Mechanism for Endogenously Expressed ApoE Modulation of Adipocyte Very Low Density Lipoprotein Metabolism

ROLE IN ENDOCYTIC AND LIPASE-MEDIATED METABOLIC PATHWAYS*

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Triglyceride-rich lipoproteins distribute energy in the form of fatty acids to peripheral tissues. We have previously shown that the absence of endogenous adipocyte apoE expression impairs adipocyte triglyceride acquisition from apoE-containing triglyceride-rich lipoproteins in vitro and in vivo. Studies were performed to evaluate the mechanism(s) for this impairment. We excluded a role for secreted apoE in accounting for the difference in very low density lipoprotein (VLDL)-induced adipocyte triglyceride accumulation using cross-incubation studies to show that secreted apoE did not enhance triglyceride synthesis in apoE knockout (EKO) adipocytes incubated with apoE-containing VLDL. Subsequent experiments established that both endocytic and lipase-mediated pathways for lipid acquisition from VLDL were impaired in EKO adipocytes. Binding and internalization of VLDL to EKO adipocytes were significantly lower due to decreased expression or redistribution of low density lipoprotein receptor family proteins. An important role for the VLDL receptor in contributing to differences in VLDL binding between wild-type and EKO adipocytes was identified. Lipoprotein lipase-dependent adipocyte lipogenesis was also significantly decreased in EKO adipocytes even though they secreted as much or more lipolytic activity. This decrease was related to impaired fatty acid internalization in EKO cells. Evaluation of potential mechanisms revealed reduced caveolin-1 and plasma membrane raft expression in EKO adipocytes. Increasing caveolin expression in EKO adipocytes increased fatty acid internalization. Our results establish a role for endogenous adipocyte apoE in VLDL-induced adipocyte lipogenesis by impacting both endocytic and lipoprotein lipase-mediated metabolic pathways. Reduced adipocyte apoE expression, for example that accompanying obesity, will suppress adipocyte acquisition of lipid from apoE-containing VLDL.

Obesity is an increasingly prevalent condition that produces increased risk for metabolic and cardiovascular disease (1–4). Parallel with the obesity epidemic, there has been increasing realization that adipose tissue is a metabolically dynamic endocrine and paracrine organ that plays a major role in regulating systemic carbohydrate and lipid flux (2, 4, 5). One important function of adipose tissue is to serve as a metabolically active depot for energy in the form of triglyceride. Adipose tissue TG2 is generated from the lipid delivered by circulating triglyceride-rich lipoproteins, such as VLDL, chylomicrons, and chylomicron remnants (6–8). Lipoprotein lipase produced by adipocytes acts on circulating TGRL in microcapillary beds to hydrolyze lipoprotein core TG, thus, producing free fatty acids for adipocyte internalization. Internalized FFAs then serve as substrates for esterification to glycerol to drive de novo adipocyte TG synthesis. Adipocytes also express a number of high affinity binding sites that facilitate endocytic uptake of entire TGRL particles (7, 9). Delivery of these internalized lipoproteins to lysosomes with subsequent degradation also produces intracellular FFA to drive de novo adipocyte TG synthesis.

Zechner et al. (10) have shown that apoE is highly expressed in human adipose tissue. We have subsequently reported physiologic regulation of human and murine adipocyte apoE expression by peroxisome proliferator-activated receptor γ agonists, angiotensin II, inflammatory cytokines, and nutritional status. For example, treatment of humans or isolated adipocytes with peroxisome proliferator-activated receptor γ agonists increases adipocyte apoE expression (11). Fasting and weight loss also increase adipocyte apoE expression, whereas angiotensin II, tumor necrosis α, and diet-induced obesity reduce it (11–14). With respect to a function for apoE in adipose tissue, we and others have reported that apoE knock-out (EKO) mice have less adipose tissue and smaller adipocytes compared with wild-type (WT) controls (15–18). The absence of apoE on circulating TGRLs in vivo clearly impacts adipocyte size, TG mass, and gene expression in EKO mice (16–18). However, we have also reported a role for endogenous adipocyte apoE expression independent of extracellular or circulating apoE for modulating adipocyte TG content, size, and gene expression (15, 19). For example, we have shown that after 10 days in culture, EKO adipocytes still contain less TG compared with WT (15). We have also demonstrated that the TG content of EKO adipose tissue does not increase after a 24-h incubation with apoE-rich VLDL, whereas that in WT adipose tissue increases almost double.

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2 The abbreviations used are: TG, triglyceride; TGRL, TG-rich lipoprotein; EKO, apoE knockout; LDL, low density lipoprotein; LDLR, LDL receptor; LRP, LDLR-related protein; VLDL, very low density lipoprotein; VLDLR, VLDL receptor; LPL, lipoprotein lipase; FFA, free fatty acid; WT, wild type; BSA, bovine serum albumin; RAP, receptor-associated protein; FATP, fatty acid transport protein; DMEM, Dulbecco’s modified Eagle’s medium; shRNA, short hairpin RNA; GM1, N-acetylenaminylditetrasaccharide.
2-fold (15). Most importantly, we have validated the importance of endogenous adipocyte apoE, separate from circulating apoE, in vivo. Using adipose tissue transplantation experiments we have shown that EKO adipocytes remain smaller and lipid-poor compared with WT adipocytes even after 12 weeks in a wild-type in vivo environment (19). In this same series of experiments, we showed that WT adipocytes transplanted into WT mice increase in size and lipid content in response to the increased circulating TG and apoE levels produced by a high fat diet but that EKO adipocytes do not. Based on these in vitro and in vivo observations, we concluded that the absence of endogenous adipocyte apoE expression impairs adipocyte acquisition of TG from apoE-containing TGRL. The current studies were undertaken to more fully evaluate the mechanism(s) for defective TGRL-induced lipid accumulation in EKO adipocytes. The results indicate that endogenous adipocyte apoE expression is important for both the lipoprotein lipase-mediated and endothelial pathways of TGRL lipid delivery to adipocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture medium and fetal bovine serum were purchased from In Vitrogen. All chemicals were from Sigma. Organic solvents were from Fisher. [3H]Cholesteryl hexadecyl ether, [14C]glucose, and [3H]acetate were obtained from PerkinElmer Life Sciences. QBT assay kits to measure fatty acid internalization were purchased from Molecular Devices (Sunnyvale, CA). A rabbit anti-LDL receptor antibody was prepared as previously described (20). A monoclonal antibody for LRP was from Fitzgerald Industries (Concord, MA). A monoclonal antibody for caveolin-1 was from BD Biosciences. Anti-mVLDLR, FATP1, FATP4, and CD59 antibodies were purchased from R&D Systems (Minneapolis, MN) or Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

**Cell Isolation and Culture**—C57BL/6 mice (WT) were purchased from The Jackson Laboratory. ApoE knock-out breeder mice were also purchased from The Jackson Laboratory. The Institutional Animal Care and Use Committee of the University of Illinois approved all animal protocols in this study. Mature adipocytes were isolated from freshly harvested adipose tissue for immediate use in experiments as previously described (11–13, 15). Preadipocytes from the adipose tissue stromovascular fraction were isolated and differentiated into adipocytes using a 3-day incubation in insulin, dexamethasone, and isobutylmethylxanthine as previously described in detail (11–13, 15). Preadipocytes differentiated in culture were used 10 days after this incubation for experiments.

**Isolation and Labeling of VLDL**—VLDL was isolated by sequential density gradient ultracentrifugation of human plasma as previously described in detail (15). VLDL particles were core-labeled with the non-hydroxylizable neutral lipid analogue, [3H]cholesteryl hexadecyl ether, according to a previously published method (21). Labeled VLDL particles were reisolated by ultracentrifugation, and the VLDL fraction was exhaustively dialyzed for 24 h against normal saline to remove unincorporated label. The specific activity of the VLDL preparations averaged $4.0 \times 10^5$ dpm/mg of VLDL cholesterol ester.

**Adipocyte VLDL Metabolism**—To measure binding of whole VLDL particles, core-labeled VLDL was added to adipocytes at the concentration of VLDL protein shown in the figures in 0.1% BSA, DMEM for 2 h at 4 °C alone or with a 20-fold excess of unlabeled VLDL. After this incubation, VLDL binding to cells was measured by washing the cells at 4 °C and extracting lipids with chloroform:methanol (2:1). Specific binding was determined by subtracting the nonspecific binding measured in the presence of a 20-fold excess unlabeled VLDL from total binding. In some cells internalization of bound VLDL was measured by incubating cells at 4 °C with labeled VLDL for 2 h, washing cells at 4 °C to remove unbound VLDL, and then incubating with 0.1% BSA, DMEM at 37 °C for an additional 4 h to allow internalization of pre-bound VLDL. At that time cells were washed, and lipids were extracted. Radioactivity in extracted lipids was measured in a scintillation counter and expressed as dpm/µg DNA for mature freshly isolated adipocytes or dpm/µg of cell protein for cultured adipocytes.

To measure adipocyte TG synthesis in response to incubation with VLDL, adipocytes were incubated with 0.5 µCi/ml [14C]glucose with or without 100 µg/ml VLDL in 0.1% BSA, DMEM for 6 h at 37 °C. After washing, cellular lipids were extracted as described above, and TGs were separated by thin layer chromatography in a solvent system of hexane:ethyl ether:acetic acid (90:30:1) (15). The TG spots were harvested, and radioactivity in spots measured in a scintillation counter.

For some experiments, receptor-associated protein (RAP) or anti-mVLDLR antibody (5 µg/ml or 25 µg/ml) was included during incubations with VLDL. A glutathione S-transferase-RAP fusion protein was produced from the expression vector pGEX-2T-RAP/TEV (kindly provided by Dr. Peter Gettins, University of Illinois at Chicago). The fusion protein was purified using GSTrap FF columns (GE Healthcare) and cleaved with AcTEV protease (Invitrogen). VLDLR, LDLR, and LRP expression was disrupted by incubation with lentivirus containing shRNA targeting VLDLR, LDLR, or LRP (Santa Cruz Biotechnology Inc., Santa Cruz, CA; catalog no. SC-35803-V, LDL shRNA; SC-36823-V, VLDL; SC-40102-V, LRP) according to the manufacturer’s instructions. Cells were incubated with virus containing targeting shRNA or a scramble control shRNA (catalog no. SC-108080) and 10 µg/ml Polybrene for 18 h. After an additional 48 h of incubation, cells were used for Western blot and to measure VLDL binding.

Adipocyte apoE expression was silenced by incubation with adenovirus containing an shRNA targeting apoE (AGG ACA CTA TGA CGG AGAGT) or a control scrambled shRNA. Mouse apoE shRNA adenoviral particles were prepared using BD TM Knock-out Adenoviral RNAi System 2 according to the manufacturer’s instructions (BD Bioscience). Cells were cultured for additional 48 h and used for Western blot and to measure VLDL-mediated TG synthesis as described above.

**Adipocyte Fatty Acid Uptake and Secreted Lipolytic Activity**—Fatty acid uptake by adipocytes was measured using a QBT fatty acid uptake assay kit according to the manufacturer’s instructions. The kit uses a biodipy-dodecanoic fatty fluorescent analog that remains quenched until it is internalized by the cell (22). Freshly isolated mature and cultured adipocytes were incubated with 0–120 µM oleic acid (fatty acid:BSA molar ratio 2:1) in the QBT fatty acid uptake solution. Readings for cellular uptake of fatty acids were recorded from 0 to 1600 s after the
addition of FFA using a Synergy HT Multi-Mode plate reader (Bio-Tek, Winooski, VT).

In separate experiments caveolin-1 expression in cultured EKO adipocytes was increased by infecting cells with cav-1 or control retroviral particles (23, 24) for 18 h. Cells were cultured for another 48 h before harvest for Western blot or for measuring fatty acid uptake. The caveolin-1 retroviral construct was generated by cloning wild-type caveolin-1 into the HindIII and HpaI sites of the pLNCX vector (Clontech, Palo Alto, CA). Caveolin-1 retroviral construct was transfected into Phoenix-Eco cells using Lipofectamine (Invitrogen). Retroviral vector-rich media was harvested 48-h post-transfection and clarified by centrifugation.

Lipolytic activity secreted by adipocytes was analyzed using a previously published method (25). Briefly, conditioned medium was collected from adipocytes in 10 mm Tris-HCL, pH 7.4, with 0.1% BSA over 90 min. Total lipolytic activity was measured by releasing cell-associated lipase using a 90-min incubation in 50 units/ml heparin (9, 22). After centrifugation to remove cellular debris, lipolytic activity was quantitated by measuring hydrolysis of the fluorogenic substrate 4-methylumbelliferyl haptanoate. The results were expressed as nmol/mg of protein for cultured cells or nmol/μg of DNA for freshly isolated mature cells.

De Novo Fatty Acid Synthesis—De novo fatty acid synthesis was measured according to previously published methods (26). Briefly, cultured adipocytes were incubated with 0.5 μCi/ml [14C]glucose in DMEM and 0.2% BSA for 6 h. After incubation, cells were washed, and lipids were extracted and saponified. Radioactivity in the lipid fractions representing the newly synthesized fatty acids was estimated by β-counter and normalized to cell protein. In separate experiments, fatty acid synthesis rate was also measured by incubating cells with 50 μCi/ml [3H]acetate in DMEM and 0.2% BSA for 2 h before being processed as described above.

Western Blot and PCR—Cells were lysed, and extracts were used for Western blot analysis of caveolin-1, FATPs, apoE, or lipoprotein receptor expression as described in detail in previous publications (11–13, 15). Western blot images were quantitated using ImageQuant TL software (GE Healthcare) and corrected using actin 2 as an internal loading control. Cell surface receptors were quantitated using biotinylation at 4 °C as previously described in detail (27). The cell surface-biotinylated proteins were purified by immobilization on streptavidin beads, eluted, and used for Western blot as described above.

Quantitative reverse transcription-PCR was performed as previously described (11–13, 15). For normalization of RNA loading, samples were standardized to β-actin message abundance. The primer sets used for this analysis were: mCaveolin-1, GGCAACATCTAAAGCCACAAC (forward) and GTCGAACTGTGTGCCCTTTC (reverse); mCD36, ATGCCCGAGTGGTTCTTAC (forward) and GAGCCGGTTTCTACTACCTCCA (reverse); mFATP1, CATGTGTTACCCACCTGCCGT (forward) and CTGGGCCGAACTTCTCTTG (reverse). Data were analyzed using the comparative critical threshold method and normalized to β-actin. -Fold change was calculated by 2−ΔΔCt.

Flow Cytometric Analysis of Adipocyte Plasma Membrane Lipid Rafts—Evaluation of plasma membrane lipid raft content was performed as previously described (28). Briefly, cells were stained with Vybrant lipid raft labeling kit (Invitrogen) according to the manufacturer’s instructions. Cells were then incubated with 1 μg/ml Alexa Fluor 594-conjugated cholera toxin-B subunit for 15 min at 4 °C. Cells were washed and incubated with rabbit anti-cholera toxin-B antibodies (1/200 dilution) for 15 min at 4 °C. After washing, cells were fixed with 4% paraformaldehyde for 20 min. Cell fluorescence was measured using a MoFlo flow cytometer (Dako, Carpinteria, CA) and analyzed with Summit Version 4.3 software (Dako).

Statistical Analysis—Results are shown as the mean ± S.D. of triplicate determinations. Experiments are representative of results obtained from experiments using cells isolated from 3–6 individual mice in each experimental group. Statistical differences were analyzed by analysis of variance using SPSS 15.0 (Chicago, IL) followed by a post hoc test for multiple comparisons. p < 0.05 was considered significant.

RESULTS

Secrated ApoE Does Not Enhance VLDL-induced TG Synthesis in EKO Adipocytes—Before examining more detailed adipocyte VLDL metabolic pathways, it was important to consider the potential for adipocyte-secreted apoE to impact adipocyte VLDL metabolism. Specifically, we needed to evaluate if cell-derived secreted apoE could contribute to more effective VLDL lipid delivery to adipocytes, as enriching VLDL with apoE is a well established approach for increasing its interaction with many cell types (16–18, 29). We, therefore, performed experiments to directly evaluate this issue (Fig. 1). ApoE-containing VLDL was incubated with apoE-secreting WT adipocytes for 18 h (designated E−·VLDL in Fig. 1). Control VLDL was incubated over the same period with EKO adipocytes. Cell culture medium containing VLDL incubated with WT adipocytes (E−·VLDL) was enriched with apoE by 25–50% over that incubated with EKO adipocytes. When added to new WT adipocytes, the E−·VLDL stimulated a greater than 2-fold increase in TG syn-

FIGURE 1. Effect of apoE secreted from adipocytes on VLDL-mediated triglyceride synthesis in adipocytes. Human VLDL (100 μg/ml) was preincubated with apoE-secreting WT adipocytes for 18 h (E−·VLDL). VLDL incubated with EKO adipocytes for the same period of time was used as a control. The conditioned medium containing VLDL was harvested and incubated with new WT or EKO mature adipocytes (as indicated on the horizontal axis) along with [14C]glucose in DMEM at 37 °C for 6 h to measure adipocyte triglyceride synthesis. **, p < 0.01 for the indicated difference.

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The results described above demonstrate that the absence of secreted apoE results in decreased triglyceride synthesis by adipocytes. Cultured adipocytes incubated with labeled apoE-containing VLDL at 4 °C, a temperature at which cell protein secretion and cell surface receptor internalization are arrested and at which there would be no LPL-mediated remodeling of the lipoprotein particle. We observed decreased high affinity binding to EKO compared with WT adipocytes (Fig. 3A). Figs. 3, B and C, show results of experiments to evaluate both binding and internalization by freshly isolated and cultured adipocytes. To measure internalization, unbound labeled VLDL was washed away after the 2 h of incubation at 4 °C, and cells were warmed to 37 °C for an additional 4 h to allow internalization of pre-bound VLDL. For both freshly isolated (Fig. 3B) and cultured adipocytes (Fig. 3C), VLDL binding and internalization were higher in WT adipocytes. Adipocytes express several members of the LDL receptor family that could mediate high affinity-specific VLDL binding (7, 9), including the VLDLR, LRP, and the LDL receptor. We used the addition of RAP to evaluate the involvement of these receptors, as the binding of ligands to these LDL receptor family members can be inhibited by RAP (30–32). We next assessed how the addition of RAP impacted VLDL uptake in WT and EKO adipocytes (Fig. 3D). The addition of RAP significantly suppressed uptake of labeled VLDL particles in WT adipocytes but had no effect in EKO adipocytes. In the presence of RAP, VLDL uptake was the same in EKO and WT cells. These results suggested a difference in LDLR family protein expression between WT and EKO adipocytes.

We next directly measured expression of VLDLR, LDLR, and LRP in WT and EKO adipocytes. In freshly isolated adipocytes, EKO cells expressed significantly lower levels of LDLR, VLDLR, and LRP mRNA and total cell protein (Fig. 3, E and F). In cultured adipocytes, however, LDLR, LRP, and VLDLR expression was not similarly reduced in total cell lysates of EKO cells (not shown). The absence of a significant reduction in the total cell content of LDLR, LRP, or VLDLR receptor protein between cultured EKO and WT, even when there is clearly reduced binding in cultured EKO adipocytes, could mean that non-LDL receptor family binding sites were involved. Alternatively, a redistribution of these receptor proteins from the plasma membrane to intracellular compartments could reduce cell surface binding and uptake of VLDL. We next evaluated this possibility by biotinylating cell surface proteins at 4 °C (27). Biotinylated proteins were then subjected to immunoblot for LRP, VLDLR, and LDLR. As a control, we evaluated biotinylation of the adipocyte cell surface protein CD59 (33). As shown in Fig. 3G, these three receptors were readily identified on the cell surface of cultured WT adipocytes. In EKO adipocytes, however, LDLR, VLDLR, and LRP could barely be detected at the cell surface. These results are consistent with a redistribution of LDL receptor family proteins away from the plasma membrane of EKO adipocytes. We next performed experiments to address the relative importance of LDLR, VLDLR, and LRP for binding VLDL in WT adipocytes. WT adipocytes were incubated with a control silencing RNA or a silencing RNA targeted to one of the above receptor proteins. Incubation with the receptor silencing RNAs led to a 58–62% reduction in expression of each of these receptors (not shown). Incubation with the LDLR- or VLDLR-silencing RNA also significantly reduced VLDL binding,
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A. VLDL binding to cultured adipocytes

B. VLDL binding and internalization by freshly isolated adipocytes

C. VLDL binding and internalization by cultured adipocytes

D. VLDL uptake by adipocytes incubated with RAP

E. Relative mRNA levels in freshly isolated adipocytes

F. Total cell protein levels in freshly isolated adipocytes

G. Cell surface LDLR, VLDLR and VLDLR expression
VLDLR substantially decreased the absolute difference in results in previous figures. The addition of antibody to the VLDLR, binding is higher to WT adipocytes, consistent with the role of antibody to the VLDLR. In the absence of any antibody addition, binding was lower to EKO adipocytes, consistent with our previous observations (19). The addition of tetrahydrolipstatin significantly reduced triglyceride synthesis in WT cells and reduced the VLDL-induced difference in TG synthesis between WT and EKO adipocytes.

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VLDL binding between WT and EKO adipocytes and completely eliminated this difference at the higher concentration of antibody. These results indicate that differences in VLDLR expression importantly contribute to differences in VLDL binding between WT and EKO adipocytes.

**LPL-mediated Generation of FFA and TG Synthesis in WT Compared with EKO Adipocytes**—LPL produced by adipocytes acts on VLDL core TG to produce FFA, and the subsequent internalization of this FFA drives adipocyte TG synthesis. A role for this pathway for contributing to the difference in VLDL-induced TG accumulation between WT and EKO adipocytes was suggested by the observation that in the presence of RAP, to equalize VLDL binding between WT and EKO adipocytes, WT adipocytes continued to synthesize more triglyceride compared with EKO adipocytes (not shown). We, therefore, used tetrahydrolipstatin, an inhibitor of LPL activity (34), to assess the importance of the LPL-mediated pathway for contributing to the increment in VLDL-induced TG synthesis associated with adipocyte apoE expression (Fig. 4). In the presence of VLDL alone, triglyceride synthesis was higher in WT compared with EKO adipocytes, consistent with our previous observations (19). The addition of tetrahydrolipstatin significantly reduced triglyceride synthesis in WT cells and reduced the VLDL-induced difference in TG synthesis between WT and EKO freshly isolated (Fig. 4A) and cultured (Fig. 4B) adipocytes. This result indicated that the generation of FFA by LPL contributed to the incremental VLDL-induced lipogenic response in WT compared with EKO adipocytes.

Reduced LPL activity in EKO adipocytes could be one potential explanation for the results shown in Fig. 4. We, therefore, next evaluated the impact of adipocyte apoE expression on lipolysis in WT adipocytes. The data in Fig. 5, A and B, demonstrate that in freshly isolated adipocytes, secreted and total (secreted plus cell-associated) lipolytic activity was actually higher in EKO compared with WT adipocytes. In cultured adipocytes, there was little difference between WT and EKO cells. These results indicated that differences in secreted or cell-associated lipolytic activity cannot explain the higher LPL-dependent TG synthesis in WT adipocytes.

LPL action on VLDL releases FFAs that require internalization by the cell to drive triglyceride synthesis. We, therefore, next evaluated the impact of adipocyte apoE expression on internalization of FFA (Fig. 5, C–F). Fatty acid internalization, measured 420 s after the addition of fatty acid, was higher in WT adipocytes over a range of extracellular fatty acid concentrations in both freshly isolated mature (Fig. 5C) and cultured adipocytes. These results indicate that differences in VLDLR and LRP expression importantly contribute to differences in VLDL binding between WT and EKO adipocytes and completely eliminated this difference at the higher concentration of antibody. These results indicate that differences in VLDLR expression importantly contribute to differences in VLDL binding between WT and EKO adipocytes.

**FIGURE 3.** VLDL binding and uptake by WT and EKO adipocytes. A, labeled VLDL was added to cultured adipocytes at the indicated concentrations for 2 h at 4 °C to measure VLDL binding. Nonspecific binding measured in the presence of a 20-fold excess of unlabeled VLDL was subtracted from total binding to yield the specific binding shown. Freshly isolated mature (B) or cultured adipocytes (C) were incubated with 10 μg/ml [3H]VLDL in 0.1% BSA, DMEM at 4 °C for 4 h. At that time, some cells were harvested for measurement of VLDL binding as described under “Experimental Procedures.” The remainder were washed to remove unbound VLDL and incubated in 0.1% BSA, DMEM at 37 °C for an additional 4 h before being harvested for measurement of VLDL internalization as described under “Experimental Procedures.” D, freshly isolated mature adipocytes were incubated with 50 μg/ml [3H]VLDL alone or with 50 μg/ml RAP, and VLDL internalization was measured. E, VLDLR, LDLR, and LRP mRNA expression in freshly isolated WT and EKO adipocytes is shown. F, total cell lysates were used to measure LDLR, VLDLR, and LRP in freshly isolated mature adipocytes by Western blot as described under “Experimental Procedures.” G, cell surface proteins of cultured adipocytes were biotinylated at 4 °C as described under “Experimental Procedures.” Biotinylated proteins were utilized for Western blot for LDLR, VLDLR, and LRP protein expression as described under “Experimental Procedures.” The double band for the VLDLR represents the 161- and 143-kDa isoforms of the receptor. H, WT adipocytes were incubated with LDLR, VLDLR, LRP, or control siRNA lentiviral particles for 18 h followed by 48 h of incubation in fresh culture medium before use in VLDL binding assay. I, VLDL binding was measured in the presence of 0, 5, or 25 μg/ml of VLDLR antibodies. p < 0.05 (*) and p < 0.01 (**) for the indicated difference.
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adipocytes (Fig. 5D). Real-time FFA internalization rates were also higher in WT cells (Figs. 5, E and F). Decreased fatty acid internalization was not compensated for by increased fatty acid synthesis in EKO adipocytes as rates of fatty acid synthesis measured using labeled acetate or glucose as a precursor were the same or decreased in EKO compared with WT adipocytes (not shown). These results indicated that reduced FFA internalization in EKO adipocytes could contribute to impaired VLDL-induced TG synthesis in EKO adipocytes.

The pathways utilized for transport of fatty acids across the adipocyte plasma membrane remain controversial in the literature. Caveolin, FATP, and CD36 have been proposed as being involved as facilitating FFA transport across cellular plasma membrane (35–37). We measured no differences in FATP1 or CD36 mRNA levels and no difference in FATP1, FATP4, or CD36 cell protein levels between WT and EKO adipocytes (not shown). The expression of caveolin-1, however, was significantly and substantially suppressed in EKO adipocytes at both the mRNA (15) and protein level (Fig. 6A). One mechanism proposed for how caveolin facilitates transmembrane fatty acid transport relates to its required role for organizing the plasma membrane into lipid-rich rafts, termed caveolae. It has been suggested that fatty acid transport across the plasma membrane may be concentrated at these lipid-rich raft microdomains. The reduced caveolin expression shown in Fig. 6A would predict a decrease in these lipid-rich microdomains. To investigate this, we fluorescently tagged a pentasaccharide chain of ganglioside GM1, a lipid associated with plasma membrane rafts (28). Analysis by flow cytometry showed a shift toward cells with less fluorescence in EKO compared with WT adipocytes (Fig. 6B). Quantitation of cell fluorescence showed it was significantly reduced in EKO cells (Fig. 6C). These results demonstrate a reduction in lipid-rich plasma membrane microdomains in EKO adipocytes. We next evaluated whether increasing caveo-

DISCUSSION

Our results establish a key role for endogenous adipocyte apoE expression in adipocyte VLDL metabolism. The lipoprotein response to apoE-containing VLDL is higher in WT compared with in EKO adipocytes in vivo and in vitro (15, 19). Our current observations establish that this difference is not because of secretion of apoE or to any difference in the remodeling of extracellular TGRL between EKO and WT adipocytes (Fig. 1) or to cellular adipocytes. Viral transduction was used to increase caveolin-1 expression in EKO adipocytes (Fig. 6D). The increase in caveolin expression substantially increased fatty acid internalization compared with control EKO adipocytes (Fig. 6E).
FATP1, or FATP4 protein expression between WT and EKO adipocytes (not shown). There were, however, substantial reductions in caveolin mRNA (15) and protein expression (Fig. 6). Increasing caveolin-1 expression in EKO adipocytes increased fatty acid internalization. Caveolin-1 expression on the plasma membrane is restricted to lipid-rich plasma membrane rafts termed caveolae, and the expression of caveolin is required to maintain the integrity of plasma membrane caveolae.
The reduction in caveolin expression that we have observed in EKO adipocytes likely leads to the reduction in caveolae, as indicated by reduction in plasma membrane lipid raft content (Fig. 6), and this reduction in caveolae could contribute to reduced FFA internalization in EKO adipocytes. A rapid flip-flop of FFA across the cellular plasma membrane has...
been described in adipocytes (45), and a primary role for caveolae in this pathway has been proposed (44, 46). Consistent with this notion, it has been reported that triglyceride synthesis can be initiated in a subtype of adipocyte caveolae (47). The mechanism by which absent apoE expression reduces caveolin expression is not immediately obvious. We have previously shown that a 90% reduction in caveolin mRNA levels can be measured in EKO compared with WT adipocytes maintained in culture for 10–14 days (15). Based on the previously established responsiveness of the caveolin gene to cell lipid (48) we have suggested that altered lipid flux accompanying absent adipocyte apoE expression could influence expression of the caveolin gene. This hypothesis is currently under investigation.

In summary, we have established a role for endogenously expressed adipocyte apoE in adipocyte VLDL metabolism for both freshly isolated and cultured adipocytes. The absence of adipocyte apoE impairs the adipocyte acquisition of triglyceride from VLDL by both LPL-dependent and endocytic pathways. The reduction of endocytic accumulation of triglyceride from VLDL can be explained by reduced total cell or cell surface expression of LDLR family proteins leading to reduced binding and internalization of VLDL. Our results implicate a primary role for changes in VLDLr expression. The LPL-dependent accumulation of triglyceride is likely reduced because of reduced transport of FFA across the adipocyte membrane. This reduction is associated with reduced expression of caveolin-1 and reduced plasma membrane raft domains in EKO cells. Increasing caveolin-1 expression by viral transduction in EKO adipocytes increases fatty acid transport across the plasma membrane. Based on previous observations (11, 13, 15, 19, 49) and on the results in this report, it is reasonable to speculate that reduction of adipocyte apoE expression could have important consequences for systemic substrate flux. Mice with systemic knock-out of apoE on circulating lipoproteins leading to the absence of adipocyte apoE expression could influence expression of the caveolin gene.

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