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DIBUTYRYL-CYCLIC AMP INHIBITS CHOLESTEROL ESTERIFICATION IN J 774 MONOCYTE-LIKE CELLS

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SUMMARY: The effect of dibutyryl-cyclic AMP (dbcAMP) and theophylline was investigated on oleic acid incorporation into cholesteryl esters and triacylglycerols in the mouse monocyte-macrophage cell line J 774. 24h pretreatment of macrophages with dbcAMP decreased cholesteryl ester formation in a dose-dependent manner (about 4 fold reduction for dbcAMP 10^{-4}M + theophylline 10^{-3}M), while oleic acid incorporation into triacylglycerols was markedly (2 to 3 fold) enhanced. The catabolism of acetylated LDL was only slightly affected (about 15-20% reduction with dbcAMP $5x10^{-4}$M + theophylline 10^{-3}M). Acyl Coenzyme A:cholesterol-O-acyl-transferase activity, measured *in vitro* on cell homogenates, was reduced in dbcAMP-treated cells, whereas diacylglycerol acyltransferase activity was increased. These results suggest that cyclic AMP can modulate cholesteryl ester and triacylglycerol formation in macrophages, and that these metabolisms are inversely regulated. Agents which increase cyclic AMP intracellular level could be of interest for reducing cholesteryl ester accumulation in macrophages.

Atherosclerotic lesions have been demonstrated to be infiltrated by macrophage-derived foam cells containing cholesteryl esters (1). Massive cholesteryl ester accumulation occurs *in vitro* when macrophages are incubated in the presence of modified low density lipoproteins (LDL): acetylated (2), or malondialdehyde-treated (3) LDL are taken up

Abbreviations: LDL: low-density lipoprotein, LDLac: acetylated low-density lipoprotein, ACAT: acyl Coenzyme A: cholesterol-O-acyltransferase, DGAT: diacylglycerol acyltransferase.

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and degraded by macrophages, leading to cholesteryl ester deposition. Enhanced-negative net charge of the LDL seems to be required for LDL processing by macrophages. Incubation of LDL with endothelial cells also induce alterations of the lipoprotein which allows its subsequent uptake by macrophages (4, 5). Modifications of LDL by endothelial cells could occur in vivo when the LDL turn over is decreased, such as in familial hypercholesterolemia. This phenomenon may be involved in accelerated atherosclerosis observed in these patients (6).

The mechanisms which regulate cholesteryl ester formation in macrophages are still poorly documented. Previous works from our laboratory (7,8) pointed out an inhibitory effect of cyclic AMP and agents increasing its intracellular level such as methylxanthines or β-agonists on cholesterol esterification in fibroblasts. We thus investigated the effect of cyclic AMP on oleic acid incorporation into cholesteryl esters in J 774 mouse monocyte-like cells, which has been previously shown to express the receptor for modified-LDL (9). Oleic acid incorporation into triacylglycerols was also comparatively studied. It was found that dibutyryl cyclic AMP (dbcAMP) + theophylline decreased cholesterol esterification by oleic acid. By contrast, triacylglycerol formation was strongly enhanced. Acetylated LDL catabolism was only slightly affected. ACAT activity, measured on cell homogenates, was decreased by dbcAMP + theophylline, while diacylglycerol acyltransferase (DGAT) activity was notably increased.

MATERIALS AND METHODS

Materials. Dibutyryl cyclic AMP and theophylline were from Sigma, St. Louis, MO, U.S.A.; [1-^{14}C] oleic acid 52 mCi/mmol, [1-^{14}C] oleyl Coenzyme A 55 mCi/mmol and ^{125}INa 13

Ci/mg from Amersham, Buckinghamshire U.K..; Dulbecco modified Minimum Essential Medium with Earle's salts and foetal calf serum from Gibco, Grand Island, NY, U.S.A.; Ultroser G from Industries Biologiques Françaises, France; J 774 mouse cells from the American Type Culture Collection, U.S.A. Silicagel plates F 1500 were from Schleicher and Schuell, Dassel, W. Germany.

Cell culture. J 774 cells were cultured in 30 mm Nunc Petri dishes containing 1 ml Dulbecco MEM medium supplemented with 20 mM Hepes buffer (pH 7.4), 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% foetal calf serum, at 37°C in a humidified atmosphere of 5% CO_2. For experiments, foetal calf serum was replaced by 2% Ultroser G and 50µg/ml acetylated LDL (LDLac) for induction of the macrophage ACAT activity.

LDL preparation and acetylation: LDL was prepared from normal human serum by 3 step-ultracentrifugation at 105000g in a L5-50 Beckman instrument, according to Havel et al. (10). The LDL was taken as the 1.024-1.050 fraction. Acetylation was performed by the method of Basu et al. (11).

LDLac labeling: LDLac was labeled by the method of Bilheimer et al. (12), using ^{125}INa. The specific radioactivity was about 300-400 dpm/ng LDL protein. Protein determination was performed according to Lowry et al. (12).

LDLac degradation: cells were preincubated 24h in Dulbecco's MEM medium supplemented with 2% Ultroser G in the presence or in the absence of the drugs. After 3 washes with a phosphate-buffered solution pH 7.4, LDLac degradation was measured during 4h at 37°C on confluent cultures according to Goldstein et al. (2), with 10µg/ml ^{125}I-LDLac. Results are expressed in ng ^{125}I-LDLac degraded/mg of cell protein.

Oleic acid incorporation into cholesteryl esters and triacylglycerols: cells were incubated 24 h in Dulbecco's MEM medium supplemented with 2% Ultroser G and 50µg/ml LDLac, in the presence or in the absence of dbcAMP and theophylline at indicated concentrations. [1-^{14}C] oleic acid 1µCi/ml was then added and incubation further performed 4h at 37°C. Cells were washed 4 times with a phosphate-buffered solution pH 7.4, harvested with rubber policemen, centrifuged, and resuspended in 100µl NaCl 0.15M. Lipid analysis was performed by thin layer chromatography after direct application of an aliquot of the cell suspension on silicagel plates F1500 by an adaptation of the technique of Dosado et al. (13). The solvent was hexane/diethylether/acetic acid 70/30/2 (v/v). Radioactive lipids, identified after autoradiography by comparison with unlabeled purified standards from Sigma, were cut out, and their radioactivity measured by liquid scintillation in an Intertechnique instrument. Results are expressed as pmol of precursor incorporated /mg of cell protein.

Acyl Coenzyme A:cholesterol-O-acyltransferase and diacylglycerol acyltransferase activities: ACAT and DGAT activities were measured on sonicated macrophage homogenates as described by Golstein et al. (14) and Mazière et al. (15), respectively. After 24 h pretreatment with drugs at indicated concentrations, cells were harvested with rubber-policemen,

centrifuged, and resuspended in NaCl 0.15M + NaF $5x10^{-3}$M. Each assay contained 150 µg of protein, phosphate buffer 10^{-1}M pH 7.4, $MgCl_2$ 5 x 10^{-3}M, bo-vine serumalbumin 0.2 mg/ml, [1-^{14}C] oleyl Coenzyme A (0.1 µCi), and non labeled oleyl-Coenzyme A (final concentration 10^{-5}M). The final incubation volume was 100µl. The reaction was carried out during 5 minutes at 37°C and stopped on ice. An aliquot of the incubation mixture was then put on a silicagel plate and separation of neutral lipids was achieved by thin layer chromatography as described above. Spots corresponding to cholesteryl esters and triacylglycerols were cut out, and the radioactivity was counted by liquid scintillation. ACAT and DGAT activities were expressed in nmol/h/mg of cell protein.

RESULTS AND DISCUSSION

Table I shows that 24h pretreatment of J 774 cells by dbcAMP + theophylline resulted in a dose-dependent decrease in oleic acid incorporation into cholesteryl esters: about 3 to 4 fold reduction was observed with dbcAMP $5x10^{-4}$M + theophylline 10^{-3}M as compared to control. By contrast, ^{14}C-oleic acid incorporation into triacylglycerols was markedly enhanced by dbcAMP + theophylline (x 2.5 for dbcAMP $5x10^{-4}$M + theophylline 10^{-3}M). It is of note that oleic acid incorpora-

Table I: Effect of dbcAMP and theophylline on ^{14}C-oleic acid incorporation into cholesteryl esters and triacylglycerols by J 774 cells. Cultures were preincubated 24h in Dulbecco's MEM medium supplemented with 2% Ultroser and 50µg/ml LDLac, in the presence or in the absence of the drugs at indicated concentrations. ^{14}C-oleic acid was then added (1µCi/ml) and incorporation further carried out during 4h at 37°C. After washing, lipid analysis was performed by thin layer chromatography. Results are expressed in pmol of oleic acid incorporated/mg of cell protein (means of 3 experimental values ± s.d.).

Addition	^{14}C-oleic acid incorporation into cholesteryl esters	^{14}C-oleic acid incorporation into triacylglycerols
None	3230 ± 410 (100%)	1250 ± 175 (100%)
dbcAMP 10^{-5}M + theophylline 10^{-3}M:	1776 ± 225 (55%)	1687 ± 225 (135%)
dbcAMP 10^{-4}M + theophylline 10^{-3}M:	1185 ± 170 (37%)	2325 ± 340 (186%)
dbcAMP $5x10^{-4}$M + theophylline 10^{-3}M:	870 ± 95 (27%)	3310 ± 540 (265%)

tion into total phospholipids was not significantly affected (data not shown).

It can be observed in Table II that LDLac degradation was only slightly modified by 24h preincubation with dbcAMP + theophylline: indeed, the maximal effect was only 15-20% reduction at concentrations which decreased cholesterol esterification by 2-3 fold.

ACAT activity has been shown to be strongly induced following LDLac uptake and degradation by macrophages (2). The fact that in our experiments, dbcAMP and theophylline did not significantly affect the degradation of LDLac suggested that the inhibitory effect of the drugs on oleic acid incorporation into cholesteryl esters was not related to a decrease in LDLac catabolism . In order to precise the mechanisms of this phenomenon, we then investigated the effect of the drugs on ACAT and DGAT activities, measured in vitro on homogenates of cells preincubated 24h in the presence of dbcAMP 10^{-4}M + theophylline 10^{-3}M. Table III shows that about a 2 fold reduction of ACAT activity was observed in treated

Table II: Effect of dbcAMP and theophylline on ^{125}I-LDLac degradation by J 774 cells. Cells were preincubated 24h in Dulbecco's MEM medium supplemented with 2% Ultroser, in the presence or in the absence of the drug at indicated concentrations. After 3 washes, LDLac degradation was measured using 10µg/ml ^{125}I-LDLac. Results are expressed in ng LDLac degraded / mg of cell protein (means of 3 experimental values ± s.d.).

Addition	LDLac degradation (ng/mg cell protein)
None	4752 ± 890 (100%)
dbcAMP 10^{-5}M + theophylline 10^{-3}M:	4657 ± 559 (98%)
dbcAMP 10^{-4}M + theophylline 10^{-3}M:	4514 ± 722 (95%)
dbcAMP $5x10^{-4}$M + theophylline 10^{-3}M:	3944 ± 552 (83%)

Table III: Effect of cell preincubation with dbcAMP and theophylline on Acyl Coenzyme A:cholesterol-O-acyltransferase (ACAT) and diacylglycerol acyltransferase (DGAT) activities measured in vitro. Cells were preincubated 24h in the presence or in the absence of the drugs, then washed 3 times and harvested with rubber-policemen. ACAT and DGAT activities were measured on cell homogenates during 5 minutes at 37°C. Results are expressed in nmol/h/mg cell protein (means of 3 experimental values ± s.d.).

Addition	ACAT activity	DGAT activity
None	13.2 ± 1.8 (100%)	3.4 ± 0.7 (100%)
dbcAMP 10^{-4}M + theophylline 10^{-3}M:	5.8 ± 0.9 (44%)	8.0 ± 1.5 (235%)

cells as compared to control, while DGAT activity was about 2.5 fold increased.

From our results, it appears that dbcAMP modulates oleic acid incorporation into cholesteryl esters and triacylglycerols by J 774 cells in an inverse manner: triacylglycerol formation from oleic acid and DGAT activity measured in vitro were markedly enhanced by dbcAMP, whereas cholesteryl ester formation and ACAT activity were reduced. We recently reported a similar observation in cultured human fibroblasts (15). It is of note that cyclic AMP has been shown to decrease triacylglycerol synthesis in hepatocytes (16). This suggests that triacylglycerol metabolism may be inversely regulated in fibroblasts or macrophages as compared to tissues which are closely implicated in energetic regulation such as liver.

Cholesteryl ester accumulation in macrophages results from the absence of down-regulation of LDLac uptake and sterol synthesis (17). The LDLac receptor is poorly regulated: neither LDL, nor hormonal factors such as insulin, or platelet-derived growth factor, which have been shown to

modulate LDL uptake by fibroblasts (18,19), exert any effect on LDLac catabolism in macrophages (20). In the present work, we demonstrate that cyclic AMP, which is an important metabolic regulatory agent in several cell types, also regulates cholesterol esterification in J 774 macrophages. It is of note that cyclic AMP seems to do not act through an effect on LDLac catabolism, as the highest studied concentrations (dbcAMP $5x10^{-4}M$ + theophylline $10^{-3}M$) only weakly affected LDLac degradation. This also confirms that the catabolism of LDLac is not sensitive to agents which modulate native LDL receptor, as it has been previously shown that dbcAMP strongly inhibits LDL binding and degradation in human fibroblasts (7,8,21).

Finally, the observation that cyclic AMP decreases cholesteryl ester formation in macrophages is of interest, in the view that cholesteryl ester deposition in macrophages is probably one of the main processes involved in atherogenesis (6). In previous works performed on cultured human fibroblasts, we suggested that agents which increased cyclic AMP intracellular level such as theophylline could be utilized to reduce cholesterol synthesis and esterification (7). Recently, Tertov et al. demonstrated that cyclic AMP and methylxanthines decrease cholesteryl ester content in cells cultured from atherosclerotic lesions (22), and these authors also suggested that cyclic AMP could be of interest in the prevention of atherosclerosis.

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