Fatty acid translocase/CD36 mediates the uptake of palmitate by type II pneumocytes

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Guthmann, Florian, Renate Haupt, A. Cornelis Loo man, Friedrich Spener, and Bernd Rüstaw. Fatty acid translocase/CD36 mediates the uptake of palmitate by type II pneumocytes. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L191–L196, 1999.—Type II pneumocytes, which synthesize, store, and secrete pulmonary surfactant, require exogenous fatty acids, in particular palmitic acid, for maximum surfactant synthesis. The uptake of palmitate by type II pneumocytes is thought to be protein mediated, but the protein involved has not been characterized. Here we show by RT-PCR and Northern blot analysis that rat type II pneumocytes express the mRNA for fatty acid translocase (FAT/CD36), a membrane-associated protein that is known to facilitate the uptake of fatty acids into adipocytes. The deduced amino acid sequence from rat type II pneumocytes reveals 98% identity to the FAT/CD36 sequence obtained from rat adipocytes. The uptake of palmitate by type II pneumocytes follows Michaelis-Menten kinetics (Michaelis-Menten constant = 11.9 ± 1.8 mM; maximum velocity = 62.7 ± 5.8 pmol·min⁻¹·5 × 10⁵ pneumocytes⁻¹) and decreases reversibly under conditions of ATP depletion to 35% of control uptake. Incubation of cells at 0°C inhibited the uptake of palmitate almost completely, whereas depletion of potassium was without effect. Preincubation of the cells with bromobimane or phloretin decreases the uptake of palmitate significantly as does preincubation with sulfo-N-succinimidyl olate, the specific inhibitor of FAT/CD36 (C. M. Harmon, P. Luce, A. H. Beth, and N. A. Abumrad, J. Membr. Biol. 121: 261–268, 1991). From these data, we conclude that FAT/CD36 is expressed in type II pneumocytes and mediates the uptake of palmitate in a saturable and energy-dependent manner. The data suggest that the uptake process is independent of the formation of coated pits and endocytotic vesicles.

Sources as free fatty acids or in the form of triacylglycerols in lipoproteins (4, 8, 15, 22). The importance of the various sources of fatty acids is not well understood and may vary with nutritional status and development (for a review, see Ref. 3). For instance, inhibitors of fatty acid biosynthesis decrease the rate of DPPC synthesis in explants of fetal rat lung even when palmitate is added (28), but in adult pneumocytes, maximum DPPC synthesis is seen only in the presence of exogenous fatty acids (4, 6, 22). The latter emphasizes the necessity for these cells to transport fatty acids across the plasma membrane.

How long-chain fatty acids move across the membrane has been a subject of controversial discussion. Because of their lipophilic character, they are thought to diffuse freely through the plasma membrane of cells as has been shown for fatty acid methyl esters (19). This concept was challenged by the finding of a rapid and saturable uptake that was reduced by a preceding heat denaturation or protease treatment of cells (36). Several plasma membrane-associated proteins capable of binding fatty acids were isolated from various tissues and cells. Most proteins identified as fatty acid transporters by diverse methods were initially described in adipose tissue and liver (11, 14, 32, 35; for a review, see Ref. 31). A plasma membrane-associated fatty acid transporter of type II pneumocytes has not been identified so far. However, from the results of Maniscalco et al. (23), which show a saturable fatty acid uptake by these cells, a protein-mediated transport across the membrane of type II pneumocytes might be hypothesized.

Once internalized, long-chain fatty acids are intracellularly solubilized and translocated bound to cytoplasmic fatty acid binding proteins (FABPs). The FABP binding of fatty acids maintains a gradient between extracellular and intracellular compartments; thus FABPs can stimulate the uptake of fatty acids (33, 37). Recently, Guthmann et al. (13) have shown that type II pneumocytes express the epidermal type of FABP.

Based on the rationale that fatty acid translocase (FAT), a member of the CD36-receptor family (18), is coexpressed with FABP for fatty acid import (25), we considered this protein to be a strong candidate for the putative fatty acid receptor of rat type II pneumocytes. Thus, in this study, we address the identity of the fatty acid receptor in these cells and examine its role in the mediation of cellular fatty acid import.

METHODS

Materials. Oligonucleotides used for amplification of FAT cDNA from alveolar type II cells were purchased from MWG Biotech (Ebersberg, Germany). The cDNA coding sequence

TYPE II PNEUMOCYTES synthesize, store, and secrete pulmonary surfactant, a phospholipid-protein complex that prevents the collapse of the alveoli by reducing surface tension at the air-liquid interface. Surfactant is highly enriched in phospholipids, particularly dipalmitoylphosphatidylcholine (DPPC). The high rate of surfactant phospholipid synthesis requires sufficient sources of long-chain saturated fatty acids. On one hand, these fatty acids are synthesized de novo within the type II pneumocyte, in which lactate is the preferred substrate compared with glucose (5, 12, 21); on the other hand, they are obtained from exogenous fatty acid uptake; uptake kinetics; lung

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was amplified in four overlapping fragments with the following primer sets (nucleotide (nt) numbering according to Abumrad et al. (1)): fragment 1 (nt 50–633), 5’-TGATTGCTGC- TGGCACGAGGAG-3’ and 5’-AAGAAGTTGATCTTGTTGAC- CCCAC-3’, annealing temperature 54°C; fragment 2 (nt 547–1059), 5’-GACAAGCTTATCAAAAAAGCT-3’ and 5’-GC- ACACATACACGCTACAG-3’, annealing temperature 54°C; fragment 3 (nt 992–1334), 5’-ACTCAAGACCAGGACAC- CAC-3’ and 5’-TCCCAAGTCTATTTAGGCCACAG-3’, annealing temperature 62°C; and fragment 4 (nt 1173–1518), 5’-ATAGGACATACTTGGATGTGG-3’ and 5’-CTGACATAA GTAGTCTATC-3’, annealing temperature 54°C. Sulfo-N-succinimidyl deitate (SSO) was prepared with the method of Harmon et al. (14); bromobimane (p-sulfobenzoxyloxybromobimane) was obtained from Calbiochem. Nigericin, valinomycin, and all other chemicals (analytic grade) were from Sigma (Deisenhofen, Germany) unless otherwise stated.

Primary culture of type II pneumocytes. Wistar rats (body weight 100–120 g) were from a local animal facility. Alveolar type II cells were isolated by elastase digestion and by “panning” cells on immunoglobulin G-coated bacteriological plastic dishes as described by Dobbs et al. (9). Viability was judged by trypan blue staining and purity by Harris-type hematoxylin staining of the isolated pneumocytes and ranged from 90 to 95 and 87 to 93%, respectively. All experiments were done after 18 h of pneumocyte culture.

Detection of FAT mRNA by PCR. Total RNA from alveolar type II cell pellets was isolated as described by Chomczynski and Sacchi (7), and cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (GIBCO, Eggenstein, Germany) and random priming. PCR was performed with Taq DNA polymerase (Promega, Heidelberg, Germany) and primers as specified in Materials at melting, annealing, and extension temperatures of 94, 54–62, and 72°C, respectively. After 35–40 cycles, PCR products were visualized by ethidium bromide staining in 1.5% agarose gels.

Sequence analysis. PCR fragments amplified from type II pneumocyte cDNA were purified from 1% agarose gels with the Qiagen protocol (Qiagen, Hilden, Germany). The fragments were sequenced from both strands with the primers as indicated in Materials in a cycle sequence reaction with biotinylated dideoxynucleotides (GATC, Constance, Germany) and Thermo Sequenase (Amersham, Braunschweig, Germany). Terminated fragments were separated on a direct-blotting sequencing apparatus (GATC) and visualized by developing the blot with streptavidin-coupled alkaline phosphatase, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate toluidin.

Northern blotting. For the generation of radiolabeled single-strand DNA probes, the amplified PCR fragment 2 was used as a template in asymmetric 33-cycle PCR (16) with 0.26 µM [32P]dCTP (3,000 Ci/mmol; ICN Biomedicals, Eischwege, Germany). The labeled probe was separated from the deoxynucleotidetriphosphates by chromatography on a Sephadex G-50 column (Pharmacia). RNA was isolated (7) from freshly prepared type II pneumocytes, and 10 µg of RNA were separated on a 1% formaldehyde-agarose gel and transferred overnight onto Hybond-N nylon membranes (Amersham) by capillary blotting (30). The blots were incubated (2 h) with prehybridization buffer [350 mM Na2HPO4, 7% (wt/vol) SDS, 30% deionized formamide, and 1.0% (wt/vol) BSA fraction V] and hybridized (overnight at 50°C) with the DNA probe. The membranes were washed twice with 150 mM Na2HPO4 containing 0.5% (wt/vol) SDS (10 min at 25°C) and once with 30 mM Na2HPO4 containing 0.1% (wt/vol) SDS (10 min at 55°C) and were finally exposed to Kodak Biomax MS X-ray film (Integra Biosciences, Fernwald, Germany) at −80°C.

Measurement of palmitate uptake. Freshly isolated type II pneumocytes were plated at a density of 5 × 104 pneumocytes/well onto 24-mm tissue culture dishes for 18 h at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (wt/vol) fetal calf serum. Pneumocytes were washed twice with serum-free DMEM and then incubated with DMEM containing BSA and [3H]palmitic acid at 37°C. To this end, 460 µl of palmitic acid (1 mg/ml) with trace [3H]palmitic acid (60 µl, 1 Ci/ml; Amersham), each dissolved in ethanol, were added to Celite (Supelco), the ethanol was evaporated under nitrogen, and 8 ml of DMEM and 880 µl of BSA (500 µM in phosphate-buffered saline, fraction V; Boehringer Mannheim) were added to the dried palmitic acid-Celite mixture. After incubation for 1 h at 37°C, Celite was removed by centrifugation, the supernatant was recentrifuged (1,500 g for 10 min), and its volume was adjusted to 100 ml by adding DMEM. The concentration of unbound palmitate was calculated according to Richieri et al. (29). The concentration of BSA remained unchanged at 4.4 µM in all experiments, whereas the ratio of palmitic acid to BSA varied from 1 to 5.

Unless otherwise stated, the concentration of unbound palmitate was 40.6 nM in the incubation medium, and pneumocytes were incubated for 30 s at 37°C. Thereupon, the incubation medium was removed, and stop solution was added for ~30 s (2 ml/well) of ice-cold DMEM containing 0.1% (wt/vol) BSA and 0.2 mM phosphoretin and subsequently replaced with ice-cold DMEM containing 0.1% (wt/vol) BSA only. After 30 s, the medium was removed, and viability of the pneumocytes was judged with trypan blue. Cells were lysed by adding 1 ml of 0.2% (wt/vol) SDS. This sample was finally dissolved in 8 ml of scintillation cocktail (Optiphase HI-Safe 2, Wallac), and radioactivity was counted.

ATP depletion. Cells were ATP depleted as previously described (24). Briefly, adherent type II pneumocytes were washed twice with buffer [140 mM NaCl, 20 mM HEPES (pH 7.4), 1 mM CaCl2, and 1 mM MgCl2 containing 5 mM NaN3 and 50 mM 2-deoxyglucose (ATP depleted) or without NaNO3 but containing 50 mM glucose (control). Subsequently, the pneumocytes were incubated for 30 min at 37°C either with NaNO3-containing buffer or with buffer without NaNO3 as described above.

In a separate experiment, type II cells were allowed to recover after ATP depletion. The cells were once washed and incubated for 60 min with NaCl-P, containing 5 mM sodium cyanide and 50 mM 2-deoxyglucose (ATP depleted) or 50 mM glucose (control). The cells were allowed to recover during incubation in the presence of DMEM containing 50 mM glucose for 90 min. Initial uptake of palmitate was measured after ATP depletion and after recovery of the cells as described in Measurement of palmitate uptake.

Temperature. Adherent type II pneumocytes were preincubated at 37 (control) or 0°C for 20 min, and uptake of palmitate was measured as described in Measurement of palmitate uptake but at the respective temperature.

Potassium depletion. Adherent type II pneumocytes were depleted of potassium. Cells were washed three times with buffer containing 140 mM NaCl, 20 mM HEPES (pH 7.4), 1 mM CaCl2, 1 mM MgCl2, and 5.5 mM D-glucose with 10 mM KCl (control) or without KCl (potassium depleted). Subsequently, the cells were incubated for 5 min at 37°C with or without potassium-containing buffer, respectively, now diluted 1:1 with distilled water in either case. Before measurement of palmitate uptake, the cells were again washed three times with the above isotonic buffer with or without potassium, respectively.

Potassium ionophores. Type II cells were incubated with DMEM containing 3 µM nigericin for 60 min or with DMEM
containing 3 µM valinomycin for 20 min. Subsequently, initial uptake of palmitate was measured as described in Measurement of palmitate uptake.

RESULTS

Identification and expression of FAT/CD36 in type II pneumocytes. Using primers specific for FAT/CD36, we obtained DNA fragments migrating as single bands of the expected sizes on an agarose gel (data not shown). We investigated the expression of FAT/CD36 thus identified in type II pneumocyte RNA with Northern blotting. Total RNA obtained from type II pneumocytes was blotted and hybridized with the FAT/CD36 probe. As shown in Fig. 1, a single band was detected at ~2.9 kb, the size reported for FAT/CD36 from rat adipocytes (1).

Sequence analysis. Analysis of four overlapping PCR fragments obtained by respective cDNA amplification from Wistar rat type II pneumocytes resulted in a sequence of 1,468 bp, which covers the entire protein coding sequence and 23 bases upstream as well as 29 bases downstream.

Fig. 1. Expression of fatty acid translocase (FAT) in type II pneumocytes. RNA (10 µg/lane) was analyzed by Northern blotting and probed with 32P-labeled fragment 2 of FAT cDNA. 18S and 28S rRNA, respectively.

Fig. 2. Time course of palmitate uptake in adherent type II pneumocytes. Before assay, pneumocytes were maintained at 37°C for 18 h in FCS-containing DMEM and then washed with serum-free DMEM twice. Uptake of [3H]palmitate was assayed at 37°C with a concentration of unbound palmitate of 40.6 nM. After 15–300 s, uptake was stopped by removing medium and adding stop solution. Pneumocytes were then washed with ice-cold DMEM-albumin and lysed in 1 ml of 0.2% SDS before radioactivity was measured.

Fig. 3. Kinetics of [3H]palmitate uptake. Initial uptake of [3H]palmitate was assayed at 37°C for 30 s with concentrations of unbound palmitate from 5.1 to 97.9 nM, determined according to Richieri et al. (29). Albumin concentration remained constant. Inset: Lineweaver-Burk plot of the same data. Linear regression revealed a Michaelis-Menten constant of 11.9 ± 1.8 nM, and maximum velocity was 62.7 ± 5.8 pmol·min⁻¹·5 × 10⁶ pneumocytes⁻¹ (r² = 0.98). Values are means ± SD of n = 4 experiments.

Fig. 4. Influence of temperature, ATP depletion (~ATP), and potassium depletion (~K⁺) on initial palmitate uptake rates. Uptake of [3H]palmitate was assayed for 1 min at a concentration of unbound palmitate of 40.6 nM as described in METHODS. Values are means ± SD of n = 3 or 2(*) experiments.
Uptake kinetics. Uptake of palmitate by adherent type II pneumocytes was saturable and followed Michaelis-Menten kinetics (Fig. 3), with a Michaelis-Menten constant of 11.9 \pm 1.8 \text{nM}. This value is \text{lower} than values found for the uptake of oleate into hepatocytes (34) and of palmitate into rabbit type II pneumocytes (23), respectively. Maximum velocity was 62.7 \pm 5.8 \text{pmol} \text{palmitate-min}^{-1}.5 \times 10^5 \text{type II pneumocytes}^{-1}, again lower than the maximum velocity for oleate uptake by hepatocytes (30) but almost the same velocity as that shown by rabbit type II pneumocytes (23).

Lowering the temperature to 0°C diminished the initial palmitate uptake to 7% of the control value (Fig. 4), and ATP depletion of the cells by sodium azide significantly lowered palmitate uptake to 28% of that for nondepleted cells (Fig. 4). To substantiate the lack of nonrelated toxic or irreversible changes as the cause of decreased palmitate uptake, we let cells recover as described in METHODS. The initial palmitate uptake of cyaniode-treated type II pneumocytes decreased to 59% of that of the control uptake. Recovery of the cells for 90 min restored the palmitate uptake rate nearly to that of the control uptake (Fig. 5). These data clearly show that movement of palmitate across the membrane of type II pneumocytes is an energy-dependent process.

The cellular uptake via the endocytic pathway depends on the intracellular concentration of potassium. We tested the effect of potassium depletion on palmitate uptake by type II pneumocytes and found that there was no significant difference between control and depleted cells (Fig. 4). In addition, incubation of the cells for 0–300 s with DMEM containing \([\text{3H}]\)palmitate was determined (open bar) as described in METHODS. Cells were allowed to recover in presence of DMEM containing 50 \text{mM} glucose for 90 min before palmitate uptake was measured (solid bar). Values are means \pm SD of \(n = 2\) experiments.

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<th>Table 1. Inhibition of palmitate uptake by bromobimane</th>
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<td>Control</td>
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Values are means \pm SD in percent of control uptake; \(n = 3\) experiments. Type II pneumocytes were incubated for 2 h at 37°C in presence of bromobimane. Uptake of \([\text{3H}]\)palmitate was assayed at 37°C for 30 s at a concentration of unbound palmitate of 40.6 \text{nM}.
cells with nigericin or valinomycin, both potent potassium ionophores, was without significant effect on initial palmitate uptake (Fig. 4). This indicates that palmitate uptake is independent of the formation of coated pits and endocytotic vesicles.

Inhibition of palmitate uptake by bromobimane. In a more direct experimental approach to prove the involvement of membrane-bound proteins in fatty acid uptake, we incubated type II pneumocytes with bromobimane (Table 1). Bromobimane reacts with thiol groups of membrane-bound proteins (17) and might inhibit in this way receptor-mediated transport processes. In contrast to other thiol group-reacting compounds, bromobimane is not able to penetrate cells; therefore its inhibition of palmitate uptake is mediated by membrane-bound proteins only. The inhibition of palmitate uptake was ~50%.

Inhibition of FAT by SSO. Finally, to attack FAT/CD36 specifically in the process of fatty acid import into the pneumocyte, we applied SSO, which was described as a specific, nontoxic, membrane-impermeable inhibitor of FAT when preincubated with adipocytes for 25 min (14). When a similar procedure was applied here, palmitate uptake by type II pneumocytes was drastically inhibited by SSO in a concentration-dependent manner (Fig. 6). The half-maximal effect was seen at a concentration < 0.5 mM SSO, and the maximal inhibition of palmitate uptake was ~80%, similar to that affected by ATP depletion.

Additive effect of phloretin and SSO on palmitate uptake. For myocardial fatty acid uptake, the involvement of several membrane proteins has been proposed (31). The uptake kinetics and the inhibition of the palmitate influx by bromobimane and SSO strongly indicate that FAT facilitates the uptake of palmitate by type II pneumocytes, but these results did not exclude the participation of other membrane-bound proteins. To address this question, we used phloretin, a potent nonspecific inhibitor of membrane transport proteins (20), and SSO, a specific, membrane-impermeable inhibitor of FAT. Table 2 shows the effect of both compounds on palmitate uptake. The combined inhibitory action of phloretin and SSO significantly exceeds the effect reached by each substance alone. From these data and from the maximal inhibition seen with SSO (Fig. 6), we conclude that FAT/CD36 is the principal protein facilitating palmitate transport into type II pneumocytes. The existence of another fatty acid carrier besides FAT/CD36 is likely.

**DISCUSSION**

We have shown here that the uptake of palmitate by type II pneumocytes is mediated by a protein of the surface membrane in a saturable, energy-dependent process. Type II pneumocytes express the mRNA of FAT, and a FAT-specific inhibitor reduced their uptake of palmitate. FAT is a CD36-related class B scavenger receptor (2) facilitating fatty acid uptake by rat adipocytes (14). It was cloned in adipocytes and detected in various tissues by Northern blot analysis (1). Most members of the class B scavenger-receptor family are localized in raftlike membrane domains (18). These microdomains are involved in numerous cellular functions including endocytosis, for which the formation of coated pits and clathrin-coated vesicles is not essential (27). Potassium depletion inhibits the formation of coated pits and clathrin-coated vesicles but does not affect the uptake of exogenous palmitate by type II pneumocytes. This reveals that the influx of palmitate is not dependent on coated pits and clathrin-coated vesicles and is consistent with the localization envisaged for the CD36-related receptor proteins.

Because five different plasma membrane-associated proteins have been described for myocardial fatty acid uptake (for a review, see Ref. 31), it might be supposed that type II pneumocytes might express additional fatty acid transporters other than FAT. However, the maximum inhibition of palmitate uptake by preincubation with the FAT-specific inhibitor was ~75%, indicating that FAT seems to be the main protein involved in fatty acid transfer across the membrane of type II pneumocytes. On the other hand, the inhibitory effect of phloretin on the uptake of palmitate was additive to the inhibition available with the FAT-specific inhibitor. Thus a second, quantitatively less important protein-mediated mechanism for type II pneumocyte fatty acid uptake may also exist.

After protein-mediated movement of fatty acids across the membrane, the water-insoluble long-chain fatty acids can leave the inner leaflet of the surface membrane only when a cytosolic protein solubilizes the fatty acids. Therefore, the intracellular binding of fatty acids to cytosolic FABP might be an important element in the regulation of the fatty acid influx. However, whether the concentration of epidermal FABP, which was shown to be expressed in type II pneumocytes (13), modulates the uptake of fatty acids is still unknown.

From our results, we conclude that the uptake of exogenous fatty acids by type II pneumocytes of adult rats is predominantly mediated by FAT. The regulation of this receptor-mediated uptake of fatty acids by type II pneumocytes might be an important factor affecting the biosynthesis of lung surfactant and could gain clinical significance. Further investigations are now possible to understand how the FAT/CD36-mediated uptake of fatty acids is regulated.

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