Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 424-431

CYCLIC AMP DECREASES LDL CATABOLISM AND CHOLESTEROL SYNTHESIS IN THE HUMAN HEPATOMA CELL LINE HepG2

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Received September 1, 1988

SUMMARY: A 24h pretreatment of the human hepatoma cell line HepG2 with dibutyryl cyclic AMP in the presence of theophylline induced a dose dependent decrease in low density lipoprotein binding, uptake and degradation. This effect is most likely due to a reduction of the LDL receptor number. Sterol synthesis from sodium acetate is markedly inhibited, either in the presence or absence of LDL, whereas synthesis from mevalonic acid is unchanged. Cyclic AMP also induced a decrease in hydroxy methyl glutaryl coenzyme A reductase activity. These effects of cyclic AMP might be involved in some hormonal regulation of the LDL pathway and cholesterol metabolism in the liver.

The control of plasma LDL cholesterol level is of importance because it is closely related to coronary heart diseases. LDL is cleared from peripheral tissus by means of the high affinity LDL receptor pathway first reported by Goldstein and Brown in fibroblasts [1], which delivers cholesterol for cell membrane constitution and down regulates cholesterol synthesis by decreasing HMGCoA reductase activity [2]. The liver also plays a central role in LDL removal: in vivo studies demonstrated that more than 60% of LDL in humans is catabolized by the liver [3,4].

Abbreviations: LDL: low density lipoprotein, dbcAMP: dibutyryl cyclic AMP, th: theophylline, HMGCoA: Hydroxy Methyl Glutaryl Coenzyme A.

0006-291X/88 $1.50

However, in hepatocytes, the down regulation of cholesterol synthesis by LDL appears to be less effective than in fibroblasts [5,6]. Furthermore, it has been suggested that the expressed human hepatic receptor for LDL differs from the fibroblast LDL receptor, both in molecular weight and in Ca^{2+} dependency [7] .

We [8] and others [9] demonstrated that the second messenger cyclic AMP decreases the LDL receptor pathway in human cultured fibroblasts. Since the regulation of the LDL receptor pathway might be different in fibroblasts and in hepatocytes, we investigated the effects of this second messenger in hepatocytes of human origin. The human hepatoma cell line HepG2 was chosen because different authors reported the presence of specific high affinity LDL receptors in these cells [10-13]. Since it was also demonstrated that in HepG2 cells, the LDL receptor and cholesterol synthesis are subject to metabolic regulation, this cell line provides a valuable model for the studies of human hepatocyte metabolism in vitro. It was demonstrated that cyclic AMP decreases the LDL receptor number and HMGCoA reductase activity in HepG2 cells.

MATERIALS AND METHODS

Materials. All chemicals were from Sigma, St.Louis, MO, USA. [2-^{14}C] sodium acetate 55 mCi/mmol, [5-^{14}C] mevalonic acid 50mCi/mmol, were from CEA, Saclay, France; ^{125}INa 13-17 Ci/mg, 3-hydroxy-3-methyl [3-^{14}C] glutaryl Coenzyme A 52mCi/mmol were from Amersham, Buckinghamshire U.K. Dulbecco's modified Minimum Essential Medium with Earle's salts (DMEM) and foetal calf serum were from Gibco, Grand Island, N.Y., U.S.A. The HepG2 cell line was from the American Type Culture Collection, Rockville, Maryland, USA. The serum substitute Ultroser G was purchased from Industries Biologiques Françaises, Villeneuve La Garenne, France.

Cell culture. Cells were cultured in 35 mm Nunc Petri dishes containing 2 ml DMEM medium supplemented with 20 mM Hepes buffer (pH 7.4), 100 units/ml penicillin, 100 µg/ml

streptomycin and 10 % (v/v) foetal calf serum, at 37°C in a humidified atmosphere of 5% CO_2. Experiments were performed on confluent cells.

LDL preparation and labeling. LDL was prepared from normal human serum by the technique of Havel et al. [14], and labeled according to Bilheimer et al. [15]. The specific radioactivity was 200-300 dpm/ng.

Effect of cyclic AMP on LDL binding, internalization and degradation. Cells were pretreated 24h with dbcAMP in the presence of theophylline 10^{-3}M in lipoprotein deficient medium supplemented with 2% Ultroser G for induction of LDL receptors. LDL binding, internalization and degradation were studied according to Goldstein and Brown [1], using ^{125}I-LDL 10µg / ml. Results are expressed in ng LDL/mg of cell protein, and calculated in percentages of control. Protein determination was done by the Lowry method.

Effect of cyclic AMP on cholesterol metabolism. Cells were preincubated 24h with dbcAMP in the presence of theophylline in medium supplemented with Ultroser G or with foetal calf serum. The radioactive precursors were then added. After a further 4 h incubation, lipid analysis is performed by thin layer chromatography after application of a cell suspension aliquot on silicagel plates as previously described [16]. Results are expressed in pmol of precursor incorporated / mg cellular protein.

Hydroxy-Methyl-Glutaryl-Coenzyme A reductase activity. Cells were pretreated with drugs during 24h in medium supplemented with 2% Ultroser G for induction of the enzyme. After harvesting, the enzyme activity was analyzed immediately as described by Beg et al.[17].

RESULTS AND DISCUSSION

The effects of dbcAMP on high affinity LDL binding, uptake and degradation are shown in Figure 1. It can be noted that dbcAMP reduces the three parameters in a dose dependent manner. At 10^{-4} M of dbcAMP, a 30% decrease was observed for binding as well as uptake and degradation. The fact that the three parameters were inhibited to a similar extent by cyclic AMP suggests that the latter exerts its action at the step of LDL binding to its receptor. This hypothesis is further confirmed by the results of Scatchard plot analysis presented in Figure 2: cyclic AMP apparently decreases the LDL high affinity receptor number. The

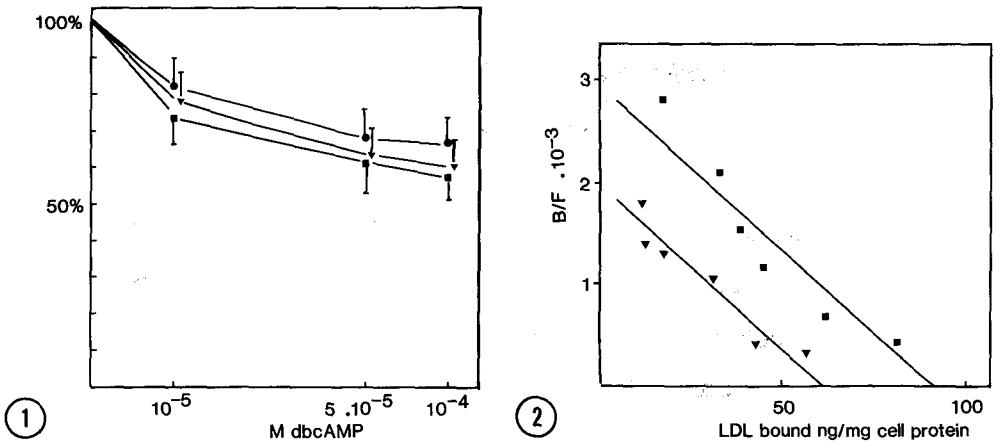

Figure 1. Effect of cyclic AMP on LDL metabolism. Cells were pretreated 24h in medium supplemented with the serum substitute Ultroser G with dbcAMP at the indicated concentrations in the presence of theophylline 10^{-3}M. After washing, LDL binding, uptake and degradation were determined as described [1], with 10µg/ml ^{125}I-LDL. Non specific values, determined in the presence of a 100 fold excess of non labelled LDL, were substracted from total values in order to obtain specific binding, uptake and degradation. Results are expressed in percentages of control values. Absolute values are 61 ± 7, 407 ± 35, 691 ± 57 ng/mg cell protein for LDL binding (■), uptake (▼), and degradation (●) respectively. Means of six determinations ± s.d.

Figure 2. Scatchard plot analysis of the effect of cyclic AMP on LDL binding. Cells were pretreated 24h in 2% Ultroser G supplemented medium with dbcAMP 10^{-4}M + theophylline 10^{-3}M. After washing, LDL binding was performed during 1h at 4°C, with 10µg/ml ^{125}I-LDL in the presence of increasing amounts of unlabeled LDL. ■ :control; ▼ :cAMP.

calculated maximum binding was 91 ngLDL/mg protein for the control and 61 ng/mg for cAMP treated cells.

In the next experiments, the effects of cyclic AMP on cholesterol synthesis were investigated. As in vivo, hepatocytes are exposed to plasmatic concentrations of LDL, the rates of sterol synthesis from sodium acetate were studied in two experimental conditions: in medium devoid of lipoproteins supplemented with the serum substitute Ultroser G, and in medium containing lipoproteins provided by foetal calf serum. The results of Table I show that cholesterol synthesis from sodium acetate is higher in

cells incubated in lipoprotein deficient medium, as expected. Furthermore, cyclic AMP decreased cholesterol synthesis in both conditions, either in the presence or in the absence of lipoproteins, with however a somewhat more marked effect in the absence of lipoproteins. At 10^{-4} M dbcAMP, the rate of sterol synthesis accounted for 10% of control in the absence of lipoproteins and 30% in their presence.

As it is well known that the cholesterol synthesis is under the control of the key enzyme HMGCoA reductase , the enzyme which catalyses the conversion of HMGCoA to mevalonic acid, we decided to comparatively investigate the effects of cyclic AMP on sterol synthesis from sodium acetate and mevalonic acid, in lipoprotein deficient medium. From the results presented in Table I, it can be

Table I. Effect of cyclic AMP on cholesterol synthesis

Precursor	Sodium acetate		Mevalonic acid
Preincubation in:	Ultroser G	Foetal calf serum	Ultroser G
Addition			
None :	2451 ± 221	1727 ± 153	264 ± 25
dbcAMP 10^{-5}M + th 10^{-3}M:	1152 ± 109 ***	1053 ± 98 **	287 ± 27
dbcAMP 5.10^{-5}M + th 10^{-3}M:	711 ± 68 ***	639 ± 57 ***	265 ± 19
dbcAMP 10^{-4}M + th 10^{-3}M:	244 ± 23 ***	518 ± 48 ***	240 ± 21

Cells were pretreated 24h either in lipoprotein-deficient medium supplemented with the serum substitute Ultroser G for induction of sterol synthesis or in foetal calf serum supplemented medium for repression of sterol synthesis. [1-^{14}C] sodium acetate (20 µCi/ml) or [5-^{14}C] mevalonic acid (5µCi/ml) were then added during 4h. Sterol separation was performed by thin layer chromatography. Results are expressed in pmol precursor incorporated per mg protein. Means of 4 experimental values ± s.d. Statistical analysis was performed by the Student's t test. **:p<0,01; ***:p<0,001.

Table II. Effect of dbcAMP on Hydroxy Methyl Glutaryl Coenzyme A reductase activity

Addition	HMGCoA reductase activity (pmol/h/mg)
None	532 ± 51
dbcAMP 10^{-5}M + th 10^{-3}M	133 ± 10 ***
dbcAMP 5.10^{-5}M + th 10^{-3}M	90 ± 7 ***
dbcAMP 10^{-4}M + th 10^{-3}M	52 ± 5 ***

Cells were pretreated 24h in medium supplemented with Ultroser G for induction of the enzyme. After washing, the enzymatic activity was determined as described [17]. Means of 4 experimental values ± s.d. ***: p<0,001 by Student's t test.

concluded that cyclic AMP most probably exerts its action on the activity of the key enzyme, since cholesterol synthesis from mevalonic acid was not impaired. This observation is further confirmed by the data given in Table II, concerning the direct effect of cyclic AMP on HMGCoA reductase activity. The activity of this enzyme is strongly decreased in the presence of cyclic AMP: the lower concentration 10^{-5} M already induced a 75% reduction of activity in our conditions.

This work demonstrates a reduction of LDL binding, uptake and degradation in the hepatoma cell line HepG2 by cyclic AMP, a result already reported in human cultured fibroblasts [8, 9], but not yet described for hepatocytes. This effect is of importance, in view of the fact that many hormones, especially insulin , might act through the activity of adenylate cyclase. Insulin, which decreases cyclic AMP intracellular level, has been reported to increase LDL binding by rat hepatocytes [18]. Results from our laboratory on the hepatoma cell line HepG2 also confirm

this effect (unpublished data). It is also of note that the direct stimulation of adenylate cyclase by the diterpene forskolin induced an effect similar to the cAMP effect on LDL metabolism (data not shown).

Our studies also pointed out that the second messenger cyclic AMP reduces cholesterol synthesis from sodium acetate by decreasing the HMGCoA reductase activity. It is well known that this enzyme is regulated by a short-term phosphorylation-dephosphorylation process [19], the reductase kinase itself is activated by a phosphorylation mechanism in the presence of reductase kinase kinase [20]. However, neither of the kinase enzymes have been identified with cAMP dependent protein kinase in rat liver [21]. Thus, it is most likely that the observed effect of cAMP on HMGCoA reductase activity results from a long term effect on the enzyme level. Kinetic studies of the effect of cAMP on the enzyme activity show that the decrease in activity became evident only after 4-5h of preincubation with the second messenger (data not shown), which confirms the absence of a short term effect. It can be concluded that cyclic AMP most likely act on the rate of enzyme synthesis or degradation.

ACKNOWLEDGMENT: We thank La Ligue Nationale Française contre le Cancer, Comité de Paris, for financial support.

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