Regulation of phospholipid biosynthesis during cholesterol influx and high density lipoprotein-mediated cholesterol efflux in macrophages

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Abstract We have studied the rate of phospholipid synthesis and turnover in mouse peritoneal macrophages in reaction to cholesterol influx and high density lipoprotein (HDL)-mediated cholesterol efflux, using three different radioactive precursors, $^{32}PO_4^{3-}$, [^{3}H]choline, and [^{14}C]oleic acid. The cells were loaded with cholesterol for up to 18 h with acetyl-low density lipoprotein (LDL), and phospholipid synthesis was measured at various time intervals and compared with nonloaded macrophages. In the first 2 h of cholesterol loading, a twofold increase in the rate of synthesis for sphingomyelin, phosphatidylcholine, phosphatidylserine-inositol, and phosphatidylethanolamine was observed. After this initial up-regulation, the rate of phospholipid synthesis continuously declined upon further cholesterol loading, while the turnover rate of cellular phospholipids was not affected under the same conditions. The lysosomal inhibitor chloroquine abolished the down-regulation, revealing a strong correlation between phospholipid synthesis and lysosomal enzyme activity which was presumably dependent on the release of cholesterol from the lysosome. The reduction in phospholipid synthesis induced by cholesterol loading is reversible by the addition of HDL_3 to the cells. When HDL_3 was added to the culture medium, a two- to threefold increase in phosphatidylcholine synthesis and a twofold increase in sphingomyelin formation was observed after 3 h. Ca^{2+} antagonists of the dihydropyridine type, which down-regulate HDL-receptor activity and promote the formation and cellular release of lamellar bodies derived from the lysosomal compartment (Schmitz, G., et al. 1988. *Arteriosclerosis.* **8:** 46–56, and Robenek, H., and G. Schmitz. 1988. *Arteriosclerosis.* **8:** 57–67), specifically enhance the synthesis of sphingomyelin in cholesterol-loaded macrophages. Inhibitors of acyl-CoA:cholesterol acyltransferase (Octimibate, progesterone) increase both the synthesis of sphingomyelin and phosphatidylcholine, and enhance HDL-receptor activity. ▪▪ The results indicate that cholesterol and phospholipid metabolism are coordinately regulated in macrophages. Moreover, the formation of phosphatidylcholine and sphingomyelin seems to be an important factor for the promotion of HDL-receptor-mediated cellular cholesterol efflux. — **Schmitz, G., M. Beuck, H. Fischer, G. Nowicka, and H. Robenek.** Regulation of phospholipid biosynthesis during cholesterol influx and high density lipoprotein-mediated cholesterol efflux in macrophages. *J. Lipid Res.* 1990. **31:** 1741–1752.

Supplementary key words phospholipid synthesis • macrophages • HDL • cholesterol efflux

Cholesterol influx and efflux in macrophages have been studied in great detail (1) and we have recently shown (2–6) that there are two major routes, in addition to physicochemical exchange, by which macrophages can release excess cholesterol.

The first is an HDL-receptor-dependent secretion of cholesterol, stimulated by ACAT inhibitors which induce the formation of lamellar bodies originating from lipid droplets or alternatively are released from the *trans* region of the Golgi apparatus. These lamellar bodies are not directly secreted by the cells. However, when HDL is added to the medium, the lamellar bodies transfer their cholesterol to HDL particles and disappear concomitantly with the HDL-receptor-mediated cholesterol efflux.

The second is an HDL-receptor-independent release of cholesterol, stimulated by dihydropyridine Ca^{2+}-antagonists which induce the formation of lamellar bodies originating from lysosomes. These lamellar bodies fuse with the cell membrane and can be secreted into the surrounding medium by an HDL-receptor-independent mechanism which appears to be promoted by apoA-IV and LCAT-rich HDL particles.

The formation of lamellar bodies by both mechanisms indicates that the translocation of cholesterol may also in-

volve phospholipid metabolism. Very little is known about the regulation of phospholipid metabolism in macrophages and there are only a few data available for other cell types.

In hepatocytes, phosphatidylcholine (PC) synthesis obviously is a necessary prerequisite for lipoprotein assembly (7–9). In these cells PC derives mainly from the CDP-choline pathway (70–80%), which is located in the endoplasmic reticulum and regulated by the cytidylyltransferase, while the methylation of phosphatidylethanolamine (PE) accounts for the remainder.

In fibroblasts, lipoprotein-mediated cholesterol uptake has been shown to modulate sphingomyelin synthesis. Incubation with LDL leads to a reduction in de novo sphingomyelin synthesis, while HDL failed to induce a similar reduction (10). The authors of this study assumed that the observed effects were due to a decrease in the synthesis of the long-chain base sphinganine. The synthesis of long-chain bases by the palmitoyl-CoA:serine palmitoyltransferase which is located in the endoplasmic reticulum is thought to be the key regulatory enzyme in ceramide synthesis (10). The final transfer of phosphocholine from PC to ceramide, the key regulatory step in sphingomyelin formation, is exclusively located in the Golgi apparatus (11, 12). Other studies have shown that HDL induces the synthesis and secretion of the glycosphingolipid lactosylceramide in leukocytes and trihexosylceramide in fibroblasts (13).

In our own studies on human monocyte-derived macrophages, we have shown that cholesterol-loading with acetyl-LDL leads to a down-regulation of phospholipid synthesis, while HDL enhances phospholipid synthesis in cholesterol-loaded cells (14). In the same studies it could be demonstrated that the cellular defect in Tangier monocyte-derived macrophages involves a dysregulation of cellular phospholipid metabolism.

All these studies prove that cholesterol and phospholipid metabolism are intimately related. Therefore, we have investigated here the coordinate control of phospholipid and cholesterol metabolism in mouse peritoneal macrophages upon cholesterol influx and HDL-mediated cholesterol efflux.

MATERIALS AND METHODS

DMEM medium was purchased from Flow Laboratories. Other biochemicals were from Sigma, Taufkirchen, or Serva, Heidelberg. $^{32}PO_4^{3-}$-phosphorus (8500–9120 Ci/mmol) was purchased from Amersham, Braunschweig and [^{3}H]methylcholine chloride (60–90 mCi/mmol), [1-^{14}C]oleic acid (52.6 Ci/mol), cholesteryl- [1,2,6,7-^{3}H(N)]-oleate (75 Ci/mmol) were obtained from New England Nuclear (NEN). The ACAT inhibitor Octimibate (sodium 8-(1,4,5-triphenyl-imidazole-2-yl)oxy octanoate) was kindly provided by Dr. Lautenschläger (Rhône-Poulenc-Nattermann, Cologne, FRG). The dihydropyridine Ca^{2+}-

antagonist, Nifedipine (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-pyridine-3,5-dicarboxylic acid dimethyl ester), was provided by Dr. Thomas, Bayer, Milan, Italy. BCEC-FAM was purchased from Molecular Probes, Junction City, OR.

Mouse peritoneal macrophage (MPM) monolayers

Murine peritoneal macrophages were obtained from unstimulated NMRI-SPF mice (20–35 g) by peritoneal lavage with PBS containing 0.5 U heparin/ml. The peritoneal fluid was pooled, and the cells were centrifuged at 400 g for 10 min at room temperature. The cells were washed once with DMEM and resuspended in DMEM containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml). Aliquots (0.5–1 ml) of the cell suspension corresponding to the number of cells from two mice (3 × 10^{6} cells) were dispensed into 35 × 10 mm dishes containing 1 ml DMEM with the additions mentioned above and then incubated in a humidified incubator at 37°C and 5% CO_2. 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 150–200 µg of total protein. Cell viability was greater than 90%, as tested by the trypan blue dye exclusion method.

Lipoproteins

Human LDL (d 1.019–1.063 g/ml) and HDL$_3$ (d 1.125–1.21 g/ml) were isolated from serum of individual normolipidemic 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, 5 mM Na$_2$EDTA (pH 7.4) at 4°C. All concentrations of lipoproteins are given in terms of their protein content with albumin as a standard.

Chemical modification of LDL and cholesterol loading of cells

LDL (d 1.019–1.063 g/ml) were acetylated by repeated additions of acetic anhydride (15) and dialyzed against PBS, pH 7.4, at 4°C. The modified LDL showed enhanced mobility on agarose gel electrophoresis at pH 8.6. Cholesterol loading of macrophages was promoted by incubation of the cells with acetyl-LDL at 37°C. Protein concentrations and the time intervals used are indicated in the legends.

Quantitation of celluar lipids

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 harvested with a rubber policeman in 1000 µl of PBS, transferred to a conical glass tube, and sonicated with a Branson sonicator three times for 20 sec in ice-water at an intensity of 30 watts. The homogenate was delipidated ac-

cording to Bligh and Dyer (16) with chloroform–methanol 1:2 (v/v) and the lipids were chromatographically separated on HPTLC-silica plates as previously described (17, 18). For all lipid determinations the coefficient of variation was between 4.7 and 7.6%. In addition, the described computer program automatically excluded all data showing >10% of variation.

Assay of acid and neutral cholesterol-esterase (ACEH and NCEH)

The activity of the cholesteryl ester hydrolases was determined by the release of [³H]cholesterol from [³H]cholesteryl oleate at different pH values as described by Etingin and Hajjar (19). One hundred µl cell homogenate (50–60 µg protein) was incubated for 1 h at 37°C with 100 µl substrate solution.

Assay for NCEH. Eighty five mM phosphate buffer, pH 7.0, 12.5 mM Na taurocholate, 6 µM [³H]cholesteryl oleate (sp act 160 mCi/mmol), 23.7 µM phosphatidylcholine, and 0.4 mg/ml BSA.

Assay for ACEH. Fifty mM Na acetate, pH 3.9, 2 mM Na taurocholate, 12.7 µM [³H]cholesteryl oleate (sp act 600 mCi/mmol), 1.3 mM phosphatidylcholine, and 50 µg/ml digitonin.

Both substrate solutions were prepared by first evaporating chloroform from cholesteryl oleate and phosphatidylcholine solutions under a stream of nitrogen. The lipids were then sonicated in the appropriate buffers until the suspension became clear. The enzyme reaction was stopped by cooling the tubes in ice-water. Samples were delipidated with chloroform–methanol as described for quantitation of cellular lipids and after separation of the lipids on HPTLC silica gel plates the radioactivity of the [³H]cholesterol spot was quantitated by liquid scintillation counting.

Assay of acyl-CoA:cholesterol-O-acyltransferase (ACAT)

ACAT activity was measured by incorporation of [¹⁴C]oleate into cholesteryl esters. At the end of each incubation period, the cells were washed with DMEM-0.2% BSA (w/v) and then further incubated for 3 h in the same medium, supplemented with 0.2 mM [¹⁴C]oleate. Afterwards the cells were harvested in 1000 µl PBS and 800-µl aliquots were delipidated as described for ACEH and NCEH. Separation of lipids was carried out as described under "Quantitation of cellular lipids." 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 (w/v) at 60°C for 30–60 min. A solution of 1.2 g BSA in 5 ml 0.9% NaCl (w/v) (adjusted to 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 (w/v). Sixteen µl of this complex was mixed with 0.5 µCi [¹⁴C]Na oleate and added

to each dish. At the end of the assay the cells were harvested, homogenized in 1000 µl PBS, and 800-µl aliquots were taken for delipidation. The extracted lipids were separated by HPTLC on silica gel plates (17, 18), and the cholesteryl [¹⁴C]oleate spot was quantitated by liquid scintillation counting.

Determination of phospholipid synthesis

Phospholipid synthesis by intact cells was determined via incorporation of ³²PO₄³⁻, [³H]choline, and [¹⁴C]oleic acid by a standard procedure described earlier (7, 20). For the incorporation experiments, 40 µCi/ml ³²PO₄³⁻, 5 µCi/ml [³H]choline (sp act 83 mCi/mmol) or 0.5 µCi/ml [¹⁴C]oleic acid (56.2 Ci/mol or 3 Ci/mol) were used. After an initial incubation with acetyl-LDL or HDL, the cells were incubated for 1 h with the different labels in the indicated concentrations. In case of ³²PO₄³⁻, an intermediate incubation for 30 min with phosphate-free medium was included prior to the addition of the labels.

Subsequent to the delipidation of the cell homogenate according to Bligh and Dyer (16), phospholipids were chromatographically separated (17, 18), scraped from the plate, and radioactivity was determined with an LKB β-counter (Mod. 1218).

Cytotoxicity measurement with BCECF

The cytotoxicity assay was performed according to Kolber et al. (21). The principle of this assay is based on the release of BCECF from dead cells, which is fluorimetrically determined in the medium. MPM were incubated for 20 min in Hank's balanced salt solution supplemented with 5% fetal calf serum and 25 µM BCECF-AM. Then, cells were washed and further incubated for 4 h with Nifedipine and Octimibate in a concentration range of 10^{-7}–10^{-4} M. The release of BCECF into the medium was fluorimetrically determined with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Total cell BCECF release was achieved by incubation of control cells with 100 µg/ml saponin. The calculation of cytotoxicity was done as previously described (20) by the following equation:

$$\text{corrected } \% \text{ cytotoxicity} = 100 \times \frac{F_{exp} - F_{med}}{F_{det} - F_{med}}$$

F_{exp} = experimental fluorescence; F_{med} = cells incubated in medium alone; F_{det} = fluorescence from cells, solubilized with 100 µg/ml saponin.

Other methods

The protein content of lipoprotein fractions and cells was determined by the method of Lowry et al. (22), using BSA as a standard. ATP was determined with the commercially available ATP bioluminescent assay kit from Sigma Chemical Co. (FLAA).

RESULTS

In order to investigate regulatory mechanisms of intracellular targeting of cholesterol, we focused on the role of phospholipids and their rates of synthesis. An experimental model using mouse peritoneal macrophages was established, and the synthesis of four different phospholipids was studied in three different sets of experiments. In the experiments, the effects of cholesterol influx (acetyl-LDL) or cholesterol efflux (HDL) on synthesis of phospholipids and the influence of the calcium antagonist Nifedipine and the ACAT-inhibitor Octimibate on the synthesis of sphingomyelin and phosphatidylcholine were addressed. (A schematic overview of the results is given in Fig. 9.)

Analysis of ACEH, NCEH, and ACAT in MPM upon cholesterol loading

ACEH, NCEH, and ACAT, which are involved in cholesterol metabolism in macrophages, were measured after exposure of MPM to acetyl-LDL for up to 18 h. Upon incubation with acetyl-LDL the cells continuously accumulated cholesterol (**Fig. 1A**). Most of the cholesterol was converted into cholesteryl esters (60–70%) by the ACAT reaction. ACAT activity increased about 4- to 5-fold during the incubation period, starting at 2.5 nmol/ mg protein × h (Fig. 1B). This increase in ACAT activity indicates that an enhanced cholesterol flux occurs from lysosomes to the microsomal enzyme ACAT. The activities of the lysosomal and cytoplasmic cholesteryl ester hydrolases ACEH and NCEH were measured under the same experimental protocol. Starting at 2.7 nmol/mg protein × h, ACEH activity was stimulated 1.4-fold within the first 2 h and then declined about 10% during

the following 16 h. Only a slight increase in NCEH activity was observed during the 18-h incubation period. Prior to the following experiments each acetyl-LDL preparation was tested for its cholesterol-loading properties in MPM. These experiments were performed to calibrate the experimental system for the following studies of phospholipid synthesis to achieve similar cholesterol loading between the experiments.

Synthesis of phospholipids in MPM upon cholesterol loading

The rate of phospholipid synthesis was studied with three radioactive labels ($^{32}PO_4^{3-}$, [^3H]choline, and [^{14}C]oleic acid) comprising different synthetic routes for the respective phospholipids.

Analysis of $^{32}PO_4^{3-}$ incorporation into phospholipids. $^{32}PO_4^{3-}$ is incorporated via the τ-$^{32}PO_4^{3-}$-nucleotide pool into the different phospholipids. **Fig. 2 A–D** shows the rate of synthesis for SPM, PC, PI/PS and PE in the presence of 100 μg/ml acetyl-LDL as measured by the incorporation of $^{32}PO_4^{3-}$. The rates of synthesis for all phospholipids were initially increased to about 10–40% within the first 2 h of cholesterol loading. After this time further cholesterol loading for up to 18 h led to a continuous decline in the rate of synthesis for SPM, PC, and PI/PS, culminating in a rate distinctly lower than determined before cholesterol loading. No changes were observed in nonloaded control cells during this time and cellular ATP remained constant (loaded: 1.8 ± 0.3 nmol ATP/mg vs nonloaded: 2.3 ± 0.5 nmol ATP/mg). Neither cellular perturbations nor fluctuations in the τ-$^{32}PO_4^{3-}$-nucleotide pool seem to be responsible for the observed changes.
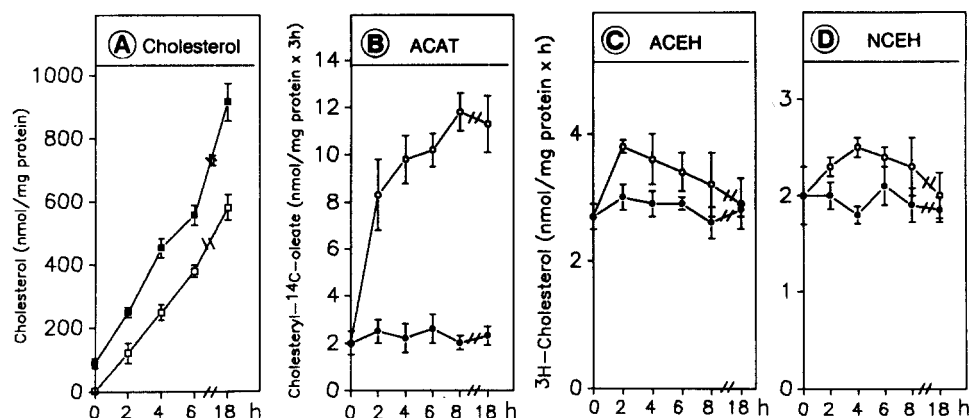

Fig. 1. Effect of acetyl-LDL incubation on cholesterol loading, ACAT, ACEH, and NCEH activity in MPM. MPM plated on 35 × 10 mm dishes were incubated for 0–18 h with 100 μg/ml acetyl-LDL. (A), Cells were delipidated and quantitation of total (■) and esterified cholesterol (□) was determined by densitometric mass analysis as described in Materials and Methods. In cells incubated with (O) and without (●) 100 μg/ml acetyl-LDL (B), ACAT activity was measured by [^{14}C]oleic acid (0.2 mM, 6600 dpm/nmol) in corporation into cholesteryl esters for 1 h after the indicated time points. ACEH (C) and (D) NCEH activities were determined via hydrolysis of [^3H]cholesteryl oleate at pH 3.9 and 7.0 as described in detail in Materials and Methods. Separation and quantitation of [^3H]cholesterol was performed by thin-layer chromatography and liquid scintillation counting. Shown are means of double determinations ± SEM of a representative experiment.
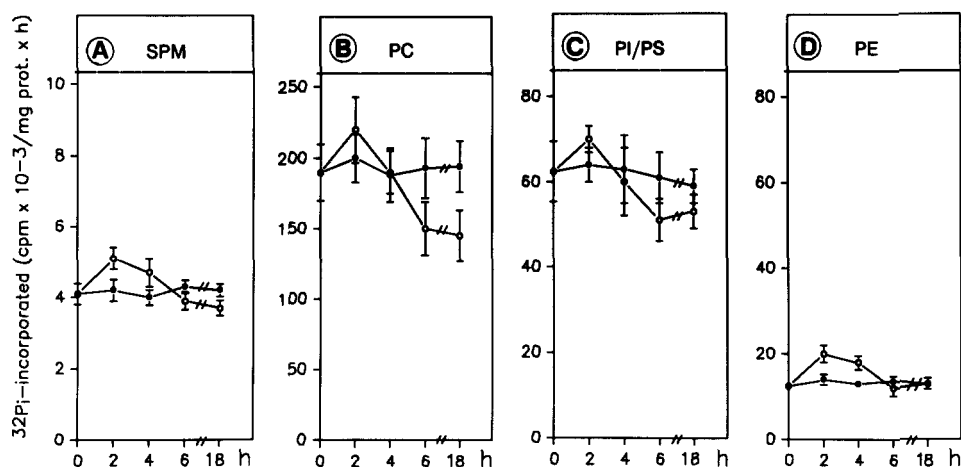
Fig. 2. Rate of phospholipid synthesis in MPM during cholesterol loading as analyzed by $^{32}PO_4^{3-}$ incorporation. MPM plated on 35 × 10 mm dishes were incubated for 0–18 h with (O) and without (●) 100 μg/ml acetyl-LDL. At the end of each indicated time point the cells were incubated for 30 min with phosphate-free medium, and then pulsed with $^{32}PO_4^{3-}$ (40 μCi/ml) for further 60 min. Analysis and quantitation of the different phospholipid classes was carried out by thin-layer chromatography and liquid scintillation counting as described in Materials and Methods. Shown are means of double determinations ± SEM of a representative experiment.

Analysis of [³H]choline incorporation. The same experiment as shown in Fig. 2 was repeated with [³H]choline, a specific precursor for PC and SPM. PC and SPM are crucial in cellular phospholipid metabolism. While $^{32}PO_4^{3-}$ is utilized for multiple cellular processes, [³H]choline provides a small precursor pool and thereby enhances the sensitivity to changes in rates of synthesis. With [³H]choline, the rates of synthesis PC and SPM (**Fig. 3 A–B**) showed kinetics similar to those in Fig. 2. However, the relative decrease of [³H]choline incorporation was higher as compared to that of $^{32}PO_4^{3-}$ indicating that this label is more sensitive.

[¹⁴C]Oleic acid incorporation. [¹⁴C]Oleic acid was selected as a third label to study phospholipid, triglyceride, and cholesteryl ester synthesis simultaneously (Fig. 4 and 5). Again following the same experimental protocol, a rapid decrease of the incorporation into all phospholipid classes was observed with oleic acid (**Fig. 4 A–D**). This decline was maintained for PC and PI/PS during the whole 18-h period, while SPM and PE synthesis tended to recover after 4–6 h of incubation. In contrast to the other two precursors, an initial activation of the rate of synthesis was not observed. This might be due to a relative high endogenous pool of acyl-CoA activated fatty acids and/or a slower activation of fatty acids as compared to $^{32}PO_4^{3-}$ and [³H]choline.

Influence of chloroquine on the incorporation of [¹⁴C]oleic acid into phospholipids. The foregoing experiments indicate a link between cellular cholesterol and phospholipid metabolism. Therefore, the influence of the lysosomal inhibitor chloroquine on phospholipid synthesis was studied in the presence and absence of acetyl-LDL. In previous studies it was shown that 25 μM chloroquine reduces acetyl-LDL

degradation to about 60% (23). The results (**Table 1**) indicate that chloroquine significantly abolishes the drop in phospholipid synthesis (Table 1, A vs B) observed upon continuous acetyl-LDL degradation in the absence of chloroquine (Table 1, A vs C). In noncholesterol-loaded
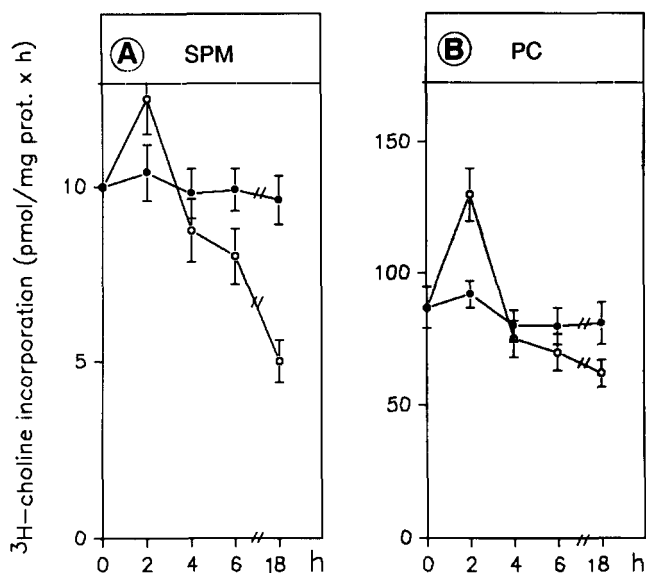

Fig. 3. Rate of SPM (A) and PC (B) synthesis in MPM during cholesterol loading as analyzed by [³H]choline incorporation. MPM plated on 35 × 10 mm dishes were incubated for 0–18 h with (O) and without (●) 100 μg/ml acetyl-LDL. At indicated times, lipoprotein-containing medium was removed and exchanged by a medium supplemented with 5 μCi[³H]choline/ml and cells were further incubated for 60 min, before analysis. Quantitation of SPM, and PC was carried out by thin-layer chromatography and liquid scintillation counting as described in Materials and Methods. Shown are means of double determinations ± SEM of a representative experiment.
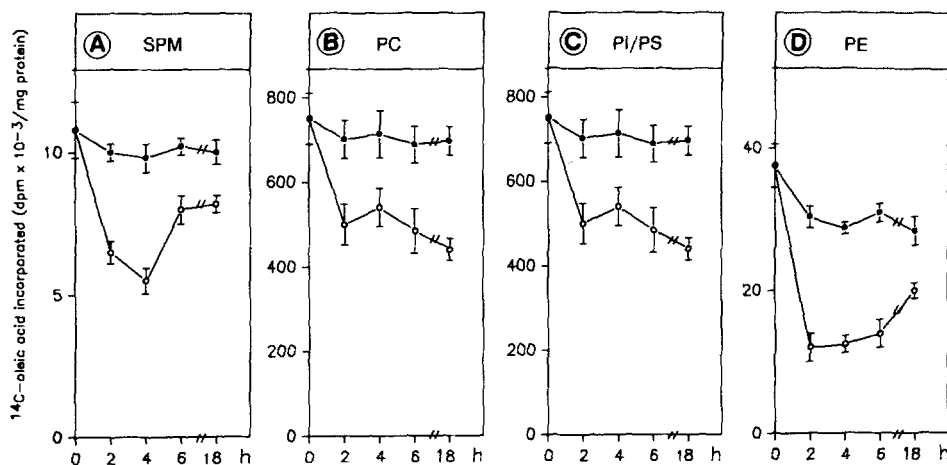
Fig. 4. Rate of phospholipid synthesis (A–D) in MPM during cholesterol loading as analyzed by [14C]oleic acid incorporation. MPM on 35 × 10 mm dishes were incubated for 0–18 h with (○) and without (●) 100 μg/ml acetyl-LDL. Subsequently, lipoprotein-free medium, supplemented with [14C]oleic acid (0.2 mM, 6,600 dpm/nmol) was added and cells were further incubated for 60 min. Analysis and quantitation of phospholipids was carried out by thin-layer chromatography and liquid scintillation counting as described in Materials and Methods. Shown are means of double determinations ± SEM of a representative experiment.

cells, chloroquine did not affect the synthesis of phospholipids (data not shown).

As an internal control for the effectivity of chloroquine, triglyceride and cholesteryl ester formation were also measured. An increase in triglyceride formation during cholesterol accumulation in MPM was completely inhibited by 25 μM chloroquine (154 ± 16 pmol vs 148 ± 14 pmol), while cholesteryl ester synthesis was reduced to about 93% (265 ± 32 vs 18 ± 7 pmol/mg protein). This indicates that lysosomal degradation of lipoproteins is strongly inhibited in MPM in the presence of 25 μM chloroquine.

Phospholipid catabolism in MPM

In order to elucidate whether an increased catabolism of phospholipids in cholesterol-loaded cells is responsible for the decreased incorporation of precursors, rather than a reduced rate of synthesis, the rate of removal of [14C]oleic acid-prelabeled phospholipids was determined in cholesterol-loaded and nonloaded MPM (**Fig. 5 A–C**). The cells were pulsed with [14C]oleic acid overnight and subsequently the rate of phospholipid degradation was measured in the following 8 h of incubation (Fig. 5 A–C). Phospholipid catabolism remained unaffected upon cellular cholesterol loading, because no significant differences in the rates of hydrolysis were found between cholesterol-loaded and nonloaded cells (Fig. 5A). During the course of the experiment the intracellular [14C]oleic acid concentration remained constant (data not shown), indicating that 14C-labeled fatty acids released were reutilized for triacylglycerol (~35 pmol/ml, Fig. 5C) and to an approximately 5-fold higher extent for cholesteryl ester synthesis (~170 pmol/mg, Fig. 5B).

Influence of HDL3 on phospholipid and cholesteryl ester synthesis in cholesterol-loaded macrophages

HDL is generally considered to be the major lipoprotein class responsible for reverse cholesterol transport from peripheral cells to the liver. Therefore, the influence of HDL-mediated cholesterol efflux on the rate of phospholipid synthesis and cholesteryl ester formation in MPM was investigated and the results are shown in Fig. 6 and Fig. 7.

Influence of HDL3 on 32PO43−-labeled phospholipids in MPM. MPM were loaded with cholesterol and subsequently pulsed with 32PO43− in the presence or absence of HDL3. Within a 3-h incubation period, the rate of synthesis for SPM, PC, and PI/PS in cholesterol-loaded

TABLE 1. Influence of chloroquine on phospholipid synthesis during cholesterol accumulation in MPM

Fraction	A − Acetyl-LDL + Chloroquine	B + Acetyl-LDL + Chloroquine	C + Acetyl-LDL − Chloroquine
SPM	130 ± 12[a]	92 ± 24	34 ± 9
PC	2316 ± 43	1434 ± 10	853 ± 45
PI/PS	417 ± 22	303 ± 4	103 ± 4
PE	364 ± 21	253 ± 14	153 ± 2
TG	154 ± 16	148 ± 14	246 ± 14
EC	5 ± 0	18 ± 7	265 ± 32

The cells were incubated for 2 h with (B, C) and without (A) acetyl-LDL (100 μg/ml) in the presence (A, B) and absence (C) of 25 μM chloroquine. Then 0.5 μCi [14C]oleic acid/ml (57 Ci/mol) was added and cells were further incubated for 2 h. Analysis of phospholipid, triglyceride, and cholesteryl ester synthesis was carried out as described in Materials and Methods.

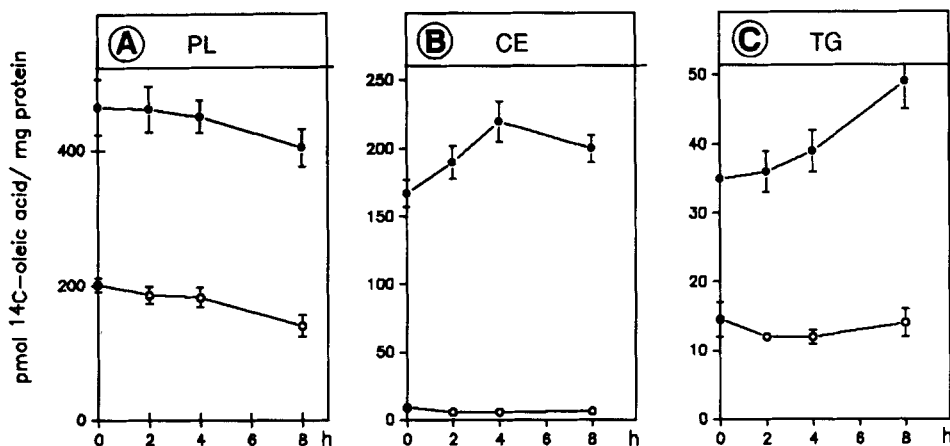[a]Results given as pmol [14C]oleic acid incorporated/mg protein ± SEM.

Fig. 5. Hydrolysis of [¹⁴C]oleic acid-labeled phospholipids (A), cholesteryl esters (B), and triglycerides (C) in MPM. MPM plated on 35 × 10 mm dishes were incubated overnight with (●) and without (○) 100 µg/ml acetyl-LDL in the presence of [¹⁴C]oleic acid (0.5 µCi/ml, 52.6 Ci/mol). Then cells were further incubated for up to 8 h with fatty acid-free DMEM. Analysis of the phospholipids, triglycerides, and cholesteryl esters at the different time points was carried out as described in Materials and Methods. Shown are means of double determinations ± SEM of a representative experiment.

MPM increased significantly in the presence of 100 µg/ml HDL₃ (**Fig. 6 A-D**). Lower HDL concentrations prolonged the incubation period to achieve a similar induction of phospholipid synthesis (data not shown). The most prominent effect of phospholipid synthesis was observed for PC and PI/PS, while SPM synthesis showed a time lag of approximately 1 h.

Activation of PE synthesis occurred only in the first 30 min of the incubation period after which time the rate of synthesis declined (Fig. 6D). In cholesterol-loaded control MPM incubated with DMEM in the absence of HDL₃,

the changes in the incorporation rates were not significant (closed circles in Fig. 6). LDL did not affect the rate of phospholipid synthesis (data not shown).

Influence of HDL₃ on [¹⁴C]oleic acid-labeled lipids in cholesterol-loaded MPM. The effect of 100 µg/ml HDL₃ on cholesteryl ester and phospholipid synthesis was investigated in cholesterol-loaded and nonloaded MPM during an incubation period of up to 6 h (**Fig. 7 A-B**). HDL-mediated cholesterol efflux is maximally stimulated under these conditions as shown earlier (3, 24). HDL stimulated oleic acid incorporation into cellular phospholipids in choles-
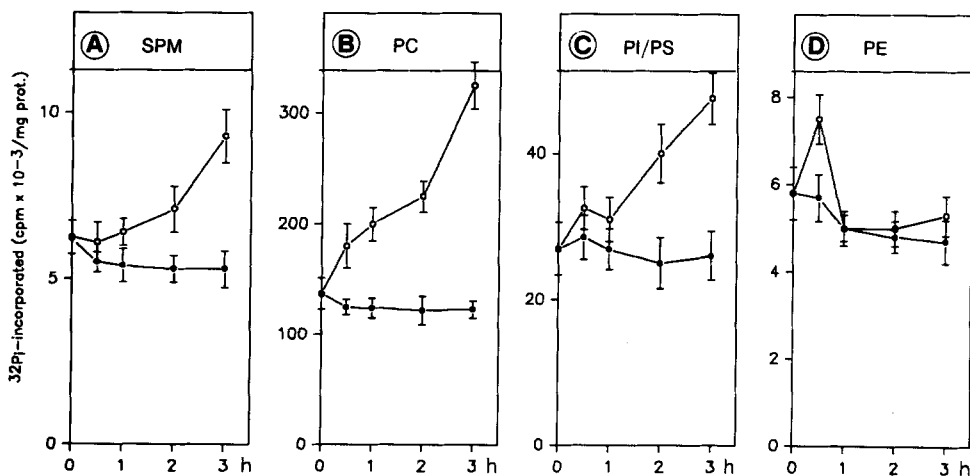
Fig. 6. Induction of phospholipid synthesis by HDL₃ in cholesterol-loaded MPM as analyzed by ³²PO₄³⁻ incorporation. MPM plated on 35 × 10 mm dishes were loaded with cholesterol by an overnight preincubation with 100 µg/ml acetyl-LDL and subsequently treated for 1-3 h with 100 µg/ml HDL₃ (○). In control dishes (●) HDL₃ was omitted. The ³²PO₄³⁻ incorporation was measured as described in legend to Fig. 2. Shown are means of double determinations of a representative experiment.

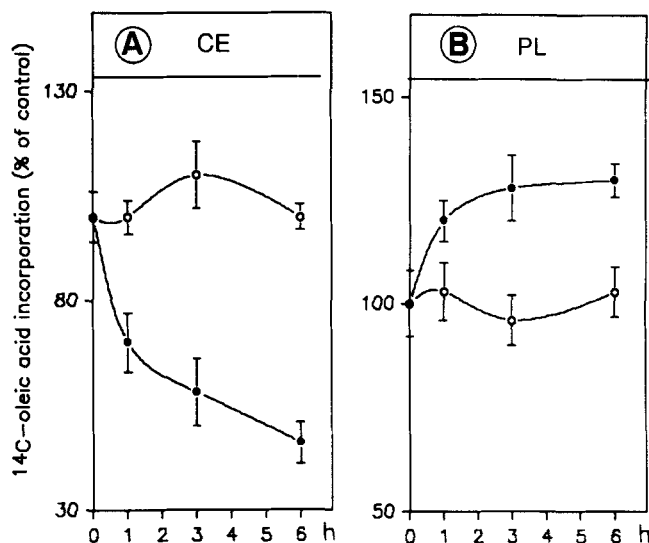**Fig. 7.** Influence of HDL$_3$ on phospholipid synthesis rates (B) and ACAT activity (A) in cholesterol-loaded and nonloaded MPM as analyzed by [^{14}C]oleic acid incorporation. MPM, plated on 35 × 10 mm dishes, were preincubated overnight with 100 μg/ml acetyl-LDL (●) or for 2 h with 10% lipoprotein deficient serum (○). Subsequently the cells were treated for 1–6 h with 100 μg/ml HDL$_3$. In control dishes HDL$_3$ was omitted. Then, medium was changed and the cells were further incubated for 1 h with [^{14}C]oleic acid (0.5 μCi/ml, 52.6 Ci/mol). Analysis of cholesteryl-[^{14}C]oleate and total phospholipids was carried out, after thin-layer chromatography, by liquid scintillation counting. Data are expressed as relative values in % of control. Control cells were incubated under the same experimental conditions without HDL$_3$ (nonloaded cells, t = 0 h: CE 0.03 nmol/mg protein × h, PL 3.4 nmol/mg protein × h and cholesterol-loaded cells, t = 0 h: CE 1.2 nmol/mg protein × h, PL 2.0 nmol/mg protein × h). Shown are means of double determinations ± SEM of a representative experiment.

terol-loaded cells by ~30% (Fig. 7B), but not in non-loaded cells pretreated with lipoprotein-deficient serum. Upon HDL incubation, cholesterol esterification was found to be reduced to ~46% in cholesterol-loaded cells, while in nonloaded cells (Fig. 7A) no effect on ACAT activity could be observed.

Influence of the dihydropyridine Ca^{2+}-antagonist Nifedipine and the ACAT inhibitor Octimibate on phospholipid synthesis

The influence of Nifedipine and Octimibate on PC and SPM synthesis in cholesterol-loaded MPM is shown in **Fig. 8**. In previous studies we described the formation of cholesterol- and phospholipid-containing lamellar bodies that originated from lysosomes upon treatment with Nifedipine. In contrast, the ACAT inhibitor Octimibate promoted the formation of a second type of lamellar body at the margin of cytoplasmic lipid droplets (4, 5).

In a concentration range between 10^{-9} and 10^{-7} mol/l, Nifedipine specifically induced SPM synthesis (200%), while Octimibate induced both SPM and PC synthesis (SPM, 120%; PC, 142%) in the concentration range from 10^{-6} to 10^{-5} mol/l. In control experiments, possible cyto-

toxic effects of these drugs could be excluded. Cytotoxicity was assayed by the release of fluorescent BCECF from MPM into the tissue culture medium during a 4-h incubation period (**Table 2**). Neither the calcium antagonist nor the ACAT inhibitor exhibits cytotoxic effects against MPM cells in the selected concentration range.

DISCUSSION

In the present study, we have investigated the effects of cholesterol influx and efflux on the rate of phospholipid synthesis in MPM cells. As a first step, a standardization of the experimental system was carried out. Cellular cholesterol and the major enzymes involved in macrophage cholesterol metabolism were analyzed upon incubation with acetyl-LDL. This type of calibration allowed us to attain comparable cholesterol-loading of MPM by use of different acetyl-LDL preparations, allowing a higher reproducibility in the measurements of phospholipid synthesis during cholesterol influx than would otherwise be possible.

A strong correlation was found between cholesterol influx and the incorporation rate of the three different radioactive precursors into phospholipids of MPM. The rate of phospholipid synthesis was increased in the first
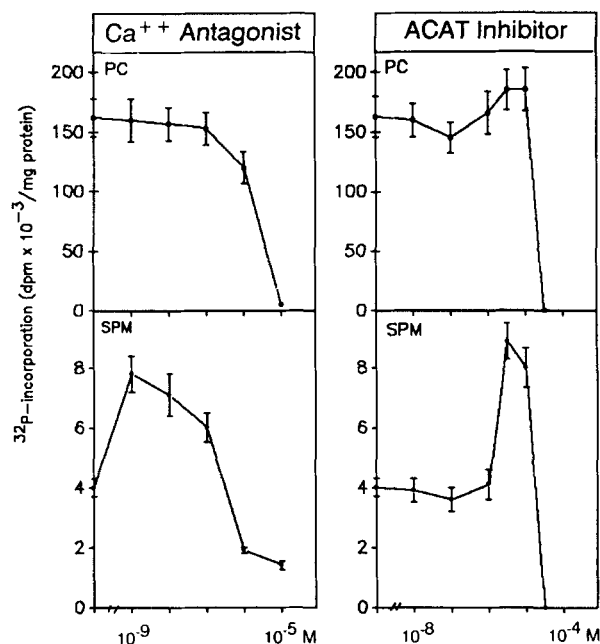
Fig. 8. Influence of the Ca^{2+}-antagonist Nifedipine and the ACAT-inhibitor Octimibate on SPM and PC synthesis in MPM. MPM, plated on 35 × 10 mm dishes, were cholesterol-loaded by preincubating overnight with 100 μg/ml acetyl-LDL before treatment for 4 h with the indicated concentrations of Nifedipine or Octimibate. Subsequently, the ^{32}PO$_4$$^{3-}$ incorporation (40 μCi/ml) was measured as described in the legend to Fig. 3. Shown are means of double determinations ± SEM of a representative experiment.

TABLE 2. Cytotoxicity assay for Nifedipine and Octimibate, determined by the release of the fluorescent dye BCECF (21)

Concentration	Nifedipine	Octimibate
mol/l		
0	100	100
10^{-8}	98 ± 3^{a}	100 ± 4
10^{-7}	100 ± 2	96 ± 6
10^{-6}	96 ± 5	95 ± 7
10^{-5}	96 ± 3	99 ± 5

MPM were incubated for 5 h in the presence of Nifedipine or Octimibate in a concentration range between 10^{-8} and 5×10^{-5} mol/l, while control cells received only Hank's balanced salt solution. Total cellular BCECF was determined with 100 µg/ml saponin, present during the entire incubation. Calculation of cytotoxicity was done as described in Materials and Methods.

[a] Results given as % corrected cytotoxicity; mean of triplicate determinations ± SEM.

2 h of acetyl-LDL incubation, followed by a continuous decline in the following 16 h, leading to lower rates than in noncholesterol-loaded cells, as observed for $^{32}PO_4^{3-}$ and [^{3}H]choline. [^{14}C]Oleic acid incorporation was reduced upon cholesterol loading without any initial activation.

Two major factors may explain the different incorporation rates observed with phosphate, choline, and oleic acid. The labels enter their specific endogenous precursor pool, which is thought to be different in size for each precursor. Depending on this pool size, the sensitivity of the assay system varies (large pool→low sensitivity and vice versa). A change in the pool size during the experiments was not ascertained. Secondly, the transport kinetics by which each label enters the direct phospholipid precursor pool (phosphate→ATP, choline→CDP-choline, oleic acid→oleoyl-CoA) show a great variation. This is obvious from the different incorporation patterns obtained with phosphate, choline, and oleic acid (Figs. 2 and 3 vs Fig. 5) during cholesterol loading.

The reduction in the rate of phospholipid synthesis upon cholesterol loading without significant changes in the hydrolysis rate of fatty acids from preformed radiolabeled phospholipids indicates that the phospholipid synthetic pathway, rather than catabolism, must be modulated upon cholesterol loading, probably triggered by unesterified cholesterol which is liberated from the lysosomes. A simple dilution of the label by the incoming lipoproteins seems to be unlikely, because high concentrations of the precursors in the medium were used (200 µM oleic acid and 70 µM choline) or intermediate incubations were included (phosphate). The data obtained with the lysosomal blocker chloroquine clearly show that hydrolysis of lipoproteins is a necessary event for the initial activation of the phospholipid synthesis rate.

We suggest from these results that macrophages need phospholipids to liberate free cholesterol from lysosomes

and that, upon continuous cholesterol influx, MPM seem to be no longer able to maintain a high phospholipid synthesis rate. Similar observations have previously been made for VLDL secretion from hepatocytes, where PC synthesis is a prerequisite (8).

Another factor might be related to cellular ATP-requiring processes, which may become a limiting factor (25–27). However, in our experiments no distinct changes in cellular ATP levels were found upon cholesterol loading. The missing initial activation of cellular phospholipid synthesis upon acetyl-LDL incubation, measured with [^{14}C]oleic acid (Fig. 5), may indicate that fatty acid activation might become a rate-limiting step in macrophage phospholipid synthesis. This could be due either to an impaired transport of fatty acids to mitochondria or to a reduction in acyl-CoA synthetase activity. Alternatively, [^{14}C]oleoyl-CoA may be incorporated, in a competitive reaction, preferentially into cholesteryl esters. Consequently, the differences in the incorporation kinetics of phosphate, choline, and oleic acid are only explicable when exogenous phospholipids are degraded in part by a phospholipase C and the resulting diacylglycerides are reutilized. This reutilization phenomenon, the exchange of the polar headgroup, is detectable only with phosphate and choline, but not with oleic acid. The observation that the majority of the [^{14}C]oleate label from degraded phospholipids was found subsequently in cellular cholesteryl esters and triglycerides is of great interest, indicating that intracellular cholesteryl esters and phospholipid degradation may promote cytoplasmic cholesteryl ester and triglyceride accumulation, as we have recently observed in Tangier-macrophages (14). However, to answer this question more precisely, further experimental work focusing on phospholipid turnover is necessary.

HDL facilitates cellular cholesterol efflux which is clearly associated with an induction of phospholipid synthesis (Fig. 6). Parallel with the HDL-mediated increase in phospholipid synthesis, cholesteryl ester synthesis is reduced in MPM (Fig. 7).

The effects of the dihydropyridine Ca^{2+}-antagonist Nifedipine, which selectively induces SPM-synthesis, and the ACAT-inhibitor Octimibate, which enhances PC and SPM synthesis, are in accordance with the differences shown in our previous studies (2–5). Nifedipine down-regulates HDL-binding, enhances SPM synthesis, and promotes the formation of electron-dense cholesterol- and phospholipid-containing lamellar bodies, which originate from lysosomes. These lamellar bodies move towards the cell periphery, attach to the cell membrane, and release their lipid components into the cell membrane and into the extracellular medium. On the other hand, ACAT-inhibitors, such as Octimibate, up-regulate HDL-binding, enhance PC- and SPM-synthesis, and promote the formation of less electron-dense lamellar bodies, which

are formed from cytoplasmic lipid droplets upon attachment of endoplasmic reticulum (4, 5, 28). All these observations support the hypothesis that the synthesis of phospholipids seems to be a prerequisite for the formation of lamellar bodies derived from lysosomes and cytoplasmic lipid droplets.

Fig. 9 integrates previous data (4, 5) and the data from the present study, proposing a hypothetical model for the possible role of phospholipids in intracellular cholesterol trafficking. For normal macrophages it is deduced that the availability of phospholipids at the site of cholesterol release from lysosomes critically determines whether cholesterol is resecreted or accumulates in cholesteryl ester-containing lipid droplets. If SPM synthesis is high, cholesterol released from lysosomes is incorporated into lamellar bodies, which are directed to the plasma membrane and can be released into the surrounding medium (Fig. 9). If cholesterol associates with PC/cholesterol liposomes, drop-like structures are formed, which are detectable by electron microscopy (for details see ref. 5, Fig. 3C and D therein) and cholesterol will be directed to ACAT (Fig. 9). The rate-limiting step in the formation of cholesterol-containing particles destined for secretion seems to be the level of sphingomyelin biosynthesis. Con-
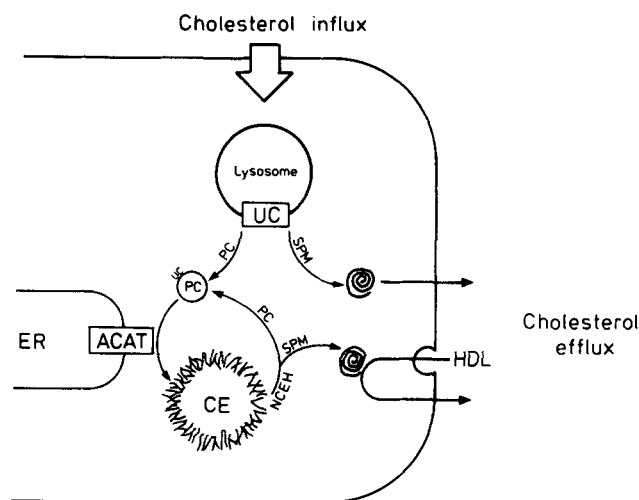
sequently most of the cholesterol is packed into PC liposomes and directed to ACAT, leading to the formation of foam cells. These cholesterol/PC liposomes represent a far better substrate for the ACAT reaction than cholesterol/SPM particles. It is of interest to note that also in Niemann-Pick type disorders cholesterol/SPM vesicles accumulate intracellularly (29–32). In whole cell assays Niemann-Pick type C and D cells have reduced ACAT activity (33) since most of the free cholesterol in the cytoplasmic compartment is associated with SPM in lamellar bodies. However, in cell homogenates, ACAT activity was found to be normal in these patients. A reduced substrate supply for ACAT by SPM/cholesterol lamellar bodies may provide an explanation of the observed low cholesteryl ester formation in Niemann-Pick type C disease. These cells seem to be defective in intracellular cholesterol and phospholipid processing, disrupting their cholesterol homeostasis (34–38).

In Tangier mononuclear phagocytes the cellular defect is associated with significant abnormalities in cellular phospholipid, triglyceride, and cholesteryl ester metabolism (14). Tangier mononuclear phagocytes express increased rates of synthesis of about 2-fold for phospholipids, about 5-fold for triglycerides, and about 3-fold for cholesteryl esters, as compared to normal mononuclear phagocytes. The turnover rate of cellular phospholipids was found to be enhanced while the turnover rates for triglycerides and cholesteryl esters were normal, leading to the formation of cytoplasmic cholesteryl esters and the failure of effective cholesterol release from lipid droplets.

It might be considered that the phospholipid composition of cholesterol-containing vesicles is important for their intracellular sorting. This has already been shown (39) for glycosphingolipids which are preferentially sorted and dispatched to the apical membranes in epithelial cells. This leads to an asymmetrical distribution of phospholipids between basolateral and apical cell membranes. It should be noted that the hypothetical model, deduced from the data of the present study and data from the literature, should be regarded as a working model, subject to further development as new findings emerge. ∎

Fig. 9. Hypothetical model for intracellular cholesterol trafficking and its effect on phosphatidylcholine and sphingomyelin synthesis. Lipoproteins taken up by the macrophage are degraded lysosomally. Unesterified cholesterol is liberated from the lysosome and usually directed towards ACAT in PC/cholesterol liposomes. The cholesterol moiety is esterified and stored in cytoplasmic lipid droplets. In cells treated with Nifedipine, SPM synthesis is induced and formation of SPM-rich lamellar bodies takes place. These particles leave the lysosomes and are directly secreted into the medium, because SPM-rich/cholesterol liposomes represent a poor substrate for ACAT. In cholesterol-loaded cells, HDL mediates the induction of phospholipid synthesis affecting only unesterified cholesterol liberated by the NCEH reaction from cytoplasmic lipid droplets. These cholesterol molecules are incorporated into cytoplasmic lamellar bodies which associate with internalized HDL. HDL is released as cholesterol-rich native particles into the medium. This is the major pathway upon inhibition of ACAT with Octimibate or other ACAT-inhibitors.

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