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Glucagon, cyclic AMP and adrenaline stimulate the degradation of low-density lipoprotein by cultured rat hepatocytes

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Rat hepatocytes were preincubated for 16 h with hormones or drugs and then for a further 8 h with ^{125}I-human low-density lipoprotein (LDL). Glucagon (via cyclic AMP) and adrenaline (via cyclic AMP and α-effects) increased the binding of ^{125}I-LDL to the LDL receptor, and the degradation of LDL to [^{125}I]iodotyrosine. The effects on degradation were antagonized by dexamethasone, and the action of cyclic AMP on binding and degradation was inhibited by actinomycin D. The results are discussed in relation to the control of lipoprotein metabolism in diabetes.

INTRODUCTION

The liver plays a central role in mammalian cholesterol metabolism. It is responsible for the uptake of 70–80% of the LDL from the circulation. This process involves receptor-mediated endocytosis [1], and it is a major factor determining the concentration of LDL in the blood [2]. The liver can resecrete the cholesterol that it acquires from LDL as VLDL or HDL. Alternatively, it can secrete it in the bile as free cholesterol, or after conversion into bile salts. This provides the major route for cholesterol elimination from the body, since reabsorption by the gut is incomplete.

Much work on the regulation of LDL-receptor activity has centred on fibroblasts, where there is stringent control according to the cholesterol content of the cell [3]. Whereas fibroblast LDL receptors are markedly decreased by cholesterol loading of the cells with LDL, much smaller differences are seen with rat hepatocytes [4] and HepG2 cells [5], unless cholesterol esterification is inhibited [6]. This relative insensitivity may allow the liver to remove cholesterol from the circulation even when LDL concentrations are high.

There is increasing evidence that the expression of the LDL receptor in liver is under hormonal control. Treatment of rats with pharmacological doses of oestrogens [7,8] increases LDL-receptor activity in membranes that are subsequently isolated from the livers. Dexamethasone, a synthetic glucocorticoid, decreases the activity of the LDL receptor in cultured rat hepatocytes [9,10]. Conversely, insulin increases this activity in cultured rat hepatocytes [9–11] and in human hepatocarcinoma cells [12]. Insulin also antagonizes the effects of dexamethasone on LDL-receptor activity and LDL degradation [9–11]. This interaction between insulin and dexamethasone could help to explain the hypercholesterolaemia that is often observed in uncontrolled diabetes [11,13–15]. Similarly, the direct action of tri-iodothyronine in increasing the activity of the LDL receptor in hepatocytes [10] might explain the hypercholesterolaemia associated with hypothyroidism [16–18].

The present study examines the effects of glucagon, adrenaline and cyclic AMP on the expression of the LDL receptor in rat hepatocytes and on the rate of degradation of LDL. These hormones and the second messenger cyclic AMP could also be particularly important in modifying the hepatic metabolism of LDL in diabetes.

EXPERIMENTAL

Animals and materials

Male Wistar rats (about 200 g) were obtained from Charles River (Québec, Canada). They were housed in a room which was lit from 08:00 h to 20:00 h, and they were fed on Wayne Rodent Blox, which contained (by wt.) 24.5% protein and 4.4% fat. The sources of most of the materials have been described [4,19,20]. 8-(4-Chlorophenylthio) cyclic AMP (CPTcAMP) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Boehringer Mannheim (Canada). Mixed bovine/porcine glucagon, (−)-adrenaline (+)-bitartrate, L-phenylephrine hydrochloride, DL-propranolol hydrochloride, (−)-isoprenaline (+)-bitartrate, bovine insulin, actinomycin D and dexamethasone were from Sigma. Human LDL (d = 1.02–1.06) was isolated from healthy male volunteers and iodinated [20].

Preparation of hepatocytes and measurement of LDL binding and degradation

Hepatocytes were prepared between 10:00 h and 13:00 h. Monolayers of hepatocytes were cultured on 60 mm-diam. collagen-coated tissue-culture dishes and incubated for the first 24 h in modified Leibovitz L-15 medium supplemented with 10% (v/v) newborn-calf serum [19]. This provides a stable model for measuring subsequent changes in LDL binding and degradation [4,9–11,20]. Thereafter the cells were cultured in serum-

Abbreviations used: CPTcAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein.

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free L-15 medium containing 0.2% (w/v) fatty-acid-poor bovine serum albumin.

After 24 h in culture, the cells were washed with 3 ml of serum-free medium and incubated in a further 3 ml for 2 h. In some experiments (Table 1 and Fig. 3) this was followed by a period of preincubation in similar medium containing hormones or drugs, without LDL. To measure ^{125}I-LDL binding and degradation at 37 °C the medium was again changed, the hormones or drugs were maintained (or introduced), and 10 μg of ^{125}I-LDL protein/ml and 1 mM-iodotyrosine were added. The latter compound was necessary to inhibit the further degradation of the liberated [^{125}I]iodotyrosine to inorganic ^{125}I by hepatic deiodinase activity [4]. Incubations at 37 °C were then performed for the times indicated. The specific appearance of [^{125}I]iodotyrosine in the medium was used as a measure of ^{125}I-LDL degradation by a modification of the method of Goldstein *et al.* [21], described by Salter *et al.* [4]. Values for the degradation of ^{125}I-LDL in the absence of cells were measured and subtracted. ^{125}I-LDL that had bound at 37 °C was then measured after cooling the cells to 4 °C. The dextran sulphate-releasable ^{125}I-LDL (assumed to be bound to the classical LDL receptor) and cell-associated ^{125}I-LDL (other surface-bound and internalized LDL) were measured as described by Salter *et al.* [20].

In experiments where the binding of ^{125}I-LDL at 4 °C was measured, cells were preincubated with hormones or drugs at 37 °C in the absence of ^{125}I-LDL for the times indicated, then cooled to 4 °C and subsequently incubated with ^{125}I-LDL for assay of binding activity [20].

Non-specific ^{125}I-LDL binding and degradation were determined in parallel incubations in the presence of a 30–50-fold excess of unlabelled LDL. These values were subtracted to yield specific binding and degradation.

LDL and hepatocyte protein concentrations were determined by the method of Bradford [22].

Statistical analysis was by Student's paired *t* test.

RESULTS AND DISCUSSION

Glucagon (100 nM), adrenaline (20 μM) and the cyclic AMP analogue CPTcAMP (100 μM) each produced increases in the rate of ^{125}I-LDL degradation which were normally apparent after 6 h and continued until 24 h (Fig. 1). CPTcAMP was the most effective. Results for dextran sulphate-releasable LDL binding at 37 °C also remained above control from 6 h until 24 h, e.g. with CPTcAMP (Fig. 2).

In further experiments hepatocytes were cultured for 16 h in the presence of various hormones and drugs. At the end of this period the medium was replaced with similar medium in which the hormone and drug concentrations were maintained, but including additionally 10 μg of ^{125}I-LDL protein/ml. Binding and degradation were measured after a further 8 h incubation. This scheme avoided the influence of any lag phase before the cells responded fully to glucagon, CPTcAMP or adrenaline. Each of these compounds produced significant increases in ^{125}I-LDL binding and degradation (Table 1). The effect of glucagon on receptor activity and on degradation was optimum at 100 nM–1 μM, and that of CPTcAMP was maximum at 100 μM (Fig. 3). This increase appears higher than those shown in Fig. 2 after 24 h. However, there was substantial variation in the extent of the increase in different experiments (Table 1). Furthermore,

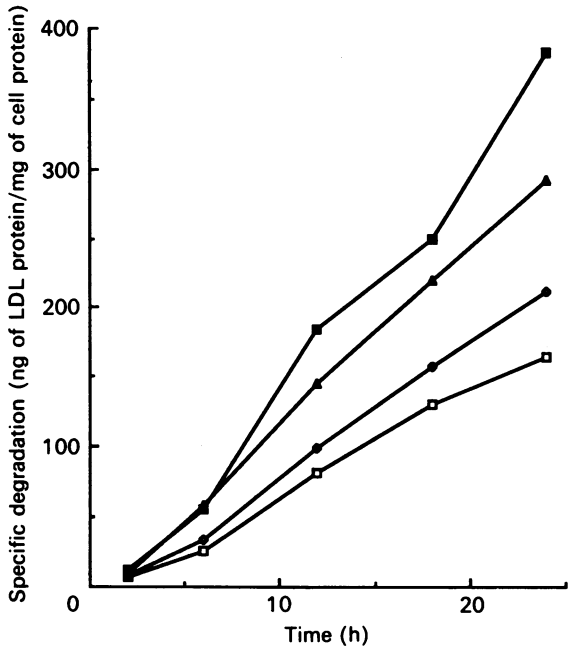

Fig. 1. Time course of ^{125}I-LDL degradation by rat hepatocytes in the presence of glucagon, CPTcAMP and adrenaline

Hepatocytes were incubated for various times in serum-free medium with 10 μg of ^{125}I-LDL protein/ml in the presence of 100 nM-glucagon (▲), 100 μM-CPTcAMP (■), 20 μM-adrenaline (◇) or no hormone (□) before measurement of ^{125}I-LDL degradation. The Figure shows means from triplicate dishes in a typical experiment. The results were reproduced in two further experiments.

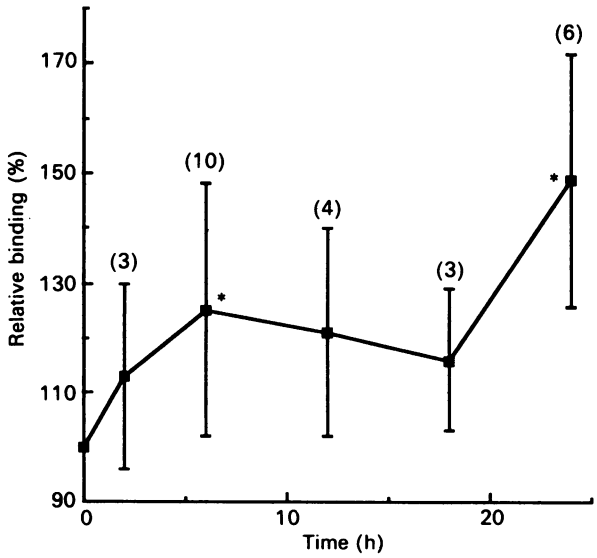

Fig. 2. Time course of ^{125}I-LDL binding to rat hepatocytes at 37 °C in the presence of CPTcAMP

Hepatocytes were incubated for the times shown with 10 μg of ^{125}I-LDL protein/ml in the presence or absence of 100 μM-CPTcAMP. Specific dextran sulphate-releasable LDL binding is shown relative to the control at each time point. Results are expressed as means ± S.D. for the numbers of independent experiments shown in parentheses. Significant difference from control is indicated by $*P < 0.01$.

Table 1. Effects of various hormones and drugs on the binding and degradation of ^{125}I-LDL by rat hepatocytes

Hepatocytes were preincubated in serum-free medium for 16 h containing the hormones and drugs indicated. The medium was then changed, maintaining the hormone or drug concentrations and including 10 μg of ^{125}I-LDL protein/ml. ^{125}I-LDL degradation and binding were measured after a further 8 h as described in the Experimental section. Results are expressed relative to incubations which contain no additions of agonists. They are given as means ± S.D. for the numbers of independent experiments shown in parentheses, or as mean ± range where only two experiments were performed. Absolute values for binding and degradation after 8 h in control incubations are 4.96 ± 1.9 and 85.8 ± 36.5 ng of LDL protein/mg of cell protein respectively (mean ± S.D., $n = 12$). Values that differ significantly from the control value in the absence of hormones or drugs are shown by $^{*}P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.01$, and $^{****}P < 0.001$, or from a similar incubation in the absence of dexamethasone by $\dagger P < 0.05$, $\dagger\dagger P < 0.02$ and $\dagger\dagger\dagger P < 0.01$; n.m., not measured.

	Dexamethasone and insulin absent		+Dexamethasone (10 nM)		+Insulin (10 nM)	
Addition	Binding	Degradation	Binding	Degradation	Binding	Degradation
None	100%	100%	81 ± 14**	56 ± 19**** (8)	125 ± 17**	137 ± 20*** (6)
Glucagon (100 nM)	215 ± 56****	169 ± 39*** (8)	161 ± 41††	113 ± 21† (4)	168 ± 66	148 ± 47 (4)
CPTcAMP (100 μM)	222 ± 40****	245 ± 53**** (9)	156 ± 45†	168 ± 44††† (4)	229 ± 100	240 ± 93 (5)
Adrenaline (20 μM)	156 ± 36****	169 ± 42*** (8)	125 ± 39	94 ± 22††† (4)	137 ± 4	148 ± 8 (2)
Isoprenaline (20 μM)	150 ± 30*	130 ± 10*** (4)	n.m.	n.m.	n.m.	n.m.
Phenylephrine (10 μM)	131 ± 25*	127 ± 6**** (5)	n.m.	n.m.	n.m.	n.m.

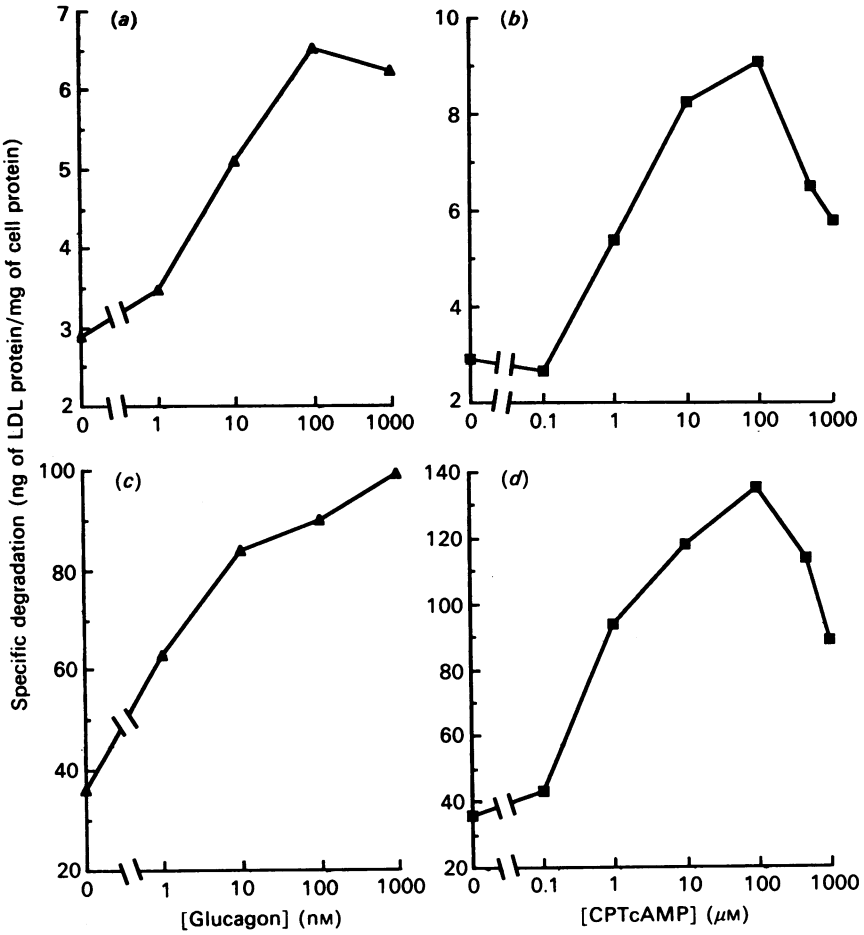

Fig. 3. Effects of increasing concentrations of glucagon and CPTcAMP on ^{125}I-LDL degradation by rat hepatocytes

Hepatocytes were pretreated with glucagon (*a*, *c*) or CPTcAMP (*b*, *d*) for 16 h. The binding and degradation of ^{125}I-LDL were measured as described in the legend to Table 1 after a further incubation for 8 h at 37 °C. The Figure shows means from triplicate dishes in a single experiment. The results were reproduced in a further experiment.

Table 2. Effect of actinomycin D on ^{125}I-LDL degradation and binding by rat hepatocytes

Hepatocytes were incubated for 6 h with 10 μg of ^{125}I-LDL protein/ml in the absence or presence of 100 μM-CPTcAMP, before measurement of ^{125}I-LDL binding and degradation. Parallel incubations included 1 μg of actinomycin D/ml. Results are expressed relative to control incubations containing no drugs (means ± S.E.M. for eight independent experiments). Absolute values for binding and degradation after 6 h in control incubations are 6.90 ± 1.05 and 41.8 ± 7.5 ng of LDL protein/mg of cell protein respectively (means ± S.E.M., $n = 8$). Significant differences are indicated by † ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$).

		Binding (%)	Degradation (%)
I	Control	100	100
II	Actinomycin D	105 ± 7	106 ± 7
III	CPTcAMP	124 ± 9 I versus III*	154 ± 15 I versus III**
IV	CPTcAMP + actinomycin D	78 ± 4 I versus IV† III versus IV†	122 ± 8 I versus IV* III versus IV**

the hepatocytes described in Fig. 2 were exposed to LDL throughout the incubation period, whereas this occurred only during the last 8 h in Fig. 3 and Table 1. The uptake and availability of cholesterol in the cells described in Fig. 2 should therefore have been greater, and this may have suppressed part of the cyclic AMP-induced increase in LDL binding [4,6]. Higher concentrations of CPTcAMP were less effective, although 1 mM-CPTcAMP still gave increases in both binding and degradation (Figs. 3*b* and 3*d*). No obvious toxic effect was observed, and recovery of cell protein was unaffected by these higher concentrations of CPTcAMP.

With each hormone or drug, cell-associated radioactivity (surface-bound LDL not displaced by dextran sulphate plus internalized LDL) varied in the same direction as binding and degradation, but to a smaller extent (results not shown).

Our previous work showed that dexamethasone decreases, whereas insulin increases, the binding and degradation of human ^{125}I-LDL by rat hepatocytes [9–11]. The results shown in Table 1 confirm this and show that 10 nM-dexamethasone can also decrease LDL degradation when this is stimulated by glucagon, CPTcAMP or adrenaline. These effects were paralleled by changes in the binding of LDL to its receptor, though here the antagonism with adrenaline did not reach statistical significance. Insulin increased the binding and degradation of ^{125}I-LDL on its own. However, there were no further significant changes in these parameters when insulin was added to incubations that also contained CPTcAMP, glucagon or adrenaline (Table 1).

To investigate whether the adrenaline effect was mediated via α- or β-adrenergic mechanisms, we performed similar experiments using 20 μM-adrenaline simultaneously with the α_1-adrenergic antagonist phentolamine (20 μM) and the β-adrenergic antagonist propranolol (20 μM). Both these compounds themselves increased degradation [respectively 127 ± 6% ($n = 2$) and 196 ± 24% ($n = 3$) relative to control], making interpretation of the results difficult. Certain α_1- and β-antagonists have been shown previously to increase LDL-receptor activity in human fibroblasts [23,24]. As an alternative approach, we incubated hepatocytes with the α-agonist phenylephrine and the β-agonist isoprenaline (Table 1). As expected from the results with glucagon and CPTcAMP, isoprenaline caused increased ^{125}I-LDL binding and degradation. Phenylephrine also increased ^{125}I-LDL uptake, but to a lesser extent than the other hormones and drugs. Also, the phenylephrine effect was not normally apparent until after more than 6 h of incubation. It is therefore possible that the action of adrenaline was mediated both by α- and β-adrenergic mechanisms.

Experiments were performed to determine if the effects of cyclic AMP were mediated by changes in the rate of synthesis of the LDL receptor. Inclusion of the mRNA-synthesis inhibitor actinomycin D had no significant effect on control incubations, but it prevented the CPTcAMP-induced increase in LDL binding and impaired the increase in LDL degradation (Table 2). In fact, in the presence of CPTcAMP and actinomycin D, LDL binding activity fell to a value below control. These results suggest that synthesis of new receptor molecules is involved in the response to CPTcAMP. We do not know if the effect of these hormones is by a direct stimulation of transcription of the LDL-receptor gene. Cyclic AMP analogues can inhibit 3-hydroxy-3-methylglutaryl-CoA reductase and hence cholesterol biosynthesis in rat liver [25,26] and in human hepatocarcinoma cells [27], and a consequent decrease in cell cholesterol content could increase LDL-receptor number.

Previous work on the effects of cyclic AMP analogues on LDL uptake by cultured cells has produced a variety of results. In human granulosa cells, 8-bromo cyclic AMP increased the degradation of LDL [28] and the synthesis of LDL receptors [29], apparently as part of the response to human chorionic gonadotropin and independent of cell cholesterol content. In human fibroblasts, dibutyryl cyclic AMP had no effect on LDL-receptor activity [3,30], or caused a decrease [31,32]. LDL degradation was decreased by dibutyryl cyclic AMP and adrenaline in human mononuclear leukocytes [33] and by dibutyryl cyclic AMP in human arterial smooth-muscle cells [32].

Incubation of HepG2 cells for 24 h with dibutyryl cyclic AMP caused a decrease either in LDL binding, as measured by a subsequent incubation with ^{125}I-LDL at 4 °C, or in LDL binding and degradation, measured during a subsequent unspecified period at 37 °C [27]. We also therefore measured ^{125}I-LDL binding at 4 °C after preincubating rat hepatocytes with 100 μM-CPTcAMP for 24 h at 37 °C in the absence of LDL. Binding of LDL to the receptor was 89 ± 30% of the control value (mean

±S.D. for seven independent experiments), which was not a significant difference. These results therefore do not necessarily indicate a difference in the effect of cyclic AMP in rat hepatocytes and HepG2 cells, since the direction of this effect might depend on the time of preincubation with cyclic AMP. The effects obtained by Mazière *et al.* [27] could reflect the relatively long incubation period. It is also important to know whether cyclic AMP can increase LDL catabolism in HepG2 cells when shorter preincubation periods are employed.

The present work establishes that glucagon (possibly via cyclic AMP) and adrenaline (possibly via cyclic AMP and α-effects) can increase LDL-receptor activity and LDL degradation at 37 °C in rat hepatocytes. However, at this stage we cannot be certain whether Ca^{2+}-mediated signalling is involved in the action of glucagon in modifying the activity of the LDL receptor. The actions of CPTcAMP, glucagon and adrenaline are superficially similar to those of insulin, whereas dexamethasone decreased the action of glucagon, adrenaline and insulin (Table 1; refs. [9–12]). Insulin failed to alter significantly the binding and degradation of LDL when added in the presence of CPTcAMP, glucagon or adrenaline. At first sight these results are unexpected, since it is normally assumed that the effects of insulin should be antagonistic towards those of glucagon and adrenaline. However, a parallel situation may also exist in the control of VLDL secretion. Thus insulin [34–40], glucagon [40,41] and adrenaline [42] have been reported to decrease this process. Conversely dexamethasone stimulates VLDL secretion and insulin counteracts its effects [39,43]. It therefore appears that the hypertriglyceridaemia and hypercholesterolaemia that are often associated with uncontrolled diabetes could be partly caused by the increased action of glucocorticoids relative to insulin [11]. Glucagon and adrenaline may, in fact, help to diminish VLDL secretion and stimulate LDL uptake by the liver.

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