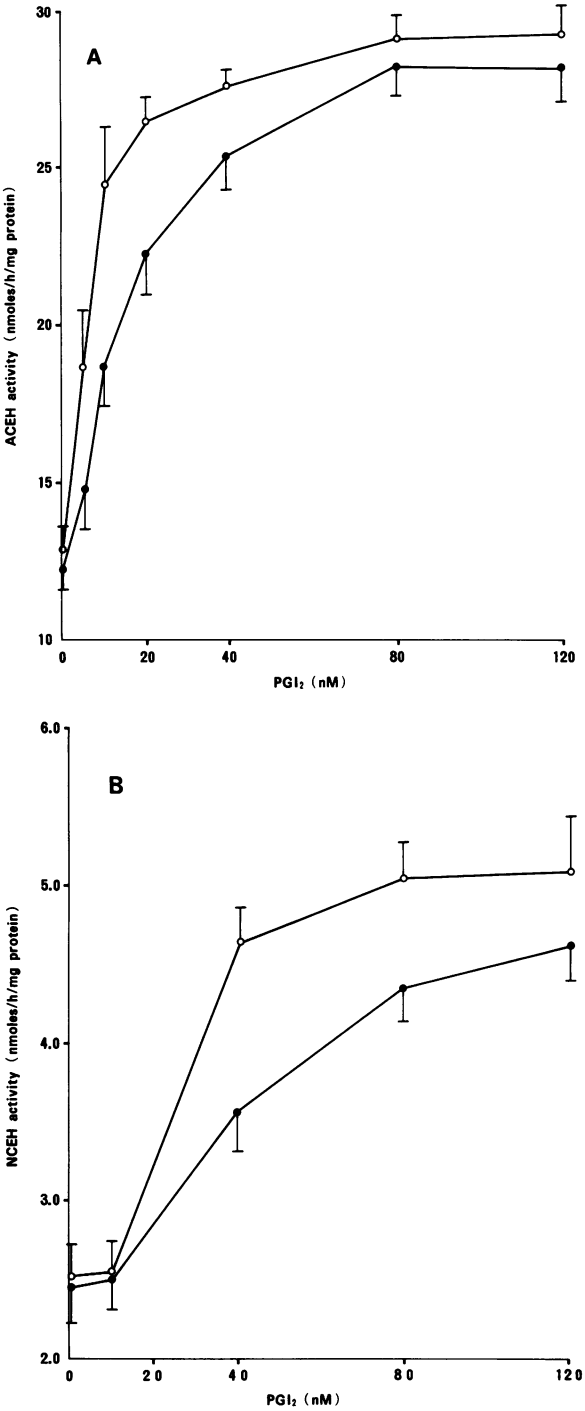

Figure 2. Enhancement of PGI_2 dose-dependent increase in ACEH and NCEH activities by HDL. Cultured smooth muscle cells were incubated for 120 min with PGI_2 at various concentrations in the presence (○) or absence (●) of HDL (50 mg/dl as cholesterol). Then, ACEH (*A*) or NCEH (*B*) activity was measured. HDL significantly ($P < 0.05$) shifted both dose response curves upward. Data are mean±SEM of mean values obtained from five separate experiments where duplicate samples were measured.

Thus, HDL was found to augment PGI_2-stimulated CE hydrolysis.

We have already demonstrated that HDL is a serum factor that can stabilize an unstable PGI_2 (9). Under the present experimental conditions, we confirmed that HDL prolonged the half-life of PGI_2 from 5 to 26 min (Fig. 4). The prolongation of the PGI_2 half-life was not seen with either LDL or VLDL. These results suggest that the effect on PGI_2 is specific to HDL (Fig. 4).

It is known that PGI_2 enhances CE hydrolase activity through the elevation of intracellular cyclic AMP levels. It is expected that augmentation of PGI_2-induced enzyme activation by HDL is also mediated through the cyclic AMP pathway. Therefore, we investigated the effect of HDL on the intracellular cAMP levels. Cyclic AMP levels rose markedly after incubation with PGI_2 and reached a peak at 15 min. Then, they declined progressively and reached near basal levels by 120 min (Fig. 5, *closed circles*). On the other hand, in the presence of HDL the cyclic AMP levels remained elevated at 120 min, although the peak levels did not increase furthermore (Fig. 5, *open circles*). These data suggest that the augmentation of the PGI_2-induced increase in CE hydrolytic activities by HDL depends on the enhancement of intracellular cAMP levels. To further confirm this point, an adenylate cyclase inhibitor, DDA, was employed to suppress the production of intracellular cyclic AMP. Cultured SMC were pretreated with DDA and incubated with PGI_2 in the presence or absence of HDL. As illustrated in Fig. 6, DDA treatment abolished the PGI_2-stimulated increases in ACEH and NCEH activities either in the presence or absence of HDL. When DDA was added to the assay mixture after preparation of the enzyme, it did not alter the activity of either enzyme (data not shown). These data indicate that enhancement of enzyme activity strictly depends on intracellular cyclic AMP levels.

The cellular cholesterol level was examined after 24 h of PGI_2 and/or HDL treatment using cultured rabbit SMC to determine whether or not increased activity of cholesteryl ester hydrolases could alter the cellular content. The SMC cholesterol pool was labeled by exposure to cLDL containing [^{3}H]-cholesteryl linoleate. Then, cells were incubated in serum free-DMEM or medium containing PGI_2 and/or HDL. HDL progressively decreased the cellular content of both [^{3}H]cholesteryl ester and total [^{3}H]cholesterol. On the other hand, PGI_2 caused a reduction in [^{3}H]cholesteryl ester alone. This is considered to be due to the absence of cholesterol acceptors in the media. In the presence of both PGI_2 and HDL, the reductions in the cellular content of [^{3}H]cholesteryl ester and total [^{3}H]cholesterol were greater than the combined reduction induced by each substance (Table I).

Discussion

Population studies have shown an inverse correlation between plasma HDL levels and the incidence and prevalence of atherosclerosis (5–8). This suggests that HDL is an anti-atherogenic factor. HDL is considered to exert its anti-atherogenic effect through reverse cholesterol transport (24, 25). HDL traps free cholesterol at the cell surface followed by reesterification and incorporation into the HDL core. Incorporated cholesterol is transferred and delivered to liver cells with eventual clearance. We have recently demonstrated that apolipoprotein A-I, a main constituent protein of HDL, can stabilize PGI_2 in circulating blood (9). PGI_2 has potent anti-aggregating and vasodilating activity (27). In addition, PGI_2 is known to elevate the activities of both lysosomal (acid) and cytoplasmic

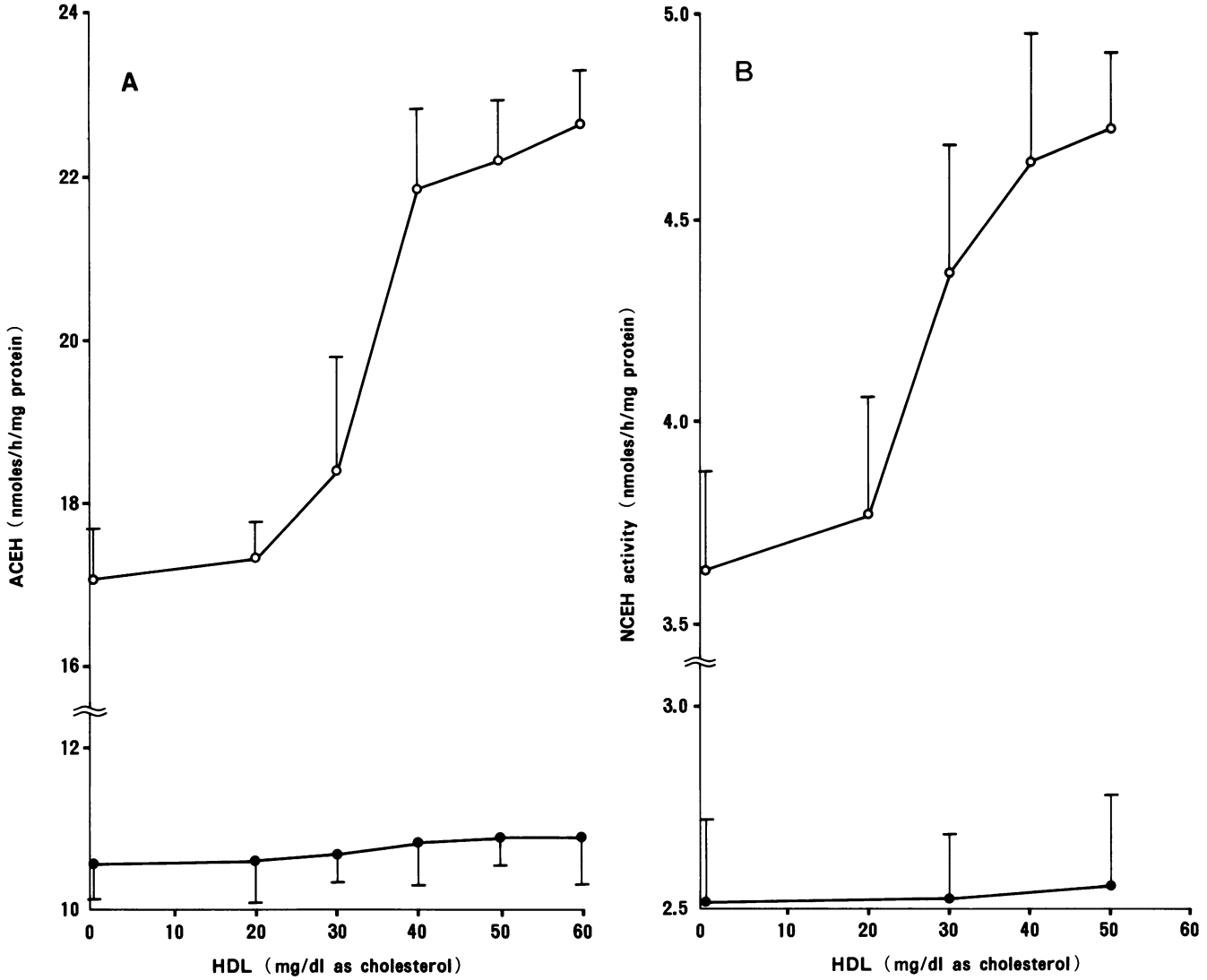

Figure 3. HDL increases dose-dependently the activities of both ACEH and NCEH in the presence of PGI_2. Smooth muscle cells were exposed to 0.1 mM ASA for 20 min and then incubated for 120 min with HDL at the concentrations indicated on the abscissa as milligrams of cholesterol/dl in the presence (○) or absence (●) of 10 nM PGI_2. After incubation, the activities of ACEH (*A*) and NCEH (*B*) were assayed. Data are presented as mean±SEM of four separate analyses.

(neutral) CE hydrolases leading to hydrolysis of esterified cholesterol accumulated in the smooth muscle cells (3, 4). These effects of PGI_2 are thought to be mediated by increases in intracellular cyclic AMP levels. Neutral CE hydrolase is activated through phosphorylation induced by cyclic AMP-dependent protein kinase (28, 29), whereas acid hydrolase is independent of such kinase (3). Taken together, these findings lead us to postulate the enhancement or prolongation of PGI_2-induced hydrolysis of cholesteryl esters as another mechanism of anti-atherogenic action of HDL. To prove this hypothesis, we have performed this study using cultured bovine arterial smooth muscle cells.

Under the present conditions, HDL prolongs the half-life of PGI_2, maintains increased cyclic AMP levels evoked by PGI_2, and enhances PGI_2-stimulated CE hydrolytic activities in smooth muscle cells. In the absence of PGI_2, HDL itself has no effect on CE hydrolytic activity. Furthermore, enhancement of hydrolase activities was abolished in the presence of an adenylate cyclase inhibitor, DDA, suggesting that the activities of both enzymes are strictly dependent on cyclic AMP levels. Thus, these data support our hypothesis on a new potential mechanism of the anti-atherogenic action exerted by HDL.

After stimulation with PGI_2, the intracellular cyclic AMP concentration increased rapidly and reached a peak around 15 min. On the other hand, the hydrolase activities increased rather slowly with a peak around 120 min. This time lag between the peaks of cyclic AMP and enzymes may represent the time required for de novo synthesis or enzyme phosphorylation.

HDL can stimulate PGI_2 production by cultured cells when arachidonic acid is provided as a substrate (29, 30). However, it is unlikely that HDL stimulates CE hydrolase activities through this mechanism under the present conditions, because endogenous PGI_2 production was inhibited with aspirin pretreatment. The possibility that HDL stimulates cholesteryl ester hydrolases by providing arachidonic acid to

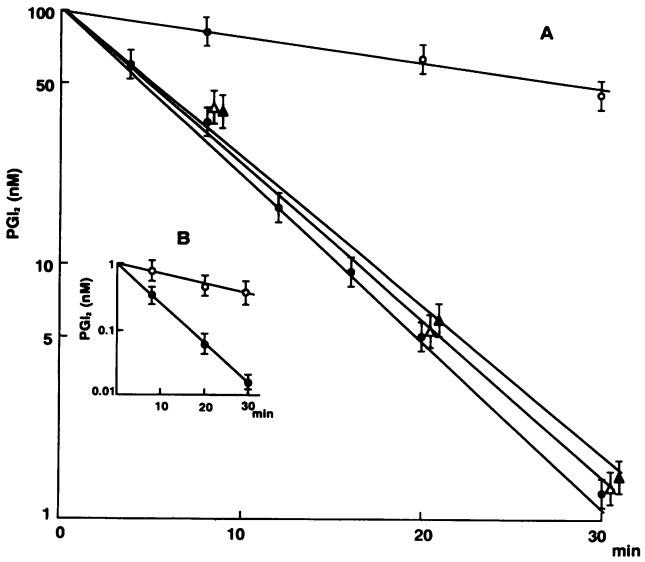

Figure 4. Effect of HDL, LDL, and VLDL on PGI_2 half-life. (*A*) The half-life of PGI_2 was measured in serum-free Dulbecco's MEM, pH 7.4 (●), or in the same buffer including HDL (○) (50 mg/dl as cholesterol), LDL (△) (200 mg/dl), and VLDL (▲) (20 mg/dl) as described in Methods. HDL significantly ($P < 0.05$) prolonged the half-life of PGI_2, but LDL and VLDL did not. (*B*) The basal level of PGI_2 was estimated to be 1 nM after 2 h incubation in SMC (2×10^5 cells/well). At this concentration of PGI_2, HDL significantly prolonged the half-life of PGI_2. Data are mean±SEM of five separate determinations.

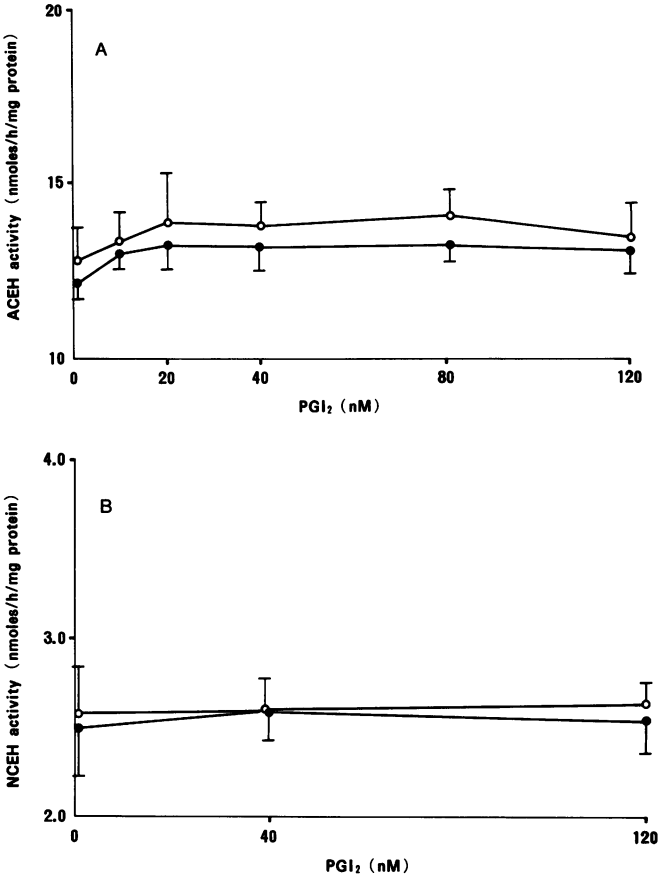

Figure 6. Adenylate cyclase inhibitor, DDA, abolishes the PGI_2-induced increase in ACEH or NCEH activity. Smooth muscle cells were preincubated for 20 min with 0.5 mM DDA followed by the addition of PGI_2 at the concentrations indicated on the abscissa in the presence (○) or absence (●) of HDL (50 mg/dl as cholesterol). After incubation, the activities of ACEH (*A*) and NCEH (*B*) were determined. Data are mean±SEM of four separate analyses.

the lipoxygenase pathway is also excluded, because ETYA did not inhibit the effect of HDL on PGI_2-induced CE hydrolase activities.

HDL enhances the PGI_2-induced increase in the activities of acid (lysosomal) and neutral (cytoplasmic) CE hydrolase activities through the stabilization of unstable PGI_2. Hydrolysis of lysosomal and cytoplasmic CE releases free cholesterol from lysosomes and cytoplasma, respectively. This free cholesterol is either reesterified in the cytoplasma by ACAT to form CE droplets again, or is transported by sterol carrier proteins to the cell surface for excretion. HDL-mediated release of unesterified cholesterol can also inhibit the activity of the intracellular esterifying enzyme, ACAT (31, 32). Therefore, HDL facilitates egress of free cholesterol from CE accumulated in the cell to the plasma membrane. Free cholesterol is trapped by HDL at the cell surface. Thus, the net effect of HDL is a decrease in the intracellular CE (33).

HDL augmented PGI_2-induced cholesteryl ester depletion not only by acting as a cholesterol acceptor, but by enhancing PGI_2-induced cholesteryl ester hydrolysis through stabilizing PGI_2. These results suggest that HDL plays an anti-atherogenic role through the stabilization of PGI_2.

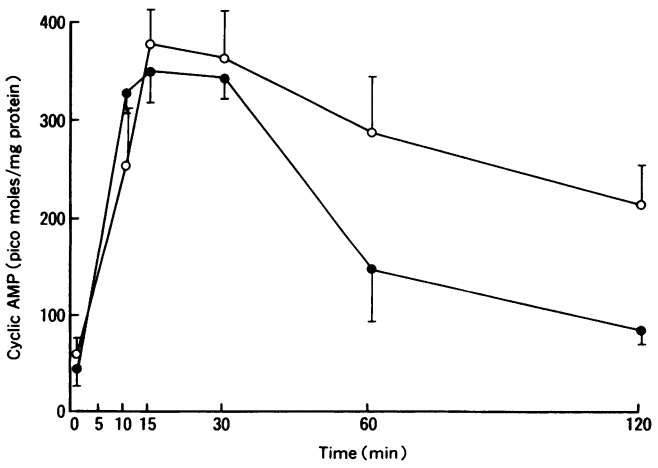

Figure 5. Time course of intracellular cyclic AMP levels. Cultured smooth muscle cells were incubated with 40 nM PGI_2 for the indicated times on the abscissa in the presence (○) or absence (●) of HDL (50 mg/dl as cholesterol). Then, intracellular cyclic AMP levels were determined as described in Methods. HDL significantly increased the intracellular cyclic AMP concentrations and maintained them at higher levels. Data are mean±SEM of four separate analyses.

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References

1. Portman, O. W. 1970. Arterial composition and metabolism: esterified fatty acids and cholesterol. *Adv. Lipid Res.* 8:41–114.

2. St. Clair, R. W. 1976. Metabolism of the arterial wall and atherosclerosis. *Atheroscler. Rev.* 1:61–117.

3. Hajjar, D. P., B. B. Wecksler, D. J. Falcone, J. M. Hefton, K. Tack-Goldman, and C. R. Minick. 1982. Prostacyclin modulates cholesteryl ester hydrolytic activity by its effect on cyclic adenosine monophosphate in rabbit aortic smooth muscle cells. *J. Clin. Invest.* 70:479–488.

4. Hajjar, D. P., and B. B. Wecksler. 1983. Metabolic activity of cholesteryl ester in aortic smooth muscle cells is altered by prostaglandin I_2 and E_2. *J. Lipid Res.* 24:1176–1185.

5. Miller, G. J., and N. E. Miller. 1975. Plasma-high-denity-lipoprotein concentration and development of ischaemic heart disease. *Lancet.* i:16–19.

6. Norum, R. A., J. B. Lakier, S. Goldstein, A. Angel, R. B. Goldberg, W. D. Block, D. K. Noffze, P. J. Dolphin, J. Edelglass, D. D. Bogorad, et al. 1982. Familial deficiency of apolipoprotein A-I and C-III and precocious coronary-artery disease. *N. Engl. J. Med.* 306:1513–1519.

7. Green, B. S., G. Heiss, B. M. Rifkind, G. R. Cooper, D. Williams, and H. A. Tyroler. 1985. The ratio of plasma high density lipoprotein cholesterol to total and low density lipoprotein cholesterol: age related change and race and sex differences in selected North American populations. *Circulation.* 72:93–104.

8. Gordon, D. J., J. Knoke, J. L. Probesfield, R. Superko, and H. A. Tyroler (for The Lipid Research Clinics Program). 1986. High density lipoprotein cholesterol and coronary heart disease in hypercholesterolemic men: the lipid research clinics coronary primary prevention trial. *Circulation.* 74:1217–1225.

9. Yui, Y., T. Aoyama, H. Morishita, M. Takahashi, Y. Takatsu, and C. Kawai. 1988. Serum prostacyclin stabilizing factor is identical to apolipoprotein A-I (apo A-I). *J. Clin. Invest.* 82:803–807.

10. Aoyama, T., Y. Yui, H. Morishita, and C. Kawai. 1990. Prostaglandin I_2 half-life regulated by high density lipoprotein is decreased in acute myocardial infarction and unstable angina pectoris. *Circulation.* 81:1784–1791.

11. 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.

12. 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.

13. Havel, R. J., H. A. Elder, and J. H. Brogton. 1955. The distribution and chemical composion of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34:1345–1353.

14. Krieger, M., M. S. Brown, J. R. Faust, and J. L. Goldstein. 1978. Replacement of endogenous cholesteryl esters of low density lipoprotein with exogenous cholesteryl linoleate. *J. Biol. Chem.* 25:4093–4101.

15. Steiner, A. L., C. W. Perker, and M. K. Kipnis. 1972. Radioimmunoassay for cyclic nucleotides. *J. Biol. Chem.* 247:1106–1120.

16. Pittman, R. C., J. C. Khoo, and D. Steinberg. 1975. Cholesterol esterase in rat adipose tissue and its activation by cyclic adenosine 3':5'-monophosphate-dependent protein kinase. *J. Biol. Chem.* 250:4505–4511.

17. Hajjar, D. P., C. R. Minick, and S. Fowler. 1983. Arterial neutral cholesteryl esterase. A hormone-sensitive enzyme distinct from lysosomal cholesteryl esterase. *J. Biol. Chem.* 258:192–198.

18. Vahouny, G. V., S. Weersing, and C. R. Treadwell. 1964. Micellar-solubilized substrate and cholesterol esterase activity in vitro. *Arch. Biochem. Biophys.* 107:7–15.

19. Wynalda, M. A., F. H. Lincoln, and F. A. Fitzpatrick. 1979. High-performance liquid chromatographic assay for prostacyclin. *J. Chromatogr.* 176:413–417.

20. Brown, M. S., Y. K. Ho, and J. L. Goldstein. 1980. The cholesteryl ester cycle in macrophage form cell. *J. Biol. Chem.* 255:9344–9352.

21. Hojnacki, J. L., and S. C. Smith. 1974. Separation of six lipid classes on thin layer chromatography. *J. Chromatogr.* 90:365–367.

22. 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.

23. Hajjar, D. P., A. J. Marcus, and O. R. Etingin. 1989. Platelet-neutrophil-smooth muscle cell interaction: lipoxygenase-derived mono- and dihydroxy acids activate cholesteryl ester hydrolysis by the cyclic AMP dependent protein kinase cascade. *Biochemistry.* 28:8885–8891.

24. Slotte, J. P., J. F. Oram, and E. L. Bierman. 1987. Binding of high density lipoproteins to cell receptors promotes translocation of cholesterol from intracellular membranes to the cell surface. *J. Biol. Chem.* 262:12904–12907.

25. Miller, N. E., A. La Ville, and D. Crook. 1985. Direct evidence that reverse cholesterol transport is mediated by high-density lipoprotein in rabbit. *Nature (Lond.).* 314:109–111.

26. Vane, J. R. 1983. Prostaglandins and the cardiovascular system. *Br. Heart J.* 49:405–409.

27. Khoo, J. C., E. M. Mahoney, and D. Steinberg. 1981. Neutral cholesterol esterase activity in macrophage and its enhancement by cAMP-dependent protein kinase. *J. Biol. Chem.* 256:12659–12661.

28. Hajjar, D. P. 1986. Regulation of neutral cholesteryl esterase in arterial smooth muscle cells: stimulation by agonists of adenylate cyclase and cyclic AMP-dependent protein kinase. *Arch. Biochem. Biophys.* 247:49–56.

29. Pomerantz, K. B., A. R. Tall, S. J. Feinmark, and P. J. Cannon. 1984. Stimulation of vascular smooth muscle cell prostacyclin and prostaglandin E2 synthesis by plasma high and low density lipoproteins. *Circ. Res.* 54:554–565.

30. Fleisher, L. N., A. R. Tall, L. D. Witte, R. W. Miller, and P. J. Cannon. 1982. Stimulation of arterial endothelial cell prostacyclin synthesis by high density lipoprotein. *J. Biol. Chem.* 257:6653–6655.

31. Evensen, S. A., J. P. Blomhoff, E. Nilsen, R. Holme, and H. Letnes. 1985. Effect of high density lipoproteins on cholesterol effulux and esterification in lipid enriched human skin fibroblasts. *Scand. J. Gastroenterol.* 20(Suppl 107):61–66.

32. Schmitz, G., H. Robeneck, M. Beuck, R. Krause, A. Schreck, and R. Niemann. 1988. Ca^{++} antagonists and ACAT inhibitors promote cholesterol efflux from macrophages by different mechanisms. *Arteriosclerosis.* 8:46–56.

33. Ho, Y. K., M. S. Brown, and J. L. Goldstein. 1980. Hydrolysis and excretion of cytoplasmic cholesteryl esters by macrophages: stimulation by high density lipoprotein and other agents. *J. Lipid Res.* 21:391–398.