Cellular Cholesterol Stimulates Acute Uptake of Palmitate by Redistribution of Fatty Acid Translocase in Type II Pneumocytes†

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ABSTRACT: Cholesterol is an abundant lipid of lung surfactant, where its concentration changes relative to phospholipids in response to certain physiological conditions. We investigated the effect of the cellular cholesterol content on uptake and esterification of palmitic acid, and on cellular distribution of fatty acid translocase (FAT/CD36) in alveolar type II cells. Incubation of type II cells with methyl-β-cyclodextrin−cholesterol complexes increased the cholesterol content of lamellar bodies. The palmitate uptake of type II cells increased in parallel with the cellular cholesterol content. The content of FAT/CD36 increased in membranes and decreased in cytosol in type II cells. The detergent-insoluble fraction (DIGs), isolated from type II cells, was enriched in FAT/CD36 and caveolin-1 after increasing the cellular cholesterol. The total incorporation of labeled palmitic acid into glycerolipids and cholesterol ester (CE) increased by a factor of about 10 when the amount of unbound ^{14}C-palmitic acid added to type II cells was increased by a factor of about 1000. Under these conditions, a small but significant increase of the palmitate incorporation into PL occurred. Independent from the amount of added palmitate, palmitate incorporation into triacylglycerol decreased and palmitate incorporation into cholesterol ester increased about 40−65-fold. The β-oxidation of palmitate significantly decreased. We conclude that alveolar type II cells respond to an increase of the cholesterol level with (i) cellular redistribution of FAT/CD36 into DIGs causing enhanced palmitate uptake and increased cholesterol ester-formation, (ii) storage of cholesterol in lamellar bodies, and (iii) induction of the formation of caveolae-like microdomains in the surface membrane, a structure possibly involved in a lamellar body-independent efflux of free cholesterol via the high-density lipoprotein-specific pathway.

Type II pneumocytes synthesize, store, and secrete pulmonary surfactant, which prevents the collapse of the alveoli by reducing surface tension at the air−liquid interface. Surfactant is highly enriched in phospholipids, particularly dipalmitoyl phosphatidylcholine. After dipalmitoyl phosphatidylcholine, cholesterol is the most abundant lipid (*1*−*3*). Cholesterol is mostly supplied by plasma lipoproteins (*4*−*6*). Alveolar type II cells interact via specific receptors with low-density lipoprotein (LDL),[1] very low-density lipoprotein (VLDL), and high-density lipoprotein (HDL) (*7*). Adult type II cells express the classic LDL-receptor (*8*), the scavenger receptor B, type I (*9*) and at least two other types of specific HDL-binding proteins, HB2 and a membrane-bound metallodipeptidase (*10*). Receptors for the specific interaction of adult type II cells with VLDL have not been found. However, fetal pre-type II pneumocytes express gp 330 (*11*), and it has been shown that lipoprotein lipase seems to be involved in the mechanism of uptake of neutral lipids from VLDL (*12*). The inhibition of this process with alpha-2-macroglobulin and receptor associated protein suggests that the LDL-receptor related protein might be the receptor mediating the lipid uptake from VLDL (*13*). The importance of the individual lipoproteins for the supply of alveolar type II cells with cholesterol is presently not known.

The phospholipid/cholesterol ratio of alveolar surfactant responds to certain clinical conditions. Maternal diabetes induces a decrease of the phospholipid/cholesterol-ratio in the alveolar surfactant of neonates (*14*), whereas exercise (*15*), hyperpnea (*16*), and low body temperature (*17*) induce an increase of cholesterol relative to dipalmitoyl phosphatidylcholine. The mechanism(s) responsible for the modulation of the phospholipid/cholesterol-ratio is (are) unknown. Surfactant lipids and other components of the newly formed surfactant are intracellularly stored as lamellar bodies (LBs). Different metabolic possibilities are imaginable to regulate

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[1] Abbreviations: FAT, fatty acid translocase; HDL, high-density lipoprotein; LCFA, long chain fatty acid; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; DIG, detergent-insoluble fraction; MβCD, methyl β-cyclodextrin; TG, triacylglycerol; DG, diacylglycerol; CE, cholesterol ester; PhtCho, phosphatidylcholine; PL, phospholipids; LB, lamellar body; SSO, sulfo-*N*-succinimidyl oleate.

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the phospholipid/cholesterol-ratio in alveolar surfactant. First, type II cells may increase the receptor-mediated uptake of cholesterol from lipoproteins. Second, changes of the phospholipid/cholesterol-ratio may be due to changed secretion of cholesterol. Third, the cellular cholesterol level may alter the biosynthesis of surfactant phospholipids in type II cells.

The latter idea was supported by the effect of cholesterol on phospholipid formation in macrophages (*18*). The formation of phospholipids can be regulated by changing the activity of enzymes of the biosynthetic pathway or by changing the availability of substrates.

In adult type II pneumocytes, the biosynthesis of phospholipid strongly depends on the availability of long chain fatty acids (LCFAs). Maximal phospholipid synthesis can occur only in the presence of exogenous LCFAs (*19*, *20*), although LCFAs can be synthesized de novo within type II pneumocytes (*21*, *22*). Exogenous sources for LCFAs are triacylglycerols (TG) in lipoproteins or free LCFAs in plasma (*20*, *23*, *19*). The latter emphasizes the necessity for type II cells to transport LCFA across the plasma membrane. However, the transmembrane transport mechanism of LCFA is discussed controversially. In general, it is thought that LCFAs enter cells by a flip-flop mechanism (*24*). In addition, there are several lines of evidence that suggest increase of cellular LCFA uptake by another mechanism that exhibits the characteristics of a protein-facilitated process (*25*). Fatty acid translocase is the rat homologue of human CD36 (*26*) that was shown to mediate uptake of LCFA (*27*, *28*). Recently, we have shown that fatty acid translocase (FAT/CD36) is also expressed in alveolar type II cells (*29*).

Cellular redistribution of FAT/CD36 may play an important role in short-term regulation of metabolic effects mediated by this protein. First evidence of different intracellular localization of FAT/CD36 was given by Berger and co-workers in human platelets. They estimated the α-granule-associated pool of FAT/CD36 to be at approximately 25% of the total cell content (*30*). Interestingly, upon activation of platelets, FAT/CD36 was shown to be redistributed to the open canalicular system and pseudopods of the plasma membrane (*30*, *31*). Tassone et al. showed a rapid, transient increase of CD36 cell surface expression upon stimulation of peripheral blood lymphocytes by phytohemagglutinin, which was not due to altered protein synthesis (*32*). Recently, Bonen et al. associated cellular redistribution of FAT/CD36 with acute regulation of LCFA uptake by giant vesicles from skeletal muscle (*33*). However, detection of intracellular FAT/CD36 may also be due to processing of newly synthesized protein (*34*, *27*).

In the present study, we show that cyclodextrin derivatives can be used to manipulate the cellular cholesterol content of alveolar type II cells. We measured uptake, β-oxidation, and esterification of palmitic acid at different concentrations of cellular cholesterol, and determined the redistribution of FAT/CD36 between cytosol, plasma membranes, and detergent insoluble membrane fraction (DIG).

EXPERIMENTAL PROCEDURES

Reagents. All chemicals used were of analytical grade. 1,2-[^{3}H]cholesterol (specific radioactivity: 48.3 Ci/mmol) and 1-[^{14}C]palmitic acid (specific radioactivity: 56.0 mCi/mmol)) were purchased from NEN (Boston, MA). Methyl-β-cyclodextrin and 2-hydroxy-β-cyclodextrin were obtained from Sigma (Deisenhofen, Germany).

Preparation of Alveolar type II Cells. Following intraperitoneal injection of 30 mg of pentobarbital, the rat lungs (Wistar rats, body mass 80−90 g) were perfused blood-free, lavaged, and the alveolar type II cells were isolated according to the method of Dobbs et al. (*35*) by elastase digestion and "panning" cells on IgG-coated bacteriological plastic dishes. The isolated alveolar type II cells were suspended in Dulbecco's Minimal Essential Medium (DMEM; Gibco BRL, Life Technologies, Paisley, Scotland). Viability was judged by trypan blue staining. Purity was judged by Harris type hematoxylin staining of the isolated cells. Viability and purity ranged from 90 to 95% and 80−85%, respectively. All experiments were done with adherent type II cells.

Electron Microscopy. Adherent alveolar type II cells were incubated without (control) or with 5 mM MβCD cholesterol complex for 2 h at 37 °C. Thereafter, the cells were washed with DMEM followed by 0.1 M sodium phosphate buffer, pH 7.3, and finally fixed with 2.5% glutaraldehyde in the same buffer for at least 2 h at 4 °C. Carefully washed cell layers were postfixed for 1 h with 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.3 followed by three further washes. The fixed cells were scraped off, collected by gentle centrifugation, and embedded in low melting agarose. Processing was continued by dehydration through a series of methanol solutions, transfer to propylene oxide, a propylene oxide/resin mixture, and embedding in Epon resin according to standard procedures.

Finally, ultrathin sections were cut from resin blocks, mounted on 200 mesh copper grids and stained routinely with uranyl acetate and lead citrate. Sections were examined on a Zeiss EM 10 transmission electron microscope at 60 kV.

Manipulation of Cholesterol Content of Alveolar Type II Cells. Cholesterol loading of adherent alveolar type II cells with cyclodextrin−cholesterol complexes was carried out as described by Christian et al. (*36*). In brief, solution of different concentrations of both cyclodextrin derivatives in DMEM, were stirred at 37 °C overnight with cholesterol, and then filtered through a 0.45 μm filter (Millipore, Bedford, MA) to remove cholesterol crystals. Adherent type II cells in tissue culture dishes (35 mm diameter/well; 1×10^6 cell/well) were incubated with 2 mL of cholesterol, complexed with cyclodextrin derivatives, for 2 h at 37 °C. The cells were washed three times with DMEM and thereafter scraped off from the dishes and used for the subsequent analysis.

Measurement of Initial Palmitate Uptake. The uptake of [^{14}C]palmitic acid was carried out as described previously (*29*). In brief, adherent type II cells were washed twice with serum-free DMEM and then incubated at 37 °C with DMEM containing fatty acid-free bovine serum albumin (Sigma, Deisenhofen, Germany; 0.33 mg/mL) and [^{14}C]palmitic acid (22.7 nmol/mL). The molar ratio albumin to palmitic acid was 1:4.5, and the concentration of unbound palmitate was 40.6 nM (specific activity: 0.28 μCi/μmol) calculated according to Richieri et al. (*37*). The initial uptake of [^{14}C]-palmitate was measured for 30 s at 37 °C. Uptake was stopped by washing the cells with an ice-cold solution of fatty acid-free albumin in DMEM containing 0.2 mM phloretin. Subsequently, cells were lyzed by addition of 1

mL of 0.2% (w/v) sodium dodecyl sulfate (SDS). Then, the sample was dissolved in 8 mL of scintillation cocktail (Optiphase HI-Safe 2, Wallace, Finland), and radioactivity was measured by scintillation counting.

The inhibitory effect of sulfo-*N*-succinimidyl oleate (SSO) on the palmitate uptake by alveolar type II cells, was measured as previously described (*29*).

Measurement of Palmitate Incorporation into Type II Cell Lipids and of β-Oxidation of Palmitate. Adherent type II cells 10^6 per well, preincubated without or with different cholesterol MβCD complexes for 2 h, were cultured in the presence of 0.041 nmol of unbound palmitate (specific activity: 29.4 μCi/μmol) or 45.8 nmol of unbound palmitate (specific activity: 18.3 μCi/μmol), respectively, at 37 °C for 1 h. The cells were washed three times with DMEM, scraped off, and lysed, using Triton X-100 in water (final concentration 0.5%; v/v). One aliquot of the cell lysates was used for the determination of the protein concentration, another aliquot was used for the extraction of lipids according to Bligh and Dyer (*38*). After one-dimensional thin-layer chromatography (TLC) of the extracts on silica gel plates (Merck, Darmstadt, Germany), the lipid fractions were scraped off and radioactivity was measured as previously described (*39*).

When the β-oxidation of palmitate had to be measured, 1×10^6 adherent type II cell in plastic dishes (diameter: 35 mm) were preincubated with 5 mM MβCD−cholesterol complex for 2 h. Thereafter, 1.5 mL of PBS containing bovine serum albumin and 40.6 nM unbound 1-[^{14}C]palmitate (specific activity: 44.1 μCi/μmol) calculated according to Richieri et al. (*37*) was added. During the incubation for 1 h at 37 °C, the plastic dishes were placed into a bigger, tightly closed vessel containing filter paper soaked with hyamine hydrochloride (*40*). The filter papers were then placed into scintillation vessels, 8 mL of scintillation cocktail was added, and the radioactivity was counted. The plastic dishes containing the cells were washed twice with phosphate buffered saline (pH 7.4), and thereafter the cells were scraped off. Aliquots of the resuspended cells were used for the determination of protein.

Labeling of Cells and Isolation of Lamellar Bodies. Long-time incubation of cells with [^{14}C]palmitic acid and [^{3}H]-cholesterol causes an incorporation of labeled palmitate into complex lipids. These labeled complex lipids and labeled cholesterol equilibrate with the cellular pools of these lipid fractions. Therefore, determinations of the ^{14}C/^{3}H ratio of individual cell fractions can be used to determine whether the relation of complex lipids to cholesterol changes. In lamellar bodies, the complex lipids containing palmitate are mainly phospholipids.

When the incorporation of labeled palmitate and cholesterol into cells and lamellar bodies had to be determined, freshly isolated alveolar type II cells were cultured in the presence of trace amounts of [^{14}C]palmitic acid and [^{3}H]-cholesterol up to adherence for 18 h at 37 °C (equilibrium; control). When the incorporation of [^{3}H]cholesterol, newly taken up from cyclodextrin−cholesterol complexes, in total cells and lamellar bodies had to be determined, 20×10^6 type II cells were cultured in the presence of 20 μCi [^{14}C]palmitic acid for 18 h at 37 °C, the cells were washed with DMEM, and thereafter 2 mL of DMEM containing the MβCD−[^{3}H]cholesterol complex were added per well containing 2×10^6 cells and cultured for another 2 h at 37 °C. The cells were washed with DMEM, and the lamellar bodies were isolated by gradient centrifugation according to Oosterlaken-Dijksterhuis et al. (*41*). In total cells and in isolated lamellar bodies, the ^{14}C/^{3}H ratio was determined by scintillation counting. An enrichment of cholesterol in lamellar bodies has to be assumed, when the ^{14}C/^{3}H ratio of lamellar bodies was lower than the ^{14}C/^{3}H ratio of total cells.

Isolation of DIGs. For the isolation of DIGs, we followed the protocol described by Gorodinsky and Harris (*42*). In brief, 10^8 adherent type II cells, preincubated in the presence of or without MβCD−cholesterol complex (5 mM cyclodextrin), were washed with serum-free DMEM. Cells were lysed by the addition of 1 mL of lysis buffer (20 mM PIPES, pH 6.5, cont. 2 mM EDTA, 0.5 mM PMSF, 1 μg/mL pepstatin, 1 μg/mL leupeptin, and 1% (v/v) Triton X-100). The lysates were kept at 4 °C for 30 min, and then, one aliquot was mixed with the same volume of 80% (w/v) sucrose in lysis buffer. Two sucrose solutions (5 and 22%) were layered over the sucrose−lysate mixture and centrifuged (Beckman SW 41 rotor). After centrifugation at 39 000 rpm at 4 °C for 18 h, DIGs were enriched in the interphase between 22 and 5% (w/v) sucrose. The material was aspirated and diluted with four volumes of lysis buffer without Triton and centrifuged at 16000*g* at 4 °C for 20 min. The precipitated DIGs were resuspended in lysis buffer without Triton and aliquots of the resuspended DIGs and of the cell lysates were used for the determination of protein and total cholesterol content. We used other aliquots for the Western blot analysis and for the extraction of lipids according to Bligh and Dyer (*38*).

The lipid extracts of the cell lysates and DIGs were separated by two-dimensional thin-layer chromatography as previously described (*43*). The phosphatidylcholine (PhtCho) and sphingomyeline (Sph) spots were scraped off from the plates, and margarinic acid (C 17:0) was added to the silica gel as an internal standard. After extraction of the phospholipids from silica gel and transmethylation with methanol/HCl, the LCFA methyl ester were separated by gas chromatography (*39*).

Preparations of Membranes and Cytosol. Adherent type II cells were scraped off from the plastic dishes and disrupted by sonication (2×20 s, 150 W at 4 °C) in 250 mM Tris-HCl buffer, 1 mM EDTA, 1 mM EGTA, 0.32 M sucrose, pH 7.4 and protease inhibitors as were in lysis buffer. Thereafter, the sonicated cells were centrifuged at 105000*g* for 1 h. The supernatants were used for cytosol, and the pellets were used for cellular membranes as previously described (*44*).

Western Blot Analysis. The content of FAT/CD36 and caveolin-1 in cytosol, membranes, and DIGs were estimated by Western blot analysis using anti CD36 monoclonal antibody Mo25 which recognizes both human CD36 (*45*) and FAT (*33*), and rabbit polyclonal anti caveolin-1 antibodies (Transduction Laboratories, Lexington, KY) as outlined in previous communications (*9*, *10*).

Other Methods. We used Bradford's reagent (Sigma, Deisenhofen, Germany) for the determination of total protein, and followed the protocol of the manufacturer. Total cholesterol was determined using a cholesterol detection kit (Boehringer, Mannheim, Germany).

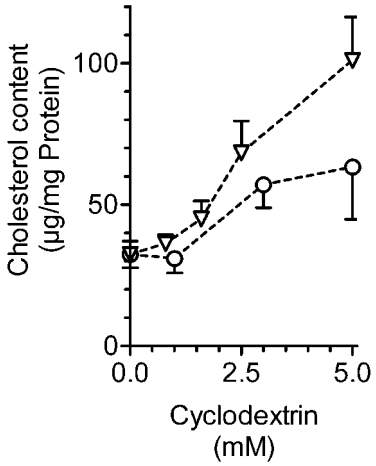

FIGURE 1: Effect of the incubation of adherent alveolar type II cells with cholesterol complexes of methyl-β-cyclodextrin and 2-hydroxy-β-cyclodextrin on the cellular cholesterol content. Legend: Alveolar type II cells were incubated with cholesterol complexes of methyl-β-cyclodextrin (triangles) or 2-hydroxy-β-cyclodextrin (circles) and the content of cellular cholesterol was determined (as described in Experimental Procedures). Values are given as mean with standard deviation of $n = 3$ independent experiments.

RESULTS

Manipulation of the Cholesterol Content in Alveolar Type II Cells. Methyl β-cyclodextrin (MβCD) and hydroxypropyl β-cyclodextrin complexed with cholesterol can be used to increase the cholesterol content of alveolar type II cells (Figure 1). Compared to MβCD, hydroxypropyl β-cyclodextrin is significantly less effective to increase the cellular cholesterol level (Figure 1).

Figure 2 shows electron micrographs of control and cholesterol-enriched type II cells. The morphometric analysis of electron microscopic images of type II cells (Table 1) showed that the areas of cells treated with 5 mM MβCD−cholesterol complex were bigger, and that the ratio of the sum of the areas of all lamellar bodies (LB) to the area of the cell nucleus increased significantly. These results indicate that cholesterol-enriched type II cells contained more and/or bigger lamellar bodies (Figure 2 and Table 1). We hypothesized that cellular cholesterol, taken up by adherent type II cells, is partly stored in lamellar bodies.

To prove this hypothesis, we isolated lamellar bodies from control and cholesterol-enriched cells that were preincubated with [^{14}C]palmitic acid and MβCD-[^{3}H]cholesterol. The relation of the ^{14}C/^{3}H ratio in LBs to the ^{14}C/^{3}H ratio in total cells suggested accumulation of cholesterol in LB (Table 3). In control cells, the ^{14}C/^{3}H ratio was higher in lamellar bodies than in the whole cell. This indicates that in relation to [^{14}C]-palmitic acid-labeled lipids, lamellar bodies contained less [^{3}H]cholesterol than total cells. In cholesterol-enriched cells, the ^{14}C/^{3}H ratio was lower in lamellar bodies than in total cells, suggesting that freshly incorporated [^{3}H]cholesterol accumulates in lamellar bodies. The ^{14}C/^{3}H ratio measured in the subfractions of the gradients where the lamellar bodies were located, showed only one peak, indicating that no special lamellar body-subfractions seems to be formed in response to an increase of the cellular cholesterol level in alveolar type II cells (results not shown).

Effect of Cholesterol Content on Uptake, Esterification, and β-Oxidation of Palmitic Acid by Alveolar Type II Cells.

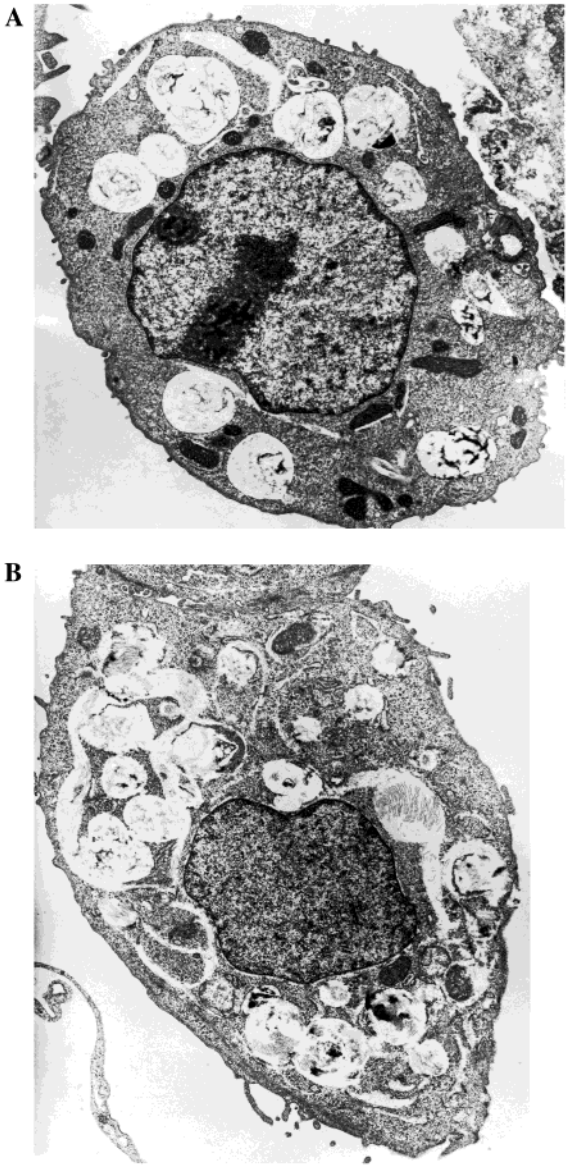

FIGURE 2: Electron microscopy of alveolar type II cells. Legend: Adherent alveolar type II cells were incubated without (A) or with 5 mM MβCD−cholesterol complex (B) for 2 h at 37 °C.

Table 1: Effect of Cellular Cholesterol Content on Size and Lamelar Body Content of Alveolar Type II Cells[a]

	control	cholesterol-enriched
area of alveolar type II cells (μm^2)	102 ± 22	128 ± 45
ratio of the sum of the LB-areas to the nucleus-area	0.8 ± 0.6	2.7 ± 2.0

[a] Alveolar type II cells were preincubated with 5 mM MβCD−cholesterol complex. Electron microscopic images of control and cholesterol-enriched alveolar type II cells ($n = 50$ per group) were digitized by using a flatbed scanner (EPSON Perfection) with a resolution of 500 pixel per micrometer. For interactive measurement of nuclear size and total area of LBs the AMBA/R image analysis system was applied (*54*). The cell areas and the ratio LB areas to nucleus area were significantly different between control and cholesterol enriched cells with at least $p < 0.05$ (Student t-test).

Table 3 shows the effect of the cellular cholesterol content on the initial uptake of [^{14}C]palmitic acid. Doubling the cellular cholesterol content caused an about 4-fold increase

Table 2: Effect of the Cellular Cholesterol Content on the Lamellar Body Composition[a]

MβCD (mM)	cholesterol content (μg (mg protein)$^{-1}$)	[^{14}C] palmitic acid/[^{3}H] cholesterol ratio		difference of ratios
		total cell	LBs	Δ ratio
0	30.7	4.8	5.9	+1.1
0	34.9	5.7	8.1	+2.4
0.8	47.3	15.6	10.9	−4.7
2.5	63.3	12.3	8.0	−4.3

[a] Alveolar type II cells were cultured in the presence of [^{14}C]palmitic acid for 18 h. Thereafter, the prelabeled cells were incubated without (control) or with a MβCD−[^{3}H]cholesterol complex for 2 h. The cellular lamellar bodies (LBs) were isolated by gradient centrifugation (see Experimental Procedures).

Table 3: Effect of the Cellular Cholesterol Content on the Initial Uptake of [^{14}C]Palmitic Acid[a]

preincubation with MβCD (mM)	cholesterol content (μg mg of protein^{-1})	[^{14}C] palmitic acid uptake (pmol mg of protein^{-1} 30 s^{-1})
control	32.4 ± 4.7	10.0 ± 0.2
0.8	36.2 ± 3.1	16.4 ± 1.0
1.6	44.9 ± 6.4	24.6 ± 1.6
2.5	69.3 ± 11.3	43.6 ± 3.9
5.0	101.0 ± 15.4	48.0 ± 3.6

[a] Adherent alveolar type II cells were incubated in the presence of cholesterol−MβCD complexes for 2 h. The cells were washed and then incubated in the presence of [^{14}C]palmitic acid for 30 s (see Experimental Procedures). The data are means ± standard deviation of n = 3 experiments.

of initial [^{14}C]palmitic acid uptake. Preincubation of cholesterol-enriched type II cells (containing 80 ± 10 μg of cholesterol/mg of protein) with SSO (final concentration 0.5 mM) reduced the palmitate uptake to the level of control cells [from 28.0 ± 0.4 to 8.5 ± 0.3 pmol (mg of protein)$^{-1}$ 30 s^{-1} in cholesterol-enriched cells; the uptake of control cells was 8.9 ± 0.9 pmol (mg of protein)$^{-1}$ 30 s^{-1}].

Increase of the cellular cholesterol content did not significantly change the incorporation of [^{14}C]palmitic acid into diacylglycerol (DG) and phospholipids, whereas the incorporation into triacylglycerol (TG) significantly decreased, and the formation of cholesterol ester (CE) increased by the factor 40 (Table 4). As a result, the CE content significantly increased in alveolar type II cells from 0.023 ± 0.002 μmol/mg of protein (n = 3) in control to 0.057 ± 0.004 μg/mg of protein (n = 3) in cholesterol-enriched type II cells. However, in these incorporation experiments unbound palmitate was added in the same concentration as used in the uptake experiments. It is possible that in relation to the high cellular content of cholesterol, the addition of 0.04 nmol of unbound palmitate/10^{6} cells was sufficient to increase the esterification of free cholesterol but too small to increase the incorporation into PL. Therefore, we increased the added amount of exogenous palmitate to 45.8 nmol of unbound palmitate/10^{6} cells. This amount of palmitic acid exceeds the highest level of cholesterol in type II cells by the factor of about 2. Incubation of type II cells with the higher amount of unbound ^{14}C-palmitic acid produced a small but significant increase in the labeling of PL, whereas the labeling of TG decreased to 50% of control. Incorporation of palmitic acid into CE further increased about 65-fold. Accordingly, the cellular phospholipid content increased (not

Table 4: Effect of the Cellular Cholesterol Content on the Incorporation of [^{14}C]Palmitic Acid into Lipids of Alveolar Type II Cells[a]

cholesterol content (μg mg of protein^{-1})	[^{14}C]palmitic acid incorporation (nmol (mg of protein)$^{-1}$ h^{-1})			
	PL	DG	TG	CE
32.4 ± 4.7	2.1 ± 0.3	0.4 ± 0.1	0.29 ± 0.01	0.012 ± 0.007
36.2 ± 3.1	1.8 ± 0.2	0.3 ± 0.1	0.22 ± 0.03	0.010 ± 0.002
44.9 ± 6.4	1.9 ± 0.4	0.3 ± 0.1	0.19 ± 0.03	0.11 ± 0.01
69.3 ± 11.3	2.1 ± 0.2	0.3 ± 0.1	0.12 ± 0.01	0.40 ± 0.02
101.0 ± 15.4	2.0 ± 0.1	0.3 ± 0.2	0.07 ± 0.02	0.49 ± 0.05
26.9 ± 5.3	14.1 ± 0.6	3.6 ± 0.1	3.0 ± 0.2	0.11 ± 0.03
71.6 ± 3.6	17.2 ± 0.3	3.4 ± 0.1	1.6 ± 0.1	7.2 ± 0.3

[a] Adherent alveolar type II cells were incubated in the presence of MβCD−cholesterol complexes for 2 h. The cells were washed and incubated in the presence of 0.04 nmol (lines 1 to 5) and 45.8 nmol (lines 6 and 7) unbound [^{14}C]palmitic acid for 60 min (see Experimental Procedures). The data are given as means ± standard deviation of n = 3 experiments.

Table 5: Effect of the Cellular Cholesterol Content on the Lipid Composition of Cell Lysates and DIGs[a]

lipid fraction (based on mg of protein)	cell lysates		DIGs	
	control	MβCD	control	MβCD
cholesterol (μmol)	0.08 ± 0.01	0.26 ± 0.04	3.78 ± 0.24	7.47 ± 0.51
PtdCho (nmol)	528 ± 89	609 ± 100	2377 ± 170	1315 ± 325
Sph (nmol)	153 ± 22	226 ± 85	496 ± 90	589 ± 164
fatty acid patterns of PtdCho (percent of total)				
16:0	53.1 ± 1.8	61.5 ± 6.0	84.0 ± 0.6	81.2 ± 6.4
16:1	5.4 ± 1.5	5.1 ± 1.6	3.3 ± 0.8	1.6 ± 1.3
18:0	10.2 ± 2.8	11.9 ± 2.3	5.1 ± 1.0	10.2 ± 2.2
18:1	7.4 ± 1.1	5.5 ± 0.8	4.0 ± 1.2	2.0 ± 1.6
18:2	8.4 ± 1.0	4.9 ± 2.3	2.7 ± 0.8	1.2 ± 0.7
20:4	11.4 ± 0.7	5.6 ± 3.8	1.7 ± 0.6	<1
22:6	1.5 ± 0.6	<1	<1	<1
ratio saturated/ unsaturated FA	1.4 ± 0.2	3.6 ± 1.3	7.0 ± 1.7	8.4 ± 2.2

[a] LCFA below 1% are not listed. The data are means ± standard deviation of n = 3 experiments. PtdCho − phosphatidylcholine; Sph − sphingomyeline; LCFA are specified by their number of carbon atoms and number of carbonEnDash−carbon double bounds.

significantly) from 776 ± 145 nmol/mg of protein (n = 3) to 901 ± 108 nmol/mg protein (n = 3). The pool size of endogenous palmitic acid in alveolar type II cells (3 to 5 nmol/mg of protein) did not change by the cholesterol-induced increase of [^{14}C]palmitate uptake (results not shown).

Effect of Cholesterol Content on FAT/CD36 Association with Membranes of Alveolar Type II Cells. To elucidate the role of FAT/CD36 in palmitate uptake, we studied the effect of cellular cholesterol on its translocation between cytosol and membranes. Treatment of alveolar type II cells with MβCD-cholesterol caused a shift of FAT/CD36 from cytosol into membranes. In relation to control, DIGs isolated from cholesterol-enriched cells, showed an increase of FAT/CD36 and caveolin-1 (Figure 4). The total amount of DIG-protein isolated from 10^{8} type II cells increased from 22 ± 14 μg (control) to 64 ± 32 μg (n = 8) in response to cholesterol loading. The lipid composition of DIGs showed high concentration of cholesterol and Sph, as expected for

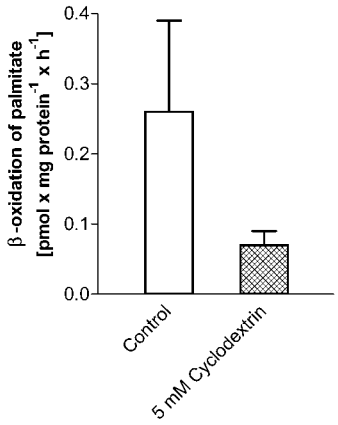

FIGURE 3: Effect of the incubation of adherent alveolar type II cells with cholesterol complexes of methyl-β-cyclodextrin on β-oxidation of palmitate. Legend: Alveolar type II cells were incubated with the methyl-β-cyclodextrin cholesterol complex. Thereafter, the cells took up 1-[^{14}C] palmitic acid. Labeled CO_2 which derived from 1-[^{14}C] palmitic acid was determined to estimate β-oxidation (see Experimental Procedures). Values are given as mean with standard deviation of $n = 3$ independent experiments. The difference of the values was significant with $p < 0.05$ (Student t-test).

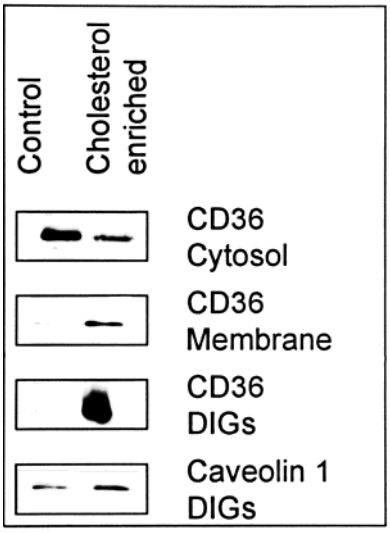

FIGURE 4: Western blot analysis of FAT/CD36 and caveolin-1 in different fractions of control and cholesterol loaded alveolar type II cells. Legend: The figure shows typical results of the Western blot analysis of different type II cell fractions. A total of 20 μg of cytosol protein, 20 μg of membrane protein, and 2.5 μg of DIG protein was applied per lane for the Western blot analysis of FAT/CD36. For the Western blot analysis of caveolin-1, we used the same protein concentration. Caveolin-1 was not detectable in cytosol and membranes under these conditions.

caveolae. The fatty acid pattern of PhtCho suggests the presence of disaturated species, that are more concentrated in DIGs than in lysates.

DISCUSSION

Cyclodextrins are cyclic oligosaccharides consisting of 7-β glucopyranose units. They have been used to deplete (*46*) and to enrich (*36*) cholesterol in a variety of cells in culture. Here we show that cholesterol complexes of both, MβCD and hydroxypropyl β-cyclodextrin can be used to increase the cholesterol content in alveolar type II cells. The measurement of the [^{3}H]cholesterol/[^{14}C]palmitic acid ratio in lamellar bodies and the electron micrographs of type II cells indicate that cholesterol, taken up from MβCD−cholesterol complexes, is mostly stored in lamellar bodies.

Schmitz et al. (*18*) showed that cholesterol loading of macrophages induces an increase of the synthesis of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, and other phospholipid classes. Likewise, cholesterol loading of alveolar type II cells increased the initial uptake of palmitic acid (Table 2). However, the incorporation of palmitate into DG and PL did not increase, and incorporation into TG and the β-oxidation decreased significantly. The incorporation of palmitic acid into CE increased in cholesterol-loaded type II cells, possibly because of allosteric activation of acyl CoA: cholesterol acyltransferase. Newly formed CE is in rapid equilibrium with the regulatory pool of free cellular cholesterol (*48*). The total incorporation of labeled palmitic acid into glycerolipids and CE increased by the factor of about 10 when the amount of unbound ^{14}C-palmitic acid added to type II cells was increased by the factor of about 1000. Under these conditions, a small but significant increase of the palmitate incorporation into PL occurred. We conclude that a high level of cellular cholesterol can induce a small increase of the surfactant lipid synthesis in type II cells only when high amounts of free fatty acids are available.

Increase of CE formation and elevated cholesterol level in type II cells were associated with an enhanced uptake of palmitate. In contrast, the pool size of endogenous palmitic acid did not change. Apparently, when the cellular cholesterol content increased above the physiological level, exogenous palmitate is primarily used for the formation of CE. This idea is corroborated by the observed decrease of TG formation and β-oxidation in the cholesterol-loaded cells. These data raise the question as to whether there is a cholesterol-induced pathway of palmitate uptake. Previously, we showed that FAT/CD36, a putative transmembrane fatty acid transporter, is expressed in alveolar type II cells (*29*). Now we show that in response to an increase of cellular cholesterol, FAT/CD36 was translocated from cytosol to membranes of type II cells (Figure 4). This provides a potential mechanism for the regulation of palmitate uptake. Recently, it was shown that fatty acid uptake induced by muscle contraction is short-term regulated by cellular redistribution of fatty acid translocase (*33*).

There are some arguments suggesting that caveolae and DIGs represent the same subcellular compartment (*50*). On the other hand, it has been shown that DIGs can be isolated from cells that do not have caveolae (*50*). Immunocytochemical studies demonstrated the presence of caveolin-1 in alveolar type II cells, but there is no ultrastructural evidence for the existence of caveolae (*51*).

Our results show that the lipid composition of DIGs isolated from type II cell was caveolae-like as far as cholesterol and sphingomyelin are concerned. Saturated PtdCho was artificially co-isolated in this fraction (Table 4). FAT/CD36 and caveolin-1, both marker-proteins of caveolae-like membrane structures, were enriched in DIGs isolated from cholesterol-loaded type II cells. In contrast, the expression of FAT/CD36 is very low and caveolin-1 could not be detected in membranes of type II cells.

In summary, the present data suggest that alveolar type II cells respond to a rise of their cellular cholesterol content with the redistribution of FAT/CD36. This may represent a

mechanism of short-term regulation of palmitate uptake and CE formation. Cholesterol-loaded type II cells form cholesterol-enriched lamellar bodies, and induce formation of caveolae-like microdomains in the surface membrane which might be involved in a lamellar body-independent efflux of free cholesterol. High levels of cellular cholesterol did not induce a proportional increase of PL synthesis. It appears that the content of PL and cholesterol in alveolar surfactant can be regulated independently of each other. This idea is corroborated by previous results (*53*) showing that vitamin E, another surfactant lipid, can be secreted as lamellar body-constituent as well as independently from lamellar bodies.

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