Ca++ Antagonists and ACAT Inhibitors Promote Cholesterol Efflux from Macrophages by Different Mechanisms

I. Characterization of Cellular Lipid Metabolism

Gerd Schmitz, Horst Robenek, M. Beuck, R. Krause, A. Schurek, and R. Niemann

The effects of the slow Ca++ channel blocker, nifedipine, and ACAT inhibitor, octirolate, on the cholesterol metabolism of cholesterol-loaded macrophages were compared. We demonstrated that apollipoprotein A-I containing high density lipoproteins (HDL) bind to specific receptor sites on macrophages, are internalized, take up cholesterol, and are then released from the cells as native lipoproteins. The ACAT inhibitor enhances HDL receptor activity and promotes HDL-mediated cholesterol efflux from cultured mouse peritoneal macrophages. In contrast, the Ca++ antagonist increases acetyl LDL-mediated cholesterol influx, abolishes the increase in HDL binding induced by cholesterol accumulation, enhances apo E synthesis, and promotes cholesterol efflux by a mechanism independent of the presence of HDL in the surrounding medium. Concomitantly, a decrease in nucleoside transporter activity, an increase in intracellular ATP hydrolysis, adenylne and cyclic AMP concentration, and a stimulation of the activities of acid and neutral cholesteryl ester hydrolases and ACAT indicated that protein kinase A-catalyzed phosphorylation reactions might be involved in the increase in cholesterol efflux. The Ca++ antagonist-induced efflux occurred only with lysosomal-associated cholesterol, while the ACAT inhibitor acted on the formation of cytoplasmic lipid droplets. The secreted lipoprotein particles contained 68% unesterified cholesterol and 21% phospholipids, 8% esterified cholesterol, and 3% triglycerides. The phospholipid components were: 72% phosphatidylcholine, 22% sphingomyelin, and 6% phosphatidyserine, phosphatidylcoline, and phosphatidylethanolamine. We conclude that macrophages release cholesterol in two ways: 1) an HDL-mediated release of unesterified cholesterol increasing upon ACAT inhibition, and 2) an HDL-independent secretion of cholesterol which can be amplified by Ca++ antagonists. (Arteriosclerosis 8:46-56, January/February 1988)

Ca++ antagonists relax vascular smooth muscle by blocking voltage-dependent or "slow" Ca++ channels.1-8 In addition, some of these drugs have been shown to exert antiatherogenic effects in animals with dietary hypercholesterolemia.9-20 However, two groups of investigators did not find antiatherogenic effects in cholesterol-fed rabbits.21,22 The reason Watanabe rabbits did not respond favorably to nifedipine23 is not yet clear, but these studies should be repeated in larger groups of animals.

Recent studies in human red blood cells showed that an increase in membrane cholesterol augments the entry of Ca++ through a channel that can be blocked with nifedipine.24,25

These findings support the hypothesis that physiological changes in membranes caused by a high cholesterol environment may favor Ca++ uptake by arterial smooth muscle. In other tissue culture studies, Stein et al.26 demonstrated that high concentrations of verapamil enhanced the receptor-mediated uptake and disposal of low density lipoprotein (LDL) by aortic cells. Etelin and Hajjar27 demonstrated that nifedipine produced a 50% loss of cholesterol and cholesteryl esters from lipid-laden rabbit aortic smooth muscle cells in culture. They concluded that the nifedipine effect on cellular cholesterol might be partly due to increased cholesteryl ester hydrolysis. Another study28 performed with cultured human monocyte-macrophages treated with verapamil, suggested that the Ca++ antagonist leads to a more efficient intracellular processing of atherogenic lipoproteins.

Whether the described antiatherosclerotic properties of Ca++ entry blockers are only caused by the Ca++ antagonistic effects of the drugs, or whether additional pharmacological actions are involved is still unknown.

There is good evidence that some of the nifedipine side effects5-8 may be due to alterations in the action of endogenously released adenosine, another Ca++ channel blocker29,30 or to the interaction of nifedipine with adenosine receptors.31 Nifedipine seems to compete with the action of adenosine, which is released in greater quantities during ischemia as the catabolic product of adenosine 5'-triphosphate (ATP) hydrolysis.31-34 Other studies have
shown that nifedipine inhibits adenosine uptake from and release into the extracellular space.35

Another interesting interaction of both nifedipine and adenosine has been described for the benzodiazepine receptors where the inhibition of adenosine uptake and subsequent prolonged adenosine action are assumed to mediate part of the benzodiazepine effects.31, 36, 37, 38 Nifedipine obviously interacts with adenosine and benzodiazepine receptors unlike the other Ca ++ channel blockers such as diltiazem and verapamil.

In summary, current evidence indicates that Ca ++ antagonists have clear antiatherogenic effects but do not reduce dietary hypercholesterolemia. However, although the precise mechanism of the Ca ++ antagonist action on cellular cholesterol metabolism is still unknown, it is possible that some side effects are involved.

We recently demonstrated that cholesterol loading of macrophages with acetylated LDL (AcLDL) in the presence of acyl-CoA: cholesterol acyltransferase (ACAT) inhibitors results in a significant increase in high density lipoprotein (HDL) binding.39, 40 In this study, we compare the influence of the ACAT inhibitor, octimibrate, and the slow Ca ++ channel antagonist, nifedipine, on macrophage cholesterol homeostasis and adenosine metabolism. In the accompanying article, we present the morphological data from these studies.

Methods

Fetal calf serum (FCS) was obtained from GIBCO Biculact (Cat no 629). Dulbecco’s Modified Eagle Medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Flow Laboratories (Cat no 10-331-25 and 18-610-24). Octimibrate (sodium 8-(1,4,5-triphenyl-imidazole-2-yl)oxy octanoate) was kindly provided by Hans-Heiner Lautenschläger (A. Nattermann GmbH, Cologne, West Germany). Nifedipine (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-pyridine-3,5-dicarbonicacid dimethyl ester) was provided by Günter Thomas, Bayer Milan, Italy. Other biochemicals were from Sigma or Serva. Sodium 125I-Iodoide was purchased from Amersham, 1-14C oleic acid (52.6 mCi/mmol), cholesteryl-1,2,6,7-3H(N)-oleate (75.3 Ci/mmol), and 3H-nitrobenzylthioinosine (3H-NBMPR) (15 to 30 Ci/mmol) were obtained from New England Nuclear, Dreireich.

Mouse Macrophage Monolayers

Murine peritoneal macrophages were obtained from unstimulated NMRI-SPF mice (25 to 35 g) by peritoneal lavage in PBS containing 0.5 U heparin/ml. The peritoneal fluid was pooled, and the cells were centrifuged at 400 g for 10 minutes at room temperature.42 The cells were washed once with DMEM and resuspended in DMEM containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Aliquots (0.5 to 1 ml) of the cell suspension corresponding to the number of cells from two mice (3 x 10⁶ cells) were dispensed into 35 x 10 mm dishes containing 1 ml DMEM with the additions mentioned above and then incubated in a humidified incubator at 37°C and 5% CO₂. On the second day, each dish was extensively washed with 1 ml of DMEM without serum until there were no nonadherent cells visible under the microscope. Each dish contained 150 to 250 μg of total cell protein. Cell viability was greater than 90%, as tested by the trypan blue dye exclusion method.

Lipoproteins

HDL₃ (d = 1.125 to 1.21 g/ml) and LDL (d = 1.019 to 1.063 g/ml) were isolated from the serum of individual normolipemic volunteers by sequential ultracentrifugation in a Beckman L 8-70 ultracentrifuge using a 50.3 Ti or 70 Ti rotor (Beckman) at 4°C. The lipoprotein fractions were dialyzed against 0.15 M NaCl, 3 mM EDTA (pH 7.4) at 4°C. To remove traces of apo E-containing lipoproteins, HDL₃ were subjected to heparin-Sepharose affinity chromatography.43 All of the lipoprotein concentrations are given in terms of their protein content.

Chemical Modification of LDL and Cholesterol Loading of Cells

LDL (d = 1.019 to 1.063 g/ml) were acetylated by repeated additions of acetic anhydride as described44 and were dialyzed against PBS (pH 7.4) at 4°C. The modified LDL showed enhanced mobility on electrophoresis in agarose gel at pH 8.6. Cholesterol loading of macrophages was performed by incubating the dishes at 37°C with AcLDL. Protein concentrations and the time intervals used are indicated in the figure legends.

Preparation of ¹²⁵I-HDL₃ and ¹²⁵I-AcLDL

Lipoproteins were labelled according to the iodobead method described by Markwell.45 The 200 μl aliquots of the appropriate lipoprotein (5 to 10 mg/ml protein in PBS, pH 7.4) were mixed with 0.5 mCi Na ¹²⁵I and 2 to 4 lodo-Beads (Pierce) and were incubated for 10 minutes at room temperature. The unbound iodine was removed by extensive dialysis against PBS. Specific activities (SA) were between 150 and 300 cpm/ng of protein. ¹²⁵I-AcLDL was diluted with nonlabelled AcLDL before use and a final SA of 30 to 60 cpm/ng was achieved.

Binding Experiments with ¹²⁵I-Labelled Lipoproteins

Dishes were chilled to 4°C for 30 minutes and then were incubated with ¹²⁵I-HDL₃ for 1 hour at 4°C. The experiments were performed according to the methods described by Goldstein and Brown46 with lipoprotein concentrations as indicated in the figure legends. After incubation the cells were washed five times with PBS/0.2% bovine serum albumin (BSA) (wt/vol) and then three times with PBS without BSA. After this standard wash, the cells were released from the dishes by incubation with 1 ml of 0.5 N NaOH at 37°C for 1 hour. The cell-associated ¹²⁵I-radioactivity was assayed in an automatic counting system (Compugamma 1282, LKB). An aliquot was used to determine the cellular protein content. Total binding activities were corrected following the suggestions of Jensen et al.47 and Renaud.48 The binding capacity of a cell-free dish was measured under the same conditions and with the same lipoprotein preparation as described above and calculated...
in cpm bound per \( \mu m^2 \) dish area. The average area of a single macrophage was determined as 240 \( \mu m^2 \), and the protein content for about 4200 cells corresponded to 1 \( \mu g \) of cell protein. The nonspecific binding activity to the cell-free area of a dish was calculated from these data and subtracted to achieve the total cellular binding activity. Specific binding was calculated from the difference between 125I-HDL$_3$ binding in the presence or absence of a 20-fold excess of nonlabelled HDL$_3$.

**Determination of Cell-Associated Radioactivity**

This procedure was carried out as described by Yatsu et al.\(^{29}\) with few modifications. Cells were incubated at 37°C for different time intervals (see figure legends), then washed five times with PBS/0.2% BSA (wt/vol) and five times with PBS. Cells were then dissolved in 0.1 N NaOH to assay for protein and radioactivity. The non-acid-soluble (peptide-bound) radioactivity was determined by protein precipitation of an aliquot of this solution with 10% TCA (wt/vol). Free iodide was extracted with chloroform after oxidation with hydrogen peroxide. Radioactivity was assayed as described above.

**Quantitation of Lipids In Cells and Medium**

At the end of each incubation period, medium was removed from the dishes and the cells were washed three times with 1 ml of DMEM and once with PBS. Cells were then harvested in 500 \( \mu l \) of medium supplemented with 0.2 mM 14C-oleate. Then the cells were harvested in 500 \( \mu l \) PBS, and 200 \( \mu l \) aliquots were delipidated as described above. For lipid separation see the section on quantitation of lipids in cells; the substrate 14C-oleate was presented in a BSA-Na-oleate complex. This complex was formed by incubation of 38.7 mg Na-oleate in 3 ml 0.9% NaCl (wt/vol) at 60°C for 30 to 60 minutes. A solution of 1.2 g BSA in 5 ml 0.9% NaCl (wt/vol) (pH 7.4, 4°C) was slowly added to the Na-oleate suspension at 40°C. The total volume was adjusted to 10 ml with 0.9% NaCl (wt/vol). Then 16 \( \mu l \) of this complex was mixed with 0.5 \( \mu l \) 14C-Na-oleate and added to the cells. At the end of the assay, the cells were harvested from the dish and homogenized in 500 \( \mu l \) PBS; then 200 \( \mu l \) aliquots were delipidated, and the lipids were separated by HPTLC on silica gel plates.\(^{49, 50}\) The radioactivity of the cholesteryl oleate spot was measured as explained above.

**Analysis of cAMP, Adenosine, and Nucleoside Transporter Activity**

cAMP was determined with a commercial radioimmunoassay from Amersham Corporation, Braunschweig and ATP with the method from Boehringer Mannheim, West Germany. Measurement of the adenosine binding sites was done according to the method of Hammond and Cianchetta\(^{51}\) with 3H-nitrobenzylthionoisonine (3H-NBMPR). Adenosine was determined by high-performance liquid chromatography (HPLC), using a Spectra Physics SP8100 liquid chromatograph equipped with a Nucleosil RP18, 5 \( \mu m \) column (4.6 \( \times \) 250 mm, Fa. Bischoff). Calculation of adenosine concentration was performed by a Spectra Physics SP4070 integrator. The running conditions were the same as those described by Rustum.\(^{52}\)

**Other Methods**

Apo E synthesis was determined by radioimmunooassay according to the method of Blum et al.\(^{53}\) The protein content of lipoprotein fractions and cells were determined by the method of Lowry et al.\(^{54}\) by using BSA as a standard.

**Calculations**

Statistical significances for the data of the drug-treated group and its respective control group were evaluated by Student's t test.
Principle of Tissue Culture Experiments

The effects of the ACAT inhibitor, octimibate, and the Ca++ antagonist, nifedipine, on macrophage cholesterol metabolism were compared under three different metabolic conditions, which are defined in Figure 1 as simultaneous incubation, sequential incubation, and sequential + intermediate incubation.

In simultaneous incubation the cells were simultaneously exposed for 0 to 8 hours to the cholesterol-loading agent AcLDL and the appropriate drug. Under the conditions of sequential incubation, the drug was added to the cells after a 12-hour period of cholesterol loading with AcLDL. During drug exposition, neither AcLDL nor a cholesterol acceptor was present in the medium. Previous investigations have shown that after a 12-hour cholesterol loading period 80% to 90% of the accumulated cellular cholesterol is associated with membranes in endosomes, multivesicular bodies, and lysosomes. Therefore, the sequential incubation studies allow analysis of drug influences on cholesterol metabolism in these organelles. In the sequential + intermediate incubation studies, the appropriate drug was added to the cells after a 12-hour cholesterol-loading period and a subsequent 12-hour incubation in AcLDL-free medium.

Under the conditions of sequential + intermediate incubation, most of the cellular cholesterol was accumulated in cytoplasmic lipid droplets (nonmembrane-associated), and only minor amounts of cholesterol were present in the lysosomal compartment (membrane-associated). Therefore, under the conditions of sequential + intermediate incubation, the influence of a drug on the cholesterol pool in the cytoplasmic lipid droplets can be studied.

Analysis of Cellular Cholesterol Content

To analyze the influence of the ACAT inhibitor and the Ca++ antagonist on cellular cholesterol metabolism in more detail, the cellular content of total, free, and esterified cholesterol were monitored under the three different experimental conditions. The simultaneous incubation of the macrophages with AcLDL alone or in the presence of the ACAT inhibitor led to a similar increase in cellular total cholesterol (Figure 4A). However, while AcLDL alone increased total cholesterol at the expense of esterified cholesterol, the increase in cellular cholesterol in the presence of the ACAT inhibitor was predominantly due to unesterified cholesterol, as expected considering the mechanism of action of the drug (Figures 4B and 4C). In contrast, the increase in total cellular cholesterol observed in the presence of the Ca++ antagonist (Figure 4A) was significantly lower. In the presence of the Ca++ antagonist, no influence on AcLDL uptake and degradation, yet a significantly observed only up to 4 hours, the ACAT inhibitor stimulated a time-dependent HDL uptake, and the Ca++ antagonist abolished the AcLDL-induced HDL internalization.

In summary, the ACAT inhibitor stimulated HDL binding and uptake, while the Ca++ antagonist abolished the AcLDL-mediated increase in HDL binding and internalization.

Results

Binding and Uptake of HDL

The incubation of macrophages in the presence of AcLDL resulted in an initial increase in HDL binding up to 4 hours. Thereafter, HDL binding decreased and returned to the basal level after 8 hours of incubation (Figure 2A). Simultaneous incubation with the ACAT inhibitor resulted in a further continuous increase in HDL binding, and the Ca++ antagonist abolished the AcLDL-induced increase in HDL binding. The time-dependent changes in 37°C cell-associated HDL (Figure 2B) resemble the 4°C binding results obtained in the absence and presence of the ACAT inhibitor and the Ca++ antagonist. Although in the control experiment an increase in cell-associated HDL could be observed only up to 4 hours, the ACAT inhibitor stimulated a time-dependent HDL uptake, and the Ca++ antagonist abolished the AcLDL-induced HDL internalization.

Figure 1. Incubation conditions of the described tissue culture experiments. DMEM = Dulbecco's Modified Eagle Medium; BSA = bovine serum albumin. Figure 2. Effect of the Ca++ antagonist, nifedipine, and the ACAT inhibitor, octimibate, on specific 4°C binding (A) and on 37°C, cell-associated, 125I-labelled HDL3 (B) of mouse peritoneal macrophages. Cells were simultaneously incubated for the indicated times at 37°C with 70 μg/ml AcLDL in DMEM/0.2% BSA (○), 70 μg/ml AcLDL and 2 μmol/l nifedipine (△) or 70 μg/ml AcLDL and 25 μmol/l octimibate (◇). Binding and cell association of 125I-HDL (20 μg/ml, SA 150 to 300 cpm/ng) were measured as described in the Methods section.
lower intracellular cholesterol content, was seen. This was surprising and led to the suggestion that the Ca++ antagonist does not significantly upregulate HDL binding and uptake, but may increase cholesterol release from macrophages by an unknown mechanism.

When the cells were sequentially exposed to the appropriate drug, a significant loss of total cholesterol (Figure 5A) at the expense of esterified cholesterol (Figure 5B) was observed within 2 hours when the cells were exposed to the Ca++ antagonist. Incubation with the ACAT inhibitor or with DMEM alone revealed no loss in cellular cholesterol content within the first 4 hours of incubation (Figures 5A to 5C).

The observed decrease in cellular cholesterol content induced by the Ca++ antagonist after sequential incubation again underlines a possible secretory mechanism of cholesterol even in the absence of an acceptor lipoprotein such as HDL. Moreover, the cholesterol decrease observed after a 6-hour incubation period with DMEM alone indicates that the Ca++ antagonist may enhance a naturally occurring HDL-independent pathway for the release of cellular cholesterol from macrophages. In the presence of the ACAT inhibitor, only a small loss of cellular cholesterol occurred. As expected, the cholesterol ester content decrease was time-dependent, while the cellular content of the free cholesterol slightly increased.

Analysis of the intracellular cholesterol content after sequential + intermediate incubation with the appropriate drug (Figure 6) revealed a smaller loss of cellular cholesterol during exposure to the Ca++ antagonist than could
be observed after sequential incubation (Figure 5A), when 80% to 90% of cellular cholesterol was associated with subcellular membranes. No significant decrease in cellular cholesterol was observed for the cells incubated with the ACAT inhibitor or DMEM alone.

**Determination of Enzymes Involved in Intracellular Cholesterol Metabolism**

To further elucidate the mechanisms of action of the drugs on macrophage cholesterol metabolism, the activity of the three key enzymes involved in cellular cholesterol metabolism (ACEH, NCEH, and ACAT) were analyzed upon simultaneous (Figure 7), sequential (Figure 8), and sequential + intermediate (Figure 9) incubations with the Ca²⁺ antagonist or the ACAT-inhibitor.

As can be seen in Figure 7, the presence of AcLDL alone in the incubation medium activated ACAT, but had no measurable effect on ACEH and NCEH. Upon simultaneous incubation, the Ca²⁺ antagonist significantly increased the activity of ACEH, NCEH, and ACAT. The ACAT inhibitor, as expected, completely inhibited ACAT activity (<2%), and reduced NCEH, but did not significantly alter the rate of ACEH.

Upon sequential incubation (Figure 8) and sequential + intermediate incubation (Figure 9), no relevant changes in enzyme activities in the presence of the Ca²⁺ antagonist were observed. The ACAT inhibitor inhibited ACAT under all experimental conditions; however, the effect was most prominent upon simultaneous incubation whereby a high flux of unesterified cholesterol occurred from the lysosomal compartment into the cytoplasm.

**Analysis of the Extracellular Lipid Content**

For the mass analysis of medium lipoproteins, cholesterol and phospholipids were determined in the d < 1.21 g/ml fraction of the culture medium.

When macrophages were incubated under sequential conditions in the presence or absence of the Ca²⁺ antagonist and the ACAT inhibitor, they secreted cholesterol into the culture medium. However, as shown in Figure 10 the highest release of cholesterol was observed after incubation with the Ca²⁺ antagonist, while only minor amounts were secreted upon ACAT inhibitor treatment. These results correspond to those from the experiments shown in Figure 5, demonstrating that Ca²⁺ antagonist treatment led to a significant release of cellular cholesterol. Incubation of the cells in DMEM alone clearly indicated a release of lipoprotein-bound unesterified cholesterol into the medium. This mechanism could have been amplified by the Ca²⁺ antagonist while the ACAT inhibitor led to a modulation of cholesterol release in favor of the HDL-mediated cholesterol transport (unpublished observations).

The relative lipid composition (molar ratio) of the d < 1.21 g/ml lipoproteins isolated after sequential incubation is shown in Figure 11. The secreted particles were rich in unesterified cholesterol (68%) and phospholipids (21%), but esterified cholesterol (8%) and triglycerides (3%) were only minor constituents. The major phospholipid components were phosphatidylcholine (72%) and sphingomyelin.

![Figure 7. Effect of the Ca²⁺ antagonist and the ACAT Inhibitor on ACEH, NCEH, and ACAT activity in mouse peritoneal macrophages on cholesterol accumulation. Cells were simultaneously incubated for the indicated times at 37°C with 70 µg/ml AcLDL in DMEM/0.2% BSA (●), 70 µg/ml AcLDL and 2 µmol/l nifedipine (▲), or 70 µg/ml AcLDL and 25 µmol/l octrimeltrate (○). Enzyme activities were determined by the procedures described in the Methods section.](http://atvb.ahajournals.org/)

![Figure 8. Effect of the Ca²⁺ antagonist and the ACAT Inhibitor on the activities of ACEH, NCEH, and ACAT in mouse peritoneal macrophages sequentially incubated with the appropriate drug. Cholesterol-loaded macrophages (70 µg/ml AcLDL in DMEM/0.2% BSA for 12 hours at 37°C) were incubated with DMEM/0.2% BSA (●), 2 µmol/l nifedipine (▲), or 25 µmol/l octrimeltrate (○) at 37°C for the indicated time intervals. Enzyme activities were determined as described in the Methods section.](http://atvb.ahajournals.org/)

![Figure 9. Effect of the Ca²⁺ antagonist and the ACAT Inhibitor on the activities of ACEH, NCEH, and ACAT in mouse peritoneal macrophages after sequential + Intermediate incubation. Macrophages were loaded with cholesterol for 12 hours at 37°C (70 µg/ml AcLDL) followed by an intermediate incubation for 12 hours with DMEM/0.2% BSA in the absence of AcLDL to transfer the majority of lysosomal cholesterol to cytoplasmic lipid droplets. After the sequential + Intermediate incubation, the cells were exposed either to DMEM/0.2% BSA (●), 2 µmol/l nifedipine (▲), or 25 µmol/l octrimeltrate (○) at 37°C for the indicated time intervals. Enzyme activities were determined as described in the Methods section.](http://atvb.ahajournals.org/)
Phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine amounted to only 6%. There was no significant difference in the relative lipid composition of the d < 1.21 g/ml lipoproteins when isolated from the medium of DMEM-, Ca++ antagonist-, or ACAT inhibitor-treated cells.

Determination of Apoprotein E Synthesis

The rate of apo E synthesis was continuously monitored upon simultaneous incubation (Figure 12). The Ca++ antagonist increased cellular apo E synthesis significantly above the rate achieved with AcLDL alone or in the presence of the ACAT inhibitor.

Determination of Nucleoside and Nucleotide Metabolism

To find out whether alterations in macrophage nucleoside and nucleotide metabolism are involved in the mechanism of action of the Ca++ antagonist, the effect of this drug on cellular adenosine, ATP, and cAMP concentration and nucleoside transporter activity was analyzed upon simultaneous incubation experiments (Figure 13). The Ca++ antagonist significantly increased the intracellular concentration of adenosine (Figure 13A) and inhibited the nucleoside transporter activity (Figure 13C). Cellular cAMP levels (Figure 13D) were elevated and ATP degradation (Figure 13B) was enhanced by the Ca++ antagonist. The ACAT inhibitor did not alter these four parameters compared to cells chased only with AcLDL.

Discussion

The effects of the slow Ca++ channel blocker, nifedipine, and the ACAT inhibitor, octimibate, on macrophage cholesterol metabolism were compared. In contrast to the ACAT inhibitor, which increased the HDL receptor activity and stimulated HDL-mediated cholesterol efflux, the Ca++ antagonist did not significantly enhance HDL binding and uptake upon cholesterol accumulation. Treatment with the Ca++ antagonist increased AcLDL-mediated cholesterol influx as well as the activity of the enzymes involved in intracellular cholesterol metabolism. Interestingly, the accumulation of cellular cholesterol was significantly lower as compared to the cells incubated under simultaneous conditions with AcLDL or in the presence of the ACAT inhibitor alone. Even in the absence of a cholesterol acceptor in the surrounding medium, a significant loss of cellular cholesterol was observed within the first 2 hours of sequential incubation.

With respect to previous studies from other laboratories with verapamil and nifedipine on various cell types, these data led to the assumption that the Ca++ antagonist...
The HDL-independent promotion of cholesterol efflux was studied in detail, and we showed that the Ca\(^{++}\) antagonist induced the formation of membrane-surrounded lamellar bodies originating from multivesicular bodies and lysosomes.\(^{41}\) The macrophages secreted these particles which were rich in unesterified cholesterol (68%) and phospholipids (21%) and from which phosphatidylcholine (70% to 80%) was determined as the major phospholipid in the surrounding medium even in the absence of cholesterol acceptors. A similar secretion of lamellar bodies has recently been reported for pulmonary surfactant.\(^{56}\) There is evidence from intra- and extracellular mass analysis (Figure 5 and Figure 10), as well as from our morphological studies,\(^{41}\) that the Ca\(^{++}\) antagonist-inducible secretion of cholesterol-containing lamellar bodies represents a naturally occurring pathway in macrophages which is enhanced by nifedipine. Elgin and Hajjar\(^{27}\) in studies on smooth muscle cells from cholesterol-fed rabbits observed that treatment of cultured cells with nifedipine resulted in a significant loss of cellular cholesterol. However, these experiments were performed in the presence of 10% serum over a 1-week period. Therefore, it was impossible to differentiate between a lipoprotein- and a nonlipoprotein-mediated release of cellular cholesterol. Our three different incubation procedures allowed the analysis of drug influences on the two major intracellular cholesterol pools. The membrane-associated cholesterol pool in endosomes, multivesicular bodies, and lysosomes was studied upon simultaneous and sequential incubation, and the nonmembrane-associated cholesterol pool in the cytoplasmic lipid droplets were monitored after sequential + intermediate incubation.

Analysis of the cellular cholesterol content (Figures 4 to 6) and the intracellular lipolytic enzymes (Figures 7 to 9) under the three incubation procedures clearly indicated...
that the Ca\(^{2+}\) antagonist predominantly influenced macrophage cholesterol release at the level of membrane-associated cholesterol in the lysosomal route. In contrast, the ACAT inhibitor did not significantly influence cellular cholesterol efflux in the absence of a cholesterol acceptor in the medium within the first 4 hours under all experimental conditions, but enhanced HDL binding and promoted the lipoprotein-mediated release of cholesterol.\(^{40}\) ACAT inhibition by the known inhibitors (Sandoz 58.035, octimibate, and cyanamide) is most effective under conditions of enhanced intracellular lipid flux through the lysosomal route (simultaneous incubation). However, under sequential + intermediate conditions when most of the cholesterol was already associated with cytoplasmic lipid droplets, the kinetics of the intracellular unesterified cholesterol formation were critically determined by the slow kinetics and the low substrate specificity for cholesterol esters of neutral cholesterol ester hydrolase. This mechanism obviously diminishes the influence of ACAT inhibitors on the cholesterol ester content in cytoplasmic lipid droplets under sequential + intermediate incubation conditions. As demonstrated in our morphological studies,\(^{41}\) the ACAT inhibitor induced the formation of lamellar bodies that originated from the margin of the cytoplasmic lipid droplets and were surrounded by a newly formed membrane. The vesicles containing this type of lamellar bodies were not secreted by the macrophages but were stored in the cytoplasmic compartment in the absence of HDL in the culture medium. When HDL was added to the medium, the vesicles with the lamellar bodies specifically interacted with endosomes containing the internalized HDL particles and rapidly disappeared concomitantly with an enhanced HDL-mediated cholesterol efflux.\(^{40}\)

The intracellular accumulation of myelin-like structures was observed by McGockey and Anderson,\(^{59}\) when they incubated cholesterol-loaded macrophages with progesterone which, in addition to a number of other metabolic effects, inhibits ACAT. They postulated that these myelin-like structures might shuttle cholesterol from the lipid droplet to the cell surface. The Ca\(^{2+}\) antagonist enhanced the rate of apo E synthesis and secretion significantly above the levels achieved with AcLDL alone or in the presence of the ACAT inhibitor (Figure 12). From these data and an analysis of the particle composition secreted into the culture medium, we conclude that apo E might be associated with the formation and secretion of cholesterol-containing lamellar bodies. Further work is necessary to demonstrate whether the Golgi-derived apo E molecules interact intracellularly with the lamellar bodies or associate with these particles after secretion into the culture medium. We observed that nifedipine competes in various cells, predominantly those of neuronal origin, with the action of adenosine and benzodiazepine receptors and this drug inhibits adenosine uptake from and release into the extracellular space.\(^{29-35}\) This prompted us to analyze these effects of nifedipine in macrophages upon cholesterol loading. The data in Figure 13 indicate that nifedipine exerts in macrophage similar effects on nucleoside and nucleotide metabolism leading to an increase in intracellular adenosine due to enhanced ATP hydrolysis and inhibition of the nucleoside transporter activity. From our data we do not know precisely the origin of the nifedipine-induced increase in intracellular cAMP (Figure 13D), which enhances cholesterol mobilization from various cells.\(^{58-63}\) This could be either due to a modulation of adenosine receptors and by interaction with the inhibitory P-site of the adenylate cyclase catalytic subunit\(^{64}\) or by inhibition of cAMP degradation.\(^{65}\) Recent data from Hajjar\(^{66}\) on smooth muscle cells indicate that in addition to cAMP-dependent phosphorylation, the activity of NCEH is influenced by a Ca\(^{2+}\)-dependent phosphoprotein phosphatase.

Further experimental work is necessary to clarify whether the rate of cAMP degradation and phosphoprotein phosphatase activity are coordinately regulated.

We conclude that macrophages release cholesterol by two major pathways: 1) an HDL-mediated release of unesterified cholesterol increasing with ACAT inhibition, and 2) an HDL-independent secretion of cholesterol which can be amplified by Ca\(^{2+}\) antagonists.

References

17. Hollander W, Peddock J, Nagral S, Colombo M, Kirkpatrick B. Effects of anticalcifying and antifibrotic drugs on...
preestablished atherosclerosis in the rabbit. Atherosclerosis 1979;33:111–123


57. Halje DP, Weaker BB, Falcone DJ, et al. Prostacyclin modulates cholesteryl ester hydrolytic activity by its effect on


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