Effects of prostaglandins on LDL receptor activity and cholesterol synthesis in freshly isolated human mononuclear leukocytes

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Abstract The effects of prostaglandin (PG) E_1, PGE_2, the stable prostacyclin analogue Iloprost, and $PGF_{2\alpha}$ on low density lipoprotein (LDL) receptor activity and cholesterol synthesis were investigated in freshly isolated human mononuclear leukocytes. Incubation of cells for up to 45 hr in a lipid-free medium resulted in an increase in the rate of cholesterol synthesis from [^{14}C]acetate and the high affinity accumulation and degradation of ^{125}I-labeled LDL. Addition of PGE_1 in increasing concentrations to the incubation medium inhibited cholesterol synthesis and the specific accumulation and degradation of ^{125}I-labeled LDL; at a concentration of 10 μM, the inhibitions were 61%, 70%, and 67%, respectively, after an incubation of 20 hr. The effects of PGE_2 and Iloprost were similar. The action of the prostaglandins on LDL receptor activity appeared to be mediated by a decrease in the number of LDL receptors and not by a change in the binding affinity. The prostaglandins yielded sigmoidal log concentration-effect curves. In contrast, $PGF_{2\alpha}$ had no influence on cholesterol synthesis or LDL receptor activity up to a concentration of 10 μM. PGE_1, PGE_2, and Iloprost, but not $PGF_{2\alpha}$, led to an increase in the concentration of intracellular cyclic AMP. Dibutyryl cyclic AMP mimicked the effects of the E-prostaglandins and Iloprost on the LDL receptor activity. The results suggest that PGE_1, PGE_2, and prostacyclin affect LDL receptor activity and cholesterol synthesis and, therefore, may play a role in the regulation of cholesterol homeostasis and in the development of atherosclerosis. — **Krone, W., A. Klass, H. Nägele, B. Behnke, and H. Greten.** Effects of prostaglandins on LDL receptor activity and cholesterol synthesis in freshly isolated human mononuclear leukocytes. *J. Lipid Res.* 1988. **29:** 1663–1669.

Supplementary key words low density lipoprotein • cholesterol • lanosterol • squalene • Iloprost • prostaglandin E_1 • prostaglandin E_2 • prostaglandin $F_{2\alpha}$ • cyclic AMP

Accumulation of cholesterol is considered the hallmark in the pathogenesis of atherosclerosis (1). Circulating monocytes are presumed to be the precursors of the lipid-laden cells in the intima (2). There are several mechanisms whereby cholesterol and cholesteryl ester in these cells are derived: i) through the cellular cholesterol synthesis de novo; ii) through endocytosis of plasma low density lipoprotein (LDL), an event that is mediated by the LDL receptor (for review see ref. 3); and iii) possibly through other routes not yet well defined (e.g., β-VLDL and acetyl-LDL). Both cholesterol biosynthesis and LDL receptor activity are affected by diet (4, 5), lipoproteins (6, 7), hormones (8–10), and drugs (11–14). Although evidence has been provided that prostaglandins may play a major role in atherogenesis (for reviews see refs. 15 and 16), little is known about their role in the regulation of lipid metabolism. Preliminary work from our laboratory has demonstrated a suppressive effect of the prostacyclin analogue Iloprost and prostaglandin E_1 (PGE_1) on the synthesis of sterols (17). In the present study the influence of several prostaglandins, i.e., Iloprost, PGE_1, PGE_2, and $PGF_{2\alpha}$, on LDL receptor activity and cholesterol biosynthesis was investigated in freshly isolated human mononuclear leukocytes.

MATERIALS AND METHODS

Materials

Sodium ^{125}I, [2-^{14}C]acetate and [1,2-^{3}H]cholesterol were purchased from Amersham International (Amersham, U.K.). RPMI 1640 culture medium and penicillin/streptomycin mixture were obtained from Gibco (Glasgow, U.K.). Ficoll-Hypaque (Lymphoprep) was purchased from Nyegaard & Co (Oslo, Norway). Conical (50 ml) plastic tubes were obtained from Falcon (Oxnard, USA) and 22-mm 12-well tissue culture clusters were from Costar (Cambridge, MA). Instant scintillation gel was purchased from United Technologies Packard (Downers Grove, IL), and prechanneled silica gel TLC plates were from Whatman, Chemical Separation (Clifton, NJ).

Abbreviations: PG, prostaglandin; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; BSA, bovine serum albumin; V_{max}, maximum velocity; K_m, Michaelis constant; AMP, adenosine monophosphate.

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Prostaglandin E_1 (prostaglandin E_1-α-cyclodextrin) was a gift from Dr. Waltraud Rogatti, Sanol Schwarz GmbH (Monheim, F.R.G.). Prostaglandin E_2 and Iloprost (ZK 36 374) were gifts from Dr. E. Schillinger, Schering AG (Berlin, F.R.G.). N^6, 2'-O-dibutyryladenosine-3':5'-cyclic monophosphate was obtained from Sigma (Munich, F.R.G.).

Isolation and preparation of LDL and LPDS

Human LDL (1.019–1.063 g/ml) and human lipoprotein-deficient serum (LPDS, d > 1.215 g/ml) were isolated from plasma of normolipidemic healthy subjects by sequential ultracentrifugation (18). ^{125}I-labeled LDL was prepared by the iodine monochloride method of McFarlane (19) as modified by Bilheimer, Eisenberg, and Levy (20). After iodination, free iodine was removed by passage through a QAE-Sephadex A-50 anion exchange column and the LDL was sterilized by 0.45 μm Millipore filtration. Analysis of iodinated lipoprotein substrates showed that less than 1% of radioactivity was trichloroacetic acid-soluble non-iodine material. Specific activity of the preparation was usually in the range between 80 and 150 cpm/ng. The concentration of LDL is expressed in terms of its protein content, which was determined by the method of Lowry et al. (21).

Isolation and incubation of mononuclear leukocytes

Peripheral blood mononuclear leukocytes were isolated from heparinized blood obtained from healthy subjects, after an overnight fast, by the method of Böyum (22). Ten ml of blood was diluted with equal volumes of 0.15 M NaCl. Ten ml of Lymphoprep was layered under the mixture of blood and saline, and centrifuged at 400 *g* for 40 min at room temperature. The resultant band of mononuclear leukocytes was washed twice in RPMI 1640 and then suspended in RPMI 1640 with 100 U/ml penicillin and 100 μg/ml streptomycin. Aliquots (0.5 ml, 2 × 10^6 cells) were placed in 22-mm wells and incubated at 37°C in a humidified atmosphere with 5% CO_2. The incubation of cells for the determination of LDL receptor activity and cholesterol synthesis was performed in RPMI 1640 medium without and with 4% (v/v) LPDS, respectively.

Cell viability as assessed by trypan blue exclusion was greater than 95% before and after incubation up to 45 hr for both untreated cells or cells treated with prostaglandins. Purity of cell preparations was routinely assessed by staining smears with Wright-Giemsa. Differential counts before and after incubations showed that 85–90% of the cells were identified as lymphocytes; the rest were monocytes as identified by their ability to ingest latex particles.

Measurement of LDL receptor activity

At zero time and after incubation periods as indicated below (Results and figures), 50 μl of 20% BSA in RPMI 1640 and ^{125}I-labeled LDL were added to the cells with and without a 25-fold excess of unlabeled LDL. Cells were incubated for an additional 6-hr period at 37°C before the incubation was stopped on ice. Mononuclear cells were overlayered on a solution of 80 mg/ml BSA and sedimented by centrifugation. From aliquots of the supernatant, the content of ^{125}I-labeled trichloroacetic acid-soluble material was determined (degradation) (6). The cell pellet was washed three times to determine the total cellular content of ^{125}I-labeled LDL (cellular accumulation) (6). Specific accumulation and specific degradation were defined as the respective differences of values with and without the excess of unlabeled LDL and were expressed as nanograms of ^{125}I-labeled LDL protein that was associated with 2 × 10^6 cells or degraded to acid-soluble non-iodine material per 2 × 10^6 cells.

Determination of the incorporation of [2-^{14}C]acetate into sterols

At zero time and after incubation periods as indicated below (Results and figures), 12.5 μl of [2-^{14}C]acetate (2.5 μCi, 55 mCi/mmol) was added to each dish. After 3 additional hr at 37°C, the incubation was stopped by transferring the cells to 8.5 ml chloroform–methanol 1:2 (v/v). [1,2-^{3}H]cholesterol was added as an internal standard and lipids were extracted by the method of Bligh and Dyer (23) and saponified with methanolic potassium hydroxide (2 M) for 1 hr at 70°C. The nonsaponifiable fraction was extracted three times with 2.5 ml heptane and the extracts were combined. An aliquot, 1.5 ml, was counted, after adding 10 ml of Insta-Gel scintillation fluid, to determine the incorporation of [^{14}C]acetate into sterols. The rest of the extract was evaporated at 70°C and dissolved in dichloromethane. [^{14}C]Squalene, [^{14}C]lanosterol, and [^{14}C]cholesterol were separated by thin-layer chromatography (7). The recovery of [^{3}H]cholesterol was used to correct for procedural losses of synthesized [^{14}C]cholesterol.

Determination of intracellular cyclic AMP

For assay of cyclic AMP content (24), cells were incubated at 37°C for 10 min with or without agents. The cells were then chilled on ice and sedimented by centrifugation. The cell pellets were homogenized in 6% ice-cold trichloroacetic acid and extracted with ether four times. The aqueous phase was evaporated to dryness under a stream of nitrogen at 56°C. The residue was dissolved in Tris/EDTA-buffer and cyclic AMP was determined by the [^{3}H] cAMP Radioassay Kit from Amersham International.

Data analysis

Values were expressed as means ± SE of n experiments (indicated in the legends of figures).

RESULTS

Freshly isolated mononuclear leukocytes exhibit a low number of high affinity binding sites for LDL (6). They also exhibit a relatively low ability to degrade ^{125}I-labeled LDL. However, incubation of cells at 37°C in a medium devoid of lipoproteins led to a progressive rise in their ability to bind (6) and accordingly to accumulate and degrade ^{125}I-labeled LDL with high affinity. The specific accumulation and degradation of ^{125}I-labeled LDL was 3- and 10-fold, respectively, after an incubation of 20 hr (data not shown). This increase in both high affinity accumulation and degradation of ^{125}I-labeled LDL was inhibited by PGE_1, PGE_2, and Iloprost which were added in increasing concentrations to the incubation medium at zero time. The E-prostaglandins and Iloprost yielded sigmoidal log concentration-effect curves which are shown in **Fig. 1**. PGE_1 at concentrations of 10 μM and 0.1 μM inhibited the specific accumulation by 70% and 25%, respectively, and the specific degradation by 67% and 30%, respectively. The inhibition of LDL receptor activity caused by PGE_2 and Iloprost was similar (Fig. 1). In contrast to the E-prostaglandins and Iloprost, $PGF_{2\alpha}$ had no effect at all up to a concentration of 10 μM.

In order to determine whether the suppression of LDL receptor activity caused by PGE_1, PGE_2, and Iloprost is due to a decreased number of LDL receptors or to a decreased affinity of LDL to its receptor, LDL concentration curves were performed in the presence or absence of the agents (1 μM). As shown in **Fig. 2**, increasing concentrations of ^{125}I-labeled LDL in the incubation medium led to a saturation of its degradation. Half-maximal saturation was achieved at 6.3 μg/ml. In the presence of PGE_1, the rate of degradation was lower at all concentrations used. Double reciprocal plots (25) of the data indicate that the major effect of PGE_1 was to produce a decrease in apparent maximum velocity (V_{max}) of LDL degradation from 136 to 80 ng/2 × 10^6 cells per 6 hr. In the presence of the prostaglandin, no change was observed in apparent Michaelis constant (K_m) of degradation (Fig. 2). The experiments using PGE_2 and Iloprost gave essentially the same results (data not shown). Dibutyryl cyclic AMP, added in increasing concentrations to the incubation medium at zero time, resulted in an inhibition of both the high affinity accumulation and degradation of ^{125}I-labeled LDL (10 μg/ml) (**Table 1**). As for the prostaglandins, the analysis of LDL concentration curves revealed a decrease in apparent V_{max} from 136 to 56 ng/2 × 10^6 cells per 6 hr without any change in apparent K_m of specific LDL degradation (Fig. 2).

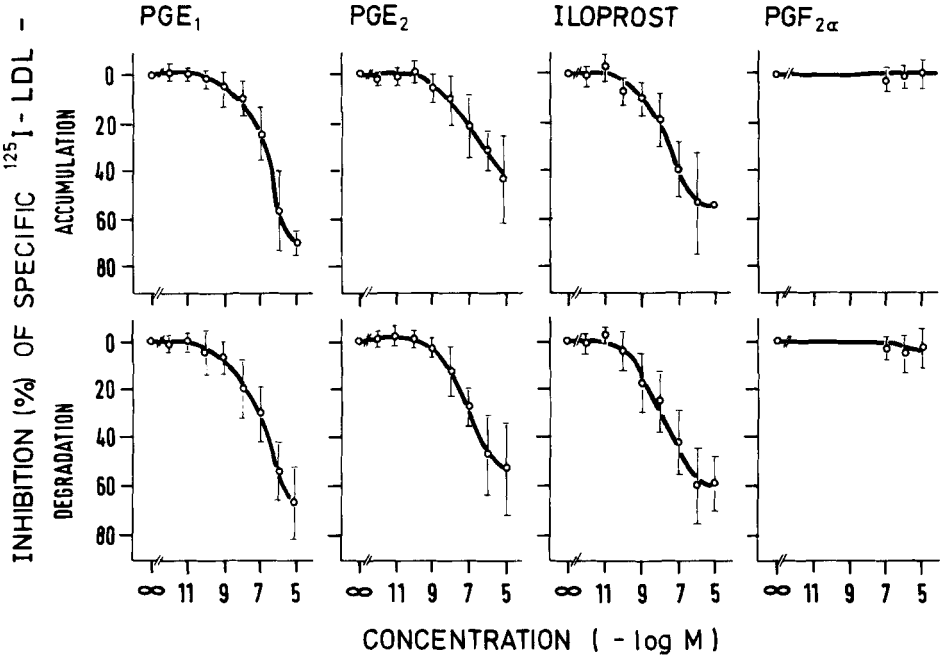

Fig. 1. Log concentration-effect curves: effect of PGE_1, PGE_2, Iloprost and $PGF_{2\alpha}$ on the rate of accumulation and degradation of ^{125}I-labeled LDL with high affinity in freshly isolated human mononuclear leukocytes. Prostaglandins were added at various concentrations at the beginning of the incubations. Cells were incubated at 37°C in RPMI 1640 with or without agents for 20 hr. ^{125}I-labeled LDL (10 μg/ml) was then added and accumulation and degradation assays were performed as described in Methods. Values without agents at 20 hr: 18 ± 3 ng/2 • 10^6 cells per 6 hr for specific accumulation, and 78 ± 6 ng/2 • 10^6 cells per 6 hr for specific degradation, were defined as 0% inhibition. Each point represents mean ± SE of four to six experiments performed in triplicate.

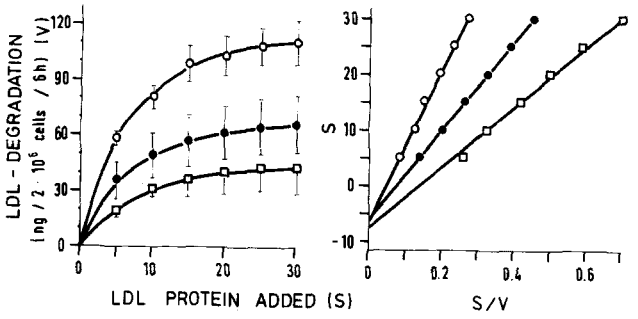

Fig. 2. Effects of PGE_1 and dibutyryl cyclic AMP on the degradation of ^{125}I-labeled LDL with high affinity in freshly isolated human mononuclear leukocytes as a function of the ^{125}I-labeled LDL concentration. Cells were incubated at 37°C in RPMI 1640 with or without PGE_1 or dibutyryl cyclic AMP for 20 hr. ^{125}I-labeled LDL was then added at the concentrations indicated for 6 hr at 37°C. Assays were performed as described in Methods. Values represent means ± SE of three to five experiments that were performed in triplicate; (○), without agent; (●), with PGE_1 1 μM; (□), with dibutyryl cyclic AMP 100 μM. The curves on the right represent reciprocal plots of the data of the curves on the left. The slope of the lines equals apparent V_{max} for degradation. The points of the intersection with the y axis equal apparent $-K_m$.

TABLE 1. Effects of dibutyryl cyclic AMP on the rate of accumulation and degradation of ^{125}I-labeled LDL with high affinity in freshly isolated human mononuclear leukocytes

Dibutyryl Cyclic AMP	LDL-Accumulation	LDL-Degradation
μM	*% inhibition*	
0	0	0
0.1	3 ± 4	2 ± 2
1	17 ± 10	12 ± 3
10	27 ± 10	41 ± 9
100	52 ± 5	73 ± 13

Dibutyryl cyclic AMP was added to the medium at the beginning of the incubations. For further details see legend to Fig. 1. Values represent means ± SE of four to five experiments performed in triplicate.

Sterol synthesis in freshly isolated human mononuclear leukocytes was measured by the incorporation of [^{14}C]acetate into nonsaponifiable lipids. Fractionation of the nonsaponifiable lipids by thin-layer chromatography showed three radiolabeled products corresponding in mobility to authentic samples of cholesterol, lanosterol, and squalene. It has been demonstrated previously that in mononuclear leukocytes [^{14}C]acetate was incorporated mainly into lanosterol and cholesterol and to a minimal extent into squalene (7 and **Fig. 3**). Incubation of cells for up to 45 hr in a lipid-depleted medium led to a rise in the synthesis of sterols, cholesterol, lanosterol, and squalene, the increases being 5- to 6-fold (data not shown), 15- to 20-fold, 10- to 12-fold, and 2- to 3-fold after 20 hr, respectively (Fig. 3). PGE_2 added in a concentration of 1 μM to the incubation medium at zero time inhibited the induction of synthesis of sterols (data not shown), cholesterol, lanosterol, and squalene (Fig. 3) for up to 45 hr. The extent of inhibition expressed as percentage of control was greatest after an incubation of 20 hr. The time curves were similar when PGE_1 or Iloprost were used. In contrast, $PGF_{2\alpha}$ had no effect on the synthesis of sterols and their subfractions up to 45 hr of incubation. **Fig. 4** shows the relationship between the concentrations of PGE_1, PGE_2, Iloprost and $PGF_{2\alpha}$ and the inhibition of cholesterol synthesis from [^{14}C]acetate when cells were incubated for 20 hr. The suppression by 10 μM PGE_2 was 47% and 39% for 0.1 μM PGE_2. The actions of PGE_1 and Iloprost on cholesterol synthesis were similar, while $PGF_{2\alpha}$ showed no effect. PGE_1, PGE_2, and Iloprost yielded sigmoidal log concentration-effect curves.

The effects of PGE_1, PGE_2, Iloprost, and $PGF_{2\alpha}$ on cyclic AMP concentration in freshly isolated human mononuclear leukocytes are shown in **Table 2**. PGE_1, PGE_2, and Iloprost in concentrations of 1 and 10 μM caused three- to four-fold increases in the level of the cyclic nucleotide. On the other hand, $PGF_{2\alpha}$ had no effect.

DISCUSSION

Freshly isolated human mononuclear leukocytes were used in this study since they reflect the in vivo milieu (26), possess receptors for prostaglandins (27), catabolize LDL

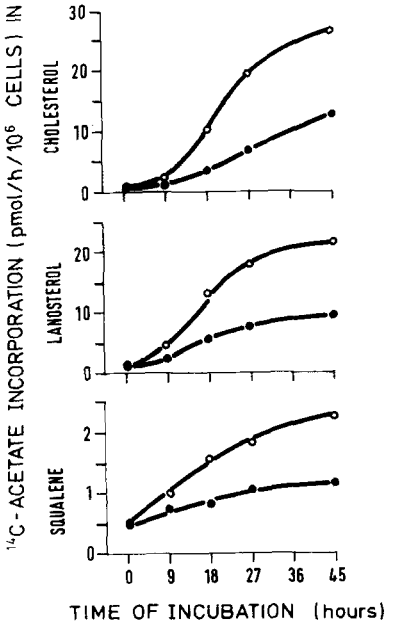

Fig. 3. Effects of PGE_2 on the ability of freshly isolated human mononuclear leukocytes to synthesize cholesterol, lanosterol, and squalene from [^{14}C]acetate as a function of the incubation time. Cells were incubated at 37°C in RPMI 1640 without lipoproteins; (○), without agent; (●), with PGE_2 1 μM. At each indicated time interval, cells were incubated for 3 hr at 37°C with [^{14}C]acetate. Assays were performed as described in Methods. Values represent means of three experiments performed in duplicate.

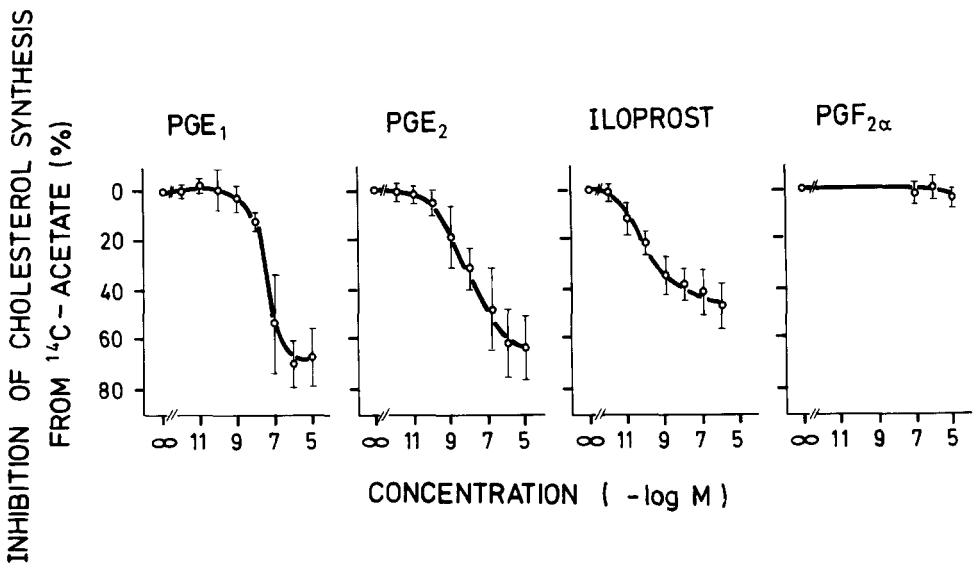

Fig. 4. Log concentration-effect curves: effect of PGE_1, PGE_2, Iloprost, and $PGF_{2\alpha}$ on the rate of cholesterol synthesis from [^{14}C]acetate in freshly isolated human mononuclear leukocytes. Prostaglandins were added at various concentrations at the beginning of the incubations. For further details see legend to Fig. 3. Values without agent at 20 hr: 16 ± 3 pmol/hr per 10^6 cells, were defined as 0% inhibition. Each point represents mean ± SE of four to six experiments performed in duplicate.

from plasma after binding to high affinity cell surface receptors (6) which can be visualized by Western blotting and immunoprecipitation techniques (28), and are able to synthesize cholesterol (29). Work from our laboratory has shown that LDL receptor activity and sterol synthesis in mononuclear leukocytes is under hormonal control; insulin stimulates (30, 31) and catecholamines suppress (10, 31) both pathways. This study demonstrates that the prostacyclin analogue Iloprost, PGE_1, and PGE_2 inhibited the rate of cholesterol synthesis and LDL receptor activity in these cells. The kinetic data and analysis (Fig. 2) indicate that prostaglandins decreased the apparent V_{max} for LDL degradation without changing the apparent K_m. Because ligand-receptor internalization and intracellular processing are very rapid, making ligand-receptor association rate-limiting, the observed decrease in V_{max} for LDL degradation may reflect a decreased number of cell surface receptors (6, 8). As to the mode of action, the prostaglandins appear to inhibit cholesterol synthesis and LDL receptor activity by a receptor-mediated process via cyclic AMP. This conclusion can be deduced from the following data: i) there is a sigmoid shape for the log concentration-effect curves (see Figs. 1 and 4) suggesting a saturable mechanism; ii) $PGF_{2\alpha}$, which has no binding affinity to mononuclear leukocytes (27), has no metabolic effect at all in our study (see Figs. 1 and 4); and iii) stimulation of prostaglandin receptors led to raised levels of intracellular cyclic AMP in mononuclear leukocytes (Table 2). Accordingly, the inhibition of cholesterol synthesis (10) and LDL receptor activity (Fig. 2 and Table 1) by Iloprost, PGE_1, and PGE_2 could be mimicked by dibutyryl cyclic AMP. A suppression of LDL receptor activity by the cyclic nucleotide has also been shown previously in fibroblasts (32, 33) and arterial smooth muscle cells (32).

A possible role of prostaglandins in the pathogenesis of atherosclerosis has been deduced from results showing

TABLE 2. Effects of PGE_1, PGE_2, Iloprost, and $PGF_{2\alpha}$ on intracellular cyclic AMP concentrations in freshly isolated human mononuclear leukocytes

Agent Added	Amount	Cyclic AMP
	μM	*pmol/5 × 10^6 cells*
None		5 ± 2
PGE_1	0.1	8 ± 2
PGE_1	1	16 ± 4
PGE_1	10	18 ± 4
PGE_2	0.1	11 ± 3
PGE_2	1	18 ± 4
PGE_2	10	19 ± 7
Iloprost	0.1	12 ± 4
Iloprost	1	15 ± 3
Iloprost	10	17 ± 3
$PGF_{2\alpha}$	0.1	5 ± 2
$PGF_{2\alpha}$	1	5 ± 3
$PGF_{2\alpha}$	10	6 ± 2

Cells were incubated in RPMI 1640 for 10 min with or without agents at the concentrations indicated. Assays were performed as described in Methods. Each value represents the mean ± SE of four experiments performed in duplicate.

that tissue derived from atherosclerotic arteries produces decreased amounts of prostacyclin (34–36). It therefore has been postulated that atherosclerosis may result from a deficiency of prostacyclin synthesis (37). Accordingly, prostacyclin production by blood vessels from humans and animals with a high risk for atherosclerosis is depressed. This has been shown for diabetes (38), smoking (39), aging (40), as well as hypercholesterolemia (34, 41) which is characterized by an increased concentration of LDL in plasma. The interaction between cholesterol metabolism and prostaglandins in atherogenesis is not well understood. On the one hand LDL, which is the major cholesterol-carrying lipoprotein in plasma, reduces prostacyclin synthesis (42). On the other hand, there are several reports that prostaglandins directly affect cellular cholesterol metabolism. Prostacyclin and some of its stable metabolites increased cholesterol hydrolase activity and cholesterol egress from cultured smooth muscle cells (43, 44), while PGE_2 inhibited the cholesteryl ester synthetic activity (45). In macrophages incubated with β-migrating very low density lipoprotein, PGE_2 decreased the accumulation of cholesteryl ester (46).

Based on these findings, it has been postulated by Hajjar (47) that the loss of cholesteryl ester from cultured smooth muscle cells of normal and atherosclerotic arteries by the addition of prostacyclin may be due to an increase in cholesteryl ester hydrolase activity. From the results reported here, it can be concluded that prostaglandins also diminish the accumulation of cellular cholesterol and cholesteryl ester by suppressing cholesterol biosynthesis as well as the endocytosis of plasma LDL. If prostaglandins can alter intracellular levels of cholesterol and cholesteryl ester in vivo as outlined above, it is reasonable to suggest that high levels of prostaglandins in arterial tissue could protect cells from cholesterol accumulation. Accordingly, decreased prostaglandin production could favor cholesteryl ester accumulation which may promote the development of atherosclerotic vascular changes.

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