Agents which increase cyclic AMP have diverse effects on low-density-lipoprotein-receptor function in human vascular smooth-muscle cells and skin fibroblasts

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Receptor-mediated binding and metabolism of low-density lipoproteins (LDL) in cultured human vascular smooth-muscle cells and skin fibroblasts are altered by increased cellular cyclic AMP concentrations. However, the LDL receptor does not respond to changes in cyclic AMP concentration in a simple manner. The activation of adenylate cyclase with forskolin, or the addition of membrane-permeant cyclic AMP analogues, initially decreases the expression of the LDL receptor, but is followed by a substantial increase in receptor expression after 24 h. This increase does not occur in the presence of inhibitors of RNA or protein synthesis, and is due to doubling of the B_{max} of the LDL receptor, without alteration of its affinity for LDL. By contrast, elevation of cyclic AMP concentration by inhibition of phosphodiesterases results in decreased receptor expression throughout the 24 h period. These two response patterns are reproducible phenomena, consistently observed in low-passaged cells derived from seven unrelated individuals.

INTRODUCTION

Proliferation of smooth-muscle cells plays an important role in the pathology of atherosclerosis [1]. Additionally, early in the disease fatty streaks expand, owing to formation of multiple layers of a mixed population of macrophage-derived foam cells and lipid-laden smooth-muscle cells [2]. Macrophages contain poorly regulated scavenger receptors for modified LDL, allowing continued lipoprotein entry and resulting in lipid accumulation [3]. By contrast, smooth-muscle cells are known to express only the 'classical' LDL receptor, which is closely controlled by the availability of intracellular free cholesterol [4]. The LDL receptor and some of the enzymes involved in cholesterol metabolism respond to changes in cholesterol concentration in a manner which maintains cellular cholesterol homeostasis [5]. However, intracellular cholesterol concentration is not the only regulatory factor. Hormones have been shown to modulate cholesterol and LDL metabolism in a number of cell types; in steroid-synthesizing tissues, trophic hormones increase LDL uptake via its receptor, their action being mediated by increased cyclic AMP concentration [6,7]. In rat hepatocytes, glucagon and adrenaline increase LDL binding and metabolism, changes which are mimicked by addition of the cyclic AMP analogue CPT-cAMP [8]. In contrast db-cAMP, another cyclic AMP analogue, decreases LDL binding in human hepatocarcinoma HepG2 cells [9]. Conflicting results were reported concerning the effect of cyclic AMP analogues on the LDL-receptor function in fibroblasts. Some authors found inhibition [10,11], but others reported no effect [12,13]. In mononuclear leucocytes, adrenaline and db-cAMP decrease LDL binding and cholesterol synthesis [14,15]. In arterial smooth-muscle cells, prostacyclin, isoprenaline (isoproterenol), db-cAMP and the adenylate cyclase activator forskolin stimulate cytoplasmic and lysosomal cholesterol ester hydrolases [16], and db-cAMP increases loss of cholesterol to the medium [17]. The cholesterol ester content of these cells is decreased after exposure to db-cAMP, cholera toxin, forskolin and the phosphodiesterase inhibitor MIX [18]. db-cAMP has

also been reported to inhibit LDL binding and degradation in cultured vascular smooth-muscle cells [10].

It is clear from these studies that cyclic-AMP-mediated hormonal effects could play a significant role in regulating lipoprotein and cholesterol metabolism. It is intriguing that, although elevated cyclic AMP concentrations alter the expression and function of the LDL receptor, the direction of the effect (i.e. inhibition or stimulation) differs between cell types.

This study attempts to clarify the effects of elevated cyclic AMP concentrations on the LDL-receptor pathway in cultured human vascular smooth-muscle cells.

MATERIALS AND METHODS

Materials

Dulbecco's minimal essential medium was purchased from Imperial Laboratories Ltd. (U.K.); foetal-calf serum was from Biological Industries (U.K.) and Flow Laboratories (U.K.); non-essential amino acids were from Flow Laboratories (U.K.); CPT-cAMP was from Boehringer-Mannheim (U.K.); $Na^{125}I$ was from Amersham International (U.K.); collagenase was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); BSA, penicillin, streptomycin, amphotericin B, db-cAMP, forskolin, MIX and heparin were all obtained from Sigma (U.K.). 1,9-Dideoxyforskolin was from Calbiochem. HL725 [9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2H-pyrimido-[6,1-*a*]isoquinolin-4-one hydrochloride] was a gift from Hoechst U.K. Ltd.

Cells

Human vascular smooth-muscle cells were released with collagenase from the veins of newly delivered umbilical cords, as described by Gimbrone & Cotran [19]. Their smooth-muscle origin was confirmed by their growth pattern, by electron microscopy and by immunocytochemistry (reaction with an antibody to desmin).

Abbreviations used: CPT-cAMP, 8-(4-chlorophenylthio)cyclic AMP; db-cAMP, N^6,O^2' -dibutyryl cyclic AMP; LDL, low-density lipoproteins; MIX, 3-isobutyl-1-methylxanthine.

The cells were cultured in 25 cm² flasks in Dulbecco's minimal essential medium supplemented with 10% (v/v) foetal-calf serum, non-essential amino acids, 100 units of penicillin/ml, 100 µg of streptomycin/ml and 2.5 µg of amphotericin B/ml. The cells remained viable after storage in liquid N₂, and could be passaged 9–14 times without changes in their morphology or in the metabolic parameters measured in this study. Routine subculturing was carried out at a split ratio of 1:2 or 1:3.

Human skin fibroblasts were from individuals less than 6 years old, with normal cholesterol metabolism. They were obtained as controls for another study [20]. Fibroblasts were grown routinely in 100 mm-diam. Petri dishes; the medium and split ratio were as described for smooth-muscle cells. All experiments with both fibroblasts and smooth-muscle cells were carried out in 6 × 35 mm cluster dishes, unless stated otherwise.

Lipoproteins

Human LDL (1.019 < *d* < 1.063) and lipoprotein-deficient serum (*d* > 1.21) were prepared by sequential centrifugation, from freshly collected blood of healthy volunteers [21]. Both fractions were extensively dialysed before use. Within 1 day of preparation, LDL was radiolabelled with Na¹²⁵I via the ICI method [21], yielding ¹²⁵I-LDL with initial specific radioactivity of 100–200 c.p.m./ng. Newly labelled LDL was used within 3 weeks. More than 98% of the ¹²⁵I-labelled material was precipitable by 10% (w/v) trichloroacetic acid, and less than 5% was extractable into lipid solvents.

Experimental procedures

All experiments involving LDL binding and metabolism were carried out in triplicate 35 mm dishes. Cyclic AMP measurements were carried out in triplicate 35 mm (Fig. 4) or 60 mm (Fig. 8) dishes. Experiments were initiated by washing the cultures with sterile phosphate-buffered saline (Dulbecco's), followed by addition of lipoprotein-deficient medium with or without the specified agents. Measurements were carried out 24 h later, unless stated otherwise. In some experiments the cultures were incubated with lipoprotein-deficient medium for 24 h before addition of the agents. Lipoprotein-deficient medium consisted of Dulbecco's minimal essential medium containing 100 units of penicillin/ml and 100 µg of streptomycin/ml, and supplemented with human lipoprotein-deficient serum at a concentration of 5 mg of protein/ml.

db-cAMP and CPT-cAMP were dissolved directly in lipoprotein-deficient medium just before use. Stock solutions of forskolin, dideoxyforskolin, MIX and HL725 in dimethyl sulphoxide were stored at –20 °C. A 2.5–5 µl portion of the appropriate solution was added per ml of medium, and the same volume of dimethyl sulphoxide was added to control cultures.

Measurement of LDL binding, internalization and degradation

LDL binding and metabolism were assayed as described in detail by Goldstein *et al.* [21]. The procedures are summarized briefly below and were applied to monolayers of human skin fibroblasts and vascular smooth-muscle cells.

LDL binding at 4 °C. At the end of the incubation with or without the agents, the cells were cooled on ice and the medium was replaced with ice-cold Dulbecco's phosphate-buffered saline, containing 5 mg of BSA/ml and 10 µg of ¹²⁵I-LDL/ml. The cultures were incubated for 2 h at 4 °C, and surface-bound LDL was subsequently released with heparin as described by Goldstein *et al.* [21]. The monolayers were rinsed in ice-cold Dulbecco's phosphate-buffered saline, dissolved in 1 ml of 0.1 M-NaOH and were assayed for protein. Non-specific binding, which was determined for each experiment in the presence of a 30–50-fold excess of unlabelled LDL, was usually about 10% of control

values and was subtracted from the total binding measured. In some binding experiments, total cell-associated LDL was assayed by omitting the incubation with heparin. Washed cultures were dissolved in 1 ml of 0.1 M-NaOH and their radioactivity was measured. It was found that the amount internalized at 4 °C was small (< 10% of the total), and changes in total cell-associated radioactivity (surface-bound plus internalized LDL) at 4 °C closely paralleled those in heparin-releasable (surface-bound) radioactivity.

LDL binding, internalization and degradation at 37 °C. Cultures were incubated in lipoprotein-deficient medium with or without the agents as described above. At the appropriate time ¹²⁵I-LDL (10 µg/ml, except in Fig. 6) was added and the incubation continued for 4 h at 37 °C. Specific LDL binding, internalization and degradation were assayed exactly as described by Goldstein *et al.* [21]. Non-specific values were no more than 10% of the total values determined with 10 µg of ¹²⁵I-LDL/ml.

Other assays

Cyclic AMP concentrations were determined by competitive binding assay using Amersham kit no. TRK432. Triplicate 60 mm or 35 mm dishes were extracted with ice-cold 65% (v/v) ethanol, and cyclic AMP was assayed in the dried extracts. Protein content was determined by the method of Lowry *et al.* [22] with BSA as standard.

Kinetic analysis (Figs. 2, 5, 6 and Table 1)

The experimental data were fitted to the Michaelis–Menten curve, by using a non-linear-regression data-analysis program for the IBM/PC (Enzfitter, from R. J. Leatherbarrow, Elsevier Biosoft, Cambridge, U.K.).

Statistical analysis

Student's unpaired *t* test was used to determine the statistical significance of differences between groups. Studies were carried out in triplicate dishes of cells, and results (presented as means ± S.D.) are typical of at least two independent experiments.

RESULTS

Cyclic AMP analogues increased the amount of LDL which bound specifically to cell-surface receptors in cultured human vascular smooth-muscle cells. LDL binding, measured at 4 °C, was stimulated after incubation for 12 h with 1 mM-db-cAMP, and further stimulation reached statistical significance by 24 h (*P* < 0.05) and 48 h (*P* < 0.01) (Fig. 1). Incubation of cultures for 24 h with different concentrations of db-cAMP and CPT-cAMP demonstrated that LDL binding increased with increasing concentrations of these compounds (Fig. 2). CPT-cAMP increased LDL binding more effectively than did an equimolar concentration of db-cAMP, though both compounds exerted their half-maximal effects at about 0.25 mM. The internalization and metabolism of LDL at 4 °C is negligible [21]; therefore the increase in specific LDL binding at 4 °C, as presented in Figs. 1 and 2, indicated an increased number of receptors present on the cell surface.

The function of the LDL receptor was assessed by measuring the amount of LDL that is degraded at 37 °C, after its specific binding and internalization. Fig. 3 shows that, after 24 h with db-cAMP (1 mM) or CPT-cAMP (0.6 mM), all three parameters were increased very significantly. CPT-cAMP doubled the amount of LDL that was bound, internalized and degraded, and db-cAMP doubled the amount bound and increased the amount internalized and degraded by 40%.

The data presented so far demonstrate that cyclic AMP analogues increase receptor-mediated binding and metabolism

Fig. 1. Time course of changes in LDL binding to human vascular smooth-muscle cells in the absence (○) or presence (●) of 1 mM-db-cAMP

Cultures were incubated in lipoprotein-deficient medium with or without 1 mM-db-cAMP for the indicated time. Specific binding of LDL was assayed at 4 °C as described in the Materials and methods section. Each value is the mean $\pm$ S.D. of measurements in triplicate dishes: * P < 0.05, *** P < 0.001 with respect to controls.

of LDL in human smooth-muscle cells. We found similar effects in cultured human skin fibroblasts. In a typical experiment, LDL binding to fibroblasts at 4 °C, after preincubation for 24 h with lipoprotein-deficient medium alone, was 61 ± 6 ng of LDL/mg of cell protein. The inclusion of 1 mM-db-cAMP in the pre-incubation medium increased the amount of LDL bound to 87 ± 7 ng/mg of protein (P < 0.01 compared with controls), and 1 mM-CPT-cAMP increased LDL binding to 149 ± 10 ng/mg of protein (P < 0.001 compared with controls).

In another set of experiments we used the adenylate cyclase activator forskolin [23] to elevate cyclic AMP concentrations endogenously, before measurement of LDL binding. Forskolin increased cyclic AMP concentrations in cultured fibroblasts in a concentration-dependent manner (Fig. 4). LDL binding at 4 °C was also elevated after 24 h exposure to increasing concentrations of forskolin (Fig. 4). These results suggest that elevation of cellular cyclic AMP concentrations is associated with increased

Fig. 2. Effect of increasing concentrations of cyclic AMP analogues on LDL binding in human vascular smooth-muscle cells

Cultures were incubated for 24 h in lipoprotein-deficient medium containing different concentrations of db-cAMP (○) or CPT-cAMP (□). Specific binding of LDL was assayed at 4 °C as described in the Materials and methods section. Each value is the mean $\pm$ S.D. of measurements in triplicate dishes.

Fig. 3. Effect of cyclic AMP analogues on LDL metabolism at 37 °C in human vascular smooth-muscle cells

Cultures were incubated for 24 h in lipoprotein-deficient medium alone (□) or with 1 mM-db-cAMP (▨) or 0.6 mM-CPT-cAMP (■). Specific binding, internalization and degradation of LDL were measured at 37 °C, as described in the Materials and methods section, and control values ($\pm$ S.D.) were respectively 202 ± 36 ng/mg, 1220 ± 180 ng/mg and 3447 ± 603 ng/4 h per mg. Values are means of measurements in triplicate dishes: * P < 0.05, ** P < 0.01, *** P < 0.001 with respect to controls.

binding of LDL to its receptor (Fig. 5). Analysis of the data presented in Fig. 5 showed a good fit to the Michaelis–Menten equation, with half-maximal increase of LDL binding at 90 ± 4 pmol of cyclic AMP/mg of cell protein (see the Materials and methods section for details of the computer program used for this analysis).

Stimulation of LDL binding by forskolin was observed also in serum-free medium. Fibroblasts incubated for 24 h in Dulbecco's minimum essential medium containing 2 mg of BSA/ml bound 37 ± 9 ng of LDL/mg of cell protein (measured at 4 °C). The inclusion of 0.1 mM-forskolin in the incubation increased LDL binding to 105 ± 9 ng of LDL/mg of cell protein (P < 0.001 compared with controls).

Fig. 4. Effect of forskolin on cyclic AMP concentration (●) and LDL binding (■) in human skin fibroblasts

Parallel cultures were incubated in lipoprotein-deficient serum and different concentrations of forskolin were added for 2 h or 24 h before assay of cyclic AMP or specific binding of LDL at 4 °C respectively. Assays were carried out as described in the Materials and methods section. Values are means $\pm$ S.D. of measurements in triplicate dishes. Where not shown, the error bars are contained within the symbols.

Fig. 5. Relationship between cellular cyclic AMP concentration and LDL binding

Experimental details were as for Fig. 4. This is a different presentation of the same data.

Forskolin also caused an increase in LDL binding at 4 °C in smooth-muscle cells. Cultures of smooth-muscle cells incubated with 0.1 mM-forskolin for 24 h bound almost twice as much LDL as did cultures incubated with lipoprotein-deficient medium alone (control, 84 ± 15 ng of LDL/mg of protein; forskolin-treated, 152 ± 21 ng of LDL/mg of protein; $P < 0.001$). In the same experiment, dideoxyforskolin (an analogue of forskolin which does not activate adenylate cyclase [23]) did not alter LDL binding significantly compared with controls (71 ± 5 ng of LDL/mg of protein). Clearly the diterpene molecule itself was not affecting the LDL receptor.

The binding of LDL to its receptor and its subsequent internalization and degradation conform to Michaelis–Menten kinetics. We measured all three processes, in the presence of increasing concentrations of ^{125}I -LDL, in cultures of smooth-muscle cells which were incubated for 24 h in lipoprotein-deficient medium with or without 0.1 mM-forskolin. The results (Fig. 6) showed that forskolin pretreatment resulted in stimulation of all three processes, at all concentrations of ^{125}I -LDL used. Furthermore, the stimulation was observed not only when the ^{125}I -LDL was added to the forskolin-containing medium (Fig. 6), but also when the forskolin-containing preincubation medium was removed before the addition of ^{125}I -LDL (results not shown). The data were fitted to the Michaelis–Menten equation by non-linear regression analysis and were used to calculate the K_m and $B_{\max}$ or $V_{\max}$ values, which are presented in Table 1. These data showed that forskolin did not alter the K_m for binding, internalization or degradation of LDL, but significantly increased the $B_{\max}$ or $V_{\max}$ for all three parameters. The unchanged K_m demonstrates that the affinity of the receptor for LDL was not affected by forskolin. Increased maximal binding indicates an increase in the number of binding sites, e.g. more LDL receptors on the cell surface. This could result from alteration in receptor recycling, increase in receptor synthesis or decrease in receptor degradation. Table 2 shows that cycloheximide and actinomycin D abolish the stimulatory effects of cyclic AMP analogues and forskolin on LDL binding. This indicates the involvement of protein and RNA synthesis, possibly the synthesis of LDL-receptor protein and its corresponding mRNA, in the stimulation of LDL metabolism by forskolin and cyclic AMP analogues.

Forskolin elevates cellular cyclic AMP concentrations by stimulating cyclic AMP synthesis. It is also possible to raise cyclic AMP concentrations by using inhibitors of cyclic AMP

Fig. 6. Effect of forskolin on LDL binding, internalization and degradation by human smooth-muscle cells, measured at different concentrations of ^{125}I -LDL

Cultures were incubated in lipoprotein deficient medium, without (○) or with (●) 0.1 mM-forskolin, for 24 h. Specific binding, internalization and degradation of LDL were measured at 37 °C, as described in the Materials and methods section. Values are means $\pm$ S.D. of measurements in triplicate dishes. Where not shown, the error bars are contained within the symbols. Non-specific values varied linearly with concentration of ^{125}I -LDL and have been subtracted from the data.

phosphodiesterase. Fig. 7 shows the effect of MIX on LDL binding. In contrast with the effects of cyclic AMP analogues and forskolin, increasing concentrations of MIX resulted in decreased LDL binding. We have ascertained that 0.25 mM-MIX does not alter the amount of [^3H]leucine incorporated into total cellular

Table 1. Effect of forskolin (0.1 mM) on the kinetics of LDL metabolism in vascular smooth-muscle cells

The constants in the Table were calculated from the experimental data presented in Fig. 6. The values are given as means $\pm$ S.E.M., determined by non-linear-regression analysis, as described in the Materials and methods section. The units for K_m are ng of LDL/ml, those for V_{max} (B_{max}) of binding and internalization are ng of LDL/mg of cell protein, and those for V_{max} of degradation are ng of LDL/4 h per mg of protein.

	Binding		Internalization		Degradation	
	B_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Control	414 $\pm$ 48	22 $\pm$ 4.8	2696 $\pm$ 168	15.4 $\pm$ 2.0	3685 $\pm$ 356	12.7 $\pm$ 2.7
Forskolin	809 $\pm$ 140	19 $\pm$ 6.5	6543 $\pm$ 678	17.7 $\pm$ 3.7	6625 $\pm$ 485	9.6 $\pm$ 1.7

protein (results not shown). This discounts the possibility that MIX decreases LDL binding as a consequence of decreased receptor synthesis, owing to non-specific effects on protein synthesis. We considered the possibility that MIX was exerting its effect via interaction with the adenosine receptor system. Methylxanthines, in addition to inhibiting phosphodiesterases, are also potent antagonists of adenosine action, preventing the stimulation of adenylate cyclase by adenosine [24]. However, we found no role for adenosine-mediated changes in LDL binding. Incubation of fibroblasts with adenosine (0.1 mM) or adenosine deaminase (1 unit/ml) for 24 h resulted in LDL binding (measured at 4 °C) similar to that found in controls incubated with lipoprotein-deficient medium alone. Furthermore, the addition of adenosine deaminase (1 unit/ml) did not alter the changes in LDL binding induced by MIX, forskolin or db-cAMP (results not shown).

To confirm that the decrease in LDL binding was due to inhibition of cyclic AMP phosphodiesterase and not to a coincidental effect of the MIX molecule, we investigated the effect of a phosphodiesterase inhibitor whose chemical structure differs from that of MIX. Rupert & Weithman [25] reported that HL725, a pyrimido-quinoline compound, was a potent inhibitor of phosphodiesterase activity in platelets. Incubation of fibro-

blasts with 0.01 mM-HL725 for 24 h decreased LDL binding to 76 % ($P < 0.05$) of control values. Also, in a similar experiment with smooth-muscle cells, 24 h with HL725 at 0.05 mM and 0.1 mM decreased LDL binding to 34 % ($P < 0.001$) and 12 % ($P < 0.001$) of controls respectively.

The changes in LDL binding and parallel changes in cyclic AMP concentrations were measured in fibroblasts, over a period of 24 h, in the absence and presence of forskolin (0.1 mM), MIX (0.25 mM) and forskolin plus MIX (Table 3; Fig. 8). Controls showed increased LDL binding in the first 5 h after changing to fresh lipoprotein-deficient medium, but this was prevented by addition of forskolin or MIX. However, after 24 h, the forskolin-treated cells showed greatly elevated LDL binding, whereas the presence of MIX continued to prevent any increase compared with controls at zero time. Incubation with both agents simultaneously resulted in an effect after 5 h which was greater than that observed with each agent alone. After 24 h, LDL binding in the presence of both forskolin and MIX was lower than that observed with forskolin alone and higher than that observed with MIX alone (Table 3). Cyclic AMP concentrations remained at basal values, around 4 pmol/mg of protein, during the 24 h incubation with lipoprotein-deficient medium alone (Fig. 8). The presence of forskolin increased this value 185-fold in 2 h, and at 24 h the cyclic AMP concentration was still 4 times basal value. MIX doubled cyclic AMP concentrations at 2 h as compared with controls, and this value was maintained over the 24 h

Table 2. Effects of cycloheximide and actinomycin D on LDL binding

Cultures of fibroblasts and smooth-muscle cells were incubated for 24 h in lipoprotein-deficient medium, followed by 24 h in fresh lipoprotein-deficient medium, with additions as specified in the Table. LDL binding was assayed at 4 °C for 2 h as described in the Materials and methods section. Values are means $\pm$ S.D. of three measurements: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with respect to control; †† $P < 0.01$, ††† $P < 0.001$ with respect to cultures incubated with forskolin or CPT-cAMP as appropriate.

Additions	LDL binding by vascular smooth-muscle cells (ng of LDL/mg of cell protein)	
	Control	CPT-cAMP (1 mM)
Control	36 $\pm$ 5	92 $\pm$ 8 ***
Cycloheximide (0.5 mM)	10 $\pm$ 10 *	13 $\pm$ 3 †††
Actinomycin D (2.5 μ g/ml)	13 $\pm$ 7 *	25 $\pm$ 12 ††

Additions	LDL binding by fibroblasts (ng of LDL/mg of cell protein)	
	Control	Forskolin (0.1 mM)
Control	53 $\pm$ 10	94 $\pm$ 12 **
Cycloheximide (0.5 mM)	42 $\pm$ 15	38 $\pm$ 5 ††

Fig. 7. LDL binding to human skin fibroblasts after exposure to increasing concentrations of MIX

Cultures were incubated in lipoprotein-deficient medium for 24 h with the specified concentrations of MIX. Specific binding of LDL was assayed at 4 °C, as described in the Materials and methods section. Values are means $\pm$ S.D. of measurements in triplicate dishes.

Table 3. Time course of changes in LDL binding in the absence or presence of forskolin and MIX

Cultures of fibroblasts were incubated for 24 h in lipoprotein-deficient medium, followed by 2, 5, 12 or 24 h in fresh lipoprotein-deficient medium with the specified additions. LDL binding was measured at 4 °C for 2 h as described in the Materials and methods section. Values are means $\pm$ S.D. of three measurements: * P < 0.05, ** P < 0.01, *** P < 0.001 with respect to controls.

Addition... Time (h)	LDL binding (ng of LDL/mg of cell protein)			
	Control	Forskolin (0.1 mM)	MIX (0.25 mM)	Forskolin and MIX
0	130 $\pm$ 12			
2	176 $\pm$ 6	136 $\pm$ 16 *	155 $\pm$ 29	138 $\pm$ 6 ***
5	204 $\pm$ 10	163 $\pm$ 12 **	158 $\pm$ 10 **	130 $\pm$ 10 ***
12	242 $\pm$ 19	261 $\pm$ 22	178 $\pm$ 10 **	188 $\pm$ 17 *
24	208 $\pm$ 21	454 $\pm$ 58 **	123 $\pm$ 4 **	377 $\pm$ 15 ***

period. Cyclic AMP concentrations in the presence of both forskolin and MIX were always higher than those with each agent alone. These results show that the mechanism by which cyclic AMP concentration was elevated was more important than the absolute value of the cyclic AMP concentration in influencing the response of the LDL receptor.

These time-dependent changes in LDL-receptor response to elevation in cyclic AMP concentration were not specific to the skin fibroblast cell. The presence of forskolin and MIX caused similar alterations in LDL-receptor expression in human vascular smooth-muscle cells. Exposure to forskolin for 3 h resulted in 25% inhibition of LDL binding, but continuation of the incubation for 24 h caused 48% stimulation (Fig. 9). MIX inhibited LDL binding after both 3 h and 24 h. Both agents together caused inhibition after 3 h, but at 24 h the combined action of the two compounds resulted in binding which was similar to that of controls.

Fig. 8. Time course of the effect of forskolin and MIX on cyclic AMP concentrations in human skin fibroblasts

Cultures were preincubated for 24 h in lipoprotein-deficient medium, followed by 2, 5, 12, or 24 h in fresh lipoprotein-deficient medium alone ($\square$) or containing 0.1 mM-forskolin ($\blacktriangle$), 0.25 mM-MIX ($\blacksquare$) or forskolin and MIX ($\bullet$). At the end of the incubation period cyclic AMP concentrations were assayed, as described in the Materials and methods section. All experimental values are significantly different from controls (P < 0.01 or less).

Fig. 9. LDL binding in human smooth-muscle cells after incubation for 3 or 24 h with forskolin or MIX

Cultures were preincubated for 24 h in lipoprotein-deficient medium, followed by 3 or 24 h in fresh lipoprotein-deficient medium alone ($\square$) containing 0.1 mM-forskolin ($\blacksquare$), 0.25 mM-MIX ($\hatched$) or forskolin and MIX ($\bullet$). At the end of the incubation period specific binding of LDL was assayed at 4 °C, as described in the Materials and methods section. Values are the means $\pm$ S.D. of measurements in triplicate dishes: * P < 0.01 with respect to controls.

DISCUSSION

Our findings show that in human vascular smooth-muscle cells and skin fibroblasts expression of the LDL receptor is affected by increased cellular cyclic AMP. However, the LDL receptor does not respond to increases in cyclic AMP concentrations in a simple manner. The activation of adenylate cyclase with forskolin, or the presence of cyclic AMP analogues, initially inhibits the expression of the LDL receptor, but is followed by a substantial stimulation at 24 h. By contrast, inhibition of cyclic AMP phosphodiesterases with MIX results in decreased receptor expression throughout the 24 h period. These two response patterns are consistent phenomena, being observed in low-passaged cells derived from seven unrelated individuals.

Some of the present observations confirm the work of Maziere *et al.* [11], who reported that the phosphodiesterase inhibitor theophylline (a methylxanthine) inhibited LDL binding in fibroblasts. However, contrary to our findings, these authors and Stout & Bierman [10] found that exposure to db-cAMP for 24 h resulted in inhibition of LDL binding in fibroblasts [10,11] and vascular smooth-muscle cells [10]. Brown and Goldstein and others reported no effects of db-cAMP [12,13] or MIX [13], thus differing from both the present study and the above authors [10,11]. None of those studies investigated the time course of the effect of these agents.

A number of experiments were carried out in an attempt to find a possible reason for these differences between laboratories. We have focused our attention on the increase in LDL binding after incubation for 24 h with lipoprotein-deficient serum with or without cyclic AMP analogues. Studies with forskolin were carried out in parallel, and showed that endogenously generated cyclic AMP increased LDL binding in the same way as added cyclic AMP analogues. It is important to note that forskolin also exerted its effects in cells incubated in serum-free medium, causing similar stimulation to that found in medium containing lipoprotein-deficient serum. Therefore this response of the LDL receptor is not likely to be the result of complex interactions involving poorly controlled variables, such as hormones present in the serum. In addition, the degree of confluence of the cultures did not affect the direction or the magnitude of the effects studied.

Table 4. Effect of increased passage number on the stimulation by db-cAMP and forskolin of LDL binding to human smooth-muscle cells and fibroblasts

Smooth-muscle cells derived from one individual and fibroblasts from another individual were subcultured for the indicated number of passages. The cultures were then incubated for 24 h in lipoprotein-deficient medium with or without the specified agents. LDL binding was assayed in triplicate dishes for 2 h at 4 °C. Results are presented as means $\pm$ S.D.: * P < 0.05; ** P < 0.02, *** P < 0.01 with respect to controls.

Cells	Passage no.	LDL bound (ng/mg of cell protein)		
		Control	1 mM-db-cAMP	0.1 mM-forskolin
Smooth-muscle	6	128 $\pm$ 29	202 $\pm$ 20 *	
	10	84 $\pm$ 22	110 $\pm$ 10	
Fibroblasts	7	86 $\pm$ 10	126 $\pm$ 6 ***	164 $\pm$ 15 **
	10	86 $\pm$ 3	92 $\pm$ 10	
	16	52 $\pm$ 1		54 $\pm$ 9

(results not shown). We have investigated the effects of cyclic AMP analogues and forskolin in cells derived from seven unrelated individuals (smooth-muscle cells from three and fibroblasts from four), using different batches of db-cAMP, CTP-cAMP, forskolin, foetal-calf serum and lipoprotein-deficient serum. In all these experiments, incubation for 24 h with db-cAMP, CTP-cAMP or forskolin consistently increased LDL binding in cells of low passage number. At high passages, results became variable; LDL binding after incubation with these agents was usually similar to that of controls, though occasionally stimulation of LDL binding was observed even in cultures of high passage. The critical passage number appeared to be between 9 and 14, and varied between cell lines. Typical results are presented in Table 4.

It seems that the nature of the interaction between cyclic AMP and the activity of the LDL receptor changes with increased subculturing. Possibly, the use of cells subcultured many times in the published reports [10–13] may account for the diverging results from different laboratories. Changes in cellular responses with increasing passage number have also been observed by Hajjar [16]. He demonstrated stimulation of cholesterol esterase by db-cAMP and forskolin in cultured bovine aortic smooth-muscle cells, and commented that cells of low passage were used because results were inconsistent in high-passage cultures.

Our data indicate that the stimulation of LDL binding and metabolism owing to prolonged elevation of cyclic AMP is caused by an increase in the number of receptors, without significant changes in the affinity of the receptor for its ligand (Fig. 6 and Table 1). This increase in receptor number and activity does not occur in the presence of cycloheximide or actinomycin D (Table 2), indicating involvement of protein and RNA synthesis. Whether the synthesis of LDL-receptor protein and LDL-receptor mRNA is stimulated could be clarified in further experiments involving direct measurement of these parameters. Golos *et al.* [26] found that in human granulosa cells 8-bromo cyclic AMP stimulated the synthesis of the LDL-receptor protein; and the involvement of RNA synthesis was also demonstrated. As far as we are aware, stimulation of LDL binding and metabolism by cyclic AMP analogues have been reported only in steroid-synthesizing tissues [6,7] and rat hepatocytes [8]. The time course and effect of forskolin on LDL binding and metabolism have not been previously investigated.

The difference between the effects of cyclic AMP analogues and

forskolin compared with that of phosphodiesterase inhibitors is intriguing, and indicates underlying complexity (Table 3 and Fig. 9). The rapidity of the initial inhibition of LDL binding, after treatment of cells with forskolin or MIX, seems to preclude effects on synthesis of the LDL receptor, since its half-life is 12–25 h [12,27]. Further work is necessary to define the mechanism of this rapid inhibition. It is possible that the primary effect of elevated cyclic AMP is inhibitory. The increased expression of the LDL receptor could be associated with subsequent breakdown of the cyclic nucleotide to 5'-AMP, thus explaining the unexpected effects of phosphodiesterase inhibitors. The existence of a 5'-AMP-dependent protein kinase, which is involved in inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase and acetyl-CoA carboxylase, has been reported recently [28]. Inhibition of LDL-receptor activity via a cyclic-AMP-mediated mechanism, and stimulation of LDL-receptor expression by a 5'-AMP-activated kinase, would be compatible with our observations.

The mechanism by which cyclic-AMP-elevating agents alter the expression of the LDL receptor is not known. The LDL receptor, like many other receptors, can be phosphorylated [29,30]. A kinase, capable of phosphorylating one of the serine residues (Ser-833) of the LDL receptor, has been identified in bovine adrenal cortex [30]. However, Davis *et al.* [31] have shown that replacement of this residue with alanine does not alter the function of the LDL receptor. Thus direct phosphorylation of the LDL-receptor molecule does not appear to modulate its function. Cyclic AMP is also known to have a regulatory function in the expression of a number of genes in eukaryotic cells [32]. It is possible that the rate of transcription of the LDL-receptor gene is altered via a mechanism which involves cyclic AMP, 5'-AMP or both.

In conclusion, we have demonstrated that elevation of cellular cyclic AMP concentrations by direct stimulation of adenylate cyclase, by addition of permeant cyclic AMP analogues or by inhibition of cyclic AMP phosphodiesterase results in changes in the activity of the LDL receptor. These are consistent phenomena in human diploid fibroblasts and smooth-muscle cells, and indicate the potential importance of this second messenger in mediating hormonal control of LDL-receptor expression in the vascular system.

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