Adipocyte Differentiation-Related Protein Promotes Fatty Acid Storage in Cytosolic Triglycerides and Inhibits Secretion of Very Low-Density Lipoproteins

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Objective—We investigated the role of adipocyte differentiation-related protein (ADRP) in triglyceride turnover and in the secretion of very low--density lipoprotein (VLDL) from McA-RH7777 cells and primary rat hepatocytes.

Methods and Results—An increase in the expression of ADRP increased triglyceride accumulation in cytosolic lipid droplets and prevented the incorporation of fatty acids into secreteable triglycerides, thereby reducing the secretion of triglycerides as well as of apolipoprotein B-100 (apoB-100) and apoB-48 VLDL. The ability of ADRP to block the secretion of apoB-100 VLDL1 decreased with increasing quantities of fatty acids in the medium, indicating a saturable process and emphasizing the importance of sequestering of fatty acids for the effect of ADRP on VLDL secretion. Knockdown (small interfering RNA) of ADRP decreased the pool of cytosolic lipid droplets but increased only the secretion of apoB-48 VLDL1. Additionally, there was an increased flow of fatty acids into β-oxidation.

Conclusions—ADRP is essential for the accumulation of triglycerides in cytosolic lipid droplets. An increase in ADRP prevents the formation of VLDL by diverting fatty acids from the VLDL assembly pathway into cytosolic triglycerides, whereas a decrease of the protein increases the sorting of fatty acids to β-oxidation and promotes the secretion of apoB-48 VLDL1. (Arterioscler Thromb Vasc Biol. 2006;26:1566-1571.)

Key Words: adipose differentiation–related protein ■ cytosolic lipid droplets ■ apolipoproteins B ■ β-oxidation ■ small interfering RNA

Cytosolic lipid droplets are ubiquitous organelles involved in the storage and turnover of neutral lipids such as triglycerides. Several proteins have been identified on these droplets, the most well known being the PAT domain proteins,1–3 including the perilipins, adipocyte differentiation-related protein (ADRP or adipophilin) and Tip 47. ADRP, which is ubiquitously expressed,4 has a central role in the formation of lipid droplets.5 These droplets are assembled at the microsomal membrane by an insulin-dependent process that requires phospholipase D1, extracellular signal regulated kinase 2, and the motor protein dynein.6,7 The assembly process involves the formation of small primordial droplets,7 which grow in size by a fusion process that is dependent on intact microtubules8 and dynein.5

The assembly of very–low density lipoproteins (VLDLs)9–12 starts with the cotranslational lipidation of apolipoprotein B-100 (apoB-100), forming a pre-VLDL particle. VLDL2 (Svedberg flotation [sf] units 20 to 60) is formed from pre-VLDL by additional lipidation,13 whereas VLDL1 (sf 60 to 80) is formed from VLDL2 by a mechanism that is dependent on an ADP ribosylation factor 1–controlled sorting/transport process14 and involves the addition of a bulk load of lipids to the particle.12,13 The triglycerides used in this assembly process are largely derived from triglycerides in cytosolic lipid droplets.15,16

In this article, we demonstrate that an increase in ADRP promotes the storage of triglycerides in cytosolic lipid droplets and inhibit their secretion as VLDL. Conversely, a knockdown of ADRP decreases the storage of triglycerides but channels the fatty acids mainly to β-oxidation.

Materials and Methods

ADRP Increases Triglyceride Storage and Decreases Its Secretion and the Secretion of VLDL in McA-RH 7777 Cells

In these experiments, McARH 7777 cells were stably transfected with ADRP in the inducible t-rex system. Induction of

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the McA-RH7777 cells with tetracycline gave rise to an increased expression of ADRP (supplemental Figure IA, available online at http://atvb.ahajournals.org). Such an induction resulted in a 4.0±1.5-fold increase (mean±SD; n=8) in the pool of cytosolic lipid droplets (Figure 1A and 1B). There was also a significant increase in the cellular triglyceride mass (measured after 48-hour induction) from 2.26±0.36 to 3.42±0.67 mmol (mean±SD; n=8; P<0.002; Mann–Whitney rank sum test).

There was no difference between induced and uninduced McA-RH7777 cells in the rate of accumulation of radioactive triglycerides after incubation with [1H]-palmitic acid (supplemental Figure IIA). Moreover, the induction of ADRP did not influence the total accumulation of radiolabeled triglycerides in the system (cell and culture medium; supplemental Figure IIB); however, the rate of secretion of radiolabeled triglycerides decreased by 60% after the increase in the amount of ADRP (supplemental Figure IIC). An increased cellular accumulation and a decreased secretion of triglycerides, after induction of the ADRP expression, were also observed after continuous labeling with [1H]-palmitic acid (data not shown) and after incubation with [14C]-glycerol (supplemental Figure III). Thus, an increase of ADRP increases the accumulation of triglycerides in cytosol and prevents their secretion.

The induction of the McA-RH7777 cells with tetracycline (increasing the amount of ADRP) reduced the secretion of radiolabeled apoB-100 in the VLDL1 and VLDL2 density ranges (Figure 1C). However, the ratio between VLDL1 and VLDL2 decreased from 1.4±0.2 to 0.9±0.06 (mean±SD; n=3; P=0.031; t test), indicating a larger effect on VLDL1. There was also a decrease in the secretion of radiolabeled apoB-48 in the VLDL1 density region but not of apoB-48 in the high-density lipoprotein density region (Figure 1C). These effects of ADRP overexpression were significantly greater than those on the secretion of transferrin and total protein, indicating that ADRP specifically affects the biosynthesis of VLDL.

**ADRP Enhances Triglyceride Storage and Reduces Secretion of Triglycerides and VLDL in Primary Rat Hepatocytes**

Infection of primary rat hepatocytes with adenovirus encoding ADRP (supplemental Figure 1B) gave rise to a 1.9±0.8-fold (mean±SD; n=7) increase in the cellular pool of lipid droplets when compared with the control (adenovirus encoding Zs-green; Figure 2A and 2B). The difference between ADRP and the control (Zs-green) was less than that observed between induced and uninduced McA-RH7777 cells (Figure 1A and 1B). The expression efficiency might contribute to this difference; whereas all McA-RH7777 cells were transfected with ADRP, the efficiency of adenovirus-mediated gene transfer (measured as the expression of Zs-green) was 80%. Another reason for this discrepancy is the effect of the virus infection per se on the formation of lipid droplets. Thus, there was a 1.4±0.5-fold (mean±SD; n=8; P=0.01; Mann–Whitney rank sum test) increase in cells transfected with the adenovirus encoding Zs-green compared with the uninfected control. Finally, the amount of lipid droplets in the untreated primary cells was much higher than in uninduced McA-RH7777 cells (compare Figures 1A and 2A). Decreasing the amount of glucose and insulin during the culture had a dramatic effect on the amount of lipid droplets and cellular triglycerides in the primary hepatocytes (supplemental Figure IVA). However, under these conditions, the levels of VLDL1...
Overexpression of ADRP in primary rat hepatocytes increases the accumulation of cytosolic lipid droplets and reduces the secretion of VLDL1. All analyses were performed 65 hours after start of infection with the adenovirus. A and B, ADRP increases the pool of lipid droplets in the cell. Primary rat hepatocytes were either left uninfected or infected with adenovirus (500 inclusion-forming units per cell) encoding Zs-green (control) or ADRP and stained with Oil Red O (A; bar=20 μm). B shows the increase in the size of the lipid droplet pool (determined as described in Figure 1 legend) after infection with adenovirus encoding ADRP when compared with the Zs-green control (set to 100%; mean±SD; n=7; ★P<0.001 vs Zs-green; Mann–Whitney rank sum test). C, ADRP reduces the secretion of VLDL1 but not of transferrin and other apoB-containing lipoproteins. Primary rat hepatocytes were infected with adenovirus encoding ADRP or Zs-green (control) and incubated with [35S]-methionine-cysteine (200 μCi/mL culture medium) for 3 hours. The medium was collected and analyzed as in Figure 1F. Values represent percentage recovery in cells overexpressing ADRP compared with those overexpressing Zs-green (mean±SD; n=3). ★★P<0.004; ★★★P<0.014 vs transferrin (1-way ANOVA).

Induction of the McA-RH7777 cells with tetracycline (which increased the amount of ADRP) decreased the turnover of the stored radiolabeled triglycerides (Figure 3A). This observation was confirmed in primary rat hepatocytes (supplemental Figure VII). However, when the formation of acylCoA and the biosynthesis of triglycerides was inhibited by Triacsin C,8 the rate of the turnover of the stored radiolabeled triglycerides increased, approaching that observed in the uninduced cells. This indicates that the ADRP-induced decrease in the turnover of cellular triglycerides is dependent on the formation of acylCoA.

Next, we investigated whether the ADRP-dependent inhibition of the apoB-100 VLDL1 secretion could be influenced by the addition of oleic acid to the culture medium. ADRP inhibited the secretion of radiolabeled apoB-100 VLDL1 at all concentrations of oleic acid investigated (Figure 3B).
However, the inhibition was greatest at oleic acid concentrations >100 μmol/L (70% to 80%); at >100 μmol/L, it decreased almost linearly to <20% at 360 μmol/L (Figure 3B; supplemental Figure VIII A), although increasing concentrations of oleic acid did not reduce the intracellular ADRP levels but rather tended to increase them (supplemental Figure V I I B and V I I C). Thus, the ADRP-induced inhibition of apoB-100 VLDL1 secretion can be overcome by increasing the amount of fatty acids in the cell.

Increasing levels of oleic acid inhibited the secretion of radiolabeled apoB-100 VLDL2 (supplemental Results; supplemental Figure V I I D). This inhibition was suppressed when the maximal ADRP induced inhibition of the apoB-100 VLDL1 secretion was observed (supplemental Results; compare supplemental Figure V I I A and V I I D). These observations are in line with the proposed precursor–product relationship between VLDL2 and VLDL1.13 Immunoblot experiments demonstrated that overexpression of ADRP did not affect the amount of the microsomal triglyceride transfer protein in the cell (supplemental Figure V I I E).

Knockdown of ADRP Influences the Storage and Oxidation of Fatty Acids and the Secretion of ApoB-48 VLDL1

Small interfering RNA (siRNA) to ADRP decreased the expression of the protein when compared with a control siRNA, whereas the control proteins were unaffected (Figure 4A). Such a knockdown of ADRP gave rise to a decrease in the accumulation of lipid droplets (Figure 4B). Thus, the size of the pool of lipid droplets in cells transfected with ADRP siRNA was 66±5% of that seen in cells transfected with control siRNA (Figure 4C). ADRP siRNA did also increase the rate of β-oxidation both of radiolabeled fatty acids supplied to the cells (data not shown) and radiolabeled fatty acids present in cytosolic triglycerides (Figure 4D). A small increase in the proportion of the intracellular pool of triglycerides that was secreted was observed in cells transfected with ADRP siRNA (supplemental Figure IX).

There was a 3.1-fold increase in the rate of secretion of apoB-48 VLDL1 when the cells were transfected with ADRP siRNA, but there was no effect on the secretion apoB-100 VLDL1 (Figure 4E). The increase in apoB-48 VLDL1 differed significantly from that observed for the controls (transferrin and the total secreted proteins; Figure 4E).

Discussion

This study shows that an increase of ADRP in liver cells increases the size of the pool of cytosolic lipid droplets. Conversely, a knockdown of ADRP by siRNA decreases this pool. An increase in the cellular amount of ADRP reduced the secretion of triglycerides and VLDL1 by converting fatty acids from leaving the cytosolic triglycerides to be incorporated into triglycerides in VLDL. The major effect of the knockdown of ADRP was an increased β-oxidation; however, there was also an increase in the rate of secretion of apoB-48 VLDL1 after such a knockdown.

Our results confirm observations by others that ADRP promotes the formation of lipid droplets in cells.5 The effects of an increased expression of ADRP were observed in both McA-RH7777 cells (stably transfected with ADRP in the inducible t-rex system) and primary hepatocytes; however, the effect was not as pronounced in the primary cells as in the McA-RH7777 cells. Possible reasons for this discrepancy have already been discussed above. Despite these differences, the results in the primary hepatocytes confirmed the observation in McA-RH7777 cells (ie, that an increase in the amount of ADRP promotes the accumulation of lipid droplets and decreases the secretion of triglycerides and VLDL1).

The major proportion of triglycerides secreted with VLDL is formed from fatty acids derived from stored triglycerides by hydrolysis15,16 (ie, the flow of fatty acids from cytosolic lipid droplets to VLDL triglycerides is of outmost importance for the regulation of the secretion of this lipoprotein). Our results indicate that an increase in the amount of ADRP in the...
The comparison was done in the presence or absence of Triacsin C, which prevents the activation of fatty acids and their incorporation into triglycerides.8 At the basal expression of ADRP, Triacsin C decreased the turnover of stored triglycerides, as we observed a large surplus of fatty acids after the treatment (approaching that rate seen in uninduced control (mean±SD; n=4; *significant difference vs control P<0.028; Mann–Whitney rank sum test). E, ADRP siRNA increases the secretion of apoB-48 VLDL1 but not apoB-100 VLDL1 from cells treated with oleic acid. Cells were labeled with [35S]-methionine-cysteine (200 μCi/mL culture medium) for 3 hours and analyzed for the radioactivity in transferrin, in total protein in the medium, and in apoB-100 and apoB-48 in VLDL1 as described in Figure 1 legend. Values represent percentage recovery in the induced cells compared with the uninduced control (mean±SD; n=12; †P<0.05 vs † and ‡; †NS vs * and **; 1-way ANOVA).

The effect of the increased expression of ADRP on the turnover of the cellular pool of triglycerides is dependent on the ability to activate fatty acids (including those fatty acids that had been released by hydrolysis) to allow them to enter into triglycerides. This, in turn, indicates that ADRP promotes the storage of newly formed triglycerides, including those formed from fatty acids released by hydrolysis.

Although an increase in the amount of ADRP gave clear effects on the secretion of both apoB-100 and apoB-48 VLDL1, the knockdown of ADRP only resulted in an increase in the secretion of apoB-48 VLDL1. This most likely reflects the very strong influence of increased amount of fatty acids on the assembly of apoB-48 VLDL1.17,18 Although the increase in the secretion of apoB-48 VLDL1 seen after ADRP knockdown was large, the amount of VLDL1 assembled by apoB-48 in these cells is only, at the most, 15% of that assembled by apoB-100. This is most likely the reason why we only detected a small increase in the secretion of triglycerides when ADRP was decreased. During the preparation of this article, an ADRP knockout mouse was published,19 showing a significant effect on the accumulation of triglycerides in the liver but not on the VLDL secretion. Where does the surplus of fatty acids disappear? We observed a large increase in the rate of the β-oxidation of fatty acids, in particular of the fatty acids that were stored in the cell. This is a plausible explanation for the loss of triglycerides from the cell when ADRP is knocked down.
Interestingly, an increase in the amount of ADRP did not influence the rate of β-oxidation (supplemental Results; supplemental Figure XA). Thus, an increase in ADRP selectively influences the assembly of VLDL, whereas a knockdown channelled fatty acids primarily into β-oxidation. This could suggest that the fatty acid pool (or pathway) that is used for β-oxidation differs from that used for VLDL assembly, and that these pools are influenced differently by variation in the cellular levels of ADRP. Thus, there may be a mechanism that secures fatty acids for β-oxidation and energy production. This needs to be addressed experimentally.

The observation that ADRP promotes the storage of triglycerides could indicate that an increase in the amount of ADRP gives rise to an increased energy consumption because the biosynthesis of triglycerides consumes energy during the activation of the fatty acids by coenzyme A and for the production of glycerol 3-P. In the case of the formation of lipid droplets, the detailed mechanism has not been established; however, the observation that it involves both the formation of a primordial droplet and a fusion of these droplets7,8 strongly suggests an energy-consuming process. Indeed, we observed that overexpression of ADRP gave rise to an increase in glucose uptake of the same magnitude as treatment with insulin20 (supplemental Results; supplemental Figure XB). This increase in glucose uptake was not just a result of the overexpression of any protein (supplemental Results; supplemental Figure XI), nor was it explained by an increased incorporation of radiolabeled glucose-carbons into triglycerides in cells overexpressing ADRP (supplemental Results; supplemental Figure XC). Based on these observations, we suggest that the ADRP-induced increase in glucose uptake is the result of an increased need for energy for the increased storage of fatty acids in lipid droplets.

In summary, increased hepatic expression of ADRP prevents fatty acids from being incorporated into triglycerides used for VLDL assembly and promotes their storage in cytosolic lipid droplets. However, together, the results from the overexpression and knockdown of ADRP point to a rather complex machinery involved in the sorting of fatty acids between storage secretion and oxidation.

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Disclosure(s)

None.

References


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SUPPLEMENTAL INFORMATION

MATERIAL AND METHODS
Trasylol (aprotinin) was obtained from Bayer Leverkusen (Leverkusen, Germany). \( N \)-Acetyl-Leu-Leu-norleucinal and rabbit immunoglobulin were from Dako (Glostrup, Denmark). Amplify, Redivue Pro-mix L\(^{35}S\) \textit{in vitro} cell-labeling mix, \(^3\text{H}\)-palmitic acid, \(^3\text{H}\)-oleic acid, 2-deoxy-d-[2,6-\(^3\text{H}\)]-glucose, d-[1-\(^{14}\text{C}\)]-glucose, [U-\(^{14}\text{C}\)]-glycerol, Rainbow molecular weight markers, PD-10 columns, and the enhanced chemiluminescence western blotting system were from Amersham Biosciences. Ready Safe was from Beckman Instruments (Fullerton, CA). Leupeptin was from Chemicon (Temecula, CA). Matrigel was from BD Biosciences (San José, CA). Anti-adipophilin (anti-ADRP) antibodies were from Research Diagnostics (Flanders, NJ). Anti-GRP-78 was from BD Biosciences and anti-actin from Abcam. MTP antibody was kindly provided by Dr. Carol Shoulders (Imperial College, London, UK).

Plasmids and Virus
The cloning of cDNA for mouse ADRP was based on the GenBank sequence (M93072). Total RNA was isolated from 3T3-L1 cells that had been allowed to differentiate for 2 days, and it was converted to cDNA using reverse transcriptase. The full-length ADRP gene was amplified with primers 5´-CCCAAGC TTGTTAGGCGTCTCTTTTCTCCA-3´ and 5´-TGCTCTAGACTGGTGACAAGGAGG GGTTTA-3´. These primers specify \textit{Hind}III and \textit{Xba}I restriction sites, and the resulting PCR products were digested and inserted into the multicloning site of the pBluescript SK (+) vector (Stratagene). The obtained construct was then digested with \textit{Hind}III and \textit{Sac}II and inserted into pEGFP-C2 (Clontech), giving rise to a sequence that encoded GFP-tagged ADRP. We digested the pEGFP-C2-ADRP construct
with HindIII and ApaI and inserted the ADRP gene into the pcDNA4/TO vector (Invitrogen), which was used to obtain stable transfections in the T-rex system. Inducible, stably transfected McARH 7777 cells were obtained as described 1

For production of recombinant adenovirus, ADRP was excised from the pEGFP-C2 vector, introduced into the pENTR vector (Invitrogen) via the pUC18 vector, and introduced into pAd/CMV/V5-DEST by recombination as described in the manual. The packaging cell line, Ad-293, was grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100,000 IU/L penicillin and 100 mg/L streptomycin. Cells were seeded in 25-cm² culture flasks, grown to 60–80% confluence, and transfected with adenoviral constructs for ADRP or the control Zs-green² using Lipofectamine in Dulbecco’s modified Eagle’s medium without serum, as described in the manual. After 4 hours of incubation, fetal bovine serum was added (final concentration, 10%). The cells were cultured for 10 to 14 days and virus was harvested by repeated freeze/thaw cycles in 10 mM Tris-HCl, pH 8.0. After large-scale amplification with Cell Factories (Nunc), recombinant adenovirus was purified by two rounds of CsCl density-gradient ultracentrifugation. The purified viral stocks were desalted over 10DG columns and eluted in sterile phosphate-buffered saline (PBS). Glycerol (15%) was added, and the stocks were batched and stored at –80°C until use. Infectious virus titers were determined with the Adeno-X Rapid Titer Kit (Clontech). Before use, purified viral stocks were screened for contamination with wild-type virus ³.

**Cell Culture and Transfections**

McA-RH7777 cells were grown as described previously ⁴ and stably transfected using the T-Rex system ¹. Two days before experiments, transfected cells were induced with tetracycline (1 µg/ml of culture medium) in ethanol (final concentration, 0.02%). The same amount of ethanol was added to the culture medium of uninduced control cells.
ADRP siRNA and control siRNA was from Ambion. Cells were transfected with lipofectamin 2000 (Invitrogen) according to manufacturers recommendation in the presence of 16%FCS. Analyses was carried out 48 h after transfection.

Primary hepatocytes were isolated from 200–300-g female Sprague-Dawley rats by nonrecirculating collagenase IV perfusion through the portal vein as described\(^5\). Cells (1.0–1.5 \times 10^6) were seeded in 12-cm\(^2\) dishes coated with 100 µl Matrigel (BD Biosciences). Four hours after seeding, the cells were infected with the virus in 1 ml of culture medium. Two hours later, 2 ml of medium was added, and incubation was continued overnight. The next morning, the medium was replaced with virus-free medium. Fresh medium was added daily as described previously\(^6\). Analyses were carried out 65 h after start of infection.

**Protein Labeling and Isolation**

For metabolic studies, McA-RH7777 and primary cells were labeled for 180 min with Redivue Pro-mix L\(^{35}S\) in vitro cell-labeling mix, and the medium and cells were collected. For analysis of VLDL secretion in presence of oleic acid, 360 uM oleic acid was present in medium 2 h before and during labeling ApoB and transferrin were isolated from whole cells as described\(^7\). Lipoproteins from the medium were isolated by ultracentrifugation as described\(^4\). Three fractions were isolated corresponding to VLDL1, VLDL2\(^8\) and lipoproteins in the high density lipoprotein (HDL) region. For the measurement of total radioactivity labelled proteins, labeled cells were lysed or the conditioned medium was collected and the proteins were precipitated with trichloroacetic acid. After a 30-min incubation at 4°C, the precipitated proteins were collected on nitrocellulose filters and the radioactivity was determined by scintillation counting.
Labeling and Isolation of Lipids

Cells were grown to 80% confluence and labeled with [3H]-palmitic acid as indicated in text or legends to figures. For pulse-chase experiments, the cells were washed three times with PBS and then chased in normal medium containing 20% fetal calf serum for different times. The medium and cells were collected, and lipids were extracted as described. Triacylglycerol, phosphatidylcholine, and cholesterol esters were separated by thin-layer chromatography in a two-phase system. Bands corresponding to the different lipids were identified with iodine vapor, cut out and placed in scintillation vials, and extracted with 1 ml of cyclohexane for 10 min before being measured by scintillation counting.

To quantify cytosolic droplets in intact cells, the cells were fixed in 3.7% formaldehyde for 10 min, washed with 60% isopropanol for 30 seconds, and stained with Oil Red O in 60% isopropanol for 20 min (5 min for primary hepatocytes), washed with 60% isopropanol for 30 sec (McA-RH7777 cells. No isopropanol wash for primary hepatocytes), stained with hematoxylin for 20 min, washed in cold water, and viewed in a Zeiss epifluorescence microscope. Ten to 20 images were captured, digitized (tiff format; 8 pixels/µm), and loaded into BioPix software (www.biopix.se), which categorizes pixels as being red or non-red and identifies adjacent red pixels as being a lipid droplet, as described in. The program can determine the number of droplets, the total lipid droplet area per cell, and the number of droplets in different size categories. For methodology see.

Other Methods

Glucose uptake was quantified with 2-deoxy-D-[2,6-3H]-glucose essentially as described. β-oxidation of fatty acids was determined as described. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot were carried out as described previously. Bound antibodies were detected with the enhanced
chemiluminescence detection kit (Amersham). Protein concentration was determined with the BCA kit (Pierce).

For statistical analysis, the Mann-Whitney rank-sum test or student t-test (as indicated in text) was used for two-group comparisons and one-way ANOVA for multiple group comparisons. Unless otherwise stated, all experiments were repeated at least once, with the same result.

**SUPPLEMENTAL RESULTS**

**An increase in ADRP influence the secretion of apoB-100 VLDL2**

In these experiments we used McA-RH7777 cells stably transfected with ADRP in the inducible t-rex system. In uninduced cells, there was a decrease in the secreted of radiolabeled apoB-100 in VLDL 2 density range with increasing concentration of oleic acid in the culture medium, i.e. oleic acid had the opposite effect on VLDL2 (Supplemental Fig VIIID) than on VLDL1 (Fig 3B and Supplemental Fig VIIIA). This effect of oleic acid on the secretion of VLDL2 was not seen when ADRP was induced and the maximal inhibition of VLDL1 was observed (i.e. at oleic acid concentrations below 100µM; Supplemental Fig VIIIA and D see also Fig 3B). When the ADRP induced inhibition of VLDL1 started to decrease (i.e. at oleic acid concentrations above 100µM), the VLDL2 secretion decreased with increasing oleic acid concentration and increasing formation of VLDL1 (Supplemental Fig VIIID compare also Fig 3B). These two observations are in line with the proposed precursor-product relationship between VLDL2 and VLDL1.

**An increase in ADRP does not affect β-oxidation.**

We investigated whether an increased expression of ADRP influenced the rate of β-oxidation. The conversion of [^3^H]-palmitic acid, to water-soluble radioactivity in induced McA-RH7777 cells (stably transfected with ADRP in the t-rex system) was 95 ± 14% of that
in uninduced control cells; similar results were obtained in primary rat hepatocytes (not shown). In pulse-chase studies, induction of the stably transfected McARH 7777 cells with tetracycline (which increases the levels of ADRP) had no effect on the conversion of $[^3H]$-palmitic acid to water-soluble radioactivity in McA-RH7777 cells (Supplemental Fig X A) during a pulse-chase experiment with $[^3H]$-palmitic acid.

**ADRP increases glucose uptake without affecting glucose incorporation into triglycerides.**

Since the effect of ADRP on the turnover of cellular triglycerides can be counteracted by Triacsin C (See Fig 3A), which prevents the activation of fatty acids, it is possible that the effect of ADRP is dependent on the re-esterification of released fatty acids into triglycerides. Such a mechanism could result in an increased consumption of glycerol-3P and ATP, and thereby cause an increase in glucose uptake. Experiments to investigate this possibility showed a 40% increase in uptake of glucose after induction of ADRP expression in McA-RH7777 cells (Stably transfected with ADRP in the inducible t-rex system; Supplemental Fig. XB). This increase was in the same range as the maximal uptake induced by high levels of insulin alone (Supplemental Fig. XI B), $^{13}$ To investigate whether the overexpression of a protein per se could induce an increase in the uptake of glucose, we expressed an inactive mutant of Phospholipase D2 - PLD 2 (K758R) - in the t-rex system in McA-RH7777 cells. The overexpression, which was in the same range as the overexpression of ADRP, did not influence the uptake of glucose (Supplemental Fig. XI). One explanation is that ADRP overproduction promoted the triglyceride biosyntheses from glucose metabolites; however, we could not detect any effect of overproduction of ADRP on the incorporation of labeled carbons from $[^{14}C]$-glucose into triglycerides (Supplemental Fig.XC). As a control, we could show that insulin had the expected effect on the production of triglycerides from glucose metabolites (Supplemental Fig. XIC).
SUPPLEMENTAL FIGURE LEGENDS

Figure I. Increased expression of ADRP in McA-RH 7777 cells and primary hepatocytes.
A, immunoblots showing ADRP expression in McA-RH7777 cells stably transfected with ADRP in the inducible t-rex system (+ induced, - uninduced; induction 48 h) and B, in primary hepatocytes infected with adenovirus encoding ADRP and harvested 65 h later. The blots were reacted with a polyclonal antibody to ADRP (diluted 1:2000). Scanning results are given below the blots.

Figure II. The influence of increased expression of ADRP on the triglyceride biosynthesis and secretion in McA-RH 7777 cells. McA-RH7777 cells stably transfected with the ADRP gene in the inducible t-rex system was used in these experiments. All experiments started after 48 hours induction with tetracycline (control cells were kept in culture without tetracycline during the same time). A, ADRP does not influence the rate of triglyceride biosynthesis. Induced (squares) or uninduced (triangles) McA-RH7777 cells were incubated with \[^{3}H\]-palmitic acid (1µCi/ml culture medium) for the indicated time, and harvested. Lipids were extracted with chloroform:methanol, triglycerides were isolated by thin-layer chromatography, and the radioactivity was determined. Values are mean ± SD (n = 3). B and C, ADRP does not influence the total amount of triglyceride in the system but reduces the secretion of triglycerides. Induced (squares) and uninduced (triangles) McA-RH7777 cells were labeled with \[^{3}H\]-palmitic acid (1µCi/ml culture medium) for 200 min and chased for the indicated times, and the amount of radioactive triglycerides in the medium and cells was determined. B, the sum of radioactive triglycerides in the cell and in the conditioned medium after each chase period. Values are mean ± SD (n = 3). C, Accumulation
of radioactive triglycerides in the conditioned medium. Values are mean ± SD (n = 3). *P < 0.001, induced versus uninduced at 480 and 1,440 min (one-way ANOVA).

Figure III. The effect of increased expression of ADRP in McA-RH 7777 cells on the accumulation and secretion of triglycerides. McA-RH7777 cells stably transfected with the ADRP gene in the inducible t-rex system was used in these experiments. All experiments started after 48 hours induction with tetracycline (control cells were kept in culture without tetracycline during the same time). Induced and uninduced McA-RH7777 cells (48 h induction) were incubated with $^{14}$C-glycerol overnight (20 n Ci/ml culture medium) and radioactivity in triglycerides in the cells and medium was determined (See legends to Supplemental Fig.II). Values are mean ± SD (n = 3). *P < 0.001 versus noninduced cells, †P < 0.001 versus medium from noninduced cells (t test).

Figure IV. The effect of different amounts of insulin and glucose on the accumulation of cytosolic lipid droplets and the secretion of apoB-100 VLDL 1 and 2 and apoB-48 VLDL1 and “HDL”. A, rat hepatocytes were recovered as described in Materials and Methods and allowed to plate in the indicated concentration of insulin and glucose. The cells were analyzed for the size of the pool of lipid droplets/cell (see legends to Fig1). Results are mean ± SD of all cells present in 6 randomly selected figures. The cells were also incubated with $^{35}$S-methionine for 3 h and the radioactivity of apoB-100 (B) in the VLDL 1 and 2 density regions and of apoB-48 (C) in the VLDL1 and “HDL” density regions determined (see Fig. 1). Results in B and C are mean ± range (n = 2).
Figure V  An increase in cellular levels of ADRP in the primary rat hepatocytes increases the accumulation and decreases the secretion of triglycerides but does not influence their biosynthesis. All analyses were carried out 65 h after the start of infection with the adenovirus. A, ADRP increases the accumulation of triglycerides. Primary rat hepatocytes were infected with adenovirus encoding ADRP or Zs-green. The cells were incubated with $[^3]$H-palmitic acid (0.1µCi/ml culture medium) for 15 h. Lipids were determined as described in the legends to Supplemental Fig II. Values are mean ± SD (n = 3). *$P < 0.027$ (t-test). B, ADRP does not influence the rate of triglyceride biosynthesis. Primary rat hepatocytes infected with adenovirus encoding ADRP (squares) or Zs-green (triangles) were incubated with $[^3]$H-palmitic acid (1µCi/ml culture medium) for the indicated times, and harvested. Lipids were determined as described in the legend to Supplemental Fig II. Values are mean ± SD (n = 3). C, ADRP reduces the secretion of triglycerides. Primary rat hepatocytes were infected with adenovirus (500 inclusion-forming units/cell) encoding either ADRP (squares) or Zs-green (triangles). They were labeled with $[^3]$H-palmitic acid (0.2µCi/ml culture medium) for 200 min and chased for the indicated period, and the medium was collected and analyzed for radioactive triglycerides. Values are mean ± SD; (n = 4). *$P = 0.002$ at 480 min, †$P < 0.045$ at 1,290 min (one-way ANOVA).

Figure VI. The effect of increased expression of ADRP on the secretion of VLDL1 and VLDL2 from McA-RH 7777 cells or from primary hepatocytes. A, McA-RH7777 cells stably transfected with the ADRP gene in the inducible t-rex system was used in these experiments. All experiments started after 48 hours induction with tetracycline (control cells were kept in culture without tetracycline during the same time). Uninduced and induced (as indicated) McA-RH7777 cells were labelled with $[^35]$S-methionine for 3 hours and VLDL1...
and VLDL2 were isolated from the culture medium by gradient ultracentrifugation. ApoB-100 was isolated by immuno- precipitated and SDS-polyacrylamide gel electrophoresis from each of these fractions and the radioactivity determined (Mean±SD; n= 3). B, uninfected primary hepatocytes and primary hepatocytes infected with adenovirus encoding Zs-green (Control) or ADRP were labelled (65 h after the infection) with $[^{35}S]$-methionine for 3h and VLDL1 and VLDL2 were isolated from the culture medium by gradient ultracentrifugation. ApoB-100 was isolated by immuno- precipitation and SDS-polyacrylamide gel electrophoresis from each of these fractions and the radioactivity determined (Mean±SD; n= 3).

**Figure VII. Increased expression of ADRP prevents the turnover of triglycerides in primary rat hepatocytes.** Primary rat hepatocytes infected with adenovirus encoding ADRP (open squares) or Zs-green (control; open triangles) were labeled with $[^3H]$-palmitate and chased for the indicated period. The radioactivity in triglycerides was determined (see legends to Supplemental Fig. II) after each chase. Values are mean ± SD (n = 4). *P < 0.045 at 480 min (one-way ANOVA). The analysis was carried out 65 h after the start of adenovirus infection.

**Figure VIII. A. The influence of different concentration of oleic acid on the secretion of apo B-100 VLDL1 and apoB-100 VLDL2 in McA-RH 7777 cell with and without increased expression of ADRP.** McA-RH7777 cells stably transfected with the ADRP gene in the inducible t-rex system was used in these experiments. All experiments started after 48 hours induction with tetracycline (control cells were kept in culture without tetracycline during the same time). A, the effect of increasing amounts of oleic acid on the ADRP-induced
inhibition of the secretion of apoB-100 VLDL1. Induced McA-RH7777 cells were incubated with the indicated amounts of oleic acid and analyzed for the secretion of apoB-100 containing VLDL1 (see legends to Fig3B) and the inhibition of apoB-100 VLDL1, relative the uninduced control, was calculated. 

B, immunoblot of ADRP in induced (+) and uninduced (−) McA-RH7777 cells cultured with the indicated amounts of oleic acid for 2 h. 

C, scanning of the blot in panel B. 

D, oleic acid reduces the secretion of VLDL 2 and this effect is partially prevented by overexpression of ADRP. Induced (squares) and uninduced (triangles) McA-RH7777 cells were treated as in Fig. 3B, VLDL2 was isolated, and apoB-100 radioactivity was determined (see legends to Fig. 3B). 

E, ADRP does not influence the expression of the microsomal triglyceride transfer protein (MTP). Immunoblot of MTP and protein disulfide isomerase (PDI) in induced and uninduced McA-RH7777 cells.

Figure IX. Transfection of McA-RH7777 cell with ADRP siRNA give rise to a small increase in the proportion of the intracellular pool of triglycerides that is sorted to secretion. The cells were transfected with ADRP siRNA or control siRNA 2 days before the experiment. The Cells (not treated with oleic acid) were labeled with [3H]-palmitic acid (1µCi/ml of culture medium) overnight. The culture medium was changed and the cells were chased for the indicated period and the radio-labeled triglycerides secreted into the medium were determined. Data are given as the proportion (per cent) of the labeled intracellular pool of triglycerides, present after the pulse, that was recovered in the medium after the different periods of chase (Mean±SD n=4; * significant difference vs control p=0.029 Mann-Whitney rank sum test)

Figure X. Overexpression of ADRP in McA-RH7777 cells does not influence the rate of β-oxidation but increases the uptake of glucose into cells without promoting the
biosynthesis of triglycerides from glucose metabolites. McA-RH7777 cells stably transfected with the ADRP gene in the inducible t-rex system was used in these experiments. All experiments started after 48 hours induction with tetracycline (control cells were kept in culture without tetracycline during the same time). A, ADRP does not influence the β-oxidation of fatty acids stored as triglycerides in the cell. Induced (squares) and uninduced (triangles) McA-RH7777 cells were labeled with [3H]-palmitic acid (1µCi/ml culture medium) for 200 min and chased as indicated in the absence of exogenous radio-labeled fatty acids, and water-soluble radioactivity (from fatty acid) in medium was determined. Values are mean ± SD (n = 3). B, Overexpression of ADRP increases the uptake of glucose to the same extent as high levels of insulin. Induced cells, uninduced cells, and uninduced cells incubated with 100 nM insulin were incubated with 2-deoxy-D-[2,6-3H]-glucose (166 nCi/ml/well) for 30 min, and cell-bound radioactivity was determined after washing. Values are mean ± SD (n = 3). *P < 0.007, †P < 0.005 versus uninduced cells (one-way ANOVA). C, Overexpression of ADRP does not promote the production of triglycerides from glucose metabolites, while the expected effect of insulin on this production can be seen. Induced cells, uninduced cells, and uninduced cells incubated with 100 nM insulin were incubated overnight with D-[1-14C]-glucose (66.7 nCi/ml/well). Triglycerides were isolated and radioactivity was determined. Values are mean ± SD (n = 3). *P < 0.001 versus induced and uninduced cells (one-way ANOVA).

Figure XI. The increase in glucose uptake induced by the increased expression of ADRP in McA-RH7777 cells is not seen when an inactive mutant of phospholipase D2 (PLD2 K758R) is overexpressed. McA-RH7777 cells stably transfected with the genes for ADRP or PLD2K758R in the inducible t-rex system was used in these experiments. All experiments started after 48 hours induction with tetracycline (control cells were kept in culture without
The effect of overexpression of ADRP (A) or PLD2 K758R (B) on the uptake of 2-deoxy-d-[2,6-3H]-glucose (166 nCi/ml/well for 30 min) is shown.

SUPPLEMENTAL REFERENCES


A

Scanning
QL-Background: 199078  48467

B

Ifu/cell  0  20  100  500

Scanning
QL-Background: 100409  131415  472120  646475

Supplemental Fig I
Supplemental Fig II

Panel A: Incorporation of \[^{3}H\]-palmitic acid into cellular triglycerides (dpm)

Panel B: Radiolabeled triglycerides in cells + conditioned medium (dpm)

Panel C: Radiolabeled triglycerides in conditioned medium (dpm)
Supplemental Fig III
A

Total area of Oil Red O-stained lipid droplets/cell

Insulin (nM)  Glucose (mM)

16  28
3  11
0.1  11

B

Radiolabeled apoB-100 (dpm)

VLDL1  VLDL2

Insulin (nM)  Glucose (mM)

16  28  163  30.1  0.1
28  28  11  11  11

C

Radiolabeled apoB-48 (dpm)

VLDL1  "HDL"

Insulin (nM)  Glucose (mM)

16  28  163  30.1  0.1
28  28  11  11  11

Supplementary Fig IV
Supplementary Fig VI

A

![Bar graph showing radiolabeled apoB-100 (dpm) for Non-induced and Induced conditions.](image)

Vertical axis: Radiolabeled apoB-100 (dpm)
- Non-induced
- Induced

B

![Bar graph showing radiolabeled apoB-100 (dpm) for Uninfected, ZS-green, and ADRP conditions.](image)

Vertical axis: Radiolabeled apoB-100 (dpm)
- Uninfected
- ZS-green control
- ADRP
Supplementary fig. VII
Supplementary Fig IX
A

Water soluble radioactivity derived from [3H]-palmitic acid (dpm)

Minutes of chase

B

Cellular uptake of 2-deoxy-D-[2,6-3H]-glucose (dpm/mg protein)

Noninduced Induced Noninduced

Insulin

C

Radiolabeled cellular triglycerides (dpm)

Noninduced Induced Noninduced

Insulin

Supplemental Fig X
Supplementary Fig XI