Essential Differences in Cholesteryl Ester Metabolism Between Human Monocyte-Derived and J774 Macrophages

Evidence Against the Presence of Hormone-Sensitive Lipase in Human Macrophages

Juan Antonio Contreras, Miguel A. Lasunción

Abstract Cholesteryl ester–laden macrophages are the hallmark of the fatty streaks that precede arteriosclerotic plaques in humans and experimental animals. This article studies several aspects of cytoplasmic cholesteryl ester metabolism in cultured human monocyte–derived macrophages. Adenosine 3′,5′-cyclic monophosphate (cAMP) consistently inhibited cholesteryl ester mobilization from cells that had been loaded with cholesteryl esters by preincubation with acetylated low-density lipoprotein. This effect was observed in both the absence and presence of extracellular cholesterol acceptors as well as with acyl coenzyme A:cholesterol acyltransferase inhibitors. In contrast, dibutyryl cAMP activated cholesteryl ester hydrolysis in J774 macrophages. Since hormone-sensitive lipase is thought to be responsible for the neutral cholesteryl ester hydrolytic activity in several cell types, we looked for the presence of its mRNA in our macrophages by means of reverse transcription coupled to the polymerase chain reaction technique. Hormone-sensitive lipase mRNA was detected in J774 macrophages but not in human monocytes or in human monocyte–derived macrophages. These results demonstrated great differences in cholesteryl ester metabolism between macrophages of different origin. While hormone-sensitive lipase may be responsible for neutral cholesteryl ester hydrolytic activity in J774 macrophages, in human monocyte–derived macrophages it is not; thus, a different and as yet unidentified enzyme must be present. (Arterioscler Thromb. 1994;14:443-452.)

Key Words • human monocyte–derived macrophages • neutral cholesteryl ester hydrolase • hormone-sensitive lipase • cAMP • J774 macrophages

Atherosclerotic lesions are characterized by the presence of cells heavily loaded with cytoplasmic droplets of neutral lipids, mainly cholesteryl esters.1–4 Histological and immunocytochemical studies show that most of these loaded cells are macrophages derived from blood monocytes that have infiltrated the subendothelial space.1–8 Understanding the mechanisms involved in cholesterol accumulation and mobilization from macrophages is significant for the clarification of the atherogenic process. Cholesteryl ester accumulation in the macrophage cytoplasm can be mediated by many mechanisms.9 Most involve the internalization of cholesterol-rich lipoproteins and the hydrolysis of the cholesteryl esters by lysosomal (acid) lipases, followed by reesterification of the lipoprotein-derived cholesterol by acyl coenzyme A:cholesterol acyltransferase (ACAT), which results in the formation of cytoplasmic cholesteryl ester droplets. These droplets are metabolically active,10 and they can be hydrolyzed by a cytoplasmic cholesteryl esterase called neutral cholesteryl ester hydrolase (NCEH). The free cholesterol can then leave the cell or be reesterified by ACAT.

Thus, NCEH allows cholesterol mobilization from cells to extracellular acceptors, which is the first step in arterial cholesterol reverse transport. Previous studies have shown that NCEH activity is highly variable depending on the origin of the macrophages used; eg, it is high in mouse macrophages and low in rabbit macrophages,11,12 which have a limited cholesteryl ester-clearing capacity. Hence, NCEH activity may be a limiting factor in the mobilization of macrophage foam cell cholesteryl esters, and thus the characterization and elucidation of its regulatory pathways are of great interest.

Several findings suggest a role for adenosine 3′,5′-cyclic monophosphate (cAMP) in the activation of NCEH in homogenates from smooth muscle cells13–15 and murine macrophages.16–19 cAMP also stimulates cholesteryl ester mobilization from intact human aortic smooth muscle foam cells,20 while the present study was being performed, Bernard et al21 reported that cAMP analogues stimulate cholesteryl ester hydrolysis in J774 macrophages. Furthermore, Small et al22 found that hormone-sensitive lipase (HSL) is responsible for NCEH activity in mouse macrophages. This would explain the activation of cholesteryl ester hydrolysis caused by cAMP in these cell types, since HSL is activated by cAMP-dependent phosphorylation.22 To our knowledge, this is the first study to report on the effects of cAMP in NCEH regulation in the human monocyte–derived macrophage model of foam cells. This study demonstrated that there are essential differ-
ences in the effects of cAMP on cholesterol ester hydrolysis in human and J774 macrophages. Our findings, at both the biochemical and molecular levels, suggested the absence of HSL in the human monocyte-derived macrophage.

Methods

Materials

Forskolin, N\textsuperscript{6},2\textsuperscript{\textprime}O-dibutryladenosine 3',5'-cyclic monophosphate (db-cAMP), 3-isobutyl-1-methylxanthine (IBMX), cyclandelate, cycloheximide, and tylosin were purchased from Sigma Chemical Co. Compound 58-035 was a gift from Sandoz Pharmaceuticals Inc. Polyscreen polyvinylidene difluoride transfer membranes, \([9,10\textsuperscript{\textprime}-\textsuperscript{3}H]\)oleic acid (7.4 Ci/mmol), \([\text{carboxyl-14C}]\)triolein (110 mCi/mmol), \([\text{octeate-1-14C}]\)cholesterole oleate (55 mCi/mmol), and \([\text{p-32P}]\)CTP (3000 Ci/mmol) were from Du Pont–New England Nuclear. L-[4,5-\textsuperscript{3}H]Leucine (150 Ci/mmol) was from Amersham Corp. Culture media, antibiotics, and fetal bovine serum were from Biochrom KG. Culture plasticware was purchased from Nunc Inter Med. Lymphoprep was purchased from Nycomed AS. LK6DF thin-layer chromatography silica gel plates were from Whatman Chemical Separation Inc. Guanidinium thiocyanate was from Fluka Chemie AG. Ficoll type 400 was from Pharmacia. Primers for the polymerase chain reaction (PCR) were ordered from Oligos Etc Inc. Avian myoblastosis virus reverse transcriptase, Taq DNA polymerase, dNTPs, and the ribonuclease inhibitor RNasin were purchased from Promega Corp. Human adipose tissue was used as a positive control for the HSL-mRNA detection experiments and was obtained from an individual undergoing abdominal surgery.

Lipoproteins were obtained from pooled human sera by sequential ultracentrifugation. Low-density lipoprotein (LDL) was isolated in the 1.019 through 1.063 g/L density range, and acetylated LDL (AcLDL) was obtained by the addition of acetic anhydride to LDL. High-density lipoprotein (HDL) was isolated in the 1.063 through 1.21 g/L density range.

Cell Culture

All media used for cell isolation and culture contained 100 U/mL penicillin, 100 g/mL streptomycin, 80 \mu g/mL tylosin, 20 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and 24 mmol/L NaHCO\textsubscript{3}. Human monocytes were obtained from transfusion units from our hospital as described by Zanella et al\textsuperscript{23} with a few modifications. Basically, 1 U blood was centrifuged at 2000g for 10 minutes to obtain a buffy coat that was transferred to the satellite bag with a plasma extractor in a sterile way. The buffy coat was centrifuged again at 250g for 10 minutes, and the resulting platelet-rich plasma was separated from the white cells. Plasma was diazylated three times in a low-molecular-weight cutoff dialysis bag from Spectrum Medical Industries Inc against 6 L (2 L per dialysis) NaCl 0.9% containing 1 g/L CaCl\textsubscript{2} 2H\textsubscript{2}O to induce the formation of a clot that was then removed by gentle agitation with a glass pipette. The resulting platelet-free serum was used for monocyte culture. The white cells were diluted with phosphate-buffered saline (PBS), and mononuclear cells were obtained by centrifugation of 250g for 30 minutes over Ficoll (Lymphoprep) according to the method of Boyum.\textsuperscript{29} After three washes with PBS, mononuclear cells were resuspended in RPMI 1640 containing 1% human serum at a density of 10\textsuperscript{6} cells/mL. This cell suspension was placed in 24-well plates (0.5 mL/well) for biochemical experiments or in 25-cm\textsuperscript{2} tissue-culture flasks (for RNA studies). After 2 hours at 37°C in a humidified 95% air/5% CO\textsubscript{2} incubator, nonadherent cells were removed by gentle aspiration, and the adherent cells were washed three times with warm PBS and cultured in RPMI 1640 supplemented with 20% autologous serum. Non-specific esterase staining showed that virtually all the adherent cells were monocytes. The medium was changed on the third day, and experiments were started after 6 days in culture, when the cells were phenotypical macrophages.\textsuperscript{27} J774 macrophages were maintained at 37°C in a 95% air/5% CO\textsubscript{2} atmosphere in RPMI 1640 containing 10% heat-inactivated fetal bovine serum. The medium was changed twice a week.

Preparation of Human Monocyte–Derived Macrophages

Human monocyte–derived macrophages were incubated for 30 hours in the presence of increasing concentrations of db-cAMP. At the end of the incubation, medium from each well was recovered, and adherent cells were gently washed three times with 1 mL 37°C PBS and dissolved in 0.1 N NaOH; their protein content was determined according to the method of Lowry et al.\textsuperscript{28} Detached cells present in the medium and washes from each well were then combined, centrifuged at 250g for 10 minutes, resuspended in PBS containing trypan blue, and counted in a Neubauer chamber.

Viability was also assessed by measuring the incorporation of \([\text{H}]\)leucine into cellular proteins during long incubations in the presence of db-cAMP. For this purpose, human monocyte–derived macrophages were preincubated for 6 hours in RPMI 1640 containing increasing concentrations of db-cAMP. Controls containing 20 \mu g/mL of the protein synthesis inhibitor cycloheximide and triplicate blanks without cells were run in parallel. \([\text{H}]\)Leucine (10 \mu Ci/mL) was added to each well, and the incubation was prolonged for 24 hours more. Cells were then disrupted by sonication in the incubation medium, and aliquots from each well were taken to measure cellular protein according to the Bradford procedure.\textsuperscript{29} Other aliquots were supplemented with bovine serum albumin (final concentration, 10 mg/mL), precipitated with 10% trichloroacetic acid (TCA), and filtered through Whatman glass microfiber filters moistened with a solution consisting of 10% TCA and 2 mg/mL leucine. Filters were thoroughly washed with the same solution and dried, and the radioactivity was counted. Mean values of triplicate blanks were subtracted from each experimental value.

Hydrolysis of Cytoplasmic Cholesterol Esters in Intact Macrophages

Human macrophages were loaded with radiolabeled cholesterol esters by preincubating the cells in RPMI 1640 containing 20% autologous serum supplemented with 200 \mu g/mL AcLDL and 5 \mu Ci/mL \([\text{H}]\)oleate for 72 hours. In some experiments, AcLDL was substituted by native LDL as indicated. At the end of the preincubation time, each well was washed six times in 1 mL PBS, and cells from at least four wells were immediately dissolved in RPMI 1640 containing 0.2% Triton X-100 and stored at -80°C. These samples were used to determine the basal content of cholesterol\([\text{H}]\)oleate and \([\text{H}]\)triglycerides. The rest of the cells were further incubated under the conditions described in the respective legends to the figures and tables. When needed, additives were added in dimethyl sulfoxide or ethanol, and control medium was supplemented with the same amount of vehicle; additions never exceeded 0.1% of total volume. Cells were then dissolved in the incubation medium by adding Triton X-100 directly to each well to obtain a final concentration of 0.2%. This procedure of dissolving the cells in the incubation medium was necessary because db-cAMP causes the detachment of human macrophages from the well (see below) and, under these circumstances, washing the monolayer would lead to the loss of most of the cellular material. A similar procedure has been previously used.\textsuperscript{21,30} In some instances, radioactive cholesterol
esters and triglycerides present in the medium after the incubation time were examined by taking an aliquot before the addition of Triton X-100. Cells were then removed by low-speed centrifugation and lipids were analyzed by thin-layer chromatography. Routinely, radioactivity in choles-
eteryl ester and triglyceride fractions from the medium accounted for less than 1.5% of that present in cells regardless of the incubation method used.

For the experiments with J774 macrophages, floating cells were cultured and washed with RPMI 1640 for the treatments with db-cAMP or forskolin as stated in the figure legends. The number of trypan blue-excluding detached cells (circles) and the amount of adhered cell protein remaining in each well (squares) were determined as indicated in "Methods." Data are mean±SEM of quadruplicate determinations.

**Analytical Procedures**

For the quantification of cellular cholesterol/[3H]oleate and [3H]triglyceride, lipids were extracted from samples using the method of Bligh and Dyer. Before lipid extraction, appropriate amounts of [oleate-4C]cholesterol oleate and [carboxyl-14C]triglycerol were added to each sample as an internal standard. Lipid extracts were dried, reconstituted in chloroform, and analyzed by silica gel thin-layer chromatography developed in heptane/diethyl ether/acetic acid (75:25:4, vol/vol/vol). Lipid spots were localized by exposing the plates to iodine vapors; once the iodine was eliminated, the spots corresponding to cholesterol esters and triglycerides were scraped into scintillation vials. All radioactive measurements were made in an LS-3800 liquid scintillation counter from Beckman Instruments Inc by using an OptiPhase scintillation cocktail from LKB. Cell protein was measured according to the method of Bradford. ANOVA and Tukey's multiple-range test were used for statistical comparison of groups by using Statgraphics, version 5 (STSC, Inc).

**Analysis of mRNA by Ribo transcriptation and PCR**

Total RNA from human monocyte-derived macrophages, J774 macrophages, and adipose tissue was extracted according to the method of Chomczynski and Sacchii and was used to obtain cDNAs by reverse transcription using oligo(dT)15 primer. For that purpose, 10 ¡tg RNA denatured with meth-

**Results**

**Effect of db-cAMP on Cell Adherence and Viability of Human Monocyte-Derived Macrophages**

Preliminary experiments showed that the adherence of human monocyte–derived macrophages to culture plasticware was impaired by long incubations in the presence of cAMP inducers (eg, forskolin, cholera toxin) or cAMP analogues. This was an important obstacle to the study of cholesteryl ester hydrolysis regulation in intact macrophages. For that reason, we investigated the effect of db-cAMP on both cell adherence and viability. db-cAMP detached the cells from the culture surface in a dose-dependent manner whether or not serum was present in the culture medium (Fig 1). This effect could be observed at doses as low as 50 ¡mol/L db-cAMP and was not attributable to cellular death, as indicated by the percentage of detached cells that excluded trypan blue (87%, 83%, 84%, and 90% for controls at 10 ¡mol/L, 50 ¡mol/L, and 500 ¡mol/L db-cAMP, respectively). To assess cell viability more specifically, we measured protein synthesis during long incubations (30 hours) in the presence of db-cAMP. No impairment in [3H]leucine incorporation into cellular
proteins was observed when concentrations up to 1 mmol/L db-cAMP were used (mean±SEM for triplicates were 4240±21 and 3817±507 cpm/μg cell protein, respectively, for controls and 1 mmol/L db-cAMP), whereas incorporation was completely blocked by the presence of 20 μg/mL of the protein synthesis inhibitor cycloheximide (less than 20 cpm incorporated per milligram cell protein). Similar results were found with forskolin and when monocytes instead of macrophages were incubated with db-cAMP (data not shown).

These results indicated that although db-cAMP impairs the human monocyte–derived macrophage adherence to plasticware after long incubations, it has no effect on cell viability, and therefore it was possible to attempt the study of the role of cAMP on cholesteryl ester metabolism in this cell type. However, since many macrophages were detached from the culture surface during incubation with db-cAMP, we were forced to design a protocol that did not require washing the monolayer at the end of the incubations. Solubilization of the cells in the incubation medium by the addition of Triton X-100 and the recovery of cells and medium as a unit were the method selected.

Effect of db-cAMP on the Hydrolysis of Cytoplasmic Cholesteryl Esters in Intact Human Monocyte-Derived Macrophages

To determine the effect of db-cAMP on cholesteryl ester mobilization, human monocyte–derived macrophages were loaded with radiolabeled cholesteryl esters and further incubated as indicated under “Methods” and in the legend to Table 1. As expected, the exposure of human monocyte–derived macrophages to AcLDL highly stimulated cholesteryl[3H]oleate formation compared with the formation in cells exposed to native LDL. When cholesteryl ester–loaded macrophages were subsequently incubated for 24 hours under control conditions, the cholesteryl[3H]oleate cell levels decreased, which is compatible with cholesteryl ester hydrolysis. When db-cAMP was present during this 24-hour incubation, the remaining cellular cholesteryl[3H]oleate was unexpectedly higher than in the cells incubated without db-cAMP (Table 1, experiment 1). This effect of db-cAMP could be due to either an inhibition of cholesteryl ester hydrolysis or to an acceleration of [3H]oleate recycling. Since HSL is present in certain types of macrophages and this enzyme is activated by cAMP-dependent phosphorylation, it was feasible that db-cAMP had provoked increased [3H]oleate availability to ACAT by means of a massive [3H]triglyceride hydrolysis. This would cause a more efficient reesterification of cholesterol with [3H]oleate that could explain the unexpected result. To test this possibility, cellular [3H]triglycerides were monitored during some experiments. [3H]Triglyceride hydrolysis was not stimulated by db-cAMP, whereas the inhibitory effect of this drug on cholesteryl[3H]oleate mobilization persisted (Table 1, experiment 2).

To prevent reuse of [3H]oleate by ACAT, in other experiments the ACAT inhibitor 58-035 was added to the incubation medium. As shown in Table 2, 58-035 led to a greater mobilization of cholesteryl[3H]oleate from the macrophages compared with control medium without the ACAT inhibitor, indicating that when ACAT is active, an important part of the hydrolyzed cholesteryl esters is efficiently reesterified with [3H]oleate. However, the strong inhibitory effect of db-cAMP on cholesterol[3H]oleate mobilization was observed both in the absence and presence of 58-035. As shown in Table 3, the inhibitory effect of db-cAMP was rigorously mimicked by the combination of forskolin and IBMX, indicating that the effect of db-cAMP can be attributed to its homology with cAMP and not to the dibutyl part of the molecule.

Next we tested the possibility of an extensive activation of ACAT by cAMP in human monocyte–derived macrophages that might even surpass the inhibitory action of 58-035. A new series of experiments was performed that omitted AcLDL from the preincubation medium to prevent ACAT prestimulation, which might have masked the hypothetical db-cAMP–mediated activation. Thus, human monocyte–derived macrophages were preincubated in medium containing 20% autologous serum and 5 μCi/mL sodium[3H]oleate for 72 hours. The monolayers were then washed, and cells from four wells were dissolved in RPMI 1640 containing 0.2% Triton X-100 and were used to measure the basal content of cholesterol[3H]oleate and [3H]triglycerides. The rest of the cells were further incubated in RPMI 1640 alone (control) or in medium containing 500 μmol/L db-cAMP for 24 hours. Triton X-100 was then added to each well, and cellular cholesterol[3H]oleate, [3H]triglycerides, and protein content were determined as described in “Methods.” Data are from two independent experiments; each value represents the mean±SEM of quadruplicate incubations and is expressed in counts per minute per microgram cell protein. In four wells from each experiment, acetylated low-density lipoprotein was suppressed from the preincubation medium and replaced by native low-density lipoprotein. The basal content of cholesterol[3H]oleate in those cells was 11±1 and 4±2 cpm/μg cell protein for experiments 1 and 2, respectively. Basal [3H]triglyceride content was 6596±90 cpm/μg cell protein in experiment 2.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cholesterol-[3H]oleate</th>
<th>Cholesterol-[3H]triglycerides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>276±18</td>
<td>ND</td>
</tr>
<tr>
<td>db-cAMP</td>
<td>213±16*</td>
<td>ND</td>
</tr>
<tr>
<td>Control</td>
<td>469±13</td>
<td>6546±162</td>
</tr>
<tr>
<td>db-cAMP</td>
<td>5728±366</td>
<td>ND</td>
</tr>
</tbody>
</table>

db-cAMP indicates dibutyryl-adenosine 3',5'-cyclic monophosphate; ND, not determined. Macrophages were loaded with radiolabeled cholesteryl esters by incubation in RPMI 1640 containing 20% autologous serum, 200 μg/mL acetylated low-density lipoprotein, and 5 μCi/mL sodium[3H]oleate for 72 hours. The monolayers were then washed, and cells from four wells were dissolved in RPMI 1640 containing 0.2% Triton X-100 and were used to measure the basal content of cholesterol[3H]oleate and [3H]triglycerides. The rest of the cells were further incubated in RPMI 1640 alone (control) or in medium containing 500 μmol/L db-cAMP for 24 hours. Triton X-100 was then added to each well, and cellular cholesterol[3H]oleate, [3H]triglycerides, and protein content were determined as described in “Methods.” Data are from two independent experiments; each value represents the mean±SEM of quadruplicate incubations and is expressed in counts per minute per microgram cell protein. In four wells from each experiment, acetylated low-density lipoprotein was suppressed from the preincubation medium and replaced by native low-density lipoprotein. The basal content of cholesterol[3H]oleate in those cells was 11±1 and 4±2 cpm/μg cell protein for experiments 1 and 2, respectively. Basal [3H]triglyceride content was 6596±90 cpm/μg cell protein in experiment 2.
was observed in the macrophage cholesterol[^3H]oleate content after a 24-hour incubation in control medium. This is not striking because there was no ACAT inhibitor in the medium, and therefore hydrolysis of the scarce cholesterol[^3H]oleate present in the cells should have been easily compensated for by basal ACAT activity, which can use the[^3H]oleate present in the cytoplasm or derived from the hydrolysis of[^3H]triglycerides. In the presence of db-cAMP, a slight but significant increase in the cellular cholesterol[^3H]oleate content was observed. This increase, however, did not occur when ACAT activity was blocked by 58-035 (Table 4). Next we explored a putative indirect mechanism for ACAT activation mediated by db-cAMP in cholesteryl ester–loaded cells. If, as proposed by others,16–19 db-cAMP does indeed activate NCEH, it is feasible that in cells heavily loaded with cholesteryl esters, a sudden increase in intracellular free cholesterol could take place during the first minutes or hours in the presence of db-cAMP and the absence of cholesterol acceptors.

### Table 3. Effect of Forskollin on Cholesterol[^3H]oleate Hydrolysis in Human Monocyte–Derived Macrophages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cholesterol[^3H]oleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>579±24</td>
</tr>
<tr>
<td>db-cAMP</td>
<td>357±17*</td>
</tr>
<tr>
<td>Forskolin+IBMX</td>
<td>568±13†</td>
</tr>
</tbody>
</table>

The magnitude of the effect of forskolin on cholesterol[^3H]oleate hydrolysis was not different from control in basal conditions.

This might activate ACAT as much as when cells are exposed to AcLDL, since the content of the intracellular free cholesterol is the main regulatory mechanism for ACAT in macrophages.36 To test this possibility, human monocyte–derived macrophages were loaded with unlabeled cholesteryl esters by preincubation with AcLDL as in the experiments shown in Table 1 except that sodium[^3H]oleate was omitted. Cells were then washed and subsequently incubated for 24 hours with 5 μCi/mL sodium[^3H]oleate in the absence or presence of db-cAMP. The results from this experiment indicated that db-cAMP causes a slight yet significant increment in cholesteryl[^3H]oleate synthesis (11.1±0.8 versus 15.6±0.9 cpm/μg cell protein in control and db-cAMP–treated cells, respectively; n=4, P<.01). The effect was found to be specific for cholesterol esterification, as no differences were observed in the[^3H]triglyceride synthesis between control (2881±206 cpm/μg cell protein) and db-cAMP–treated (2641±218 cpm/μg cell protein) cells. That stimulation of[^3H]oleate incorporation into cholesteryl esters by db-cAMP was of similar magnitude to the one observed with nonloaded cells (Table 4), thus ruling out the hypothetical indirect stimulation of ACAT by the cellular free cholesterol increase mentioned above.

Since db-cAMP detached cells from the culture well, no cholesterol-accepting proteins were included in the incubation media in the previous experiments. This allowed us to measure cell protein in each well and thus to express data in cell protein units. However, in view of the repeated and unexpected inhibitory effect of db-cAMP on cholesteryl ester mobilization from human macrophages, we sought to investigate the effects of this agent when extracellular acceptors for free cholesterol (HDL and fatty acids (BSA) were added to the system. Obviously, this experimental design made it impossible to measure cell protein at the end of the incubation, and thus results from these experiments were calculated as cholesterol[^3H]oleate content per well rather than per...
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J774 cells, we attempted the detection of the HSL gene. Analysis of mRNA by Reverse Transcription and subsequent detection of the amplified products by Southern blot hybridization using a DNA probe. Owing to the amplification by PCR, this method allows the detection of very scarce mRNA levels. As shown in Fig 2A, HSL mRNA could be detected in both J774 macrophages (lane 5) and human adipose tissue (lane 6). In contrast, no signal was obtained in human monocyte-derived macrophages from three different subjects (lanes 2, 3, and 4) or with monocytes from a fourth donor (lane 1). Specific primers for the amplification of an 825-base-long fragment of β-actin were included during the PCR process as a control for RNA integrity and reverse transcription PCR function. The amplified fragment of β-actin cDNA was visible in the agarose gel stained with ethidium bromide in human and J774 macrophages (Fig 2B; note that the amount of PCR product loaded on the agarose gel was 20-fold and 2-fold less for adipose tissue and J774 samples, respectively, than for human macrophage samples). In a separate experiment, the samples were analyzed exactly as described above except that primers for the amplification of β-actin were not included in the PCR mixture. This was done to avoid the possible competition for Taq polymerase that could occur during the simultaneous amplification of two different fragments and that could lead to a misamplification of the larger one, in this case, the HSL-cDNA fragment. In spite of this, results were exactly the same as those shown in Fig 2A: no signal was obtained in human macrophages after 1 week of autoradiography, whereas a strong signal was visible in J774 cells after 8 hours of autoradiography (data not shown).

Table 5. Effect of db-cAMP on the Cholesterol[3H]oleate and [3H]Triglyceride Hydrolysis in Human Monocyte-Derived Macrophages in the Presence of HDL and BSA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cholesterol-[3H]oleate</th>
<th>[3H]Triglycerides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>98.7 ± 2.1</td>
<td>1380 ± 44</td>
</tr>
<tr>
<td>After 24-h incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (BSA+HDL)</td>
<td>75.4 ± 3.0*</td>
<td>1075 ± 95*</td>
</tr>
<tr>
<td>db-cAMP (BSA+HDL)</td>
<td>87.7 ± 2.4†</td>
<td>971 ± 55*</td>
</tr>
</tbody>
</table>

Table 6. Effect of db-cAMP Cholesterol[3H]oleate Clearance From J774 Macrophages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cholesterol[3H]oleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>241 ± 4</td>
</tr>
<tr>
<td>After 24-h incubation</td>
<td></td>
</tr>
<tr>
<td>Control (58-035)</td>
<td>247 ± 1</td>
</tr>
<tr>
<td>db-cAMP (58-035)</td>
<td>122 ± 19*</td>
</tr>
</tbody>
</table>

db-cAMP indicates dibutyryl adenosine 3',5'-cyclic monophosphate; BSA, bovine serum albumin; and HDL, high-density lipoprotein. Macrophages were loaded with cholesterol[3H]oleate and [3H]triglycerides by preincubation for 72 hours under the conditions described in Table 1 (basal). Monolayers were then washed and incubated in RPMI 1640 containing 1% BSA and 500 μg/mL HDL in the absence (control) or presence of 500 μmol/L db-cAMP for 24 hours. Cells were then washed and triplicate wells were designated to measure the basal content of cholesterol[3H]oleate. The rest of the cells were further incubated for 24 hours in RPMI 1640 containing 10 μmol/L 58-035 (control) or the same medium supplemented with 500 μmol/L db-cAMP. Cholesterol[3H]oleate content was determined as described in "Methods." Data are mean ± SEM of triplicate incubations and are expressed as counts per minute per microgram cell protein.

*P < .05 different from control.
†P < .05 different from basal.

Discussion
The present work studied the effects of a cAMP analogue on the hydrolysis of cholesterol esters in human monocyte-derived macrophages. The results show an unexpected cAMP-mediated inhibition of the cholesterol ester mobilization from this cell type; in contrast, db-cAMP activated this process in J774 macrophages. These results correlate with the presence of the mRNA for HSL in J774 macrophages; human monocyte-derived macrophages do not express this gene. Therefore, it is concluded that hydrolysis of cytoplasmic cholesterol esters in human macrophages...
db-cAMP nor forskolin affected cell viability as far as effects of cAMP on cell adherence and viability. Neither ester metabolism were undertaken, we studied the cells. Since smooth muscle derived-foam cells only incorporation into triglycerides were concerned. These trypan blue exclusion, protein synthesis, and \[^{[3H]}\]oleate process, we considered the study of NCEH regulation artherosclerosis. Before the experiments on cholesteryl macrophages are involved during the first stages of the process,\(^7\) we considered the study of NCEH regulation in intact macrophages of interest and chose the human monocyte-derived macrophage as the closest model to the cell-like types. The possibility of activating NCEH by phosphorylation should allow rapid mobilization of cholesteryl esters from foam cells when conditions are adequate for the efflux of free cholesteryl to extracellular acceptors. In fact, Tertov et al\(^{20}\) report that cAMP induced the mobilization of cholesteryl esters from cultured foam cells obtained from human arterial explants. These cultures contained mainly smooth muscle cells.\(^7\) Since smooth muscle derived–foam cells only appear at late stages of atheroma formation while macrophages are involved during the first stages of this process,\(^5\) we considered the study of NCEH regulation in intact macrophages of interest and chose the human monocyte-derived macrophage as the closest model to the macrophage-derived foam cells present in human artherosclerosis. Before the experiments on cholesteryl ester metabolism were undertaken, we studied the effects of cAMP on cell adherence and viability. Neither db-cAMP nor forskolin affected cell viability as far as trypan blue exclusion, protein synthesis, and \[^{[3H]}\]oleate incorporation into triglycerides were concerned. These agents, however, did promote the detachment of human monocyte–derived macrophages from the culture-dish surface. But cAMP produces contrasting results in other cell lines. Thus, whereas it inhibits adherence of RAW264 cells,\(^{26}\) it stimulates adherence of J774 macrophages.\(^{21}\) CAMP induces depolymerization of actin filaments in guinea pig peritoneal macrophages,\(^{42}\) and in human monocyte–derived macrophages it interferes with the assembly and stability of microtubules.\(^{43}\) Therefore, impairment of the cellular cytoskeleton by cAMP may have caused the cell detachment observed in the present study with human macrophages. Since cell viability was unaffected by cAMP, studies on its effects on cholesteryl ester metabolism were feasible, although a special experimental design had to be adopted to prevent the loss of cellular material. For this reason, no exogenous protein was present in the incubation medium if cellular protein was to be quantified, and cells were dissolved by the direct addition of Triton X-100 to the medium at the end of the incubations.

To overload human macrophages with cholesteryl esters, the macrophages were exposed to AcLDL for 72 hours. As expected, this led to a 25- to 100-fold increase of \[^{[3H]}\]oleate incorporation into cholesteryl esters compared with incubation of the cells with native LDL. After loading, the cells were subsequently incubated in control medium or in the presence of agents affecting the cAMP system, and the changes in cholesterol\[^{[3H]}\]oleate were monitored. Under control conditions, the content of cholesterol\[^{[3H]}\]oleate tended to decrease as a result of cholesteryl ester hydrolysis. In the presence of db-cAMP, this decrease was much slower or did not even occur, since the values of cholesterol\[^{[3H]}\]oleate were always significantly higher than in control incubations. The magnitude of this effect as well as other related parameters varied in experiments with cells from different donors. However, differences between db-cAMP and control incubations were consistently observed in all experiments. This effect was reproduced by forskolin plus IBMX; it also occurred when ACAT was inhibited by 58-035 and in the presence of lipid acceptors in the medium (HDL plus BSA). These results contrasted with previous findings by others showing that cAMP stimulated cholesteryl ester hydrolysis in intact human smooth muscle cells\(^{20}\) and J774 macrophages.\(^{21}\) Moreover, in homogenates from either smooth muscle cells\(^{15}\) or murine macrophages,\(^{16,17}\) cAMP stimulates neutral cholesteryl esterase activity. Our results with human monocyte–derived macrophages showed inhibition rather than stimulation of cholesteryl ester hydrolysis by cAMP, a finding that deserves further investigation. The facts that this effect of db-cAMP could be observed in the presence of 58-035 and that db-cAMP did not stimulate \[^{[3H]}\]triglyceride breakdown in these cells reasonably ruled out the effect of db-cAMP on cellular cholesterol\[^{[3H]}\]oleate content being due to increased availability of \[^{[3H]}\]oleate for reesterification by ACAT. Next, we considered the possibility that db-cAMP stimulated ACAT in human monocyte–derived macrophages. Actually, there is controversy over the regulation of this enzyme, with evidence both for activation\(^{44,45}\) and inhibition\(^{46}\) of ACAT by phosphorylation as well as evidence against a role for such a mechanism in the regulation of ACAT.\(^{47}\) To address this question, we examined the effect of db-cAMP on the formation of cholesterol\[^{[3H]}\]oleate in human monocyte–derived macrophages and found a slight (1.2-fold)
stimulation of this process both when [H]oleate came from prelabeled intracellular stores (Table 4) and when the tracer was supplied at the same time as db-cAMP to nonlabeled, cholesteryl ester–loaded cells (see "Results"). This approach cannot distinguish unequivocally between activation of ACAT or inhibition of NCEH; however, the results in Table 4 clearly demonstrate that the modest effect of db-cAMP on cholesteryl-[H]oleate was completely prevented by 58-035. Thus, the inhibition of cholesteryl-[H]oleate mobilization from macrophages that was caused by db-cAMP even in the presence of 58-035 (Tables 1 through 3) must reasonably be attributed to a disturbance of the hydrolytic pathway and not to an activation of cholesterol esterification by ACAT.

In contrast to human monocyte–derived macrophages, J774 cells responded to db-cAMP with an increase in cholesteryl-[H]oleate mobilization, which is in agreement with previous results by others. 5-58 Murine macrophages have been reported to have HSL. 57-59 Thus, this enzyme is likely to be responsible for the cAMP-activated cholesteryl ester mobilization in these murine cells. Thus, we focused on the search for HSL mRNA in human monocyte–derived macrophages by using reverse transcription PCR with specific primers for human HSL. Results from this research confirmed the presence of mRNA for HSL in J774 macrophages, whereas no such mRNA could be detected in either monocytes or macrophages from human origin.

Therefore, all the results presented suggest that cytosolic cholesteryl ester hydrolysis in human monocyte–derived macrophages is not controlled by HSL. We have not investigated which enzyme is responsible for the NCEH activity detected. Microsomal and cytosolic NCEH have been described in tissues such as the liver, 60-62 and the mammary gland. 63,64 Reports on the regulation of those enzymes are controversial, varying from the activation, 52 to the inhibition, 53-54 of cAMP on cytosolic cholesteryl esterase. Our results with human monocyte–derived macrophages indicated that the hydrolysis of the cytosolic cholesteryl esters is inhibited by cAMP, contrary to what happens in murine macrophages. The mechanism underlying this effect remains to be elucidated. Our experimental design did not allow us to assign this effect to a direct action of cAMP on NCEH phosphorylation, as long incubations with this compound may induce changes in cell metabolism that might indirectly affect cholesteryl ester hydrolysis. The influence of the described cAMP-induced cell detachment on cholesteryl metabolism should be investigated in the future. Ruiz and coworkers 55-56 describe the presence of an NCEH inhibitable by cAMP in rat liver, and it is tempting to speculate that an analogous enzyme may exist in human macrophages.

The presence of macrophages in atheromatous lesions could be due to a defensive mechanism against the extracellular lipid accumulation that occurs in the artery wall from the very first stages of hypercholesterolemia, even before the presence of macrophage foam cells. 56-58 Thus, macrophages would probably be recruited to mobilize those extracellular deposits and to store the neutral lipids in their cytoplasm. Macrophage activation leads to increased lipoprotein uptake and intracellular cholesteryl ester formation. 62-66 It was believed that this could contribute to the development of atherosclerosis, but Inoue et al 67 have recently demonstrated that, even though monocyte colony-stimulating factor promotes cholesteryl ester accumulation in human monocyte–derived macrophages in vitro, 68 the activation of macrophages in vivo by chronic injection of macrophage colony-stimulating factor to hyperlipidemic rabbits prevents the progression of atherosclerosis. In this context, the possibility of inhibiting NCEH and blocking basal cholesteryl ester hydrolysis from cytoplasmic lipid droplets would be advantageous in a situation in which degrading phagocytosed lipoproteins are liberating large amounts of free cholesterol into the cytoplasm. Macrophages thus loaded with cholesteryl esters may abandon the lesioned area, taking their cholesterol load with them, 69-70 or excrete free cholesterol to extracellular acceptors once the external conditions become adequate for such an efflux and NCEH activity is restored.

In conclusion, the hydrolysis of cytoplasmic cholesteryl esters is affected by different enzymes in different types of macrophages. Thus, while HSL may be the enzyme responsible for this activity in murine macrophages, in human monocyte–derived macrophages it is not, and thus a different enzyme must be present.

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J A Contreras and M A Lasunción

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