Regulation of ABCA1 expression and cholesterol efflux during adipose differentiation of 3T3-L1 cells

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Abstract **Adipose cells specialized in energy storage, contain large intracellular triglyceride-rich lipid droplets, are enriched with free cholesterol, and express sterol-regulated transcription factors such as liver X receptor (LXR). The recent identification of the LXR-dependent ATP binding cassette transporter A1 (ABCA1) pathway for cholesterol release from peripheral cells has led us to address the question of the expression and function of ABCA1 in adipocytes. In 3T3-L1 adipose cells, we observed a strong induction of ABCA1 mRNA during adipose differentiation, but only limited variations in ABCA1 protein. Lipid efflux onto apolipoprotein A-I (apoA-I), which depends on ABCA1, was comparable in adipocytes and preadipocytes, demonstrating a differential regulation of ABCA1 mRNA and cholesterol efflux. We also found that total cell cholesterol remained stable during differentiation of 3T3-L1 cells, but membrane cholesterol was lower in adipocytes than in preadipocytes, suggesting redistribution of cholesterol to the lipid droplet. Finally, we show that under standard lipolytic stimulation, 3T3-L1 adipocytes do not release cholesterol onto apoA-I, a process that required long exposures to lipolytic agents (24 h).** **In conclusion, despite large induction of ABCA1 mRNA during differentiation, cholesterol efflux through the ABCA1 pathway remains limited in adipocytes and requires prolonged lipolysis. This is consistent with the view of the adipocyte behaving as a cholesterol sink, with plasma cholesterol-buffering properties.**—Le Lay, S., C. Robichon, X. Le Liepvre, G. Dagher, P. Ferre, and I. Dugail. **Regulation of ABCA1 expression and cholesterol efflux during adipose differentiation of 3T3-L1 cells.** ***J. Lipid Res.*** **2003.** 44: **1499–1507.**

Supplementary key words adipocyte • lipid droplet • ATP binding cassette transporter A1

Cholesterol is an essential membrane constituent that maintains the integrity of structural domains for signal transduction and vesicular trafficking. Almost all cells from vertebrates are able to synthesize cholesterol from acetyl-CoA through a well-defined regulated process in which the sterol regulatory element binding protein (SREBP) family of transcription factors plays a key role (1). On the other hand, because cholesterol cannot be catabolized in most cells, cellular homeostasis also greatly depends on the efflux of sterols to extracellular acceptors in the plasma (2).

The ability of cellular lipids to participate in HDL formation through an apolipoprotein-dependent process has been recognized for many years (3). A key player in this process has been identified recently, the ATP binding cassette transporter A1 (ABCA1). It was described originally that a mutation in the *abc1* gene was responsible for the drastic reduction in HDL-cholesterol in patients with Tangier disease (4–7). ABCA1 is believed to play a significant role in the efflux of cellular lipids to lipid-poor apolipoprotein A-I (apoA-I) molecules from peripheral cells (8), which represents the initial step of reverse cholesterol transport (9). Indeed, raising the expression of ABCA1 in transfected cells (10–12) by cAMP treatment (11) or cholesterol loading of cultured macrophages (13) can significantly increase cholesterol efflux to apoA-I, the main apolipoprotein in HDL. Furthermore, it has also been established that the liver X receptor (LXR), which belongs to the nuclear receptor superfamily, potently activates ABCA1 gene expression and cholesterol efflux (14–18). This suggests that specific LXR agonists might be used to reduce cholesterol accumulation in cells, especially in the atherosclerotic lesions of arterial walls. Indeed, transgenic overexpression of ABCA1 in mice has proven useful in reducing the development of aortic atherosclerosis in apoE-knockout animals (19).

ABCA1 promotes active cholesterol efflux from cells onto lipid-free apoA-I, a process that differs from the diffusion route facilitated by the scavenger receptor class B, type I (SR-BI) (20). However, the precise molecular mechanism of ABCA1 action still remains unclear. A well-established feature is that ABCA1 does not act specifically on cholesterol, but is also able to promote phospholipid efflux from cell membranes (10). In addition, direct apoA-I

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binding to ABCA1 has been established (12). ABCA1 was found not to be associated with cholesterol and sphingolipid-rich membrane raft domains (21), and the subcellular origin of the cholesterol translocated to the cell exterior remains elusive. In fact, ABCA1 is abundant at the cell surface, but when expressed as a green fluorescent protein fusion, it has been reported to reside also in intracellular sites (22).

The liver is an important organ for cholesterol homeostasis. It is considered the principal cholesterol biosynthetic site, and exhibits a unique ability to catabolize cholesterol through bile acid production. However, when considering the distribution of cholesterol at the whole-body level, it is obvious that white adipose tissue can also be distinguished by its remarkably high cholesterol content (23). In a normal human subject, for example, 25% of body cholesterol is found in fat tissue, a proportion that can increase to well over half in obese patients (24). Thus, in addition to its well-known function in the managing of energy stores in the form of triacylglycerol, adipose tissue also contains the largest pool of cholesterol in the body. In fat tissue, cholesterol synthesis is extremely low (25), and most cholesterol is taken up from the circulation and is deposited in the free nonesterified form. In this regard, cholesterol storage in fat can be considered a "buffering" process, which might help to avoid hypercholesterolemia.

Thus, given *i*) the quantitative importance of adipose tissue in whole-body cholesterol homeostasis, *ii*) the newly discovered LXR-ABCA1 axis for the regulation of cholesterol efflux, and *iii*) the recently published observations that adipocytes express LXRα in a differentiation-dependent manner (26), we addressed the question of the regulation of ABCA1 expression and function in adipose cells.

MATERIALS AND METHODS

Cell culture

3T3-L1 cells (a kind gift from Dr. J. Pairault, Paris, France) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Perbio) and antibiotics. At confluence, adipocyte differentiation was induced by adding methyl-isobutylxanthine (100 μM), dexamethasone (0.25 μM), and insulin (1 μg/μl) for 2 days. Then, cells were maintained in high-glucose DMEM supplemented only with 10% calf serum and insulin alone for an additional 5 days. At that time, a vast majority of cells (more than 90%) had accumulated lipid droplets.

When appropriate, confluent undifferentiated preadipocytes or fully differentiated adipocyte cells were shifted to a serum-free medium containing DMEM and 0.2% bovine serum albumin (BSA), and treated for 24 h with 8-Bromo (8-Br) cAMP (0.3 mM) or LXR-retinoid X receptor (RXR) agonists (0.1 μM LG100268 and 1 μg/ml 22-R hydroxycholesterol, alone or in combination, a kind gift from S. Commans, GlaxoSmithKline, les Ulis, France).

RNA preparation and real-time RT-PCR

Total RNA was extracted from 100 mm dishes as described (27) from 3T3-L1 preadipocytes or adipocytes treated or not with effectors.

For RT-PCR purposes, cDNA was synthesized from total RNA RQ1 DNase-treated (Promega) using random hexamers and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The primers were designed using the primer express software (Perkin Elmer Life Sciences), and are described elsewhere (28).

Real-time quantitative RT-PCR analyses were performed starting with 50 ng of reverse-transcribed total RNA, with 200 nM sense and antisense primers (Genset) in a final volume of 25 μl using the Sybr Green PCR core reagents in an ABI PRISM 7700 sequence detection system instrument (Perkin Elmer Life Sciences). Because we used Sybr Green in measurements of amplification-associated fluorescence for real-time quantitative RT-PCR, we ensured that generated fluorescence was not overestimated by contaminations resulting from residual genomic DNA amplification (using controls without reverse transcriptase) and/or from primer dimers formation (controls with no DNA template or reverse transcriptase). RT-PCR products were also analyzed on ethidium bromide-stained agarose to ensure that a single amplicon of the expected size was indeed obtained. To measure PCR efficiency, serial dilutions of reverse-transcribed RNA (0.1 pg to 200 ng) were amplified, and a line was obtained by plotting cycle threshold values as a function of starting reverse-transcribed RNA, the slope of which was used for efficiency calculation using the formula $E = 10^{|(1/\text{slope})|} - 1$. Relative quantification for a given gene, expressed as fold variation over control, was calculated after normalization to 18S ribosomal, and correction for interplate variation using an internal calibrator. Experiments were repeated three to five times.

Lipid efflux assays

Assays for cholesterol and phospholipids efflux were performed in confluent preadipocytes and fully differentiated adipocytes grown on 24-well dishes, essentially as described by Rothblat and colleagues (29–31). Briefly, cells were incubated for 24 h in DMEM containing 0.5 μCi/well of [1,2-^{3}H]cholesterol (48 Ci/mmol, NEN Life Science) in DMEM supplemented with 0.2% BSA. The labeling medium also contained 1 μg/ml Pfizer ACAT inhibitor 113818 (kind gift of Nathalie Fournier, HEGP, Paris) to ensure that all the labeled cholesterol was present as free cholesterol (data not shown). Following the labeling period, cells were washed and allowed to equilibrate for 16 h in DMEM containing 0.2% BSA, in the presence or absence of effectors. After equilibration, which ensured that label cholesterol would be distributed in all intracellular pools, 3T3-L1 preadipocytes or adipocytes were washed twice with DMEM and incubated for 4 h in the presence or absence of 10 μg/ml lipid-free apoA-I (Sigma). Culture media were harvested and centrifuged for 10 min at 10,000 *g* to eliminate remaining cell debris. The cell monolayer was washed with PBS and extracted with isopropanol to provide cell total [^{3}H]radioactivity. apoA-I-mediated cholesterol efflux was calculated as the percent of total [^{3}H]cholesterol released into the medium after subtraction of values obtained in the absence of apoA-I.

Phospholipids efflux was also assayed in cells labeled with 0.5 μCi/ml methyl-[^{3}H]choline (75 Ci/mmol, NEN Life Science) for 24 h. The experimental scheme was the same as that described above, except that radioactivity associated with choline-containing phospholipids in cells or culture medium was determined by scintillation counting following extraction with chloroform-methanol (2:1; v/v).

Western blot analysis

Aliquots of membrane preparations (10–30 μg) were separated by SDS-PAGE after denaturation at 70°C in 0.5 vol of 8 M urea, 5% SDS, 0.5 M β-mercaptoethanol, and 0.125 M Tris-HCl, pH 6.8. After transfer onto nitrocellulose membranes and prob-

ing with antibodies against ABCA1 (Novus Biologicals), specific protein signals were revealed using the ECL detection system (Amersham Pharmacia Biotech).

Membrane preparation

Total membranes were prepared by lysing cells with a Teflon potter in 20 mM HEPES, 1 mM EDTA, and 250 mM sucrose in the presence of a protease inhibitor cocktail. The postnuclear supernatant (600 *g* for 10 min at 4°C) was collected and centrifuged at 200,000 *g* for 2 h. The final pellet was resuspended in 30 mM HEPES, and protein content was determined using the Bio-Rad Protein assay.

Lipolysis

In some experiments using differentiated 3T3-L1 cells, lipolytic activity was assessed concomitantly with cholesterol efflux, in the same batch of cells. In this case, increasing amounts of isoproterenol, a nonspecific β-adrenergic agonist, were added, together with apoA-I at the beginning of the efflux reaction. After 4 h, 200 μl aliquots of the culture medium were precipitated with 30 μl of 10% TCA. The TCA-soluble supernatant was neutralized with 33% KOH, and glycerol content was determined using the Peridochrome Triglycerides Kit (Boerhinger Mannheim). The presence of apoA-I and/or ACAT inhibitor during the incubation did not affect glycerol release from cells (data not shown).

Other determinations

Cholesterol or phospholipids content in cell lysates or in membrane fractions were determined after extraction of total lipids as described by Folch, Lees, and Sloane-Stanley (32). Commercially available kits (Cholesterol, Sigma, and Phospholipides Enzymatiques PAP 150, Bio Merieux, France) were used.

Statistical analysis

Statistical significance was assessed by paired *t*-test analysis. $P < 0.05$ was considered to be the threshold of significance.

RESULTS

Coordinate induction of LXRα and ABCA1 mRNAs during adipocyte differentiation

The existence of high-affinity oxysterol ligands for the nuclear receptor LXR has allowed the identification of LXR transcriptional targets, among which is ABCA1 (16). It has been shown recently that LXRα gene expression was highly induced during the course of adipocyte differentiation (26), but no data are available for ABCA1 expression in adipose cell systems. We first measured the levels of ABCA1 mRNA during differentiation of 3T3-L1 preadipocytes to adipocytes (**Table 1**). For LXRα mRNA, we observed a strong 44-fold induction, in agreement with the previous report of Luo and Tall (26). This was accompanied by remarkable changes in the expression of ABCA1, which was induced more than 30-fold upon differentiation. It is noteworthy that ABCA1 mRNA levels measured here in differentiated 3T3-L1 adipocytes were very similar to those present in rat fat pads (26 ± 2.2, normalized expression relative to 18S RNA), suggesting that ABCA1 mRNA is expressed in the same range in 3T3-L1 adipocytes and in real fat cells from adipose tissue. Thus, 3T3-L1 provides a cell system suitable for studying adipocyte ABCA1 regulation and function.

TABLE 1. mRNA levels of LXRα and ABCA1 during the course of 3T3-L1 adipocyte differentiation

	Normalized mRNA Levels	
	ABCA1	LXRα
	arbitrary units	
Preadipocytes	1.19 ± 0.45	0.10 ± 0.09
Adipocytes	43.0 ± 24.1	4.6 ± 1.7
Fold induction	36	44

LXR, liver X receptor; ABCA1, ATP binding cassette transporter A1. Preadipocytes (confluent cells harvested before treatment with differentiation inducers) and fully differentiated 3T3-L1 adipocytes (harvested at Day 7 after confluence) were used for total RNA extraction. mRNA levels for the indicated genes were measured using real-time RT-PCR. Values were expressed as a ratio to 18S RNA relative expression, and represent mean ± SEM obtained from at least three independent cultures. For ABCA1 and LXRα, differences between preadipocytes and adipocytes are statistically different by Student's *t*-test, at the $P < 0.001$ level.

We next tested the effect of exogenous LXR ligands in differentiated adipocytes. **Figure 1** shows that activating LXR-RXR dimers by providing LG 100268 (a specific RXR agonist) and 22R OH-cholesterol (an LXR ligand) increased ABCA1 mRNA by 4-fold in differentiated adipocytes. This effect could not be observed in preadipocytes, possibly because LXRα expression is extremely low. In these experiments, we also observed that activation of LXR-RXR dimers could also induce SREBP1c mRNA, another LXRα-responsive gene (33–35), with the same potency as ABCA1. Together, these data establish that ABCA1 gene expression is induced during fat cell differentiation, and can be modulated in differentiated adipocytes by the presence of exogenous LXR ligands. This suggests that ABCA1 might participate in the function of fully differentiated fat cells.

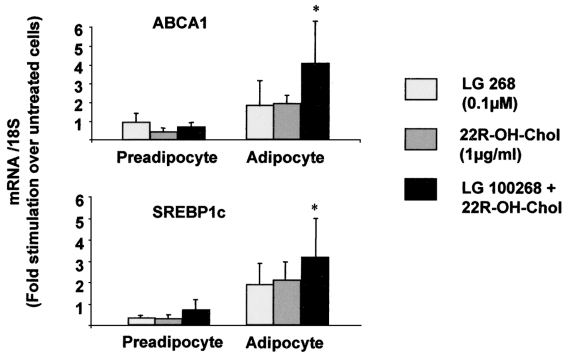

Fig. 1. Effect of liver X receptor (LXR)-retinoid X receptor (RXR) ligands on the expression of ABCA1 and sterol regulatory element binding protein (SREBP)1c genes in 3T3-L1 cells. Confluent preadipocytes or fully differentiated adipocytes (Day 6 postconfluence) were treated or not by the RXR ligand LG 100268 (0.1 μM), 1 μg/ml of 22-R hydroxycholesterol [a potent LXR agonist], or both for 16 h. mRNA levels for ABCA1 were measured by real-time RT-PCR from at least 3 independent cultures. Results are normalized to 18S RNA levels, and expressed as fold variations ± SEM relative to untreated control cells. * Indicates a significant difference versus untreated controls ($P < 0.05$).

Moderate changes in ABCA1 protein content during differentiation

We next examined ABCA1 expression at the protein level in preadipocytes and adipocytes. **Figure 2** shows that Western blotting of crude membrane fractions with a polyclonal ABCA1 antibody revealed immunoreactive bands of high molecular weight (>200 kDa), with higher intensities in differentiated adipocytes than in preadipocytes. Quantitative scanning of the ACBA1 signal in Western blots showed that the relative content of the ABCA1 protein significantly increased (2- to 3-fold) in differentiated adipocytes versus preadipocytes. Such a moderate increase in the content of ABCA1 protein in membranes adipocytes is strikingly contrasted with the large induction in ABCA1 mRNA during fat cell differentiation. This indicated that some posttranscriptional mechanisms might limit the consequences of the differentiation-dependent induction of ABCA1 mRNA. These mechanisms might involve reduced ABCA1 synthesis or decreased stability of the protein. Recently, regulation of ABCA1 by proteolysis has been described, and a protective effect of apoA-I against degradation of ABCA1 was reported in THP-1 cells (36) and mouse macrophages (37). In order to investigate whether such a mechanism was effective in adipocytes, we performed experiments in which differentiated adipocytes were incubated as described by Arakawa and Yokoyama (36) in a serum-free medium containing 0.2% BSA for 16 h and then for 1 h in the presence of apoA-I or protease inhibitors ALLN, or pepstatin. As shown in **Fig. 3**, Western blot analysis did not revealed any variation of ABCA1 protein content in cells treated with either apoA-I or the protease inhibitors. Such treatments were also ineffective to increase ABCA1 protein or cholesterol efflux onto apoA-I after longer (3 h to 16 h) incubation periods (data not shown). These data point out a striking difference in the regulation of ABCA1 between adipocytes and macrophages, in which ABCA1 stability is of crucial importance in establishing the levels of ABCA1 protein.

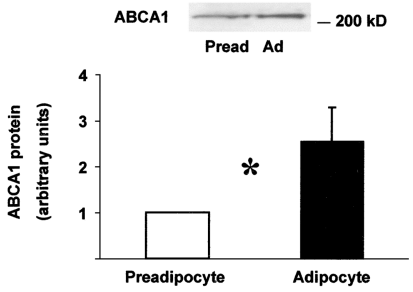

Fig. 2. ABCA1 protein levels in 3T3-L1 cells. Western blot analysis of ABCA1 was performed as described in Materials and Methods on total membrane pellets prepared from preadipocytes (Pread) or adipocytes (Ad). Equal quantities of protein (30 μg) were run in each lane. Western blots were obtained from three independent cultures. Samples from one representative experiment are shown. Relative quantification was obtained by densitometric scanning of the autoradiograms. The values obtained for the signals in preadipocytes were arbitrarily set to 1. Fold changes ± SEM are shown. * Indicates a significant difference by paired *t*-test, at the $P < 0.05$ level.

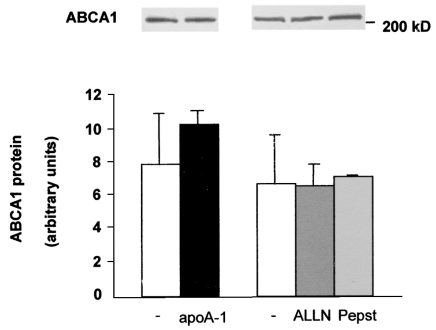

Fig. 3. ABCA1 protein levels in differentiated adipocytes in the presence of apolipoprotein A-I (apoA-I) and ALLN. Differentiated adipocytes were cultured in serum-free medium containing 0.2% BSA for 16 h and then in the presence of 10 μg/ml apoA-I, 50 μM ALLN, or 20 μM pepstatin (Pepst) for 1 h. Cells were collected and Western blot analysis of ABCA1 was performed on total membrane pellets. A representative autoradiogram is shown at the top, and quantitative results were obtained by densitometric scanning of blots from three independent experiments. Values are mean ± SEM.

Functional apoA-I-mediated lipid efflux in 3T3-L1 adipocytes

We next examined the function of ABCA1 in 3T3-L1 cells. We tested the ability of 3T3-L1 cells to release cellular lipids (mainly phospholipid and cholesterol) onto lipid-poor apoA-I, a process that is largely dependent on ABCA1 activity. Results shown in **Fig. 4** show that choles-

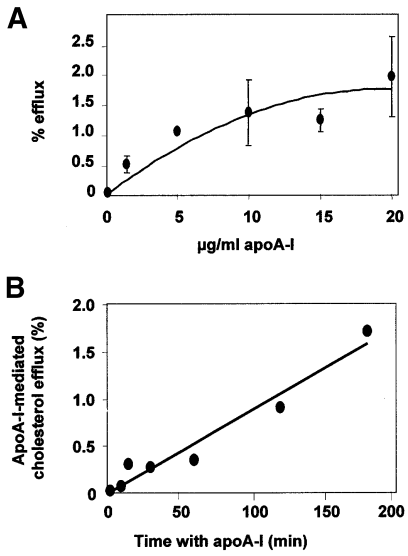

Fig. 4. Efflux of cholesterol on apoA-I in 3T3-L1 adipocytes. Differentiated cells were incubated in serum-free medium containing 2% BSA and labeled cholesterol (0.5 μCi) for 16 h, and were allowed to equilibrate in the same medium for 24 h. Then, apoA-I was added and the medium and cells were collected after 4 h. The radioactivity in media and that remaining in the cells were counted, and cholesterol efflux was expressed as the percentage of radioactivity in the medium relative to total radioactivity (medium and cells). An ACAT inhibitor was present at all times (see Materials and Methods) to prevent cholesterol esterification. Concentration dependence on apoA-I (A), and time course of apoA-I-mediated cholesterol efflux (B) are shown. Values are mean ± SEM obtained from at least three independent determinations.

terol efflux increased with increasing concentrations of apoA-I in the medium, reaching a plateau at 10 μg/ml. In addition, cholesterol efflux from 3T3-L1 adipocytes onto apoA-I was a linear function of time. These data indicate a significant ability of adipocytes to transfer cellular cholesterol onto apoA-I as an extracellular acceptor.

ApoA-I-mediated lipid efflux is unchanged during the preadipocyte-to-adipocyte transition

We next examined whether apoA-I-mediated lipid efflux from 3T3-L1 cells was modulated during adipocyte differentiation. Preadipocytes and adipocytes were studied at the same differentiation state as above. Because the ABCA1 transporter has a dual ability to promote the efflux of cellular cholesterol and phospholipids (10), cells were labeled with either tritiated cholesterol or choline, and lipid efflux onto apoA-I was determined. Under the conditions used here, preadipocytes and differentiated cells were labeled to the same specific activities for cholesterol (64,680 ± 14,800 dpm/μg total cell cholesterol in preadipocytes and 62,450 ± 12,850 dpm/μg in fully differentiated fat cells) and phospholipids (7,022 ± 544 and 7,604 ± 619 dpm/μg phospholipid in preadipocytes and adipocytes, respectively), allowing direct comparison of apoA-I-mediated efflux between nondifferentiated and differentiated fat cells. As shown in **Fig. 5**, apoA-I-mediated phospholipid efflux increased from 0.36% to 0.98% during adipose conversion. This variation was in the same range as that of ABCA1 protein in Western blots. In contrast, we observed that the efflux of cholesterol remained unchanged during the preadipocyte-to-adipocyte transition. In summary, our data point out some unique features of the adipocyte cell system with regard to the regulation of ABCA1 and lipid efflux. First, the large induction of ABCA1 gene expression that occurs during fat cell differentiation is followed by only modest changes in ABCA1 at the protein level. At the functional level, very small or no variations were detected in the ability of adipocytes and preadipocytes to efflux lipids to extracellular acceptors.

Cell cholesterol is redistributed during fat cell differentiation

The ongoing adipocyte differentiation program induces dramatic changes in the phenotype of 3T3-L1 cells, the most striking of which is the accumulation of large intracellular triglyceride droplets. Because cholesterol efflux from cells is largely influenced by their degree of lipid loading, we evaluated cell cholesterol content and/or distribution during fat cell differentiation. **Table 2** shows that more than 90% of cell cholesterol in both preadipocytes and adipocytes was essentially found in free, nonesterified form, in agreement with previous reports in the literature (23). This proportion did not change during differentiation, ruling out the possibility that an increased proportion of cholesterol was used for esterification in mature adipocytes. Importantly, we found that on a per-cell basis, total cholesterol in crude lysates remained unchanged during the differentiation process.

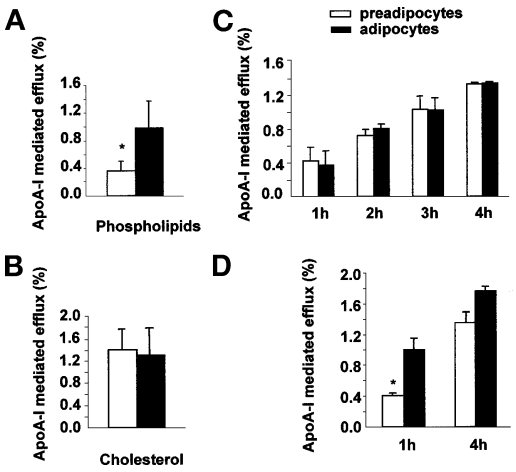

Fig. 5. Comparison of lipid efflux on apoA-I in preadipocytes and adipocytes. ApoA-I-mediated efflux of phospholipids (A) and cholesterol (B) in preadipocytes (white bars) and differentiated (black bars) 3T3-L1 cells was measured as described after 4 h in the presence of 10 μg/ml apoA-I. Values are mean ± SEM from at least five independent determinations. C: Shows the time dependence of apoA-I-mediated cholesterol efflux in preadipocytes and adipocytes labeled with the standard protocol. Values are mean ± SEM from at least three independent determinations. D: Shows apoA-I-mediated cholesterol efflux in cells labeled with the protocol described by Lange et al. (38), in which plasma membrane cholesterol was labeled during a short incubation with tritiated cholesterol loaded into cyclodextrins. The preparation of the labeled cholesterol-cyclodextrin mix was as described elsewhere (28), except that tritiated cholesterol was used. The final concentration of cyclodextrin in the medium was 5 mM, containing 0.5 μCi of cholesterol. Immediately after labeling, cells were rapidly rinsed, and cholesterol efflux was initiated by the addition of apoA-I and followed over time. Values are mean ± SEM. * Indicates a statistically significant difference between preadipocytes and adipocytes (Student's *t*-test, $P < 0.05$ level).

In addition, when considering total membranes, we observed a nearly 2-fold decrease in cholesterol content in adipocytes compared with undifferentiated cells (Table 2). On the other hand, no change in phospholipid content of total membranes occurred during differentiation. This resulted in a specific decrease in the cholesterol-to-

TABLE 2. Lipid content of 3T3-L1 preadipocytes and fully differentiated cells

	Preadipocytes	Adipocytes
Free cholesterol (% total)	93.3 ± 4.4 (3)	92.5 ± 1.9 (4)
Total cholesterol (μg/10^6 cells)	10.2 ± 1.4 (4)	10.2 ± 1.8 (4)
Cholesterol in total membranes (μg/mg protein)	51 ± 2 (3)	27 ± 1 (5)[a]
Phospholipids in total membranes (μg/mg protein)	97 ± 10 (3)	83 ± 3 (5)

Values are mean ± SEM, with the number of independent determinations in parentheses. Total cholesterol was measured in whole-cell lysates, and the ratio of free to esterified cholesterol in total lysates was calculated by counting the radioactivity incorporated into cholesterol and its ester after separation on thin-layer chromatography plates in petroleum ether-ethylic ether-acetic acid (85:15:1; v/v/v). Unlabeled standards were run in parallel to identify free and esterified cholesterol bands.

[a] Significant difference by Student's *t*-test, at the $P < 0.001$ level. Cholesterol and phospholipid content were measured on total membranes.

phospholipid ratio (w/w) in total membranes of adipocytes versus preadipocytes (0.53 ± 0.05 and 0.31 ± 0.03, respectively). Together, these data strongly suggest a specific redistribution of cholesterol in a nonmembrane (cytosolic?) pool in differentiated adipocytes.

The specific redistribution of cholesterol but not phospholipids to intracellular pools during adipocyte differentiation might be an important factor contributing to the different efflux patterns of phospholipid versus cholesterol during differentiation. To investigate this question, we designed an experiment in which the possible effects of cholesterol redistribution could be evaluated. To this aim, we compared the kinetics of cholesterol efflux in the classical protocol, in which all cellular cholesterol pools were labeled, with a protocol first described by Lange et al. (38), in which only plasma membrane cholesterol is labeled. In this latter protocol, plasma membrane cholesterol labeling was achieved during a short incubation (20 min) of cells with tritiated cholesterol incorporated into cyclodextrins. Immediately after labeling, cholesterol efflux was followed over time. Results in Fig. 5C show that using the classical protocol, cholesterol efflux increased linearly as a function of time in both preadipocytes and adipocytes, with no differences between the two cell types. This is in agreement with results in Fig. 5B. In contrast, when the plasma membrane cholesterol labeling protocol was used (Fig 5D) cholesterol efflux at an early time point (1 h of exposure to apoA-I) was doubled in adipocytes versus preadipocytes. After 4 h, a time sufficient for labeled cholesterol to redistribute from the plasma membrane, no more difference could be observed between preadipocytes and adipocytes. This demonstrated that under conditions that solely evaluate the ability for exchange of cholesterol from the plasma membrane onto apoA-I, higher rates of cholesterol efflux could be measured in adipocytes compared with preadipocytes. Moreover, under these conditions, a close parallelism could be observed between differentiation-dependent variations in ABCA1 protein content and cholesterol efflux. This parallelism is no longer observed with the standard protocol (all cellular cholesterol pools labeled) or after redistribution of labeled membrane cholesterol to other cellular pools (efflux measurement after longer time points in the cyclodextrin labeling protocol). Together, these results strengthen the importance of intracellular cholesterol distribution for efflux.

ApoA-I mediated cholesterol efflux under lipolytic conditions

Because the above data suggest that cholesterol redistribution to the lipid droplet in adipocytes might influence cholesterol efflux, we next examined the ability of adipocytes to release cholesterol under lipolytic conditions in which triacylglycerols are mobilized from the lipid droplet. The breakdown of adipocyte triglyceride stores is physiologically activated by lipolytic agents through the β-adrenergic system and the cAMP-signaling pathway. Thus, we treated 3T3-L1 adipocytes with isoproterenol (a nonselective β agonist), and during the same incubation, we measured the concomitant release of cholesterol onto apoA-I and that of glycerol produced by triglyceride breakdown. In this experiment, both the lipolytic stimulating agent and the extracellular cholesterol acceptor were added for 4 h. **Figure 6** shows that under these conditions, ongoing lipolysis occurred, as judged by the dose-dependent release of glycerol to the medium. However, isoproterenol was not able to increase cholesterol efflux onto apoA-I. The same results were obtained using the physiological lipolytic stimulating agent, epinephrine (data not shown). This indicates that lipolysis and cholesterol release are not necessarily associated during cAMP stimulation of fat cells.

We next tested the effect of an extended treatment with cAMP-raising agents. Cells were incubated for 24 h in the presence of 8-Br cAMP, a stable analog of cAMP. During a 24 h period of cAMP exposure, ongoing lipolysis could not be measured accurately because of reutilization or disappearance of lipolitic products in the culture medium. However, apoA-I-mediated cholesterol efflux at the end of the incubation period was increased 2-fold in adipocytes treated with 8-Br cAMP compared with untreated controls (Fig. 5A). After 24 h with 8-Br cAMP, ABCA1 mRNA levels were stimulated 2-fold as compared with untreated controls (Fig. 6B). This indicated that the stimulatory effect of cAMP on cholesterol efflux in adipocytes is caused by

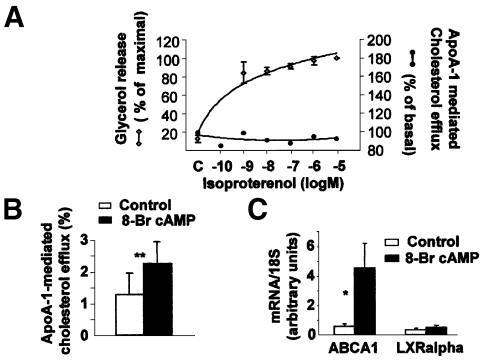

Fig. 6. ApoA-I-mediated cholesterol efflux during lipolytic stimulation of adipocytes. A: Concomitant measurement of cholesterol efflux onto apoA-I and glycerol release in 3T3-L1 adipocytes stimulated by isoproterenol. Cells were labeled with cholesterol, and processed for cholesterol efflux as above, except that isoproterenol was added at indicated concentrations during incubation with or without apoA-I. After 4 h, the culture medium was collected and used for glycerol determination. An aliquot from the same medium also served to measure cholesterol efflux by scintillation counting of labeled cholesterol. The presence of apoA-I did not affect glycerol release (data not shown). Each point represents mean values ± SEM, obtained from three separate experiments. B: Effects of a 24 h treatment with 8-Bromo (8-Br) cAMP on apoA-I-mediated cholesterol efflux from fully differentiated adipocytes. Cholesterol efflux was evaluated as above, in the presence of 8-Br cAMP (0.3 mM) added during the equilibration period and onwards (24 h). Significant differences compared with control untreated cells ± SEM were tested by Student's *t*-test. ** Indicates significant difference at the $P < 0.01$ level. C: Effect of a 24 h treatment of differentiated adipocytes with 8-Br cAMP (0.3 mM) on ABCA1 and LXRα mRNA levels. Each point represents mean values ± SEM, obtained from three separate experiments. * Indicates significant difference at the $P < 0.05$ level.

stimulation of the expression of the ABCA1 gene. Together, these data suggest that cholesterol mobilization from the fat cell onto apoA-I is not easily achieved during short-term activation of lipolysis, but rather requires sustained stimulation and cAMP-induced up-regulation of ABCA1 gene expression.

DISCUSSION

The present study examines the aptitude of fat cells to release their cholesterol via the recently described LXR-ABCA1-apoA-I pathway. The question of the contribution of adipose tissue to apoA-I-mediated cholesterol efflux has not been examined so far, but is worth considering, based on the high cholesterol content of adipocytes and on recent observations that fat cells express LXRα, a transcriptional regulator of ABCA1, in a differentiation-dependent manner (26). We show here for the first time that the ABCA1 gene, like LXRα, is strongly induced upon differentiation of 3T3-L1 preadipocytes to mature fat cells, and that ABCA1 mRNA abundance in differentiated adipocytes can be modulated by treatment with LXR-RXR agonists. Thus, regulation of ABCA1 mRNA in adipocytes closely resembles that described in monocyte-macrophage cell systems. However, in striking contrast with the induction of ABCA1 gene expression during fat cell differentiation, an important finding in this study is that ABCA1 protein does not increase more than 2-fold during adipose conversion. Accordingly, phospholipid efflux was doubled between preadipose and fully differentiated states. In most cell lines studied so far (8), ABCA1 mRNA correlated well with ABCA1 protein or function. The reasons for the inadequate ABCA1 protein content relative to ABCA1 gene expression in adipocytes deserve further investigation. Recently, stabilization of ABCA1 through an apoA-I-mediated process was described in macrophages (36). In similar experiments, we observed no effect of apoA-I or protease inhibitors on ABCA1 content in adipocytes. In this regard, the present data suggest the existence of adipocyte-specific mechanisms regulating ABCA1 expression at the posttranscriptional level.

An important point is that adipocyte differentiation proceeds in the 3T3-L1 cell system without changes in total cholesterol content (expressed on a per-cell basis). Cholesterol efflux is largely influenced by cell cholesterol content, although the underling mechanisms remain unclear. Although no variation in total cell cholesterol content occurred during adipose conversion in the 3T3-L1 cell line, we observed a marked decrease in the cholesterol content of total membranes during differentiation. This observation fits with the lower lipid order of the plasma membrane previously reported in 3T3-F442A adipocytes compared with undifferentiated fat cells (39). Together, these data suggest that cholesterol is redistributed toward a nonmembrane pool in differentiated cells, presumably the lipid droplet.

Such a shift in cholesterol intracellular distribution during differentiation might be a key feature to explain the ineffective release of cholesterol relative to phospholipids from adipocytes. In this regard, our results showing that the ability of cholesterol to transfer from the plasma membrane were not altered in adipocytes and proceeded in close proportion to the ABCA1 protein content and reinforced the importance of cholesterol distribution for efflux. This idea is in line with previous studies that observed the absence of a relationship between cellular cholesterol levels and incorporation of cholesterol to HDL (40). Also, experiments that used cyclodextrin preincubation to deplete membrane cholesterol showed that ABCA1-mediated cholesterol efflux was abolished but phospholipid efflux was unaffected (10).

The adipocyte lipid droplet is mainly formed with a triacylglycerol core surrounded by a free cholesterol-containing phospholipid monolayer. No information is available on the coupling of triacylglycerol and cholesterol during the lipolytic process that leads to the mobilization of energy from the adipocyte. Our results indicate that a short-term stimulation of lipolysis does not induce concomitant adipocyte cholesterol release onto apoA-I, suggesting independent mobilization of cholesterol and triacylglycerols. However, under conditions of sustained lipolytic stimulation, a boost in the mobilization of adipocyte cholesterol can be observed. This is likely the result of increased ABCA1 gene expression, which was reported to be regulated by cAMP (11). Consistent with this view, we show here that ABCA1 mRNA levels in adipocytes are induced after 24 h of cAMP treatment. This raises the question, still unresolved, of the intracellular pathways through which cholesterol can traffic from the lipid droplet to the plasma membrane. In rodents, under physiological conditions in which lipolysis is stimulated (i.e. fasting), increased levels of HDL cholesterol have been described (41). The responsible mechanisms, however, might involve interactions between lipoproteins in the circulating compartment or decreased clearance by the liver, rather than increased peripheral production.

Under normal feeding conditions, in vivo studies have examined the contribution of adipose tissue to plasma cholesterol levels, and have concluded that adipose tissue does not significantly contribute to hypercholesterolemia (23). In addition, one observation from the literature reported normal cholesterol content in adipocytes isolated from a patient with Tangier disease (42), indicating little, if any contribution of the ABCA1 pathway in adipose tissue cholesterol homeostasis. Rather, it has been suggested that adipose tissue might be a sink for circulating cholesterol, which could help to buffer the high levels of circulating cholesterol frequently found in obese states. Because adipose tissue cholesterol content is strongly correlated with fat cell size in humans or rodents (23, 25), we have proposed that intracellular cholesterol in adipocytes might act as a cell size-dependent intracellular signal, and might serve as a sensor for the level of intracellular triglyceride stores (28). In that view, the low ability of adipocytes for cholesterol efflux fits with cholesterol being a signaling molecule in fat cells, linked to the levels of energy stores.

In conclusion, this study provides new information on cholesterol efflux from peripheral cells. In particular, it points out the unique ability of the adipocyte, a cholesterol-rich cell type, to differentially regulate ABCA1 mRNA and protein expression. Whether this property is brought about by the presence of an intracellular triglyceride droplet rich in free cholesterol or is related to fat cell function per se remains to be investigated.

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