Stimulation of Vascular Smooth Muscle Cell Prostacyclin and Prostaglandin E sub2 Synthesis by Plasma High and Low Density Lipoproteins

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SUMMARY. We studied the effects of plasma high density and low density lipoproteins upon the synthesis of prostacyclin and prostaglandin E sub2 by vascular smooth muscle cells. Prostaglandin synthesis was measured in 24-hour cultures by radioimmunoassay of the stable metabolites of prostacyclin, 6-keto-prostaglandin F sub12, and of prostaglandin E sub2. High density lipoproteins induced dose-dependent increases in the release of 6-keto-prostaglandin F sub12, and of prostaglandin E sub2 from smooth muscle cells to values 14- and 50-fold above control. Incubations with low density lipoproteins at comparable cholesterol concentrations also induced dose-dependent release of 6-keto-prostaglandin F sub12, and prostaglandin E sub2, but to a lesser extent. Rat high density lipoprotein, which contained 2.5 times more cholesteryl arachidonate than human high density lipoproteins, stimulated 6-keto-prostaglandin F sub12, and prostaglandin E sub2 release 2- to 3-fold more than human high density lipoproteins, whereas the delipidated apoproteins of high density lipoproteins had no significant effect on prostaglandin synthesis. Recombinant high density lipoproteins containing cholesteryl-[1-14C]arachidonate stimulated release of [14C]-6-keto-prostaglandin F sub12, and [14C]-prostaglandin E sub2 from cells preincubated with recombinant high density lipoprotein containing cholesteryl-[1-14C]arachidonate. Unlabeled high density lipoproteins, in contrast, did not cause release of radioactive prostaglandins from cells preincubated with [1-14C]-arachidonate. Phospholipase activators were synergistic (bradykinin) or additive (angiotensin II) with high density lipoprotein in stimulation of prostaglandin synthesis. The data indicate that both high and low density lipoproteins stimulate the synthesis of prostacyclin and prostaglandin E sub2 by vascular smooth muscle cells. The results suggest that the lipoproteins provide arachidonate to a phospholipase-sensitive pool accessible to cyclooxygenase. (Circ Res 54: 554-565, 1984)

PROSTACYCLIN (PGI sub2) and prostaglandin E sub2 (PGE sub2) are vasodilator prostaglandins that are synthesized by blood vessels and vascular endothelial and smooth muscle cells grown in tissue culture (Moncada et al., 1977, 1979; Weksler et al., 1977; Baenziger et al., 1979). Studies in perfused organs and in intact animals have indicated that the synthesis of PGI sub2 and PGE sub2 in vessels of organs such as the heart and kidneys, modulates the effects of vasoconstrictor stimuli, thus contributing to the maintenance of tissue perfusion (McGiff et al., 1970; Needleman et al., 1978; Oliver et al., 1980; Gunther and Cannon, 1980). Furthermore, Moncada and Vane have postulated that the balance between the production of thromboxane A sub2 by platelets and the synthesis of PGI sub2 by endothelial cells is important in the maintenance of vascular integrity (Moncada and Vane, 1979).

The principal substrate for prostaglandin synthesis is arachidonate present in the phospholipids of cellular membranes; various mechanical and hormonal stimuli activate cell membrane phospholipases which liberate arachidonate, making it available to cyclooxygenase and other enzymes of the prostaglandin cascade (Ramwell et al., 1977; Whorton et al., 1982; and Hong and Deykin, 1982). However, arachidonate might also be derived from exogenous sources, and, in plasma, large amounts of arachidonic acid are present in esterified form in the phospholipids and cholesteryl esters of the plasma lipoproteins. In a previous study, we demonstrated that high density lipoproteins (HDL) markedly stimulated the synthesis of prostacyclin by vascular endothelial cells grown in tissue culture, whereas low density lipoproteins (LDL) had little effect (Fleisher et al., 1982a). Indirect evidence suggested that HDL may provide endothelial cells with arachidonate.

Both HDL and LDL are normally transported through the intact endothelium of blood vessels by the process of diacytosis and come into contact with vascular smooth muscle cells present in the media (Stein et al., 1973; Stein and Stein, 1973; Leake and Bowyer, 1981). HDL and LDL are also present in interstitial fluid and in lymph (Roheim et al., 1976). At sites of endothelial denudation where platelet
aggregation can initiate the formation of experimental atherosclerotic lesions, prostaglandin formation is initially reduced (Eldor et al., 1981). However, medial smooth muscle cells migrate through the internal elastic membrane and proliferate to form a neointima; these cells are capable of synthesizing PGI₂ and are in direct contact with plasma HDL and LDL (Eldor et al., 1981). The effects of plasma lipoproteins upon prostaglandin synthesis by vascular smooth muscle cells are unknown. The present study was designed to investigate the influence of plasma lipoproteins, particularly HDL, upon the synthesis of PGI₂ and PGE₂ by vascular smooth muscle cells grown in tissue culture.

Methods

Reagents

Plasma lipoproteins were obtained by preparative ultracentrifugation of human and rat plasma in a Beckman 40.3 rotor at densities between 1.019 and 1.063 g/ml (LDL), 1.125 and 1.21 g/ml (human HDL), and 1.075 and 1.21 g/ml (rat HDL). Lipoproteins were recentrifuged once at the upper density limit, and were assayed for protein by the method of Lowry et al. (1951), and for cholesterol by the method of Zlatkis and Zak (1969). Before incubation with cells, the lipoproteins were dialyzed once against 200 volumes of 0.9% saline overnight, and once against 100 volumes of M-199 tissue culture media [Grand Island Biological Company (Gibco)] containing penicillin (100 U/ml), streptomycin (100 µg/ml), and 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, Sigma Chemical Company, pH 7.4, at 4°C. Apo-HDL was prepared by delipidation of native HDL in 20 volumes of ethanol:diethyl ether (3:2), followed by washing in diethyl ether at -15°C. Lipoprotein-deficient serum was prepared as previously described (Havel et al., 1955).

All reagents were prepared in M-199 tissue culture media containing 25 mM HEPES (pH 7.4) containing 1% penicillin-streptomycin, Sodium arachidonate (10 µM; Sigma) and chloroquine (45 µM; Sigma) were prepared in stock solutions of 1.0 mg/ml in absolute ethanol, and stored at -15°C. Lipoprotein-deficient serum was prepared by the method of Zlatkis and Zak (1969). Before incubation with cells, the lipoproteins were dialyzed once against 200 volumes of 0.9% saline overnight, and once against 100 volumes of M-199 tissue culture media [Grand Island Biological Company (Gibco)] containing penicillin (100 U/ml), streptomycin (100 µg/ml), and 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, Sigma Chemical Company, pH 7.4, at 4°C. Apo-HDL was prepared by delipidation of native HDL in 20 volumes of ethanol:diethyl ether (3:2), followed by washing in diethyl ether at -15°C. Lipoprotein-deficient serum was prepared as previously described (Havel et al., 1955).

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Isolation and Culture of Rabbit Aortic Smooth Muscle Cells

Aortic smooth muscle cells were isolated and cultured by a modification of the techniques of Gunther et al. (1982). Thoracic aortas were removed from pentobarbital-anesthetized New Zealand white rabbits [male, 2.5 kg (Vrana Research Animals)] using aseptic technique. Aortas were placed in modified minimal essential media (mm: CaCl₂ 0.19; KCl 5.36; KH₂PO₄ 0.44; NaCl 135; Na₂HPO₄ 0.65; glucose 5.5; HEPES 25; and 20 ml/liter of 50x nonessential amino acids) (Gibco). The adventitia was stripped off by blunt dissection, and the vessel war split longitudinally. The endothelium was removed by scraping with a no. 10 scalpel. The tissue was then minced into pieces approximately 1.0 mm², and transferred to a 50-ml Costar flask (Costar Data Packaging) containing 12 ml of enzyme dissociation medium (enzyme carrier medium containing 2.0 mg/ml bovine serum albumin (Pentex, Miles Laboratories), 360 U/ml collagenase (CLS, lot no. 42C287, Worthington Biochemical Corp.), 96 U/ml elastase (E-0217, lot no. 100F8075, Sigma), and 0.375 mg/ml of soybean trypsin inhibitor (type US, lot no. 11F8085, Sigma). After incubation for 75 minutes at 37°C in a shaking (120 rpm) water bath, the tissue suspension was aspirated into a 10-ml syringe, and triturated 10 times through a 13-gauge stainless-steel cannula. The resulting suspension then was sieved through a 100-µm steel mesh, transferred to a silanized conical test tube, and centrifuged at 1500 rpm (250 g) for 10 minutes. The supernatant was discarded, and the cell pellet was suspended in M-199 containing 10% fetal bovine serum (Hyclone, Sterile Systems, Inc.), 1% penicillin-streptomycin, 25 mM HEPES, pH 7.4. The undigested material present on the mesh was subjected to a second digestion, as described above. The resulting cell suspensions were pooled and inoculated into 75-cm² culture flasks (Costar), and incubated at 37°C in 5% CO₂ in air at 90% humidity. The culture media were changed every 3 days until the cells were confluent; the cells were subcultured (via trypsinization) into their third passage. Cells then were pooled, suspended into M-199 tissue culture media containing 10% dimethyl sulfoxide (Sigma), and frozen in liquid nitrogen.

Verification of Cells

Cells were determined to be vascular smooth muscle by (1) their hill-and-valley configuration at confluence (Ross, 1971); (2) positive fluorescence staining for smooth muscle actin and myosin (Groschl-Stewart et al., 1975; Chamley et al., 1977), and (3) the presence of thick and thin filaments, and dense bodies, as demonstrated by electron microscopy (Gimbrone and Cotran, 1975).

For phase contrast microscopy, cell monolayers on coverslips were prefixed in cacodylate-buffered glutaraldehyde for 15 seconds, then exposed to 0.25% NP-40 (Shell Oil Co.) in phosphate-buffered saline (PBS), pH 7.0, for 1–5 minutes, rinsed in PBS, and the fixation continued for 10 minutes more in 2% glutaraldehyde in 0.06 M cacodylate buffer, pH 6.8, containing 170 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, and 0.5% tannic acid. After fixation, washed coverslips were postfixed in osmium tetroxide, stained for 60 minutes in 1% aqueous uranyl acetate, mounted in buffered glycerol, and observed with a Leitz Ortholux phase contrast microscope.

For immunocytology, cells were fixed in 2.5% purified formalin in phosphate-buffered saline, made permeable with acetone/water (1:1) and then acetone at -20°C before staining. Sites of F-actin were identified with rabbit antibody to human platelet myosin (Godman et al., 1980).

For electron microscopy, cells growing in plastic dishes were fixed in phosphate-buffered 2.5% glutaraldehyde (pH 7.2) containing 1% sucrose for 20 minutes, postfixed in 2% osmium tetroxide, stained in 0.25% aqueous uranyl acetate overnight, dehydrated in ethanol, and embedded in Epon 812. After overnight incubation at 55°C, the
partially polymerized casts containing the monolayer was removed from the dish and polymerization was continued for 48 hours at 60°C. Selected areas of the cast were cut out, glued to Epon blocks, and sectioned on an LKB Ultramicrotome II. Sections were stained with uranyl acetate and lead acetate and viewed in a Phillips 200 electron microscope.

**Incubations**

For each experiment, cells were thawed rapidly, and plated into 24-well cluster plates in M-199 (containing 10% fetal bovine serum, 1% penicillin-streptomycin, 25 mM HEPES, pH 7.4), and incubated at 37°C. The media were changed 24 hours after plating, and every 3 days thereafter. Cell viability, as determined using the trypan blue exclusion technique 24 hours after plating, was 88% with a plating efficiency of 40%. Before study, subconfluent cells (5.2 x 10³ cells/well) were washed twice with 1.0 ml of serum-free M-199. Control cultures (in triplicate wells) received 1.0 ml of M-199 (which did not contain 10% fetal bovine serum), and experimental cultures (in triplicate wells) received 1.0 ml of M-199 containing the test agent. After 24 hours of incubation at 37°C, media were removed and assayed for 6-keto-PGF₁α, the stable hydrolysis product of prostacyclin, and PGE₂ by radioimmunoassay (RIA). Results of these experiments were analyzed by analysis of variance after log transformation of the data. Data are reported as mean ± se. Cell number was determined by counting the cells directly, using a calibrated ocular within the phase-contrast microscope (Olympus).

**Radioimmunoassay**

The RIA for 6-keto-PGF₁α was performed essentially as previously described (Fleisher et al., 1982a), with the modification that M-199 was used for the preparation of the standard curves in place of Dulbecco’s modified Eagle’s medium (DME). PGE₂ was measured by RIA as previously described (Oliver et al., 1980). The PGE₂ antibody (Sigma) achieved approximately 40% binding of [³H]PGE₂ in the absence of unlabeled PGE₂. Cross-reactivity with other prostanoids was as follows: PGE₁, 3.2%; PGD₂, 0.2%; 13,14-dihydro-PGE₂, 0.15%; 13,14-dihydro-15-keto-PGE₂, 0.11%; and less than 0.1% for 6-keto-PGF₁α, PG₁α, 6-keto-PGF₁β, PGB₁, and PGB₂. All samples were assayed in duplicate without extraction. Each assay tube received 100 μl of standard [2.0-200 pg of authentic PGE₂ (Upjohn Co.)] in M-199, or unknown, 100 μl of 0.1 mM phosphate-buffered saline (pH 7.4) containing 0.1% gelatin (PBS-G), with [³H]PGE₂ [5,6,8,11,12,14,15-³H(N)-PGE₂, 8000 cpm (New England Nuclear)] and 100 μl of antiserum prepared in PBS-G. Samples were incubated for 2 hours at 4°C. Antibody-bound PGE₂ was separated from free ligand by addition of 1.0 ml of ice-cold PBS-G containing 0.025% dextran T-70 (Pharmacia Fine Chemicals) and 0.25% activated charcoal (Norit “A.” Amend Drug and Chemical Co.). After 15 minutes, samples were centrifuged at 5000 rpm (4810 g) for 10 minutes at 4°C. The supernatants were decanted into polypropylene scintillation vials (Kimble); after addition of 15 ml of Hydrofluor (National Diagnostics) radioactivity was counted in a Tri-Carb model 3330 liquid scintillation spectrometer.

**Radioisotope and Recombinant HDL Experiments**

Smooth muscle cells were prelabeled with [¹⁴C]arachidonate (50-60 μCi/mmol) (Amersham/Searle) by meth-
FIGURE 1. Morphology of cultured rabbit aortic smooth muscle cells. Panel A: phase-contrast photomicrograph of confluent smooth muscle cell culture, illustrating cell alignment with developing multilayers (X85 bar 50 μm); (panel B) actin, as revealed by staining with NBD-coupled phallacidin. (X675 bar 25 μm); (panel C) myosin, as revealed by indirect immunofluorescence staining with rabbit antimyosin antibodies. (X675 bar 25 μm); (panel D) electron micrograph of an aortic smooth muscle cell, sectioned through a peripheral aspect. Thin filaments (TF), with associated dense bodies (arrow) divide the cytoplasm, which is rich in mitochondria, vesicles, and rough endoplasmic reticulum (X18970, bar 5.0 μm).

The Influence of Plasma Lipoproteins on Prostaglandin Synthesis by Vascular Smooth Muscle Cells

Figure 2 illustrates the dose-response relationship of HDL and LDL on prostaglandin release by vascular smooth muscle cells. Control cells (incubated in media alone) released 0.24 ± 0.07 ng 6-keto-PGF₁₀/ml, and 0.16 ± 0.03 ng PGE₂/ml (Fig. 2B), into the media at the end of the 24-hour incubation period. HDL₃ induced a dose-dependent stimulation of 6-keto-PGF₁₀ and PGE₂ release by the smooth muscle cells (Fig. 2, A and B). At highest doses, incubation with HDL₃ (0.375 mg cholesterol/ml),
induced a 14-fold increase of 6-keto-PGF$_{1\alpha}$ production to 3.48 ± 0.06 ng/ml ($P < 0.01$). PGE$_2$ synthesis was increased by this concentration of HDL$_3$ to values 50 times control (8.07 ± 0.75 ng/ml, $P < 0.001$). LDL, at equivalent cholesterol concentrations, also produced dose-dependent stimulation of 6-keto-PGF$_{1\alpha}$ and PGE$_2$ (Fig. 2, A and B). However, the stimulatory effect of LDL on prostaglandin release was considerably less than that of HDL$_3$. The concentrations of 6-keto-PGF$_{1\alpha}$, and PGE$_2$ found in incubations containing LDL were approximately 4-10 times those of the control cultures.

Figure 3 summarizes three experiments depicting the time-course of lipoprotein-induced prostacyclin release by the smooth muscle cells. In the control cultures, the concentration of 6-keto-PGF$_{1\alpha}$ in the media (0.25 ± 0.03 ng/ml) did not increase significantly over the 24-hour time period. In contrast, both HDL$_3$ (0.15 mg cholesterol/ml) and LDL (0.15 mg cholesterol/ml) produced a slowly progressive accumulation of 6-keto-PGF$_{1\alpha}$ in the media. HDL$_3$ produced a 10-fold, and LDL, a 4-fold, increase in 6-keto-PGF$_{1\alpha}$ concentration in the media at 24 hours.

The relationship between cell density and lipoprotein-induced 6-keto-PGF$_{1\alpha}$ release is depicted in Figure 4. Cells were plated at variable density and exposed to media alone (control) or media containing HDL$_3$ (0.15 mg cholesterol/ml), LDL (0.15 mg cholesterol/ml), or sodium arachidonate (10 $\mu$M). The stimulation of 6-keto-PGF$_{1\alpha}$ per $10^4$ cells, induced by HDL$_3$, LDL, and arachidonate was greater in cells at lower densities than in cells at higher densities. As the cells approached confluence ($10^5$/well), the synthesis of prostacyclin induced by all three agents was significantly less than at lower cell densities.

Gospodarowicz et al. and other investigators have reported that plasma factors, including the lipoproteins, may influence the growth characteristics and morphology of vascular smooth muscle cells grown in tissue culture (Gospodarowicz et al., 1981; Tauber et al., 1980, 1981a). Figure 5 shows phase-contrast photomicrographs illustrating the effects of serum-free M-199, and serum-free M-199 containing HDL$_3$, LDL, or lipoprotein-deficient serum (LPDS), on the morphology of the cultured vascular smooth muscle cells over a 24-hour incubation period. In serum-free M-199, the cells lost their spindly shape and appeared rounded, but were not detached from the plating surface (panel A). When HDL$_3$ (0.15 mg cholesterol/ml) was present in the media, the normal morphology of the cells was preserved (panel B). In contrast, when LDL (0.15 mg cholesterol/ml) was
the adverse effects of media containing LDL on cell morphology (panel D). HDL3 had similar effects as LPDS (not shown). However, despite these changes in morphology, neither basal nor lipoprotein-induced PG12 and PGE2 release was altered by the presence of LPDS (Fig. 6). When the cells were incubated in 5% platelet-poor lipoprotein-deficient serum (2.5 mg protein/ml), they did not secrete significantly more 6-keto-PGF1α into the media than in the basal state [control = 0.18 ± 0.07 vs. LPDS = 0.37 ± 0.05 ng/ml (n = 2)].

To determine whether lysosomal degradation is necessary for lipoprotein-induced prostaglandin synthesis, cells were incubated with HDL3 (0.15 mg cholesterol/ml) and LDL (0.15 mg cholesterol/ml) during exposure to chloroquine (45 μM), an inhibitor of lysosomal enzyme activity (Goldstein et al., 1975). Chloroquine did not cause a significant alteration in either basal or lipoprotein-induced 6-keto-PGF1α release (Fig. 7). In further experiments, LDL coincubated with dextran sulfate stimulated 6-keto-PGF1α release to the same degree as native LDL (2.59 vs. 2.21 ng/ml, respectively).

Table 1 summarizes experiments in which the effects of two cyclooxygenase inhibitors on HDL3-induced 6-keto-PGF1α release were evaluated. Cultures were pretreated with aspirin (20–100 μg/ml), and meclofenamate (5.0, 10.0, and 20.0 μg/ml) for 30 minutes before exposure to media alone (control) or media containing HDL3 (0.15 mg cholesterol/ml). Aspirin and meclofenamate inhibited the basal and HDL3-induced release of 6-keto-PGF1α by vascular smooth muscle cells.

The effects of agents which stimulate phospholi-
pases upon prostaglandin synthesis by the vascular smooth muscle cells were evaluated. Cells were exposed to bradykinin, (1.0 µg/ml), and angiotensin II (5.0 µg/ml), in the presence and absence of HDL 3, (0.15 mg cholesterol/ml) and to the calcium ionophore, A-23187 (10 µM) and sodium arachidonate (10 µM) (Table 2). In the basal state, the cells released similar amounts of PGI 2 and PGE 2. All agents stimulated PGI 2 and PGE 2 release significantly. Bradykinin, angiotensin II, and sodium arachidonate induced an approximately equal stimulation of PGI 2 and PGE 2 while A-23187 stimulated PGI 2 release more than PGE 2. In contrast, HDL 3 stimulated PGE 2 synthesis more than that of PGI 2. In coincubations with HDL 3, the effects of HDL 3 and of angiotensin II on prostaglandin synthesis were additive, whereas the effects of bradykinin and HDL 3 were synergistic.

In order to determine whether esterified arachidonic acid in HDL 3 might be involved in the stimulation of prostaglandin synthesis, we examined the effects of rat HDL, which has a greater content of arachidonate in its cholesteryl esters than human HDL 3. GLC analysis of fatty acid methyl esters revealed that rat HDL contained 38% of its cholesteryl esters as arachidonate, compared to 12% for human HDL 3. The arachidonate content of phospholipids was similar in the rat and human particles, 10%. Smooth muscle cells were exposed to human and rat HDL and their respective apoproteins at a constant protein concentration (0.4 mg protein/ml—equivalent to 0.15 mg cholesterol/ml in native HDL 3). Control cultures received media alone. At comparable protein concentrations, rat HDL induced significantly greater synthesis of PGI 2 and PGE 2 (approximately 2-fold) (P < 0.05) than did human HDL 3, whereas their respective apoproteins did not stimulate significantly the synthesis of either prostaglandin (Fig. 8).

To investigate more directly whether HDL lipids might provide arachidonate to smooth muscle cells as substrate for prostaglandin synthesis, recombinant human HDL containing cholesterol [1-14C]arachidonate was prepared and incubated with the smooth muscle cells for 24 hours. At 24 hours, the media was removed, acidified, extracted and analyzed by thin layer chromatography. Figure 9A shows the densitometric analysis of an autoradiogram of the TLC plate. Radioactivity was incorporated by the cells into the 6-keto-PGF 1α and PGE 2 peaks. The cells then were washed and incubated
for 30 minutes in medium alone (control) or medium containing A-23187. Figure 9B shows that, after incubation with the ionophore, significant amounts of label appeared in the 6-keto-PGF1α, and PGE2 bands. Cells incubated with media alone did not elaborate prostaglandins containing label. Analysis of the cell lipids in these experiments indicated that 78% of the 14C was present in cell phospholipids; only 3% was present in cellular cholesteryl esters. These data suggest that HDL cholesteryl arachidonate undergoes hydrolysis upon interaction with smooth muscle cells and provides cell phospholipids with arachidonate which may be available for prostaglandin synthesis.

Experiments were also performed to investigate whether HDL mobilized cellular arachidonate stores for prostaglandin synthesis. Smooth muscle cells were preincubated with [1-14C]arachidonate for 18 hours, then washed and incubated with unlabeled HDL for 24 hours or with A23187 for 15 minutes; the media were extracted and analyzed by TLC. Table 3 indicates that there was no measurable incorporation of radioactivity into 6-keto-PGF1α or PGE2 after incubation with HDL, in contrast to the significant incorporation of label into prostaglandins after exposure to A23187.

Discussion

These results indicate that plasma high and low density lipoproteins stimulate the release of 6-keto-PGF1α, and of PGE2 from vascular smooth muscle cells grown in tissue culture. Since 6-keto-PGF1α is

Table 2

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<th>Treatment</th>
<th>6-Keto-PGF1α (ng/ml)</th>
<th>PGE2 (ng/ml)</th>
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<tr>
<td>Control</td>
<td>0.65 ± 0.13</td>
<td>0.62 ± 0.11</td>
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<tr>
<td>Bradykinin</td>
<td>2.20 ± 0.42*</td>
<td>2.50 ± 0.86*</td>
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<tr>
<td>Angiotensin II</td>
<td>1.06 ± 0.09†</td>
<td>1.21 ± 0.32†</td>
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<tr>
<td>HDL3</td>
<td>2.98 ± 0.66*</td>
<td>3.95 ± 1.06*</td>
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<tr>
<td>HDL3 plus bradykinin</td>
<td>6.04 ± 1.14†</td>
<td>8.38 ± 1.04†</td>
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<tr>
<td>HDL3 plus angioteins II</td>
<td>3.57 ± 0.74</td>
<td>4.54 ± 1.14</td>
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<tr>
<td>A-23187</td>
<td>5.17 ± 2.50*</td>
<td>3.24 ± 1.05*</td>
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<tr>
<td>Arachidonate/bovine serum albumin</td>
<td>0.81 ± 0.29*</td>
<td>0.69 ± 0.40*</td>
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<td>Rat HDL</td>
<td>11.72</td>
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Cells were grown to post-confluent density and exposed to media alone or media containing bradykinin (1.0 μg/ml) or angiotensin II (5.0 μg/ml) in the absence and presence of HDL3 (0.15 mg cholesterol/ml) for 24 hours at 37°C. (n = 3, mean ± se). Cells were also exposed to A-23187 (10 μM), sodium arachidonate (10 μM), and sodium arachidonate (10 μM) complexed to bovine serum albumin (n = 2). Differences between groups were determined by analysis of variance followed by Duncan’s multiple range test after log transformation of the data.

* P < 0.001 from control.
† P < 0.01 from treatment interactions alone.
‡ P < 0.02 from control.

the major stable product of prostacyclin (Johnson et al., 1976) and since prostaglandins are not stored in cells (Piper and Vane, 1971), the data indicate that HDL3 and LDL stimulate the synthesis of PGI2 and PGE2 by the cultured vascular smooth muscle cells. Incubation of the cells with HDL3 for 24 hours produced a significant dose-dependent accumulation of 6-keto-PGF1α and PGE2 in the culture media. Although the release of 6-keto-PGF1α by untreated cells was larger than the release of PGE2, the release of PGE2 from the cells induced by HDL3 was proportionately larger than that of 6-keto-PGF1α. LDL also induced dose-dependent increases in the release of 6-keto-PGF1α, and of PGE2 by the vascular smooth muscle cells into the culture medium; the degrees of stimulation of 6-keto-PGF1α release and PGE2 release induced by LDL were equivalent. However, the magnitude of the vascular smooth muscle prostaglandin synthesis induced by LDL was significantly less than that produced by HDL3 at the same cholesterol concentrations. The in vitro stimulation of prostaglandin synthesis produced by both lipoproteins in these experiments was observed over a range of concentrations of HDL3 and LDL that was similar to those present in normal serum and lymph.

In our previous study, HDL3 produced a marked stimulation of prostaglandin synthesis by vascular endothelial cells grown in tissue culture (Fleisher et al., 1982a). The magnitude of 6-keto-PGF1α release induced by HDL3 was greater in endothelial cells
than in smooth muscle cells; this observation is consistent with the previously reported data of Moncada, who reported that there was a gradient of 6-keto-PGF1α synthesis from the intima toward the media and adventitia of intact blood vessels (Moncada et al., 1977). In contrast to the modest stimulation of 6-keto-PGF1α and PGE2 release by smooth muscle cells induced by LDL in the present study, LDL at equivalent cholesterol concentrations (0.015–0.30 mg/ml) produced no significant stimulation of 6-keto-PGF1α release by endothelial cells cultured under similar conditions (Flesher et al., 1982b). In subsequent studies, however, higher doses of LDL (>0.3 mg cholesterol/ml) have been found to stimulate PG12 synthesis by endothelial cells. However, even at similar cholesteryl arachidonate content, HDL stimulated prostaglandin synthesis to a greater extent than LDL (Pomerantz et al., unpublished observations). These observations indicate that this is a relative but not absolute specificity of the effect of HDL on prostaglandin synthesis.

Inhibition of HDL3-induced PG12 synthesis by cyclooxygenase inhibitors implies an effect of lipoproteins prior to cyclooxygenase, the enzyme responsible for the conversion of arachidonate to the endoperoxide precursors of PG12 and PGE2. Our data differ from those showing divergent effects of HDL and LDL on PG12 synthesis by aortic microsomes, where effects of the two lipoproteins on prostacyclin synthase were suggested (Beitz and Forster, 1980). The slow progressive time course of lipoprotein induced prostaglandin synthesis differs markedly from the rapid effects of the phospholipase activators, angiotensin II, bradykinin and A23187 (Whorton et al., 1982; Hong and Deykin, 1982), and the rapid stimulation of prostaglandin production produced by arachidonate coupled to human serum albumin, suggesting a different more complex mechanism of action.

Results from several of the present experiments suggest that HDL stimulates prostaglandin synthesis, at least in part by providing the cells with arachidonate. The observations that the delipidated apoproteins of human and rat HDL did not significantly stimulate the production of 6-keto-PGF1α and PGE2 by the smooth muscle cells—whereas the native HDLs from both species had significant stimulatory effects—suggest that the lipid portion of the HDL particle is important in the stimulation of prostaglandin synthesis. When incubated at equivalent protein concentration, rat HDL induced significantly greater release of 6-keto-PGF1α and PGE2 than hu-

### Table 3

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<th>HDL and Calcium Ionophore Effect on [1-14C]Arachidonate Metabolism by prelabeled Rabbit Aortic Smooth Muscle Cells</th>
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<tr>
<td><strong>6-Keto-PGF1α</strong></td>
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<tr>
<td>Control</td>
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<tr>
<td>HDL3 (0.4 mg protein/ml)</td>
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<tr>
<td>A-23187 (5.0 μM)</td>
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Cells at confluent density were preincubated for 18 hours in [1-14C]arachidonate (3.0 μCi/plate) The cells were washed and then exposed to media alone (control) or media containing HDL3 for 24 hours. Cells also were exposed to A-23187 for 15 minutes The media were removed, extracted, and analyzed by thin layer chromatography, as described (n = 3, mean ± se). * P < 0.05.
man HDL₃ (Fig. 8). Rat HDL contains a much higher proportion of arachidonate esterified in its cholesteryl esters than human HDL (38 vs. 12%), whereas the phospholipid arachidonate content of rat and human HDL are similar (~10%); this suggests that the marked effect of rat HDL may be due to its higher content of cholesteryl arachidonate.

Direct evidence for incorporation of arachidonate from HDL as substrate for smooth muscle cell prostaglandin synthesis was obtained in the experiments showing the incorporation of label from cholesteryl [1-¹⁴C]arachidonate from HDL recombinants into 6-keto-PGF₁α and PGE₂ (Fig. 9A). The experiments with cells preincubated with the labeled recombinant that were exposed to A23187 (Fig. 9B) indicate that [1-¹⁴C]arachidonate was transferred from the lipoprotein into cellular phospholipid pools that were available for prostaglandin synthesis via the action of phospholipases. The studies with cells prelabeled with [1-¹⁴C]arachidonate indicate that native HDL does not significantly liberate arachidonate from endogenous cellular stores for prostaglandin synthesis. Additional evidence that lipoprotein stimulates prostaglandin synthesis by providing arachidonate has been obtained in experiments with endothelial cells: (1) when HDL₃ recombinants containing cholesteryl [³H]arachidonate were incubated with cells, the label was incorporated into newly synthesized 6-keto-PGF₁α, and (2) the degree of HDL-induced 6-keto-PGF₁α release was directly proportional to the cholesteryl arachidonate content of the HDL particle (Fleisher et al., 1982b).

Data from two other experiments are also consistent with the hypothesis that lipoproteins may provide arachidonate to the smooth muscle cells for prostaglandin synthesis. First, the differential response of prostaglandin release induced by the lipoproteins (PGE₂ > 6-keto-PGF₁α) differed from that induced by the three activators of phospholipases (PGE₂ ≤ 6-keto-PGF₁α, Table 2), suggesting that HDL does not act in a similar fashion. Second, phospholipase activators were either synergistic (bradykinin) or additive (angiotensin II) with HDL in stimulating prostaglandin release (Table 2). These results can be explained by incorporation of lipoprotein arachidonate into a pool of cellular lipid which is accessible to cyclooxygenase.

Although the potential mode of entry of lipoprotein lipid containing arachidonate into the cells remains to be explored, several observations of the present study indicate that it is not via receptor-mediated endocytosis involving the LDL receptor. First, HDL₃ induced significant stimulation of PGI₂ and PGE₂ synthesis by the smooth muscle cells, despite the fact that HDL₃ does not contain the apoproteins recognized by the LDL receptor, i.e., apoB and apoE (Innerarity et al., 1978). Second, chloroquine, an agent which inhibits the activity of lysosomal enzymes such as acid cholesteryl hydrolyases by raising lysosomal pH (Goldstein et al., 1975), did not affect either the basal or the lipoprotein induced 6-keto-PGF₁α release from smooth muscle cells (Fig. 6). Third, LDL coupled to dextran sulfate so it could not undergo receptor-mediated endocytosis (Basu et al., 1979), stimulated prostaglandin synthesis by smooth muscle cells. Alternative mechanisms whereby lipoprotein lipid could enter cells might involve specific HDL binding sites similar to those described on endothelial cells, fibroblasts, and human smooth muscle cells (Bierman and Albers, 1975; Tauber et al., 1981b; Biesbroeck et al., 1983) or the poorly understood "scavenger pathway" which allows LDL at high concentrations to enter cells (Goldstein and Brown, 1977).

The effects of HDL and LDL on the morphology of smooth muscle cells were similar to those reported previously by Gospodarowicz and co-workers (Gospodarowicz et al., 1981; Tauber et al., 1980, 1981a). When smooth muscle cells were incubated for 24 hours in serum-free, lipoprotein-free media (M-199), the cells appeared rounded but did not detach from the plates. Incubation with HDL (0.15 mg cholesterol/ml) preserved the normal spindly shape, whereas incubation with LDL (0.15 mg cholesterol/ml) caused the cells to appear to be irregular, vacuolated, and swollen. Both HDL and lipoprotein-deficient serum ameliorated the adverse effects of LDL upon cell morphology. The mechanisms of these effects of lipoproteins upon cell morphology are incompletely understood. However, in our study, it was found that lipoprotein stimulation of PGI₂ synthesis was independent of these effects on morphology (Fig. 5). The observation (Fig. 4) that the 6-keto-PGF₁α release/10⁴ cells induced by HDL and LDL sodium arachidonate was greater in subconfluent than confluent cells is similar to our previous findings with HDL in endothelial cell cultures (Fleisher et al., 1982a). Since this effect was also observed with arachidonate, there may be a decrease in cyclooxygenase activity in confluent cells (Ager et al., 1982).

The present in vitro experiments suggest that the lipoproteins provide arachidonate to the smooth muscle cells as substrate for prostaglandin synthesis. Whether lipoprotein arachidonate, plasma-free arachidonic acid bound to albumin, or endogenous cellular stores are the most important sources of substrate for prostaglandin synthesis in vivo remains to be elucidated. Similarly, the physiological significance of the stimulation of vascular smooth muscle cell PGI₂ and PGE₂ synthesis by HDL and LDL remains to be determined. Enhanced synthesis of vasodilator prostaglandins by medial smooth muscle cells might influence vascular tone in regional circulations such as in heart or kidneys (McGiff et al., 1970; Oliver et al., 1980; Gunther and Cannon, 1980). Epidemiological studies have shown that the concentration of LDL cholesterol in plasma correlates positively with the risk of coronary artery dis-
ease, whereas the concentration of plasma HDL correlates negatively with risk of coronary artery disease (Miller and Miller, 1975). The mechanism of apparent protective effect of HDL is unknown, but could conceivably relate to a reduction in thrombotic complications of atherosclerotic disease (Heiss et al., 1980) or to effects of HDL on mobilization of cholesterol from peripheral tissue (Carew et al., 1976). In this regard, Stein et al. (1975, 1976, 1980), and Hajjar et al., showed that HDL can mobilize cholesteryl esters from lipid-laden smooth muscle cells and macrophages (Stein et al., 1975, 1976, 1980), and Hajjar et al., showed that PGI2, 6-keto-PGE, and 6-keto-PGFα activated acid-cholesteryl hydrolase and implicated PGI2 in the process of cholesterol removal from smooth muscle cells.

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