Effect of human plasma lipoproteins on prostacyclin production by cultured endothelial cells

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Abstract Prostacyclin (PGI$_2$) production by bovine aortic or human umbilical vein endothelial cells increased when either human high density lipoproteins$_3$ (HDL$_3$) or low density lipoproteins (LDL) were added to a serum-free culture medium. At low concentrations and short incubation times, HDL$_3$ produced more PGI$_2$ than LDL, but LDL was just as effective as HDL$_3$ in 18-hr incubations with high concentrations of lipoproteins. Neither lipoprotein was toxic to the cultures as assessed by [^{3}H]leucine incorporation into cell protein. The stimulatory effect of HDL$_3$ and LDL on PGI$_2$ production decreased as growing cultures became confluent. Incubation with lipoproteins neither enhanced arachidonic acid release nor increased PGI$_2$ formation when the cells were stimulated subsequently with ionophore A23187, indicating that the lipoproteins do not affect the intracellular processes involved in PGI$_2$ production. The addition of albumin reduced the amount of PGI$_2$ formation elicited by HDL$_3$ or LDL. As compared with albumin-bound arachidonic acid, from 6- to 13-fold less PGI$_2$ was produced during incubation with the lipoproteins. Furthermore, the amount of PGI$_2$ formation elicited by the lipoproteins in 18 hr was 4-fold less than that produced during incubation with a fatty acid mixture containing only 5% arachidonic acid, and 3-fold less than when the cells were stimulated with the ionophore A23187 for 20 min.■■ Taken together, our results indicate that human HDL and LDL contribute to endothelial PGI$_2$ production only in a modest way and suggest that this process is not specific for either of these two plasma lipoproteins. In view of the greater participation of albumin-bound arachidonic acid in PGI$_2$ production, plasma lipoproteins may not play as important a role in endothelial prostaglandin formation as has been suggested. —Spector, A. A., A. M. Scanu, T. L. Kaduce, P. H. Figard, G. M. Fless, and R. L. Czervionke. Effect of human plasma lipoproteins on prostacyclin production by cultured endothelial cells. *J. Lipid Res.* 1985. **26:** 288–297.

Endothelial cells are an important source of prostacyclin (PGI$_2$), a prostaglandin that inhibits platelet aggregation and is an arterial vasodilator (1, 2). These actions of PGI$_2$ are thought to protect against thrombosis and ischemic heart disease (3). PGI$_2$ production by cultured endothelial cells is stimulated by exposure to thrombin, trypsin, calcium ionophore A23187, arachidonic acid, and prostaglandin H$_2$ (4–7). Recently, Fleisher et al. (8) have shown that human and rat high density lipoproteins (HDL) stimulate PGI$_2$ synthesis by porcine aortic endothelial cells in culture, apparently by providing arachidonic acid to the cells. A time-dependent formation of PGI$_2$ was observed during the course of a 24-hr incubation with HDL, as opposed to the very rapid release of PGI$_2$ that occurs when cultured endothelial cells are exposed to other stimulating agents (4–7). Relatively large amounts of PGI$_2$ synthesis occurred when the porcine aortic endothelium was incubated with HDL; the quantity was greater than that formed when the cultures were incubated with 10 μM arachidonic acid (8). HDL also was much more potent than LDL in stimulating PGI$_2$ production by the porcine endothelium. These observations linking HDL to PGI$_2$ synthesis in a concentration-dependent manner are of great interest because HDL appears to exert a protective effect against the development of ischemic heart disease (9, 10). Since the findings of Fleisher et al. (8) are potentially so important, we have further explored the effects of human plasma lipoproteins on PGI$_2$ production in two additional endothelial cell culture systems, bovine aortic endothelium and human umbilical vein endothelium.

METHODS

Tissue culture

Human endothelial cells were obtained from umbilical veins (11), and primary cultures were prepared according

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Abbreviations: PGI$_2$, prostaglandin I$_2$ or prostacyclin; HDL, high density lipoproteins; LDL, low density lipoproteins; 18:2, linoleic acid; 18:1, oleic acid; 20:4, arachidonic acid; 6-keto PGF$_{1\alpha}$, the 6-keto derivative of prostaglandin F$_{1\alpha}$.

to a slight modification of the method of Jaffe et al. (12) as previously described (13). Briefly, the cells were suspended in a modified medium 199 containing 20% heat-inactivated fetal bovine serum, counted with a hemocytometer and seeded in 25-cm^2 flasks at a concentration of 2.25×10^6 cells per flask. After incubation for 24 hr at 37°C in an atmosphere containing 5% CO_2, this medium was replaced with 3 ml of medium 199 containing 25 mM HEPES, pH 7.4, plus 20% fetal bovine serum, and the confluent cultures were continued in the 5% CO_2 atmosphere.

Bovine endothelial cell cultures were isolated from the aorta (14, 15). All experiments were performed with cultures below passage 20. The cells were maintained in medium 199 supplemented with 10% fetal bovine serum, BME vitamins, MEM nonessential amino acids, 2 mM glutamine, 10 mM HEPES, pH 7.4, and 100 μl/ml neomycin sulfate in an atmosphere containing 5% CO_2. Seeding was performed following detachment of the cells from the flask with 0.25% trypsin and 0.02% EDTA in a solution containing 150 mM NaCl, 5 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4. The cells were suspended in maintenance medium and seeded at a concentration of $5-7 \times 10^3$ cells/cm^2. Cultures were fed every 3 days and reached confluence within 5-7 days after seeding. Most of the studies were done with cultures that were either subconfluent or had just reached confluence. In general, the results reported in a single table or figure were obtained with cells prepared at the same time, but different preparations of cells usually were used for each of the different types of experiments.

Lipoprotein preparation

Lipoproteins were prepared from pooled plasma of human donors. The plasma was obtained by drawing blood into a solution containing Na_2EDTA, 1-1.5 g/l; NaN_3, 0.1 g/l; chloramphenicol, 50 mg/L; gentamicin sulfate, 100 mg/l; kallikrein inactivator, 10,000 units/l; and DFP, 1×10^{-4} M. LDL (1.019-1.063 g/ml) and HDL_3 (1.12-1.21 g/ml) were then prepared by sequential flotation. The HDL_3 fraction was washed twice in a solution of density 1.21 g/ml to remove other plasma protein contaminants. After dialysis against 1,000 volumes of 0.15 M NaCl, 0.01% Na_2EDTA, and 0.01% NaN_3, the LDL solution was adjusted with solid CsCl to 4% by weight and fractionated by centrifugation on the self-generating CsCl gradient in the Ti-60 rotor at 59,000 rpm for 20 hr at 20°C. A narrow cut from the peak was pooled after it was shown to be free of Lp(a) by gradient gel electrophoresis on 2.5-16% linear polyacrylamide gradient gels (Isolab). Salt was removed by dialysis against three changes of 300 volumes of phosphate-buffered saline, pH 7.4, containing 0.01% Na_2EDTA. Aliquots of each lipoprotein fraction were taken for analysis, and the remain-

der of the lipoprotein preparations was dialyzed against 300 volumes of medium 199 buffered to pH 7.4 with $NaHCO_3$. Protein content was determined by a modification of the Lowry method (16), and total cholesterol content was measured enzymatically using the Calbiochem Behring reagent.

PGI$_2$ production

Endothelial cell cultures were incubated with various media, and the cell-free medium was collected for measurement of PGI_2 content (13, 15). PGI_2 was measured by a radioimmunoassay for the 6-keto derivative of prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), a stable catabolic product of PGI_2. In this assay, 100 μl of either standard or samples was mixed with 100 μl of [^{3}H]6-keto-$PGF_{1\alpha}$ (New England Nuclear, Boston, MA) and 10 μl of antiserum at a dilution that bound 50% of the radioactivity in the absence of standard. After 1 hr of incubation at 37°C, 50 μl of IgGsorb (The Enzyme Center Incorporated, Tufts University School of Medicine, Boston, MA) was added, and the radioactivity contained in a 100-μl sample of the supernatant solution was measured in a liquid scintillation spectrometer. Assay detection limits were 0.3 pmol of 6-keto-$PGF_{1\alpha}$ and 50% inhibition was obtained with 3.3 pmol of 6-keto-$PGF_{1\alpha}$. The assay has negligibly small amounts of cross-reactivity with prostaglandins other than 6-keto-$PGF_{1\alpha}$ (17).

Arachidonic acid incorporation and release

After the growth medium was removed and the cultures were washed, medium containing 15 μM fatty acid-free bovine albumin (Miles Laboratories Incorporated, Elkhart, IN), 30 μM [1-^{14}C]arachidonate (600,000 dpm/ml) and, in some cases, a lipoprotein solution containing 100 μg cholesterol was added. The cultures were incubated at 37°C in a 5% CO_2 atmosphere (13, 15). Following this incubation, the cells were washed with a solution containing 50 μM bovine serum albumin and then phosphate-buffered saline, pH 7.4. Representative cultures were harvested by scraping (18), and then they were extracted with chloroform-methanol 2:1 (v/v) to obtain the cellular lipids (19). The chloroform phase was isolated by adding 4 mM HCl in 0.15 M NaCl and then evaporated under N_2 in a liquid scintillation counting vial. After 4 ml of Budget Solve scintillator solution (Research Products International, Mount Prospect, IL) was added, the radioactivity was measured in a liquid scintillation spectrometer. Quenching was monitored with a ^{137}Cs external standard. Additional cultures that were exposed initially to [1-^{14}C]arachidonic acid were subsequently incubated in media containing either lipoproteins or the calcium ionophore A23187. These media also contained 50 μM bovine serum albumin. The radioactivity released from

the cells during these incubations was also measured by extraction and analysis of the medium as described above.

Fatty acid analysis

The fatty acyl composition of the bovine aortic endothelial cell lipids and the human lipoproteins was analyzed by gas–liquid chromatography. Cell cultures were incubated with media containing 200 μg of lipoprotein cholesterol for 20 hr. Other cultures were incubated in similar media containing no added lipoproteins. Lipoprotein solutions also were incubated for 20 hr under these conditions in the absence of cells. The medium was removed following incubation and, after washing, the cells were isolated by scraping (18). The lipids were extracted from the cells or medium with chloroform–methanol 2:1 (v/v) and separated into individual classes by thin-layer chromatography (20). A solvent system containing hexane–diethyl ether–methanol–acetic acid 85:20:2:2 (v/v/v/v) was utilized. After the silica gel was inactivated with 4 ml of acidic NaCl, the lipids were extracted with 10 ml of chloroform–methanol 1:1 (v/v). After each fraction was transesterified with 14% BF$_3$ in methanol at 100°C for 1 hr (21), the fatty acid methyl esters were separated by gas–liquid chromatography (21). Separation was made using a 2 mm × 1.9 m glass column packed with 10% SP2330 on 100/120 mesh Chromosorb W-AW (Supelco, Bellefonte, PA). The Hewlett-Packard model 5700 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) was equipped with a flame ionization detector. N$_2$ served as the carrier gas at a flow rate of 25 ml/min, and the oven temperature was programmed from 176° to 220°C. Peak areas were determined with a Hewlett-Packard model 3380 S integrator-recorder, and the areas are reported as weight percentages.

Leucine incorporation

Bovine aortic endothelial cultures were incubated for 18 hr with either HDL$_3$ or LDL. After removal of this medium, the cultures were washed with Dulbecco's phosphate-buffered saline solution and then incubated for 1 hr with 1 ml of a medium containing 1 μCi of [4,5-^{3}H]leucine (147 Ci/mmol). The medium was removed, the cultures were washed, and 0.5 ml of 10% trichloroacetic acid was added. After 10 min, the material was harvested by scraping. The culture flasks were washed once with 1 ml of 10% trichloroacetic acid, and this was added to the first extract. Each tube was heated for 20 min at 95°C, cooled in ice, and the precipitate was collected on glass filters with suction. The tubes were rinsed twice with 2 ml of 10% trichloroacetic acid and the rinses were also filtered. After the filters were heated for 1 hr at 70°C and cooled, they were added to 10 ml of Budget-Solve solution and the radioactivity was measured in the liquid scintillation spectrometer.

RESULTS

PGI$_2$ production

Fig. 1 illustrates the time-dependence of PGI$_2$ production when subconfluent bovine aortic endothelial cell cultures are incubated in a serum-free medium supplemented with either human HDL$_3$ or LDL. The PGI$_2$ that accumulated in the medium after 4, 8, and 16 hr of incubation was measured by radioimmunoassay of the inactivated product, 6-keto-PGF$_{1\alpha}$. With HDL$_3$, increasing amounts of PGI$_2$ accumulated throughout the 16-hr period, and about 50% of the total production occurred during the first 4 hr of incubation. By contrast, relatively little PGI$_2$ production occurred during the first 8 hr of incubation with LDL, but a marked increase was observed between 8 and 16 hr.

Fig. 2 illustrates the effect of lipoprotein concentration on PGI$_2$ production. A 16-hr incubation time was employed for these experiments. As shown on the left side of Fig. 2, very little PGI$_2$ production occurred when subconfluent bovine endothelial cells were incubated in a serum-free medium without supplemental lipoproteins.
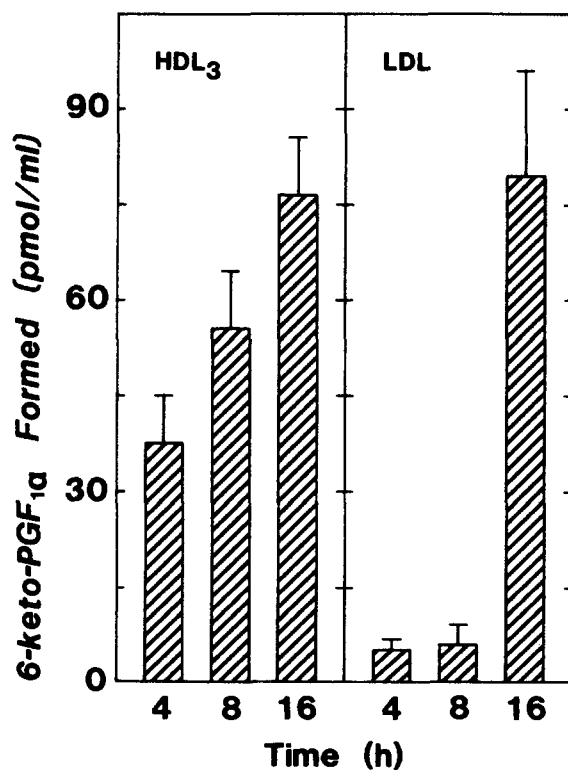

Fig. 1. Time-dependence of PGI$_2$ production by endothelial cells during incubation with lipoproteins. Cultures of bovine aortic endothelial cells were incubated with 0.6 ml of either HDL$_3$ containing 75 μg/ml of cholesterol or LDL containing 313 μg/ml of cholesterol. Each value for PGI$_2$ production, measured by radioimmunoassay of 6-keto PGF$_{1\alpha}$, is the mean ± SE of results obtained from three separate cultures, each from the same passage and incubated with the same lipoprotein preparations.
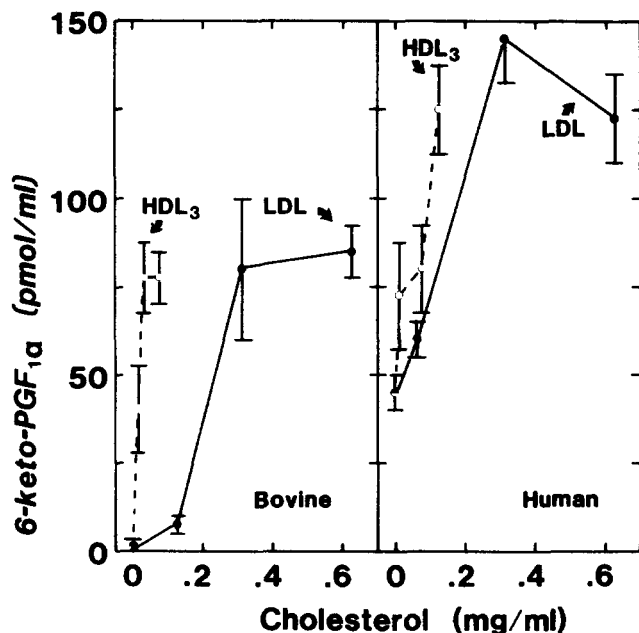
Fig. 2. Effect of lipoprotein concentration on PGI₂ production by cultured bovine aortic and human umbilical vein endothelial cells. The time of incubation was 16 hr. Each value is the mean ± SE of results obtained from three separate cultures. The bovine endothelial cells were from the same passage, and the human cells were prepared at the same time from a pool of umbilical veins. Both types of cells were incubated with the same preparations of lipoproteins.

PGI_2 production increased as larger amounts of the lipoproteins were added, the lipoprotein concentration being measured as total cholesterol content. At low concentrations, HDL_3 stimulated PGI_2 production to a much greater extent than LDL. At the highest concentrations of each lipoprotein that were tested, however, the amounts of PGI_2 produced were roughly similar. When concentrations of HDL_3 above 100 μg/ml cholesterol were added, PGI_2 formation was depressed (data not shown).

Results with human umbilical vein endothelial cultures are shown on the right side of Fig. 2. These cultures produced about 40 pmol/ml PGI_2 in 16 hr when no lipoproteins were added to a serum-free medium, accounting for the larger total PGI_2 output as compared with the bovine endothelial cultures. As observed with the bovine cells, the human cultures produced more PGI_2 as the lipoprotein concentration was raised. Although HDL_3 was more effective at low concentrations than LDL in stimulating PGI_2 production by the human cells, the difference between HDL_3 and LDL was not as marked as in the case of the bovine cells.

Leucine incorporation into cell protein

To determine whether incubation with these lipoproteins produced any toxic effect on the cultures, protein synthesis as assessed by [4,5-³H]leucine incorporation was tested. Bovine aortic endothelial cells were incubated

initially for 18 hr with increasing amounts of either HDL_3 or LDL. Control cultures were incubated without any added lipoprotein. After removal of this medium, the cultures were incubated for an additional 1 hr with [4,5-³H]leucine, and incorporation of radioactivity into cell protein was measured. As shown in **Table 1**, endothelial cells that had been incubated with either lipoprotein up to a concentration of 250 μg/ml cholesterol incorporated somewhat more radioactivity into proteins than the control cultures. At the highest lipoprotein concentration examined, 500 μg/ml cholesterol, the amounts of radioactivity incorporated were similar to the control values. There was no consistent difference at any of the lipoprotein concentrations between the cultures that had been incubated with HDL_3 as compared with LDL. Therefore, based on leucine incorporation into cellular protein, neither of these lipoproteins appears to be overtly toxic to the endothelial cultures.

Effect of culture status

The ability of HDL_3 and LDL to stimulate PGI_2 production was compared during several stages of culture growth. Bovine aortic endothelial cultures that were sparse, subconfluent, or confluent at the beginning of the incubation were exposed for 18 hr to either HDL_3 or LDL at a concentration of 50 μg/ml cholesterol. Control cultures were incubated without added lipoproteins. As seen in **Table 2**, HDL_3 and LDL stimulated less PGI_2 production as the confluency of the cultures increased. The small inherent PGI_2 formation in the absence of added lipoproteins also decreased as the degree of confluency increased.

Effect of lipoprotein-albumin mixtures

The presence of fatty acid-poor bovine serum albumin in the incubation medium reduced the amount of PGI_2

TABLE 1. Incorporation of [4,5-³H]leucine into bovine aortic endothelial cell protein

Cholesterol Content of Added Lipoprotein	Leucine Radioactivity Incorporated[a]		
	None[b]	HDL₃[c]	LDL[c]
μg/ml		*dpm*	
0	39,100 ± 360		
50		48,400 ± 400	56,300 ± 130
100		36,000 ± 650	50,500 ± 150
250		49,100 ± 910	49,600 ± 800
500		41,400 ± 560	37,800 ± 220

[a] Amount of [4,5-³H]leucine incorporated into cell protein in a 1-hr incubation. Each value is the mean ± SE of results obtained from three separate cultures, each prepared from the same passage and tested at the same time.
[b] No lipoproteins were added during the initial 18-hr incubation.
[c] Cultures were incubated for 18 hr with these human plasma lipoproteins prior to measurement of [4,5-³H]leucine incorporation.

TABLE 2. Degree of culture confluency influences PGI$_2$ production

Degree of Confluency[a]	PGI$_2$ Formed[b]		
	None[c]	HDL$_3$[d]	LDL[d]
		pmol/ml	
Sparse	9.3 ± 0.3	45.6 ± 9.9	58.0 ± 7.0
Subconfluent	4.4 ± 0.2	29.0 ± 8.6	25.8 ± 4.3
Confluent	1.6 ± 0.7	4.7 ± 2.1	6.1 ± 1.2

[a]The extent to which the bovine aortic endothelial cultures were confluent was estimated by microscopic inspection. Sparse cultures were 25 to 50% confluent; subconfluent cultures were 60 to 80% confluent.

[b]After incubation for 18 hr, the amount of 6-keto-PGF$_{1\alpha}$ present in the medium was measured by radioimmunoassay. Each value is the mean ± SE of results from three separate cultures. All cultures were from the same passage, and they were incubated with lipoproteins from a single preparation.

[c]No lipoproteins were present during the 18-hr incubation.

[d]The concentrations of HDL$_3$ and LDL were 50 μg/ml of cholesterol.

production elicited by lipoproteins (**Table 3**). Bovine aortic endothelial cultures were incubated for 16 hr with either HDL$_3$ or LDL, in the presence or absence of 50 μM albumin. In every case with HDL$_3$ and all but one case with LDL, less PGI$_2$ was formed in those media containing albumin.

Arachidonic acid effects

The amounts of PGI$_2$ produced by cultures incubated with arachidonic acid bound to albumin were compared with those produced during incubation with HDL$_3$ or LDL. At the arachidonic acid concentration tested, 200 μM bound to 100 μM albumin, 90% of the maximal PGI$_2$ output that can be obtained with these endothelial cell cultures occurs. Likewise, the concentrations of HDL$_3$ and LDL tested produce close to the maximal amounts of PGI$_2$ that can be obtained by incubation of these cultures with lipoproteins. As shown in **Fig. 3**, much more PGI$_2$ was produced in 16 hr when the cultures were incubated with arachidonic acid as compared with either HDL$_3$ or LDL. With bovine endothelial cells, the difference was about 6-fold; with the human cells, it was almost 13-fold.

The time-dependence of PGI$_2$ production during incubation of bovine endothelial cultures with albumin-bound arachidonic acid was also examined. Cultures in this study were incubated with 100 μM albumin containing a mixture of fatty acids that resembled the composition of the plasma free fatty acid under ordinary physiological conditions. The total free fatty acid concentration was 300 μM, only 5% being arachidonic acid. As seen in **Fig. 4**, PGI$_2$ formation increased during the 24 hr of incubation. The amounts of PGI$_2$ produced at the longer incubation times were considerably larger than those ordinarily elicited by incubation with lipoproteins.

Agonist-stimulated PGI$_2$ production

The ionophore A23187 stimulates PGI$_2$ production by

TABLE 3. Effect of albumin on PGI$_2$ production during incubation with lipoproteins

Experiment	Cholesterol Concentration of Added Lipoprotein	PGI$_2$ Formed[a]			
		HDL$_3$	HDL$_3$ plus Albumin[b]	LDL	LDL plus Albumin[b]
	μg/ml		pmol/ml		
1	50	80	3	9	16
	100	26	4	42	25
	200	43	12	69	45
2	50	60	3	9	3
	100	NT[c]	NT	22	5
	200	NT	NT	49	25

[a]After incubation for 16 hr, the amount of 6-keto-PGF$_{1\alpha}$ present in the medium was measured by radioimmunoassay. Each value is the average of two separate cultures. Each experiment was done with a culture from a different stock of bovine aortic endothelial cells and with a different lipoprotein preparation.

[b]In addition to the lipoprotein, these media contained 50 μM fatty acid-poor bovine serum albumin.

[c]Not tested in Experiment 2.

cultured endothelial cells (4, 13, 15). **Fig. 5** shows the time course of PGI$_2$ release following exposure of bovine endothelial cells to 10 μM A23187. After a 2-min lag period, a rapid output of PGI$_2$ occurred. PGI$_2$ formation after 5 min was about equal to that occurring following 16 hr of incubation with either HDL$_3$ or LDL. After only 20 min of incubation, the PGI$_2$ output was twice as large as that ordinarily occurring in 16 hr with either HDL$_3$ or LDL.
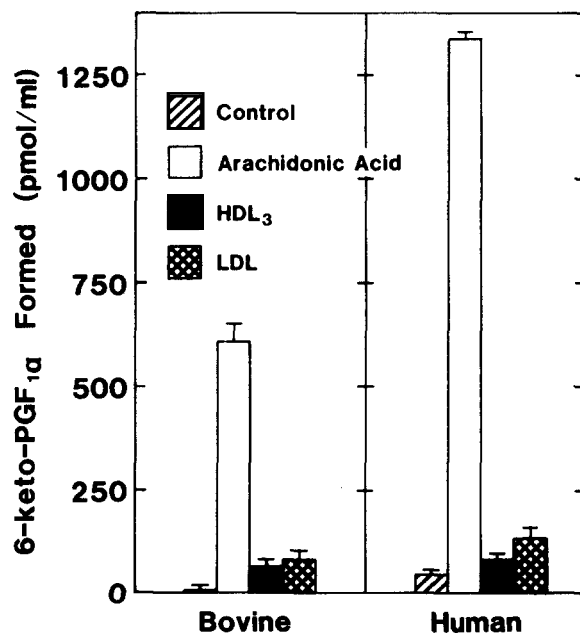

Fig. 3. Comparison of PGI$_2$ production by bovine aortic endothelial cells incubated with lipoproteins or arachidonic acid bound to albumin. The time of incubation was 16 hr. HDL$_3$ added was equivalent to 100 μg of cholesterol; LDL was equivalent to 300 μg of cholesterol. The arachidonic acid concentration was 200 μM bound to 100 μM bovine serum albumin. Each value is the mean ± SE of results obtained from three separate cultures, each from the same passage.
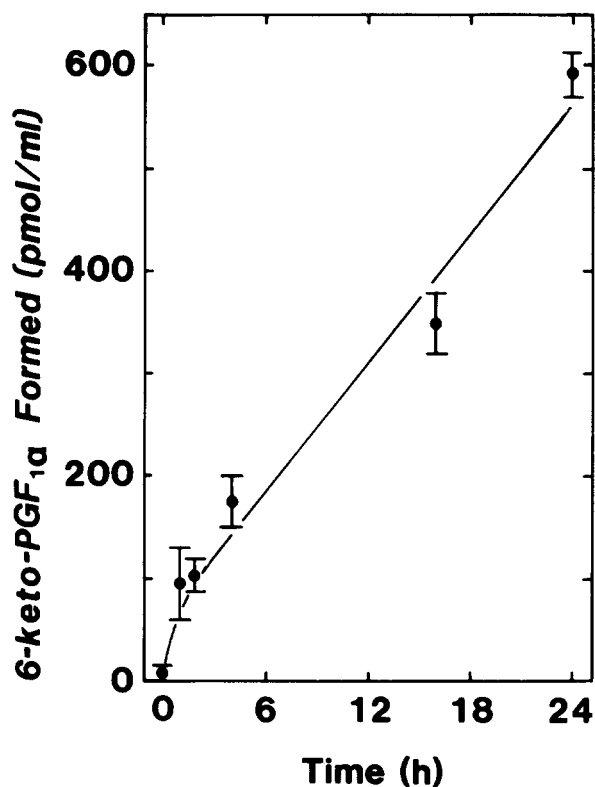
Fig. 4. Time-dependence of PGI_2 production by bovine endothelial cells incubated with free fatty acid. The medium contained 100 μM albumin, and a mixture containing 300 μM of free fatty acid in a total volume of 0.6 ml. The mixture contained 5% arachidonic acid, 25% palmitic acid, 10% stearic acid, 45% oleic acid, and 15% linoleic acid. Each value is the mean $\pm$ SE of results obtained from three separate cultures, each from the same passage.
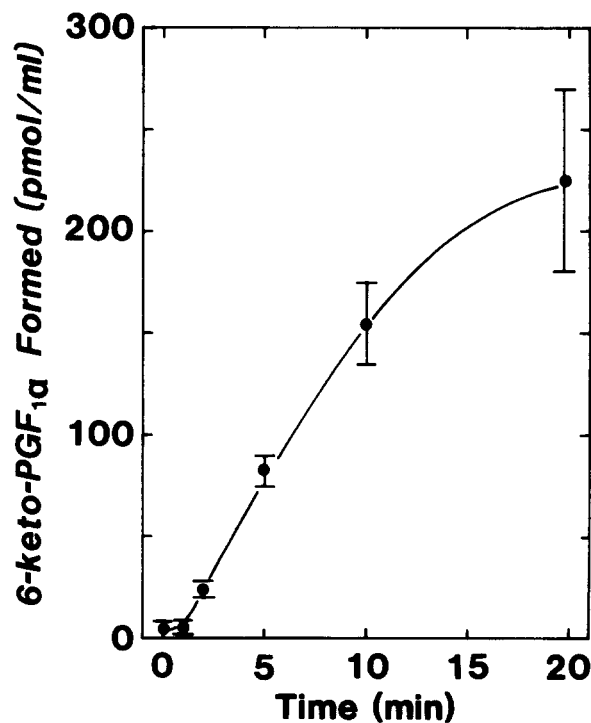
The effect of a prior incubation with lipoproteins on the capacity of the bovine endothelial cultures to release PGI_2 in response to ionophore A23187 was also tested. The cells were incubated initially with either HDL_3 or LDL for 16 hr, the lipoprotein concentration being between 50 and 200 μg/ml of cholesterol. After the lipoproteins were removed and the cultures were washed, the ionophore was added for 10 min. As seen in **Fig. 6**, a prior incubation with either HDL_3 or LDL inhibited subsequent PGI_2 production in response to the ionophore. Inhibition increased as the lipoprotein concentration was raised, with LDL producing a somewhat greater degree of inhibition than HDL_3. To determine whether the inhibition might have resulted from cyclooxygenase inactivation (22), a reversible cyclooxygenase inhibitor, ibuprofen (23, 24), was added during incubation with the lipoproteins. Previous work demonstrated that the presence of ibuprofen during an initial incubation with arachidonic acid prevents cyclooxygenase inactivation, and cultures treated in this way are completely responsive to stimulation as soon as the ibuprofen is removed (25). Fig. 6 shows, however, that the presence of ibuprofen during the incubation with either HDL_3 or LDL did not prevent

the subsequent reduction in PGI_2 release when the cultures were stimulated with ionophore A23187.

Cell lipids

Isotopic studies were done to determine whether incubation with HDL_3 or LDl might stimulate PGI_2 production by mobilizing arachidonic acid contained in cell lipids. Bovine endothelial cells were labeled by incubation for 30 min with tracer amounts of $[1\text{-}^{14}C]$arachidonic acid. After washing, the cultures were incubated in a serum-free medium for 16 hr, with or without added lipoproteins. As shown in **Table 4**, 11% of the radioactivity present initially in the cell lipids was recovered in the culture medium as free fatty acid after 16 hr when no lipoproteins were added. There was no significant increase in the amount of radioactivity released when either HDL_3 or LDL was added to the medium. The amounts of radioactivity released during these 16-hr incubations were slightly less than those released when cultures labeled in this way were incubated for only 10 min with ionophore A23187.

Additional studies were done to determine whether the fatty acid composition of the bovine aortic endothelial cell lipids changed in response to incubation with the lipoproteins. After exposure of the cultures to either HDL_3 or LDL for 20 hr and washing, the fatty acid composition of

Fig. 5. Time-dependence of PGI_2 release by bovine endothelial cells stimulated with ionophore A23187. The concentration of the ionophore was 10 μM, and the incubation medium contained 0.6 ml. Each value is the mean $\pm$ SE of results obtained from three separate cultures, each from the same passage.
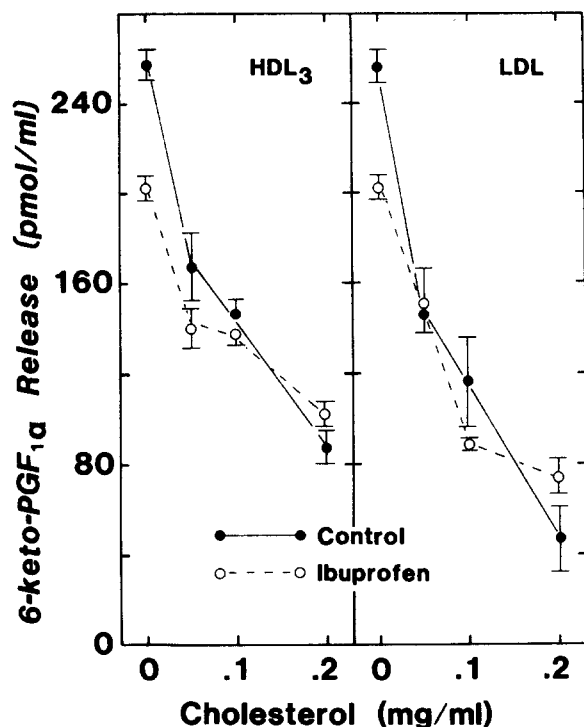
Fig. 6. Effect of incubation with lipoproteins on the capacity of bovine aortic endothelial cells to release PGI₂ in response to subsequent stimulation with the calcium ionophore A23187. The cultures were incubated with either HDL₃ or LDL for 16 hr. In each case, half of the cultures also were exposed to 100 μM ibuprofen during this 16-hr incubation. These media were removed and the cultures were washed. Following this, the cultures were exposed to 10 μM A23187 for 10 min, and the media were assayed for PGI₂ production by the radioimmunoassay against 6-keto PGF₁α. Each value is the mean ± SE of results obtained from three separate cultures, each from the same passage.

TABLE 4. Release of [1-^{14}C]arachidonic acid from bovine aortic endothelial cells

Parameter	Incubation Time	Radioactivity[a]
		dpm
Cell lipids after loading		42,100 ± 150
Release to unsupplemented medium[b]	16 hr	4,500 ± 300
Release to medium with HDL₃[c]	16 hr	4,450 ± 280
Release to medium with LDL[c]	16 hr	4,260 ± 160
Release to medium with A23187[d]	10 min	4,850 ± 100

[a]Mean ± SE of values obtained from three separate cultures, each from the same cell passage.
[b]Medium contained 50 μM bovine serum albumin in a total volume of 0.6 ml.
[c]Medium contained 50 μM bovine serum albumin plus 100 μg of lipoprotein cholesterol.
[d]Medium contained 50 μM bovine serum albumin plus 10 μM ionophore A23187.

and 21% less monoenoic fatty acid in the cell phospholipids. The higher polyenoic fatty acid content was accounted for almost entirely by an increase in linoleic acid (18:2). Although there also appeared to be a small increase in the arachidonic acid (20:4) content of the cell phospholipids, this difference was not statistically significant ($P > 0.1$). Similar but somewhat smaller changes in the cell phospholipid fatty acid composition occurred when the cultures were incubated for 16 hr with LDL, and the apparent increase in cellular 20:4 content also was not statistically significant. There was no appreciable difference in the fatty acid composition of the cell neutral lipids as a result of incubation with either HDL₃ or LDL.

The fatty acid composition of the major lipid classes contained in these human HDL₃ and LDL preparations was also determined. **Table 6** lists the values only for 18:2 and 20:4. HDL₃ phospholipids and cholesteryl esters contained appreciable amounts of 20:4, the highest per-

the cell phospholipids was determined by gas–liquid chromatography. **Table 5** shows that, as compared to cultures incubated without added lipoproteins, those incubated with HDL₃ contained 55% more polyenoic fatty acids

TABLE 5. Fatty acid composition of bovine aortic endothelial cell lipids after a 20-hr incubation

Fatty Acid	Composition[a]					
	Phospholipids			Neutral Lipids[b]		
	No Lipoprotein Supplement[c]	HDL₃[c,d]	LDL[c,d]	No Lipoprotein Supplement	HDL₃	LDL
	%					
16:0	27.4 ± 0.3	28.4 ± 1.4	25.2 ± 0.6	41.1 ± 2.5	41.0 ± 1.8	42.8 ± 1.4
18:0	25.0 ± 1.0	25.1 ± 1.3	25.7 ± 0.6	28.7 ± 1.5	28.2 ± 1.7	29.1 ± 1.4
16:1	5.7 ± 0.5	3.5 ± 0.3	3.8 ± 0.2	5.5 ± 2.0	4.4 ± 0.6	4.9 ± 1.0
18:1	23.3 ± 0.2	19.8 ± 0.4	23.1 ± 0.1	9.5 ± 1.4	10.2 ± 1.7	8.2 ± 0.8
18:2	3.2 ± 0.2	7.7 ± 0.4	4.9 ± 0.1	2.1 ± 0.5	1.8 ± 0.1	2.0 ± 0.3
20:4	5.5 ± 0.2	6.3 ± 0.6	6.6 ± 0.2	N.D.[e]	N.D.	N.D.

[a]The values do not add up to 100% because only the major fatty acids are listed. Each value is the mean ± SE of the results obtained from three separate cultures, each from the same cell passage and incubated with the same preparation of human lipoproteins.
[b]This fraction contains primarily triglycerides.
[c]Refers to the culture medium.
[d]These media contained 100 μg of lipoprotein cholesterol.
[e]Not detected.

TABLE 6. Linoleic and arachidonic acid composition of human plasma lipoprotein lipids

	Composition[a]			
	HDL₃		LDL	
Lipid Fraction	18:2	20:4	18:2	20:4
	%			
Phospholipids	21.9	10.8	20.0	5.4
Cholesteryl esters	48.6	7.1	41.4	2.4
Triglycerides	7.1	0.9	5.3	0.7

[a] Average of two samples that were in agreement within 10%.

centage being present in the phospholipids. The reverse was observed for 18:2; this fatty acid accounted for almost 50% of the cholesteryl ester fatty acids. Similar results were obtained with LDL, although the percentages of 18:2 and 20:4 were slightly lower in both phospholipids and cholesteryl esters as compared with HDL₃. Both of these lipoproteins contained only a very small amount of triglyceride, and relatively little 18:2 and only a trace amount of 20:4 were present in this fraction. The fatty acid composition of the HDL₃ and LDL phospholipids and cholesteryl esters also was determined after the 20-hr incubation with the endothelial cultures (data not shown). In neither case was there any statistically significant change as a result of incubation with the cells.

DISCUSSION

These findings demonstrate that a small amount of PGI₂ is formed by bovine aortic and human umbilical vein endothelial cultures during prolonged incubation with either human HDL₃ or LDL. This is consistent with the observations of Fleisher et al. (8) in porcine aortic endothelial cultures, and suggests that stimulation of PGI₂ production by plasma lipoproteins may be a general property of endothelium. The amounts of PGI₂ produced by the bovine and human cultures, however, are considerably less than those reported for the system investigated by Fleisher et al. (8), porcine aortic endothelium incubated primarily with rat plasma lipoproteins. Fleisher et al. (8) utilized the entire HDL fraction; we utilized HDL₃ because it is a relatively more homogeneous preparation. This apparently is not the main reason for the quantitative differences, for it recently has been reported that HDL₃, like HDL, stimulates prostaglandin formation in rabbit aortic smooth muscle cultures (26). A more likely explanation is species variation, either in the properties of the endothelium or the plasma lipoproteins. With regard to the latter, Fleisher et al. (8) suggest that HDL stimulates PGI₂ production by providing arachidonic acid to the cells and find that rat HDL cholesteryl esters contain 54% arachidonic acid. By contrast, we find that human HDL₃ cholesteryl esters contain only 7% arachidonic acid (Table 6).

Another important difference between our findings and those of Fleisher et al. concerns the lipoprotein specificity. Fleisher et al. (8) concluded that HDL is much more effective than other lipoproteins in stimulating PGI₂ formation by porcine aortic endothelial cells. This is also the case in our system when the lipoprotein concentrations are low. At higher concentrations, we find that human LDL becomes as effective as HDL₃, suggesting that the process is not specific for a single class of lipoproteins. A number of studies indicate that endothelium can bind LDL and utilize LDL lipids (27–32). Although the percentage composition is less than in HDL₃, the phospholipids and cholesteryl esters of LDL also contain arachidonic acid (Table 6). Therefore, it is not surprising that human LDL, like HDL₃, can stimulate a small amount of PGI₂ formation. Based on cholesterol content, there ordinarily is 2- to 3-fold more LDL than HDL in human plasma. When 2- to 3-fold more LDL was added (based on cholesterol content), LDL was about as effective as HDL₃ in eliciting PGI₂ formation (Fig. 2).

Human LDL is reported to inhibit the proliferation of bovine vascular endothelial cells at concentrations between 100 and 250 μg/ml protein (33), which corresponds to about 170 to 420 μg/ml cholesterol. Based upon leucine incorporation into cellular protein, however, we did not observe any clear evidence of toxicity when the cultures were incubated for 18 hr with LDL at concentrations up to 250 μg/ml cholesterol, and possibly up to 500 μg/ml cholesterol (Table 1). It is possible that the injurious effect of LDL noted in other studies resulted from oxidative damage to the lipoproteins during culture (34) and that such damage was prevented by the conditions that we employed.

The observation that PGI₂ formation decreases as cell density increases (Table 2) can be explained, at least for LDL, by the fact that the number of LDL binding sites on bovine aortic endothelial cells also decreases as cell density increases (32). PGI₂ formation in response to HDL₃ appears to reach a maximum between 50 and 100 μg/ml cholesterol, depending on the lipoprotein preparation. In response to LDL, PGI₂ production continues to increase up to 200 to 250 μg/ml cholesterol. Since the specific binding of LDL to bovine aortic endothelial cells is saturated at about 20 μg/ml LDL (32), corresponding to about 35 μg/ml cholesterol, it is unclear why PGI₂ production continues to increase. Perhaps the effect of LDL occurs indirectly and nonspecific binding, which is not saturated at LDL concentrations up to 80 μg/ml (32), plays a role in the PGI₂ response. It also is difficult to explain why PGI₂ formation in response to LDL exhibits a lag period (Fig. 1). One possibility is that the PGI₂ is produced in response to a product that is released during intracellular LDL catabolism. Such products continue to accumulate linearly over 12 hr when cultured fibroblasts are incubated with LDL (35). Alternatively, PGI₂ pro-

duction may be secondary to some metabolic effect of LDL on the cells.

Our findings indicate that albumin-bound arachidonic acid stimulates PGI$_2$ production by the bovine aortic endothelial cells to a greater extent (Fig. 3) and much more rapidly (Fig. 4) than either of the human lipoproteins. This is in contrast to the results of Fleisher et. al (8) showing that HDL is much more effective than arachidonic acid in the porcine cultures. Although the molar ratio of fatty acid to albumin was 2.0 in the experiment shown in Fig. 3, a value within the physiologic range (31), all of the fatty acid was in the form of arachidonic acid. Under physiologic conditions arachidonic acid accounts for 5%, at most, of the plasma free fatty acid (36–40). To determine whether this may be a factor in the apparent discrepancy, the ability of arachidonic acid to stimulate PGI$_2$ production was also tested when it comprised only 5% of a mixture containing the most prevalent plasma free fatty acids. Even under these conditions (Fig. 4), we found that more PGI$_2$ was formed by the endothelial cultures incubated with the albumin-bound fatty acid mixture than either HDL$_3$ or LDL, and the production occurred more rapidly. These findings suggest that while both plasma lipoproteins and free fatty acids can stimulate the formation of some PGI$_2$ over relatively long periods of time, free fatty acid probably plays a more important role in this process.

Fleisher et al. (8) concluded that HDL stimulates PGI$_2$ synthesis primarily by providing some of the arachidonic acid contained in its lipids to the endothelial cells. Although indirect, our data are consistent with this interpretation. Incubation with HDL$_3$ did not enhance arachidonic acid release from endothelial cell lipids (Table 4) or PGI$_2$ production in response to ionophore A23718 (Fig. 6), processes that might be expected to occur if HDL$_3$ acted by stimulating endogenous PGI$_2$ synthesis. Since the amount of PGI$_2$ formed is in the pmol range, enough arachidonic acid could be made available by HDL$_3$ to support a small amount of prostaglandin synthesis without concomitantly producing any appreciable increase in the cellular arachidonic acid content (Table 5). Linoleic acid, which is present in large amounts in the lipoprotein phospholipids and cholesteryl esters (Table 6), is increased in the cell phospholipids following incubation with HDL$_3$ and, to a lesser extent, LDL (Table 5). The fact that arachidonic acid did not increase appreciably, even though the intracellular linoleic acid content increased, is consistent with previous results indicating that the conversion of linoleic to arachidonic acid is minimal when endothelial cells are cultured in a lipid-rich medium (13, 15, 18, 41).

What is important in considering the possible physiologic role of plasma lipoproteins in endothelial prostaglandin synthesis is that lipoproteins stimulate only a small amount of PGI$_2$ production and that this occurs continuously over a long period of time. By contrast,

endothelial cultures release PGI$_2$ much more rapidly and to a greater extent after exposure to stimuli like ionophore A23187 (Fig. 5) or thrombin (4). This rapid release is thought to constitute the protective effect of PGI$_2$ against acute events such as platelet aggregation and arterial vasoconstriction (1–4). It is possible that the small but continuous production of PGI$_2$ elicited by plasma lipoproteins may also contribute to these protective actions or have other important functional roles. The fact that the presence of albumin reduces the amount of PGI$_2$ produced during incubation with either HDL$_3$ or LDL (Table 3), however, suggests that the process may occur to only a limited extent under physiological conditions. Therefore, it seems likely that plasma lipoproteins may not play as large a role as has been suspected in endothelial PGI$_2$ production. ▪

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