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The β -subunit of ATP synthase is involved in cellular uptake and resecretion of apoA-I but does not control apoA-I-induced lipid efflux in adipocytes

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Abstract

Cellular uptake and resecretion of apoA-I (apoA-I recycling) could be an important factor in determining the circulating plasma levels of apoA-I and/or HDL. Using a novel method to study protein recycling, we have recently demonstrated recycling of apoA-I by adipocytes and suggested that this is a receptor mediated process independent of ABCA1 function. In the present study, it is shown that apoA-I recycling by adipocytes can be blocked by a monoclonal antibody against the β -subunit of ATP synthase, a protein that had been previously identified as an apoA-I receptor. Investigation of the cellular recycling of two other proteins, an apolipoprotein and a small globular protein, showed that recycling of apoA-I is a selective process. The present study also shows that blocking apoA-I recycling has no effect on the rate of apoA-I-induced cholesterol or phospholipid efflux. It is concluded that cellular recycling of apoA-I is a selective process that involves the ectopically expressed β -subunit of ATP synthase. The physiological function of apoA-I recycling remains to be elucidated. However, this study shows that the process of apoA-I uptake and resecretion is not required for apoA-I lipidation.

Keywords

apoA-I; ATP synthase; Cholesterol; ABCA1; Lipid efflux; Adipocytes

Introduction

ApoA-I is the major protein component of HDL and is involved in the processes of HDL formation and cholesterol removal from peripheral tissues [1,2]. One of the main proteins

affecting the rate of loading of apoA-I with lipids is the membrane protein ABCA1 [3–6]. Genetic studies have shown that this protein plays a major role defining the levels of circulating HDL and individuals carrying certain mutations in ABCA1 develop Tangier's disease [7–9]. On the other hand, biochemical studies have shown that cells obtained from Tangier disease patients are defective in releasing cholesterol when incubated with apoA-I [10,11]. Although the fact that ABCA1 plays a major role in HDL biogenesis is strongly supported, the mechanism by which apoA-I acquires cellular lipids remains to be elucidated.

The cellular location where apoA-I lipidation takes place does not seem to be clearly defined. Some studies have suggested that apoA-I lipidation occurs on the surface of the plasma membrane by binding of the apolipoprotein to lipid perturbations caused by ABCA1 [12–15]. Conversely, other reports have suggested that lipidation involves endocytosis of apoA-I. The role of endocytosis is supported by colocalization studies of apoA-I and ABCA1 [16–18] and by studies showing a correlation between cellular uptake and lipidation of apoA-I [19–25].

Using a novel method to monitor apoA-I uptake and re-secretion (apoA-I recycling), we have recently reported apoA-I recycling by adipocytes [26]. We have also shown that lipidation of apoA-I can be effectively inhibited by BFA, which affects intracellular vesicular and ABCA1 transport. However, this inhibition does not affect cellular recycling of the apolipoprotein suggesting that apoA-I recycling is not affected by ABCA1 dynamics and, also, that lipidation and recycling could be independent and unrelated processes. The method used to monitor apoA-I recycling was based on the use of a recombinant apoA-I (pka-apoA-I) that contains a consensus phosphorylation site for protein kinase A, PKA. Incubation of pka-apoA-I with adipocytes leads to the appearance of phosphorylated apoA-I in the cell culture medium indicating that the protein entered the cell, was phosphorylated by PKA, and then re-secreted [26]. The ability of the method to monitor actual apolipoprotein recycling was strongly supported in the previous study. However, the specificity of the protein recycling process has not been extensively studied. To investigate further the specificity of the process of apoA-I recycling in adipocytes, the studies presented here have examined cellular recycling of two additional proteins. For this purpose, we constructed and tested a recombinant apolipoprotein-III (pka-apoLp-III), which is an exchangeable apolipoprotein of similar properties to apoA-I, and thioredoxin (pka-Thrx), a small globular protein, whose function is unrelated to that of apoA-I. Cellular recycling of these proteins and pka-apoA-I was tested in adipocytes. We are interested in studying adipocytes because, due to their large reservoirs of intracellular cholesterol [27,28] and their ability to efflux cholesterol to apoA-I [29,30], they are likely to contribute to the overall levels and/or composition of HDL.

On the other hand, although we have shown that inhibition of the lipidation of apoA-I does not prevent apoA-I recycling, this fact did not prove that apoA-I recycling is not needed for apoA-I lipidation [26]. To evaluate the role of apoA-I recycling in lipidation, it is necessary to observe the consequences of blocking apoA-I recycling on apoA-I lipidation. In the present study, we show that it is possible to inhibit apoA-I recycling with an antibody against the β subunit of ATP synthase. We tested the effect of this antibody because the ATP synthase β subunit has been previously identified as an apoA-I receptor involved in HDL uptake [31].

The study of the effect of blocking apoA-I recycling on apoA-I-induced phospholipid and cholesterol suggested that lipidation of apoA-I is independent of apoA-I recycling.

In summary, the present study provides experimental evidence that advances our understanding of the specificity of the process of apoA-I recycling, identifies a membrane

protein that plays a significant role in recycling and, also, evaluates the role of apoA-I recycling in HDL biogenesis.

Materials and methods

Materials

3T3L-1 cells were purchased from American Type Cell Culture (Manassas, VA). Isoproterenol, fatty acid free bovine serum albumin (BSA), isobutyl methyl xanthine (IBMX), dexamethasone, trypsin, sodium pyruvate, insulin, streptomycin, and penicillin were purchased from Sigma Chemicals Co. (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM) was purchased from Cellgro Mediatech, Inc (Herndon, VA). Infinity triglycerides reagent was from Thermo (Louisville, CO). Monoclonal antibodies to the β subunit of ATP synthase clone "3E8" was purchased from NOVUS Biologicals NB600 (Littleton, CO) and clone "3D5" was purchased from Abcam ab14730 (Cambridge, MA). [^{32}P]-orthophosphate (carrier free) was from MP Biomedicals (Irvine, CA). [^3H]-cholesterol was purchased from Perkin Elmer (Waltham, MA). Densitometric analysis was done with AlphaEase Software (Santa Clara, CO). Graphs and statistical calculations were performed on GraphPad (La Jolla, CA).

Cell culture

3T3 L-1 pre-adipocytes were cultured at 37°C in 8% CO₂ atmosphere in DMEM supplemented with 10% FBS and 0.01% antibiotics. One day after confluence, the differentiation into adipocytes was induced by addition of IBMX (111 $\mu\text{g/ml}$), dexamethasone (0.46 $\mu\text{g/ml}$), and insulin (1.5 $\mu\text{g/ml}$) in the medium [32]. After 48 h, the cells were incubated in DMEM/10% FBS containing insulin for additional 48 h. Afterward, the cells were maintained in DMEM/10% FBS. All experiments were conducted 12–14 days after completion of the differentiation period.

Cloning and purification of pka-apoLp-III, pka-Thrx, and pka-apoA-I

Full-length mature human apoA-I was cloned into a vector which incorporates an N-terminal tag containing six-His residues and a five amino acid recognition sequence (RRASV) for the catalytic subunit of cAMP-dependent protein kinase A (PKA), as previously described [26].

L. migratoria apolipoprotein-III (apoLp-III), a well-characterized exchangeable apolipoprotein that shares a number of physical properties with apoA-I, and thioredoxin (Thrx) were independently cloned into a pET30 (Novagen, Inc.) vector, which incorporates an N-terminal six-His residue tag and a PKA recognition site RRASV. The final sequence of pka-apoLp-III construct encoded a protein of 216 residues with a mass of 23 kDa, whereas the pka-thrx construct encoded a protein of 162 residues with a mass of 17.7 kDa. The proteins were expressed separately in *Escherichia coli* and purified by Ni-affinity chromatography using standard procedures. The protein sizes and identities were confirmed by SDS-PAGE and Maldi-TOF peptide mass fingerprinting on a Voyager DE-Pro mass spectrometer. The ability of the recombinant proteins to become phosphorylated by PKA was confirmed by in vitro phosphorylation with purified PKA and γ -[^{32}P]-ATP as previously described [33].

Cellular recycling of apoA-I, apoLp-III, and thioredoxin

3T3L-1 adipocytes cultured in six well dishes were radio-labeled by incubation for 4–5 h at 37°C with 50–75 $\mu\text{Ci/well}$ of [^{32}P]-orthophosphate (carrier free) in phosphate-free DMEM containing 0.05% BSA. At the end of the labeling period, the cell medium was replaced with

1 ml of fresh medium containing the indicated amounts of proteins (pka-thrx, pka-apoLp-III, or pka-apoA-I) and 10 μ g/ml isoproterenol (to enhance PKA activity). The amounts of proteins added to the wells are indicated in the figure legends. To determine cellular recycling of individual proteins, or the competition between apoLp-III and apoA-I, or between thrx and apoA-I, aliquots of the cell media were collected after a 60 min incubation period. The proteins found in the media were separated by SDS-PAGE on 4–20% gels. The gels were dried and phosphorylation was visualized by autoradiography and quantified by densitometry. Aliquots of the media were also used to determine glycerol levels.

To study the effect of the anti-ATP synthase β (either clone 3E8 or 3D5) antibodies, 1 h before the end of the labeling period the cell medium was replaced with 1 ml of fresh medium containing the indicated amounts of anti-ATP synthase β (either clone 3E8 or 3D5). Pka-apoA-I was added at the end of the labeling period (zero time) and incubated with the cells for 60 min.

Phospholipid efflux and apoA-I recycling

[32 P]-radiolabeled adipocytes were incubated with apoA-I in the presence or absence of anti-ATP synthase β antibody. After 60 min of incubation, the cell media were collected and pka-apoA-I purified by Ni-affinity chromatography. Aliquots of the purified apoA-I were subjected to SDS-PAGE to determine the concentration of protein and its phosphorylation. [32 P]-labeled phospholipids associated to purified apoA-I were extracted, and separated from free phosphate, using Folch's procedure [34]. The organic phase containing phospholipids was completely dried and the radioactivity associated to phospholipids determined by scintillation counting. Counts were normalized by the amount of apoA-I recovered. Thin layer chromatography was used to confirm incorporation of [32 P] into phospholipids.

Cholesterol efflux and apoA-I recycling

To monitor the effect of anti-ATP synthase β on apoA-I-dependent cholesterol efflux, apoA-I recycling and cholesterol efflux assays were performed in parallel. In both studies, before the experiment, adipocytes were incubated for 24 h in 2.5% FBS-DMEM, but wells to be used for cholesterol efflux the media contained [3 H]-cholesterol. Five hours prior to the experiment, the cell media were replaced by media containing [32 P]-phosphoric acid in 0.05% BSA-DMEM (for wells used in recycling assays) and [3 H]-cholesterol in 0.05% BSA-DMEM (for wells used in efflux assays).

For apoA-I recycling, anti-ATP synthase β clone 3D5 (60 μ g) was directly added to some wells 1 h prior to the addition of pka-apoA-I. At the starting time, recombinant pka-apoA-I was added to cell media.

Phosphorylation of pka-apoA-I was assessed and quantified as described above by SDS-PAGE and autoradiography using media collected 1 h after the addition of apoA-I.

The assays of cholesterol efflux were started by replacing the media of the wells by fresh media containing apoA-I, with or without antibody, and no radiolabeled cholesterol. Aliquots of the media were taken at the indicated time points, centrifuged for 2 min at 10,000 rpm in a micro-centrifuge, and radioactivity of the supernatant determined by scintillation counting. After 5 h, the remaining cell media were removed and cholesterol was extracted from the cell monolayers with isopropanol. An aliquot of the cholesterol extract was used to determine the [3 H]-cholesterol content of the cells. ApoA-I-induced [3 H]-cholesterol efflux was estimated as the difference (Δ cpm) between the radioactivity determined in medium containing apoA-I and the radioactivity found in the medium of wells

containing no apoA-I. The percentage of cellular cholesterol efflux at each time point was calculated as:

$$100 * \Delta \text{cpm media} / (\text{cpm media} + \text{cpm homogenate}).$$

The rate of cholesterol efflux was calculated from the slopes of the time courses by linear regression.

Results

Specificity of apoA-I recycling

We have recently shown that adipocytes internalize and resecret apoA-I and have also gathered evidence suggesting that this process of apoA-I recycling is mediated by a receptor and is characterized by a K_m of $\sim 1-2 \mu\text{M}$. It has also been shown that human apoA-I competes with and prevents the recycling of recombinant pka-apoA-I [26]. In spite of this information, the specificity of the protein recycling process can be investigated further by extending the studies to other proteins. Therefore, two additional recombinant proteins containing a PKA recognition site were constructed, expressed and purified to investigate further the specificity of the mechanism of apoA-I recycling observed in adipocytes. One of these proteins, pka-apoLp-III, is an insect exchangeable apolipoprotein that shares a number of properties with apoA-I. ApoLp-III is an α -helical protein [35] and, as apoA-I, it has the ability to form discoidal lipoprotein particles upon incubation with multilamellar liposomes of phospholipid [36,37]. A second protein, pka-Thrx was chosen as a model of small globular protein of function unrelated to apoA-I. These two proteins allow testing whether the specificity of apoA-I recycling is high, intermediate, or low. For example, if the specificity of the recycling process is high we would only observe recycling of apoA-I. Contrarily, if the specificity of the process of cellular recycling of apoA-I is low, we would expect to observe recycling of all proteins. Finally, if only the apolipoproteins (apoA-I and apoLp-III) are recycled it would suggest that the recycling process would have an intermediate specificity, selective for apolipoproteins.

Recycling of the pka constructs of apoA-I, apoLp-III, and Thrx was individually assessed by incubating the proteins with [^{32}P]-radiolabeled adipocytes as previously described for apoA-I [26]. With this approach, the rate of protein recycling is determined from the extent of radiolabeling of the protein recovered from the cell culture medium after a period of incubation with the cells. For this purpose, the experimental medium is subjected to SDS-PAGE and the phosphorylation visualized by autoradiography. The rationale of the method is that only those proteins that are internalized will be phosphorylated by intracellular PKA, and, if re-secreted, they can be recovered in the cell culture medium.

The Fig. 1 shows the results of a representative experiment in which we tested the ability of adipocytes to recycle apoA-I, apoLp-III, and Thrx. From the extent of phosphorylation observed, this study suggests that apoA-I is recycled at a faster rate than the other two proteins. The extent of apoA-I phosphorylation, normalized by number of moles, indicates that apoA-I is recycled ~ 20 and ~ 7 times faster than apoLp-III and Thrx, respectively. The ability of the proteins to become phosphorylated by PKA was confirmed by in vitro phosphorylation with purified PKA and γ -[^{32}P]-ATP. This assay showed that the three proteins are phosphorylated to similar extents by PKA (Fig. 1d) suggesting that the differences observed in the recycling experiments are actually due to differences in the rate of recycling.

The specificity of the recycling process was also studied by analyzing a possible inhibition of apoA-I recycling by either apoLp-III or Thrx. For this purpose, the cells were incubated with pka-apoA-I in the absence or in the presence of an excess of thrx or apoLp-III. Figure 2 shows that neither apoLp-III nor Thrx affect the extent of apoA-I recycling. We had previously shown that human apoA-I (no pka-tag) competes with the recombinant pka-apoA-I decreasing the extent of recycling (phosphorylation) of the later in proportion to the molar ratio. Thus, an inhibition of ~50% was observed when the cells were incubated with a 1:1 molar ratio of human apoA-I to pka-apoA-I [26]. The fact that Thrx or apoLp-III, at a 4:1 molar ratio, produced no significant reduction in apoA-I phosphorylation suggests that the process of apoA-I recycling is quite specific. Confirming the results shown in the Fig. 1, the results shown in Fig. 2 also show that pka-apoLp-III and pka-Thrx are only slightly phosphorylated, or recycled by adipocytes.

Inhibition of apoA-I recycling by an antibody to ATP synthase beta subunit

Since cell surface-expressed ATP synthase β has been identified as a high-affinity receptor for apoA-I in adipocytes [31] and binding of apoA-I to this receptor could be blocked by a monoclonal antibody against the β subunit [31], we decided to investigate a possible role of this receptor in the process of apoA-I recycling by adipocytes. For this purpose, the apoA-I recycling assays were performed as before, incubating [32 P]-radiolabeled adipocytes with pka-apoA-I, but in the presence of increasing amounts of anti-ATP synthase β . Following the incubation, pka-apoA-I was purified from the media by affinity chromatography and its intensity of phosphorylation determined after SDS-PAGE and autoradiography, Fig. 3a and b, respectively. As shown in Fig. 3c, the intensity of phosphorylation of pka-apoA-I progressively decreases with increasing antibody dose until it levels off at approximately 35% of maximum phosphorylation. This level of inhibition is comparable to the inhibition of purified F₁ ATP synthase and endothelial cell surface ATP synthase activities observed in a previous study by binding of a monoclonal antibody (clone 3D5AB1) to the domains near the active site of the beta subunit [38]. We would like to note that, although anti-ATP synthase β clone 3D5 successfully blocked apoA-I recycling, another monoclonal antibody tested (clone 3E8) did not have a significant effect on apoA-I phosphorylation levels (data not shown).

We have previously shown that the cellular PKA activity has a direct influence on the intensity levels of pka-apoA-I phosphorylation [26]. Therefore, a possible effect of the antibody on PKA activity of adipocytes was monitored by determining the rate of glycerol release (lipolysis) into the media. The rate of lipolysis, which is highly affected by PKA activity, showed no significant changes with increasing concentrations of antibody (Fig. 4) suggesting that the changes in pka-apoA-I phosphorylation were in fact due to inhibition of the apoA-I recycling, rather than to changes in the intracellular PKA activity.

The same cells used to study the effect of the antibody on apoA-I recycling were simultaneously used for the estimation of the effect of the antibody on phospholipid efflux. This was possible because radiolabeling of the cells with [32 P]-phosphate leads to the radiolabeling of the cellular phospholipids. Thus, aliquots of the samples of pka-apoA-I purified from the cell culture medium that were used for the study of protein phosphorylation, were also used for estimating changes in the rate of phospholipid efflux. The phospholipids associated to apoA-I were extracted with organic solvents and their radioactivity determined by scintillation counting. As shown in Fig. 4, increasing concentrations of antibody did not promote significant changes in the levels of radioactive phospholipid (normalized by pka-apoA-I protein content). Therefore, the study suggests that the antibody does not affect the rate of apoA-I-induced phospholipid efflux.

Overall, the study with the anti-ATP synthase β clone 3D5 suggests that the β -subunit of ATP synthase is involved in the process of apoA-I recycling but does not affect the rate of phospholipid efflux.

Effect of the inhibition of apoA-I recycling on apoA-I-induced cholesterol efflux

Since it is not fully clear whether or not cholesterol and phospholipids are loaded by the same mechanism, we also studied the effect of the anti-ATP synthase β antibody on the rate of cholesterol efflux. The effect of the antibody on the rate of cholesterol efflux and apoA-I recycling was studied in parallel. The cells used were from the same batch and were identically treated. The only difference was that wells used for apoA-I recycling were incubated with [32 P]-phosphate and those used for cholesterol efflux with [3 H]-cholesterol. This consideration may be important because cholesterol efflux experiments involve radiolabeling of the cells with [3 H]-cholesterol by incubation for 24 h in a media containing less serum (2.5%) than the normal medium (10% FBS) and previous studies have shown that the serum levels of the medium can influence the expression levels of ectopic ATP synthase in HepG2 hepatocytes and the human T cell line CEM [39–41].

For this study, [32 P]-phosphate labeled or [3 H]-cholesterol labeled cells were pre-incubated for 1 h with or without 60 μ g anti-ATP synthase β clone 3D5. The time courses of [3 H]-cholesterol efflux obtained in the presence or in the absence of antibody showed no significant differences, suggesting that the ATP synthase β subunit is not involved in the mechanism of apoA-I-induced cholesterol efflux. Under identical conditions, however, the antibody reduced the rate of apoA-I recycling by 40% (Fig. 5b).

Discussion

The mechanisms underlying the process of apoA-I-induced lipid efflux are not completely clear. The studies described here intended to evaluate the contribution of apolipoprotein A-I uptake in the process of apoA-I lipidation in adipocytes using a method to study protein uptake and re-secretion that was recently developed in our lab [26]. This method monitors the appearance of phosphorylated protein in the cell culture medium and, since PKA-catalyzed phosphorylation is an intracellular process, it provides an unambiguous way to determine cellular recycling of proteins. We have previously shown that incubation of adipocytes with a recombinant apoA-I containing a pka-consensus site leads to the appearance of phosphorylated protein in the cell culture medium. Although the evidence of apoA-I recycling in adipocytes is strong, we still know very little about the mechanism involved in this process. One of the aspects that required further studies was the selectivity of the recycling process. The study of this issue had been limited mostly to the demonstration that human apoA-I competed and, therefore, inhibited pka-apoA-I phosphorylation [26]. Here, we extended the study of the specificity of the recycling process to two additional proteins that were selected and constructed on the basis of their small size and properties. The study suggested that the process of protein recycling that is being monitored in our studies is considerably selective for apoA-I. The apolipoprotein, pka-apoLp-III, and the small and compact globular protein, pka-Thrx, were both recycled at a much lower rate than pka-apoA-I.

A second important aspect of the current study is the finding that apoA-I recycling can be blocked by an anti-ATP synthase β -subunit antibody. This result suggests the involvement of the β -subunit of ATP synthase in apoA-I recycling. However, it also suggests that the process of apoA-I recycling monitored is related to previous studies in hepatocytes that linked HDL endocytosis via a non-SRBI receptor to the interaction of the β -subunit of ATP synthase with apoA-I [31]. ATP synthase-mediated ADP production has been proposed to modulate HDL endocytosis via the G-protein coupled receptor P2Y₁₃ on the hepatocyte

surface [42,43]. Also significant is the fact that niacin, which is used to raise plasma levels of HDL, has been shown to reduce the ectopic expression of the β -subunit of ATP synthase in hepatocytes and inhibit cellular uptake of HDL [39]. Our study builds on these previous studies and suggests that β -ATP-synthase could also play a significant role in the metabolic function of apoA-I in adipocytes. The involvement of β -ATP-synthase is somewhat surprising. However, cell surface expression of ATP synthase has been reported for several cell lines [31,44–46] and, in addition, β -ATP-synthase has been shown to bind to several alpha helical proteins [45,47–49].

The third topic of this study related to the role of apoA-I recycling in apolipoprotein-induced lipid efflux from adipocytes. Several studies have observed cellular uptake of apoA-I [20,21,23,25] and it has also been suggested that apoA-I promotes the removal of cholesterol from intracellular sites by a mechanism involving ABCA1 [50]. Early studies showed defective intracellular vesicular trafficking in macrophages from patients with Tangier's disease [51]. Moreover, Tangier's fibroblasts were shown to be defective in vesicular trafficking of both cholesterol and ABCA1 and also in internalization of apoA-I [17]. These studies, the apparent high-affinity binding of apoA-I to ABCA1 [15,52], and the co-localization of apoA-I with ABCA1 in endocytic vesicles [16–18] suggested that trafficking of apoA-I bound to ABCA1 could mediate cholesterol efflux from late endosomes or lysosomes. The evidence supporting a role of β -ATP-synthase in apoA-I uptake and re-secretion leads to a possible scenario in which ABCA1 together with β -ATP-synthase could constitute a complex receptor involved in apoA-I recycling and lipidation. This model could also apply to adipocytes. In fact adipocytes express high levels of ABCA1 protein and the rate of apoA-I-induced cholesterol efflux increases with the levels of ABCA1 protein [53]. However, studies in adipocytes suggest that ABCA1 and β -ATP-synthase work independently of each other. Brefeldin A (BFA), a well known inhibitor of cholesterol efflux, affects intracellular vesicle transport and also ABCA1 recycling between plasma membrane and intracellular vesicles [54]. Incubation of adipocytes with BFA was shown to promote a strong inhibition of apoA-I-induced lipid efflux [26]. However, suggesting that apoA-I recycling would be an ABCA1 independent process, BFA did not inhibit apoA-I recycling [26]. In spite of these studies, the purpose of apoA-I internalization and whether recycling is necessary for apoA-I lipidation remained unclear. Clarification of this issue required determining the role of intracellular trafficking of apoA-I in apoA-I lipidation. This is difficult to prove because normally only a small fraction of apoA-I is actually internalized and most apoA-I is found associated to the plasma membrane [13,14]. However, by monitoring apoA-I recycling through the appearance of phosphorylated apoA-I in the cell culture the background of apoA-I resecretion, normally associated to the methods used to monitor apolipoprotein uptake and resecretion, is effectively removed. Thus, in the present study, it was possible to observe that inhibition of apoA-I recycling does not lead to a decrease in the rate of apoA-I-induced cholesterol or phospholipid efflux. Therefore, the current data suggest that the β -ATP-synthase dependent process of apoA-I uptake and re-secretion is not related to the process of apoA-I-induced cholesterol efflux. In this regard, our study provides experimental support to previous studies which have suggested a model in which lipidation of apoA-I is an ABCA1 dependent process that takes place on the cell surface [12–14,19,55].

The relationship of apoA-I recycling to efflux as well as the localization of apoA-I lipidation are likely to remain controversial and highly studied topics [56,57]. We have studied a β -ATP-synthase dependent process of apoA-I recycling. However, the possibility of other mechanisms also contributing to apoA-I uptake and resecretion cannot be fully dismissed. Moreover, given the fact that β -ATP-synthase dependent apoA-I recycling is not related to lipid efflux, future studies should investigate the physiological role of this process. A possible role could be apoA-I degradation. Preliminary studies suggest that this is not the

case in adipocytes. However, studies in other cell lines could shed some light into the physiological function of apoA-I recycling.

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Fig. 1.

Cellular recycling of apoA-I, apoLp-III, and Thrx. Adipocytes were incubated for 4 h with labeling media containing 50 $\mu\text{Ci/ml}$ [^{32}P]-phosphoric acid. Media containing recombinant pka-apoA-I (1.5 nmol), or pka-apoLp-III (3 or 6 nmol), or pka-Thrx (3 or 6 nmol) was added to cell monolayer for recycling assays. After 1 h, the media were collected and samples were subjected to SDS-PAGE and autoradiography to detect protein phosphorylation. **a** Representative Coomassie-stained gel of media samples (15% volume loaded) showing location of pka-apoA-I, pka-apoLp-III and pka-Thrx. **b** Autoradiogram of the gel showing [^{32}P]-phosphorylation of pka-recombinant proteins. **c** Average pka-apoLp-III and pka-Thrx phosphorylation (as percentage of pka-apoA-I phosphorylation). Phosphorylation was quantitated by densitometry of the autoradiography, and normalized by the number of moles of recombinant protein. Bars represent the average $\pm$ s.d. of two independent experiments for apoA-I:apoLp-III ($n = 6$) and one experiment for apoA-I:Thrx ($n = 4$). **d** In vitro phosphorylation of pka-apoA-I, pka-apoLp-III and pka-Thrx; purified proteins were incubated with bovine PKA and γ -[^{32}P]-ATP and subsequently analyzed by SDS-PAGE and autoradiography. The top panel shows the Coomassie blue stained gel, whereas the bottom panel displays the autoradiography of the gel

Fig. 2.

Competition experiments of apoA-I recycling. [^{32}P]-prelabeled adipocytes were incubated for 1 h with pka-apoA-I (1.5 nmol) in the absence or in the presence of pka-apoLp-III (3 or 6 nmol) or pka-Thrx (3 or 6 nmol). The cell media were recovered and subjected to SDS-PAGE and autoradiography to estimate protein recycling. **a** Representative coomassie-blue stained gel of media samples; **b** Autoradiography of the gel (from *panel A*) showing [^{32}P]-phosphorylation of pka-recombinant proteins. **c** Average phosphorylation of pka-recombinant proteins as determined by autoradiography of media gels. Bars represent the mean $\pm$ S.D. of two independent experiments for apoA-I: apoLp-III ($n = 5$) and one experiment for apoA-I:Thr ($n = 3$). The differences between pka-apoA-I densities of phosphorylation were found to be not significant by student's t -test ($P > 0.05$) for both apoLpIII and Thr competition assays

Fig. 3.

The effect of antibody to ATP synthase (β subunit) on cellular recycling of apoA-I. [32 P]-prelabeled adipocytes were incubated for 1 h in labeling media containing 75 μ Ci/ml [32 P]-phosphoric acid and anti-ATP synthase β (monoclonal 3D5). Subsequently, 40 μ g of pka-apoA-I was added to cell media and incubated for an additional 90 min period. Afterward, the media were collected and apoA-I purified by Ni-affinity chromatography. Samples were run on SDS-PAGE and exposed to film to detect phosphorylation. **a** Representative Coomassie blue stained gel; **b** autoradiogram displaying phosphorylation of purified pka-apoA-I protein. **c** Effect of antibody concentration on the phosphorylation intensity of pka-apoA-I. The intensities of phosphorylation were normalized by protein recovery (as

determined by densitometry of the coomassie blue stained gels). The data points in **c** represent the mean $\pm$ S.D. with $n = 2-6$ from two independent experiments

Fig. 4.

Effect of anti-ATP synthase β on apoA-I-dependent phospho-lipid efflux. [^{32}P]-prelabeled adipocytes were pre-incubated with different concentrations of anti-ATP synthase β clone 3D5 for 1 h. Recombinant pka-apoA-I was added to cell media (40 $\mu\text{g/ml}$ final concentration) and after incubation for 1 h the media was collected and apoA-I purified by Ni-affinity chromatography. Lipids were then extracted from apoA-I and the radioactivity associated to [^{32}P]-labeled phospholipids determined by scintillation counting. The radiolabeling intensities of phospholipids (*open squares*) were normalized by apoA-I content. The graph also shows the effect of the antibody on the lipolytic rate (glycerol output) of adipocytes (*closed squares*). Error bars represent mean $\pm$ S.D. of $n = 2-6$ from two independent experiments

Fig. 5.

Effect of anti-ATP synthase β on apoA-I-induced cholesterol efflux. **a** Cholesterol efflux was studied in adipocytes radiolabeled with [^3H]-cholesterol and pre-incubated for 1 h with or without 60 μg anti-ATP synthase β clone 3D5. Pka-apoA-I was then added to some wells. Efflux media were collected from control apoA-I containing wells at the indicated time points and cholesterol efflux estimated as indicated in “Materials and methods” section. The data points represent the mean $\pm$ S.D. ($n = 4$). **b** The rates of efflux (*Solid bars*) were calculated from the time courses shown in **a**. ApoA-I recycling (*Open bars*) was studied in the same batch of cells, but the cells were radiolabeled with [^{32}P]-phosphate. Apo A-I recycling was determined from the intensity of phosphorylation of apoA-I purified from the medium after 1 h of incubation with the cells. Samples were separated by SDS-PAGE stained with coomassie blue. Gels were dried and subjected to autoradiography and densitometry to calculate apoA-I phosphorylation. The intensities of phosphorylation were normalized by apoA-I content. The data in **b** represent the mean rates of cholesterol efflux (obtained from the slopes of the graph shown in **a**) and their corresponding standard error. The data of apoA-I recycling represent the means $\pm$ s.d. ($n = 4$)