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

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Running title: ABCA1 and cholesterol efflux in 3T3-L1 adipocytes

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Summary.
Adipose cells are specialized in the storage of energy and contain large intracellular triglyceride-rich lipid droplets. They are also enriched with free cholesterol, and express sterol-regulated transcription factors such as LXR. Recently ABCA1 has been identified as a key protein controlling the release of cholesterol from peripheral cells. Moreover, the transcriptional control of ABCA1 expression by LXR has been established. Here, using the 3T3-L1 adipose differentiation cell system, we addressed the question of the expression and function of ABCA1 in adipocytes. We observed that ABCA1 mRNA was strongly induced during adipose differentiation, but only limited variations (2 fold) were detected for ABCA1 protein. Accordingly, only minor changes were observed in phospholipid and cholesterol efflux between preadipocytes and adipocytes. This demonstrates that adipocytes differentially regulate ABCA1 mRNA and cholesterol efflux. We also found that, on a per cell basis, total cholesterol content remained stable during adipocyte differentiation of 3T3-L1 cells. However, cholesterol contents in membranes of differentiated cells were lower than those of preadipocytes, suggesting redistribution of cholesterol to the lipid droplet. Finally, we show that under standard isoproterenol-induced lipolytic stimulation, 3T3 L-1 adipocytes do not release cholesterol onto apoA-I. Longer exposure to lipolytic agents (24 hours) is needed to detect an increased rate of apoA-I-mediated cholesterol efflux from fat cells. In conclusion, despite large induction of ABCA1 mRNA during differentiation, cholesterol efflux through the ABCA1 pathway remains limited in differentiated adipocytes. Moreover, stimulation of the ABCA1 pathway for cholesterol efflux from fat cells requires prolonged exposure to lipolytic agents. This is consistent with the view of the adipocyte behaving as a cholesterol sink, with plasma cholesterol-buffering properties.
Introduction.

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 acetylCoA, through a well-defined regulated process in which the Sterol Regulatory Element Binding Protein (SREBPs) family of transcription factors plays a key role (1). On the other hand, since 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 to 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 protein 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 apo A-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), or by cAMP treatment (11) or cholesterol loading of cultured macrophages (13), can increase significantly cholesterol efflux to apo A-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 suggested 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 to reduce the development of aortic atherosclerosis in apoE-Knockout animals (19).

ABCA1 promotes active cholesterol efflux from cells onto lipid-free apo A-I, a process that differ from the diffusion route facilitated by the Scavenger Receptor Class B, type I (SR-B1) (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 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 GFP 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 well over half in obese patients (24). Thus, beside 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 non-esterified 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 LXR alpha in a differentiation dependent -manner (26), we addressed the question of the regulation of ABC1 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 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 (1µg/µl) for 2 days. Then, cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented only with 10% calf serum and insulin alone for 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 adipocytes cells were shifted to a serum-free medium containing DME and 0.2% Bovine Serum Albumin (BSA), and treated during 24 hours with 8-Br cAMP (0.3 mM) or LXR/RXR agonists (0.1 µM LG100268 and 1µg/ml 22-R Hydroxycholesterol, alone or in combination, kind gift from S. Commans, Glaxosmithkline, les Ulis, France)

RNA preparation and Real-time RT-PCR
Total RNA was extracted from 100mm 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 nor 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 (C<sub>T</sub>) values as a function of starting reverse transcribed RNA, the slope of which was used for efficiency calculation using the formula E=10<sup>(1/slope)</sup>-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 24h in DMEM containing 0.5 μCi/well of [1,2-<sup>3</sup>H] cholesterol (48 Ci/m mole, NEN life Science) in DMEM supplemented with 0.2% BSA. The labeling medium also contained 1μg/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 16h in DMEM containing 0.2% bovine serum albumin, in the presence or absence of effectors. After equilibration, which ensured label cholesterol to be distributed of in all intracellular pools, 3T3-L1 were washed twice with DMEM and incubated for 4h in the presence or absence of 10 μg/ml lipid-free apolipoprotein A-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 [<sup>3</sup>H] radioactivity. ApoA-I-mediated cholesterol efflux was calculated as the percent of total [<sup>3</sup>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-[<sup>3</sup>H]-choline (75 Ci/m mole, NEN Life science) for 24h. The experimental scheme was the same than 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, vol/vol).

**Western Blot analysis**

Aliquots of membrane preparations (10-30 µg) were separated by SDS-PAGE after denaturation at 95°C in 0.5 volume of 8M urea, 5% SDS, 0.5M β-mercaptoethanol, and 0.125 M Tris-HCl pH 6.8. After transfer onto nitrocellulose membranes and probing 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, 1mM EDTA, 250 mM sucrose in the presence of a protease inhibitor cocktail. The post nuclear supernatant (600 X g for 10 min at 4°C) was collected and centrifuged at 200 000 g for 2h. The final pellet was resuspended in 30 mM Hepes and protein contents were determined using the Bio-Rad Protein assay.

**Lipolysis**

In some experiments using differentiated 3T3-L1 cells, lipolytic activity was assessed concomitantly to cholesterol efflux, on the same batch of cells. In this case, increasing amounts of isoproterenol, a non-specific β-adrenergic agonist, was added, together with apoA-I at the beginning of the efflux reaction. After 4 hours, 200µl aliquots of the culture medium was precipitated with 30 µl of 10% TCA. The TCA-soluble supernatant was neutralized with 33% KOH, and glycerol content was determined using Peridochrome Triglycerides Kit (Boehringer 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 contents in cell lysates or in membrane fractions were determined after extraction of total lipids as described by Folch (32). Commercially available kits (Cholesterol, Sigma and Phospholipides enzymatiques PAP 150, Bio Merieux, France) were used.

**Statistical analysis**


Statistical significance was assessed by pair t test analysis. P<0.05 was considered to be the threshold of significance.
Results.

Coordinate induction of LXR alpha and ABCA1 mRNAs during adipocyte differentiation.

The existence of high affinity oxysterols 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 LXR alpha 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 LXR alpha mRNA, we observed a strong forty-four fold induction, in agreement with the previous report from Luo and Tall (26). This was accompanied by remarkable changes in the expression of ABCA1, which was induced more than thirty-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 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 suitable cell system to study adipocyte ABCA1 regulation and function.

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 (a LXR ligand) increased ABCA1 mRNA by 4 fold in differentiated adipocytes. This effect could not be observed in preadipocytes, possibly because LXR alpha expression is extremely low. In these experiments, we also observed that activation of LXR/RXR dimers could also induce SREBP1c mRNA, another LXR alpha responsive gene (33-35), with the same potency than 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.

Moderate changes in ABCA1 protein contents 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 >200kD, with higher intensities in differentiated adipocytes than in preadipocytes. Quantitative scanning of the ABCA1 signal in western blots showed that the relative content of the ABCA1 protein significantly increased 2-3 fold in differentiated adipocytes versus preadipocytes. Such a moderate increase in the content of ABCA1 protein in membranes adipocytes strikingly contrasted with the large induction in ABCA1 mRNA during fat cell differentiation. This indicated that some post-transcriptional 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) or 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 in (36) in a serum-free medium containing 0.2% BSA for 16 hours and then for 1 hour in the presence of apo-AI or protease inhibitors ALLN, or pepstatin. As shown in Figure 3, western blot analysis did not revealed any variation of ABCA1 protein contents in cells treated with either apoA-I or the protease inhibitors. Such treatments were also ineffective to increase ABCA1 protein or cholesterol efflux onto apo-AI after longer (3 to 16 hours) 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.

Functional ApoA-I-mediated lipid efflux in 3T3-L1 adipocytes.

We next examined the function of ABCA1 in 3T3L1 cells. We tested the ability of 3T3L1 cells to release cellular lipids (mainly phospholipid and cholesterol) onto lipid-poor ApoA-I, a process that is largely dependent on ABCA1 activity. Results in Figure 4 show that cholesterol 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 adipocytes 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 than above. Since the ABCA1 transporter has a dual ability to promote the efflux of cellular cholesterol and phospholipid (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 same specific activities for cholesterol (64680 +/- 14800 dpm/µg total cell cholesterol in preadipocytes and 62450 +/- 12850 dpm/µg in fully differentiated fat cells) and phospholipid (7022 +/- 544 and 7604 +/- 619 dpm/µg phospholipid in preadipocytes and adipocytes respectively), allowing direct comparison of apoA-I-mediated efflux between non-differentiated and differentiated fat cells. As shown in Figure 5, ApoA-I mediated phospholipid efflux increased from 0.36 % to 0.98 % during adipose conversion. This variation was in the same range than 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 being the accumulation of large intracellular triglyceride droplets. Since 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 non-esterified form, in agreement with previous reports in the literature (23). This proportion did not changed 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. In addition, when considering total membranes, we observed a nearly two-fold decrease in cholesterol contents in adipocytes compared to undifferentiated cells (Table 2). On the other hand, no change in phospholipid contents of total membranes occurred during differentiation. This resulted in a specific decrease in the cholesterol to 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 non-membrane (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 (38), in which only plasma membrane cholesterol is labeled. In this latter protocol, plasma membrane cholesterol labeling was achieved during a short incubation (20min) of cells with tritiated cholesterol incorporated into cyclodextrins. Immediately after labeling, cholesterol efflux was followed over time. Results in figure 5 panel C 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 panel B. In contrast, when the plasma membrane cholesterol labeling protocol was used (panel D) cholesterol efflux at an early time point (1hour of exposure to apo-AI) was doubled in adipocytes versus preadipocytes.
After 4 hours, 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 to 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 when all cellular cholesterol pools are labeled, or when labeled membrane cholesterol is allowed to redistribute to other cellular pools, by measuring efflux 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.**

Since 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 Beta-Adrenergic system and the cAMP-signaling pathway. Thus, we treated 3T3-L1 adipocytes with isoproterenol (a non selective beta agonist), and during the same incubation, we measured the concomitant release of cholesterol onto apo A-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 four hours. 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. 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, and cells were incubated for 24 hours in the presence of 8BrcAMP, a stable analogue of cAMP. During a 24-hour period of cAMP exposure,
ongoing lipolysis could not be measured accurately, because of reutilization or disappearance of lipolitic products in the culture medium. However, apo A-I-mediated cholesterol efflux at the end of the incubation period was increased two-fold in adipocytes treated with 8-Br cAMP compared to untreated controls (figure 5A). After 24 hours with 8-Br cAMP, ABCA1mRNA levels were stimulated 2 fold as compared to untreated controls (figure 6B). This indicated that the stimulatory effect of cAMP on cholesterol efflux in adipocytes is brought by stimulation of the expression of the ABCA1 gene. Altogether these data suggest that cholesterol mobilization from the fat cell onto apo A-I is not easily achieved during short-term activation of lipolysis, but rather requires sustained stimulation and cAMP-induced upregulation 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 apo-Al-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 LXR alpha, a transcriptional regulator of ABCA1, in a differentiation-dependent manner (26). We show here for the first time that the ABCA1 gene, like LXR alpha, 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 ABCA1protein does not increase more than two 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 apo-AI 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 post-transcriptional 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 underlying mechanisms remain unclear. Although no variation in total cell cholesterol content occurred during adipose conversion in the 3T3L1 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 to undifferentiated fat cells (39). Together, these data suggest that cholesterol is redistributed towards a non-membrane pool in differentiated cells, presumably the lipid droplet.

Such a shift in cholesterol intracellular distribution during differentiation might be a key feature to explain ineffective release of cholesterol relative to phospholipids from adipocytes. In this regard, our results showing that the ability of cholesterol transfer from the plasma membrane was not altered in adipocytes and proceeded in close proportion to the ABCA1 protein content, reinforce the importance of cholesterol distribution for efflux. This idea is in line of other previous studies that observed the absence of 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 apo-A1, 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 hours 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 concluded that adipose tissue did not significantly contributed to hypercholesterolemia (23). In addition one observation from the literature reported normal cholesterol contents 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, that could help to buffer the high levels of circulating cholesterol frequently found in obese states. Since 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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Reference List


Chemistry 226, 497-509. 1956.
Ref Type: Generic


Figure legends.

**Figure 1**: Effect of LXR/RXR ligands on the expression of ABCA1 and SREBP1c genes in 3T3-L1 cells. Confluent preadipocytes or fully differentiated adipocytes (day 6 post confluence) 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 hours. 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 relative to untreated control cells. * indicates a significant difference versus untreated controls (p<0.05).

**Figure 2**: ABCA1 protein levels in 3T3-L1 cells.
Western blot analysis of ABCA1 was performed as described in “Methods” on total membrane pellets prepared from preadipocytes (pread) or adipocytes (Ad). Equal quantities of protein (30µg) 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. * indicates a significant difference by paired t test, at the p<0.05 level.

**Figure 3**: ABCA1 protein levels in differentiated adipocytes in the presence of apoA-I and ALLN
Differentiated adipocytes were cultured in serum-free medium containing 0.2% BSA for 16 hours and then in the presence of 10μg/ml apoA-I, 50μM ALLN or 20μM Pepstatin for 1 hour. Cells were collected and Western blot analysis of ABCA1 was performed on total membrane pellets. A representative autoradiogram is shown on the top, and quantitative results were obtained by densitometric scanning of blots from 3 independent experiments.

**Figure 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 (0.5µCi) for 16 hours, and were allowed to equilibrate in the same medium for 24 hours. Then, apoA-I was added and the medium and cells were collected after 4 hours. 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 all the time (see methods), to prevent cholesterol esterification.

Concentration-dependence on apoA-I (Panel A), and time course of apoA-I-mediated cholesterol efflux (Panel B) are shown. Values are means +/- sem obtained from at least three independent determinations

Figure 5: Comparison of lipid efflux on apoA-I in preadipocytes and adipocytes.
ApoA-I-mediated efflux of phospholipids (panel A) and cholesterol (panel B) in preadipocytes (white bars) and differentiated (black bars) 3T3-L1 cells was measured as described after 4 hours in the presence of 10µg/ml apoA-I. Values are means +/- sem from at least five independent determinations. Panel C shows the time dependence of apoA-I-mediated cholesterol efflux in preadipocytes and adipocytes labeled with the standard protocol. Values are means +/- sem from at three independent determinations. Panel D shows apoA-I-mediated cholesterol efflux in cells labeled with the protocol described in (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 5mM, containing 0.5µCi of cholesterol. Immediately after labeling, cells were rapidly rinsed, cholesterol efflux was initiated by the addition of apoA-I and followed over time. A statistically significant difference between preadipocytes and adipocytes (Student’s t test, p<0.05 level) is indicated by *.

Figure 6: ApoA-I mediated cholesterol efflux during lipolytic stimulation of adipocytes.
Panel 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 hours, 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. Panel B: Effects of a 24-hour treatment with 8-Bromo cAMP on apoA-I mediated cholesterol efflux from fully differentiated adipocytes. Cholesterol efflux was evaluated as above, in the presence of 8-Bromo cAMP (0.3mM) added during the equilibration period and onwards (24hours). Significant differences compared to control untreated cells were tested by Student’s t test, and indicated for p<0.01: **. Panel C: Effect of a 24-hour treatment of differentiated adipocytes with 8-Bromo cAMP (0.3mM) on ABCA1 and LXR alpha mRNA levels. Each point represents mean values +/- sem, obtained from three separate experiments Significant difference at the p<0.05 level (*) is indicated.
Table 1: mRNA levels of LXRalpha and ABCA1 during the course of 3T3-L1 adipocyte differentiation.

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<tr>
<td>Preadipocytes</td>
<td>1.19 +/- 0.45</td>
<td>0.10 +/- 0.09</td>
</tr>
<tr>
<td>Adipocytes</td>
<td>43.0 +/- 24.1</td>
<td>4.6 +/- 1.7</td>
</tr>
<tr>
<td>Fold induction</td>
<td>36</td>
<td>44</td>
</tr>
</tbody>
</table>

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 means +/- sem obtained from at least 3 independent cultures. For ABCA1 and LXR alpha, differences between preadipocytes and adipocytes are statistically different by student’s t test, at the p<0.001 level.

Table 2: Lipid contents of 3T3-L1 preadipocytes and fully differentiated cells.

<table>
<thead>
<tr>
<th></th>
<th>Preadipocytes</th>
<th>Adipocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cholesterol (% total)</td>
<td>93.3 +/- 4.4 (3)</td>
<td>92.5 +/- 1.9 (4)</td>
</tr>
<tr>
<td>Total cholesterol (µg/10^6 cells)</td>
<td>10.2 +/- 1.4 (4)</td>
<td>10.2 +/- 1.8 (4)</td>
</tr>
<tr>
<td>Cholesterol in total membranes (µg/mg protein)</td>
<td>51 +/- 2 (3)</td>
<td>*** 27 +/- 1 (5)</td>
</tr>
<tr>
<td>Phospholipids in total membranes (µg/mg protein)</td>
<td>97 +/- 10 (3)</td>
<td>83 +/- 3 (5)</td>
</tr>
</tbody>
</table>

Values are means +/- sem with the number of independent determinations in parenthesis. 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 TLC plates in 85/15/1 (vol/vol/vol) Petroleum Ether/ Ethylc Ether/ Acetic Acid. Unlabeled standards were run in parallel to identify free and esterified cholesterol bands. *** indicates a significant difference by student’s t test, at the p<0.001 level. Cholesterol
and phospholipid contents were measured on total membranes.
Figure 1

mRNA /18S
(Fold stimulation over untreated cells)
Figure 3

ABCA1 protein (arbitrary units)

- apoA-1
- ALLN Pepst

-200 kD
Figure 4

A

\[
\text{% efflux vs. } \mu\text{g/ml apoA-I}
\]

B

\[
\text{ApoA-I-mediated cholesterol efflux vs. Time with apoA-I (min)}
\]