Stimulation of Arterial Endothelial Cell Prostacyclin Synthesis by High Density Lipoproteins*

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Prostacyclin (PGI2) is a vasoactive prostaglandin synthesized by vascular endothelial and smooth muscle cells. In order to investigate whether plasma lipoproteins influence the biosynthesis and release of prostacyclin by vascular tissues, human high density lipoproteins (HDL) and low density lipoproteins (LDL) were incubated with porcine arterial endothelial cells grown in tissue culture. PGI2 production was measured by radioimmunoassay of its stable metabolite, 6-keto-PGF1α. In incubations of HDL with endothelial cells for 24 h, levels of 6-keto-PGF1α in the medium increased significantly in a dose-dependent fashion to values 2-4-fold above control. This effect was less pronounced in confluent than in subconfluent cultures and did not occur in the presence of an inhibitor of cyclooxygenase. No significant stimulation of 6-keto-PGF1α was observed when the endothelial cells were incubated with LDL. In time course experiments with HDL, 6-keto-PGF1α levels increased continuously over 24 h. Rat HDL, containing a high content of arachidonate in its cholesterol ester fatty acids, caused a 2-4-fold greater release of 6-keto-PGF1α than human HDL. The delipidated apoprotein of both human and rat HDL caused similar stimulation of 6-keto-PGF1α production, but much less than intact HDL. The data indicate that HDL stimulates PGI2 synthesis by cultured arterial endothelial cells, possibly by providing the cells with arachidonate.

Prostacyclin is a vasodilator and inhibitor of platelet aggregation that is synthesized by a variety of tissues including isolated perfused hearts, blood vessels, and vascular endothelial and smooth muscle cells grown in tissue culture (1-4). Thromboxane A2, an arachidonic acid metabolite produced by platelets, promotes aggregation and is a potent vasoconstrictor (4). Moncada and Vane (4) have postulated that a balance between the production of thromboxane A2 by platelets and the production of PGI2 by endothelial cells is important in the maintenance of vascular integrity. Deficient PGI2 production at sites of endothelial injury has been implicated in the platelet aggregation which characterizes early stages in the formation of atherosclerotic lesions (4). PGI2 is synthesized from arachidonate derived from cellular lipids (3). Endothelial cells lack enzymes for adequate synthesis of arachidonic acid from linoleic acid and thus may ultimately need to acquire arachidonate from exogenous sources (5). In plasma, arachidonate exists in low concentrations as free fatty acid or in esterified form in lipoproteins (6). The present study was designed to test the hypothesis that plasma lipoproteins, particularly high density lipoproteins, might stimulate the synthesis of vasoactive prostaglandins by vascular tissue.

EXPERIMENTAL PROCEDURES

Preparation of Plasma Lipoproteins—Plasma lipoproteins were prepared by preparative ultracentrifugation of human and rat plasma in a Beckman 40.3 rotor (Beckman Instruments) between densities 1.019-1.063 g/ml (LDL) and 1.063-1.210 g/ml (HDL) (1.075-1.210 for rat HDL). The lipoproteins were recentrifuged once at their upper density limit. The total protein content was determined by the method of Lowry et al. (7) and the total cholesterol content by the method of Zlatkis and Zak (8). Prior to incubation with cells, the lipoproteins were dialyzed against 3 changes of 40 volumes of Dulbecco's modified Eagle's medium containing penicillin, 100 units/ml, and streptomycin, 100 μg/ml. For preparation of apo-HDL, HDL was delipidated in 20 volumes of ethanol/ether, 3:2, and washed 3 times with diethyl ether at 0°C.

Preparation of Cell Cultures—Porcine aortic endothelial cells obtained by mild collagenase digestion from aortae of freshly slaughtered pigs were cultured and frozen in liquid nitrogen at the seventh passage. These cells were verified to be endothelial by the presence of cobblestone morphology at confluence, by positive immunofluorescence for factor VIII antigen using specific antifactor VIII antiserum, and by the presence of Weibel-Palade bodies by electron microscopy. For each experiment, the endothelial cells were thawed rapidly and plated into Petri dishes (16 x 10 cm, Falcon) at 8 x 10⁶ cells/dish in DME (Grand Island Biological Co.) containing 10% fetal calf serum (Sterile Systems, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine. The plates were incubated for 48-54 h at 37°C in an atmosphere containing 5% CO2/95% air.

Incubations—Prior to each experiment, confluent cells were washed, the subconfluent cells were used. The experimental cultures were incubated in 2 ml of DME (also lacking fetal calf serum) containing HDL, LDL, sodium arachidonate, or indomethacin (10 μg/ml). At 24 h, the DME was removed and the concentration of 6-keto-PGF1α was measured by radioimmunoassay. The results of these experiments as well as all others were analyzed by analysis of variance following a log transformation of the data. Data reported are mean ± S.E.

Radioimmunoassay of 6-Keto-PGF1α—The supernatants from the incubations were assayed directly for 6-keto-PGF1α using a specific antibody obtained as a generous gift from Dr. Lawrence Levine of Brandeis University. The cross-reactivity of various prostaglandins or prostaglandin metabolites was less than 0.1% for prostaglandins E1, E2, A1, and F1α, and for 13,14-dihydro-15-keto-prostaglandin E2, 6-keto-PGF1α, thromboxane B2, or sodium arachidonate. 6-Keto-5,6,9,11,12,14,15-dihydro-15-keto-PGF1α was obtained from New England Nuclear (120 Ci/mmol) and the unlabeled 6-keto-PGF1α was from Upjohn Diagnostics.

Each assay tube (polypropylene tubes, 12 x 75 mm, Sarstedt 55.596) received approximately 6000 cpm of 6-keto-[14C]PGF1α dissolved in 100 μl of Tris-buffered saline (10 mM, pH 7.4) with 0.1% gelatin, followed by 100 μl of antiserum diluted 1:4000 in the Tris buffer. Standards containing 8-2000 pg of unlabeled 6-keto-PGF1α dissolved in 100 μl of DME were treated in the same manner as samples. The tubes were incubated in a shaking bath at 37°C for 60 min and placed on ice for 5 min. Antibody-bound 6-keto-PGF1α was separated from the unbound ligand by the addition of 1 ml of an ice-
were centrifuged at 4800 g for 10 min. The supernatant (containing antibody-bound 6-keto-PGF\(_1\alpha\)) was decanted into glass scintillation vials. After the addition of 15 ml of Hydrofluor, the vials were counted in a Packard Tri-Carb Model 3330 liquid scintillation spectrometer.

Standard curves were analyzed by the method of Rodbard and Hutt (9) which is a nonlinear transformation of the logit-log relationship. The initial (zero point) binding was 57%; nonspecific binding was 1.5%. The amount of unlabelled 6-keto-PGF\(_1\alpha\) required to displace zero point binding by 10% was 20 pg; the amount required to displace zero point binding by 50% was 145 pg. The accuracy of the assay was determined by adding known amounts of unlabelled 6-keto-PGF\(_1\alpha\) (25-800 pg) to DME. Linear regression analysis of the recovery of standards gave

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y = 0.979 x - 16 \quad (r = 0.99; n = 3)
\]

where \(y\) is the amount of 6-keto-PGF\(_1\alpha\) recovered and \(x\) is the amount added. The intraassay coefficient of variation was 2%; the interassay coefficient of variation was 5%.

RESULTS AND DISCUSSION

The data from 4 experiments are summarized in Fig. 1. The concentration of 6-keto-PGF\(_1\alpha\) in the medium of control endothelial cell cultures was 2.4 ± 0.03 ng/ml at 24 h. When endothelial cells were incubated with media containing human HDL, the production of 6-keto-PGF\(_1\alpha\), was stimulated 5-fold (\(p < 0.01\)). At 24 h, the concentration of 6-keto-PGF\(_1\alpha\) in the medium of endothelial cell cultures containing HDL at 0.15 mg of cholesterol/ml was 12.6 ± 0.18 ng/ml; it was 5.5 ± 0.9 ng/ml in the endothelial cell medium containing HDL at 0.015 mg of cholesterol/ml. In contrast, medium from endothelial cells incubated with human LDL at two concentrations (0.15 and 0.015 mg of cholesterol/ml) did not contain significantly increased concentrations of 6-keto-PGF\(_1\alpha\), (Fig. 1). Cells incubated in medium with sodium arachidonate (5 and 10 \(\mu M\)) also produced significantly more 6-keto-PGF\(_1\alpha\), than control cells (Fig. 1). Indomethacin inhibited completely the stimulation of 6-keto-PGF\(_1\alpha\), by HDL and by sodium arachidonate (Fig. 1). No 6-keto-PGF\(_1\alpha\), was formed when HDL was incubated without cells for 24 h. Because prostaglandins are not stored in cells (9), increasing levels of a prostaglandin or its metabolites in the incubation medium are evidence for prostaglandin biosynthesis. Thus, these results indicate, at similar concentrations, that HDL but not LDL stimulates prostacyclin synthesis by cultured arterial endothelial cells.

Fig. 2 summarizes 4 dose-response experiments performed with human HDL and LDL. Over the concentration range from 0.015 to 0.3 mg of cholesterol/ml, HDL stimulated the production of 6-keto-PGF\(_1\alpha\), in a dose-dependent fashion; \((r = 0.97; p < 0.01)\). Although in some individual experiments there was stimulation of 6-keto-PGF\(_1\alpha\), by LDL (0.015 to 0.3 mg of cholesterol/ml), the effects were small and not statistically significant at any dose. In mixing experiments, LDL was co-incubated with HDL in the endothelial cell cultures, HDL (0.4 mg of protein/ml) increased 6-keto-PGF\(_1\alpha\), concentration in the medium from a control of 0.54 ± 0.08 to 5.05 ± 0.74 ng/ml, \(p < 0.01\) (\(n = 2\)). However, co-incubation of the HDL (0.4 mg of protein/ml) with human LDL at concentrations of 0.16, 0.08, and 0.04 mg of protein/ml did not significantly alter the HDL response; 6-keto-PGF\(_1\alpha\), concentrations in the media were 5.98 ± 0.42, 5.55 ± 0.48, and 4.38 ± 0.46 ng/ml, respectively. Thus, LDL did not inhibit stimulation of prostacyclin synthesis by HDL, suggesting that these effects were independent of LDL receptor-mediated uptake.

Fig. 3 presents the mean results from 2 time course experiments performed with DME (control) and DME containing HDL (0.15 mg of cholesterol/ml). In the control experiments, the accumulation of 6-keto-PGF\(_1\alpha\), in the medium containing HDL was much more pronounced and increased steadily throughout the 24 h.

To determine the effects of cell confluence on production of 6-keto-PGF\(_1\alpha\), HDL (0.15 mg of cholesterol/ml) and AA (10 \(\mu M\)) were incubated for 24 h with confluent and subconfluent cultures of endothelial cells (\(n = 3\)). The HDL-induced production of 6-keto-PGF\(_1\alpha\), was 870 ± 293% of control for subconfluent cells (\(p < 0.05\)) and 185 ± 63% for confluent cells (increase not significant). The arachidonate-induced stimulation was 797 ± 210% for subconfluent cells (\(p < 0.01\)) and 213 ± 38% for confluent cells (increase not significant). Tauber et al. (10) have reported that HDL enhanced the proliferation of endothelial cells cultured over a 2-5-day period in serum-free medium. To determine if the stimulation of 6-keto-PGF\(_1\alpha\), production in our studies was due to an effect of HDL on proliferation or viability, subconfluent cells were incubated with medium (control), HDL (0.015 mg of cholesterol/ml), AA (10 \(\mu M\)), and HDL + AA. In 3 experiments, the number of viable cells (\(10^{12} \) per plate (cells excluding trypan blue quantitated by hemocytometer) were 209 ± 49 (control), 216
The fact that rat HDL has a high content of arachidonate in its cholesterol ester fatty acids by gas-liquid chromatography. At similar concentrations (0.4 mg of protein/ml), rat HDL caused a 4-fold greater stimulation (p < 0.01) of 6-keto-PGF\(_1\alpha\) release into the medium of cultured endothelial cells than human HDL (control = 0.60 ± 0.17 ng/ml; human HDL = 3.80 ± 0.37 ng/ml; rat HDL = 15.82 ± 3.76 ng/ml; n = 4). In additional experiments, human and rat apo-HDL caused an approximately equal, small stimulation of 6-keto-PGF\(_1\alpha\) release while the stimulation of 6-keto-PGF\(_1\alpha\) release produced by intact rat HDL was much greater than that produced by intact human HDL (Table 1). These results suggest that the marked effect of rat HDL resulted from a high content of arachidonic acid in its cholesterol ester fatty acids and not from differences in its apoprotein composition.

In summary, the results of this study indicate that HDL stimulates prostacyclin synthesis by cultured arterial endothelial cells. The possibility raised by these in vitro results, i.e. that the beneficial effects of HDL in cardiovascular disease (12, 13) might involve stimulation of the release of vasoactive prostaglandins by blood vessels, awaits further investigation in intact animals and man. Stimulation of endothelial cell prostaglandin production by HDL could perhaps explain the results of Nordoy et al. (14) who showed that HDL could partially reduce the inhibitory effect of LDL on the ability of endothelial cells to inhibit platelet aggregation. Beitz and Forster (15) showed that HDL increased the production of 6-keto-PGF\(_1\alpha\) from PGH\(_2\) by aortic microsomes and suggested that this effect was due to stimulation of prostacyclin synthetase by HDL. Our results suggest that the mechanism of action of HDL may be complex, perhaps involving both its lipid and apoprotein moieties. The fact that stimulation of 6-keto-PGF\(_1\alpha\) was produced by HDL but not by LDL suggests that specificity may reside in the apoprotein moiety, since the fatty acid composition of the human LDL and HDL lipids are not markedly different. However, the apoprotein of HDL caused only a small stimulation of 6-keto-PGF\(_1\alpha\) release, a finding which implies that the intact lipoprotein is required to achieve the full effect. The much greater stimulation of 6-keto-PGF\(_1\alpha\) release by rat HDL than human HDL suggests that HDL may be providing substrate (AA) to the endothelial cells for synthesis of prostacyclin.

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