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Cholesterol depletion inhibits fatty acid uptake without affecting CD36 or caveolin-1 distribution in adipocytes

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Abstract

Caveolin-1 and CD36 are plasma membrane fatty acid binding proteins that participate in adipocyte fatty acid uptake and metabolism. Both are associated with cholesterol-enriched caveolae/lipid rafts in the plasma membrane that are important for long chain fatty acid uptake. Depletion of plasma membrane cholesterol reversibly inhibited oleate uptake by adipocytes without altering the amount or the cell surface distribution of either caveolin-1 or CD36. Cholesterol levels thus regulate fatty acid uptake by adipocytes via a pathway that does not involve altered cell surface localization of caveolin-1 or CD36.

Keywords: Adipocyte; Caveolae; Caveolin-1; CD36; Cholesterol; Fatty acid; Lipid raft

Differentiation of preadipocytes to adipocytes is accompanied by increased long chain fatty acid (LCFA) uptake and increased expression of a number of proteins implicated in the process [1–3]. These include caveolin-1 and CD36, which are enriched in caveolae/lipid rafts, which are abundant in the adipocyte plasma membrane [4–7]. Caveolin-1 is a LCFA and cholesterol binding protein and participates in cholesterol trafficking [1,8–10]. Caveolin-1 deficient mice lack caveolae and exhibit reduced adipocyte mass and increased serum free fatty acids, suggesting that adipocyte LCFA uptake may be impaired [11]. LCFA uptake is increased by caveolin-1 overexpression and inhibited by overexpression of a dominant-negative caveolin mutant [12,13]. Disruption of lipid raft/caveolae structure also impairs LCFA uptake implicating these membrane domains in this process [12,14].

How lipid rafts/caveolae contribute to LCFA uptake remains unclear. Cholesterol depletion in fibroblasts leads to translocation of caveolin-1 from the cell surface to the Golgi apparatus [15]. LCFA uptake in myocytes may be regulated by CD36 translocation between the PM and intracellular sites [16]. We therefore tested the hypothesis that depletion of cellular cholesterol inhibits LCFA uptake in adipocytes by altering levels or localization of either caveolin-1 or CD36. We demonstrate that cellular cholesterol depletion reversibly inhibits oleate uptake in adipocytes, without altering levels or cell-surface localization of either CD36 or caveolin-1.

Abbreviations: BSA, bovine serum albumin; D-MEM, Dulbecco's modified Eagle's medium; $FABP_{PM}$, plasma membrane fatty acid binding protein; FATP, fatty acid transport protein; FBS, fetal bovine serum; HRP, horse radish peroxidase; IM, internal membranes; LCFA, long chain fatty acid; M-β-CD; methyl-β-cyclodextrin; NCLPDS, newborn calf lipoprotein deficient serum; PM, plasma membrane; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.

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Materials and methods

Materials. Reagents were obtained from sources previously described [1]. 3T3-F442A cells were from Kathleen Cook. Antibodies (and sources): Rabbit anti-caveolin-1 (BD Biosciences); mouse monoclonal anti-mouse CD36 (Maria Febbraio, Lerner Research Institute, Cleveland Clinic); rabbit anti-ε-COP (Monty Krieger, Massachusetts Institute of Technology); alexa-488-goat anti-rabbit (Molecular Probes). All other reagents were from Sigma Chemical Corp.

Cell culture. Preadipocytes were maintained in complete D-MEM containing 10% calf serum, glutamine and antibiotics (medium A) [1]. 3T3-F442A cells were stimulated to differentiate by culture in medium B (medium A in which calf serum was replaced with FBS) containing insulin (5 μg/ml). Two days post confluence, the medium was replaced with medium B or medium C (medium B in which FBS was replaced with newborn calf lipoprotein deficient serum (NCLPDS) [17]). Differentiation of 3T3-L1 cells was described previously [1]. For fluorescence staining, cells were cultured on poly-D-lysine coated cover slips [18].

Cholesterol depletion. Cells cultured in medium B were treated with filipin (5 μg/ml) in medium B for 30 min at 37 °C. Cells cultured in medium C for the last 2 days of differentiation were treated with 5 mM methyl-β-cyclodextrin (M-β-CD) in medium C for 2 h at 37 °C.

Filipin staining. Cells were fixed (1 h; 0.1 M Na-Cacodylate, pH 7.4 + 1% paraformaldehyde and 12.5% glutaraldehyde), washed and incubated (2 h; room temp.) with filipin (50 μg/ml) in cPBS (PBS + 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$). Fluorescence microscopy was performed using a DAPI filter set.

Uptake assays. [9,10-^{3}H]Oleate or 2-[^{3}H]deoxyglucose uptake was measured using a filtration assay [1] or in adherent cells after incubation with D-MEM containing 100 μM each of [9,10-^{3}H]oleate and BSA for 15 min at 37 °C. After incubation, cells were washed twice with ice cold PBS containing 0.1% BSA and 100 μM phloretin and lysed by incubation with 0.1 M NaOH for 20 min at room temp. A portion of each lysate was used for scintillation counting and protein determination (BCA Protein Assay, Pierce Chemical Corp.).

Immunoblotting. Total cell homogenates, plasma membranes (PM), and internal membranes (IM) were prepared and analyzed by SDS–PAGE and immunoblotting as described previously [1,8].

Immunofluorescence. Immunofluorescence in paraformaldehyde fixed, permeabilized cells was done as described previously [19]. Fluorescence microscopy was performed using a FITC filter set. Z-stack images (75), separated by 0.75 μm were subjected to deconvolution (Zeiss Axiovision software).

Flow cytometry. Cells were released from dishes with 1 mM EDTA in PBS, washed and fixed in suspension with 2% paraformaldehyde in PBS at

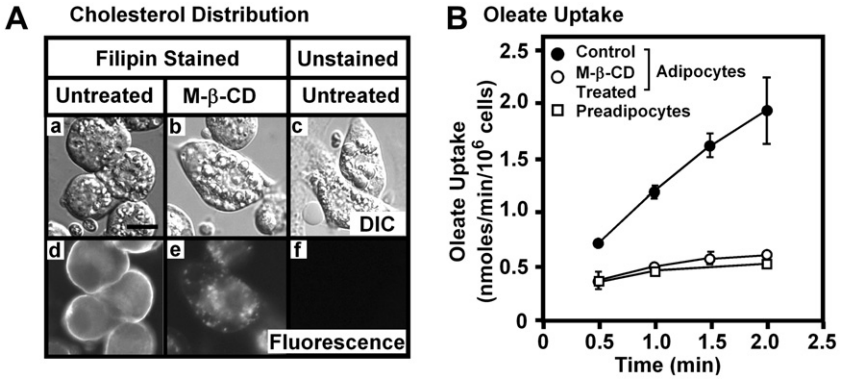

Fig. 1. M-β-CD depletes cellular cholesterol and inhibits oleate uptake. (A) 3T3F-442A cells were either untreated (a, c, d, and f) or treated with 5 mM M-β-CD (b and e) and then either stained with filipin (a, b, d, and e) or unstained (c,f). Representative DIC (a–c) and fluorescence (d–f) images are shown (bar = 10 μm). (B) Preadipocytes (open squares), untreated adipocytes (closed circles) or adipocytes treated with 5 mM M-β-CD (open circles) were assayed for oleate uptake using a filtration assay. Data are means ± SD of triplicates. $P < 0.01$ for untreated adipocytes vs. either treated adipocytes or preadipocytes.

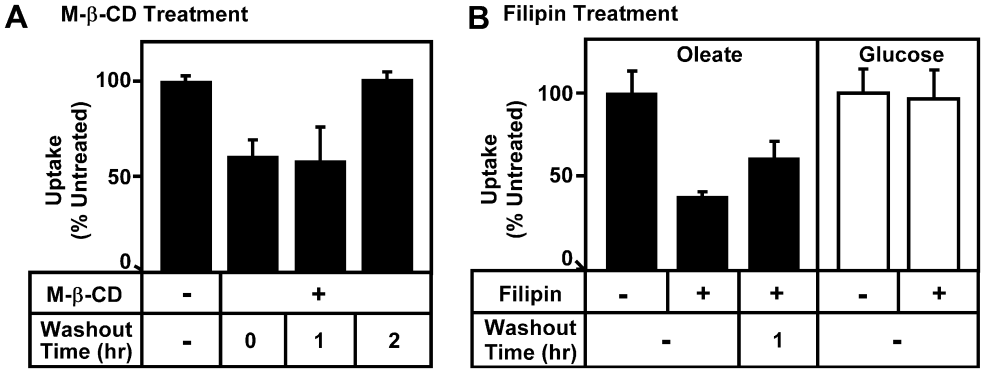

Fig. 2. Inhibition of oleate uptake by M-β-CD or filipin is reversible. (A) Oleate uptake in adherent adipocytes treated ±5 mM M-β-CD was measured either immediately (washout time 0) or after replacement of the media with medium B (washout time 1 or 2 h). Data are the means ± ranges of duplicate independent experiments. (B) Adipocytes treated ±5 μg/ml filipin were assayed immediately or 1 h after replacement of the media with medium B (washout). Oleate (left panel) and 2-deoxyglucose (right panel) were assayed using the filtration assay. Data are the means ± SD of triplicates. $P \leqslant 0.01$ for oleate uptake by cells treated with filipin versus untreated or treated + washout. Uptake rates are expressed as a percentage of the rate in untreated cells.

4 °C for 30 min. All steps were carried out at room temp. in PBS. Cells were washed, blocked with 10% FBS, and incubated (30 min) without or with anti-CD36 antibody (1:100) followed by FITC-goat anti-mouse IgA (1:500) for 30 min. Flow cytometry was done using a Beckman–Coulter Epics XL.

Statistical analysis. Data were subjected to Student's *t*-test and considered statistically significantly different if $P < 0.05$.

Results and discussion

Filipin staining [20] revealed that the majority of unesterified cholesterol in adipocytes was at the cell periphery, consistent with localization at the PM (Fig. 1A). M-β-CD-treated adipocytes displayed reduced filipin staining at the cell periphery, consistent with loss of PM cholesterol. M-β-CD-treatment of adipocytes resulted in a 3.5-fold reduction in the rate of oleate uptake, almost to the low level of uptake observed in undifferentiated cells (Fig. 1B). To test if M-β-CD mediated inhibition of oleate uptake was reversible, cells were treated with or without 5 mM M-β-CD and assayed immediately or after 1 or 2 h incubation in media B lacking M-β-CD (Fig. 2A). In this experiment, oleate uptake was reduced by 40% after M-β-CD treatment and recovered partially by 1 h and fully by 2 h after removal of M-β-CD (Fig. 2A). Oleate uptake was also reduced by treatment with 5 μg/ml filipin, which disrupts PM-cholesterol dependent processes [21,22]. Removal of filipin and incubation of cells in the presence of FBS for 1 h also resulted in a partial recovery of oleate uptake (Fig. 2B). Neither cell viability (not shown) nor non-insulin stimulated uptake of 2-deoxyglucose (Fig. 2B) were affected by this treatment, consistent with other reports [23]. Therefore cholesterol depletion inhibits adipocyte LCFA uptake in a reversible manner, consistent with other reports [12,24].

To explore the molecular basis for reduced fatty acid uptake by cholesterol depleted adipocytes, we examined the effects of cholesterol depletion on the level and distribution of caveolin-1. Treatment of adipocytes with filipin, M-β-CD, or culture in the absence of lipoproteins (NCLPDS) did not alter caveolin-1 levels measured by immunoblotting (Fig. 3A). Furthermore neither culture in the absence of lipoproteins, nor treatment with filipin or M-β-CD altered the distribution of caveolin-1 between PM and IM-enriched fractions (Fig. 3B). This was confirmed by immunofluorescence microscopy (Fig. 3C). Caveolin-1 was detected primarily at the cell periphery and peri-nuclear regions in 3T3-F442A cells regardless of the condition employed. Therefore, cholesterol depletion did not alter the amount or localization of caveolin-1.

We also investigated the subcellular distribution of CD36 following cholesterol depletion of adipocytes. As for caveolin-1, neither culture of cells in the absence of lipoproteins nor cholesterol depletion with filipin or M-β-CD altered the amount of CD36 associated with PM or IM fractions (Fig. 4A). To confirm these results, cell surface CD36 in untreated and M-β-CD-treated 3T3-L1 adipocytes was detected by indirect fluorescence immunostaining with anti-CD36 antibody and analysis

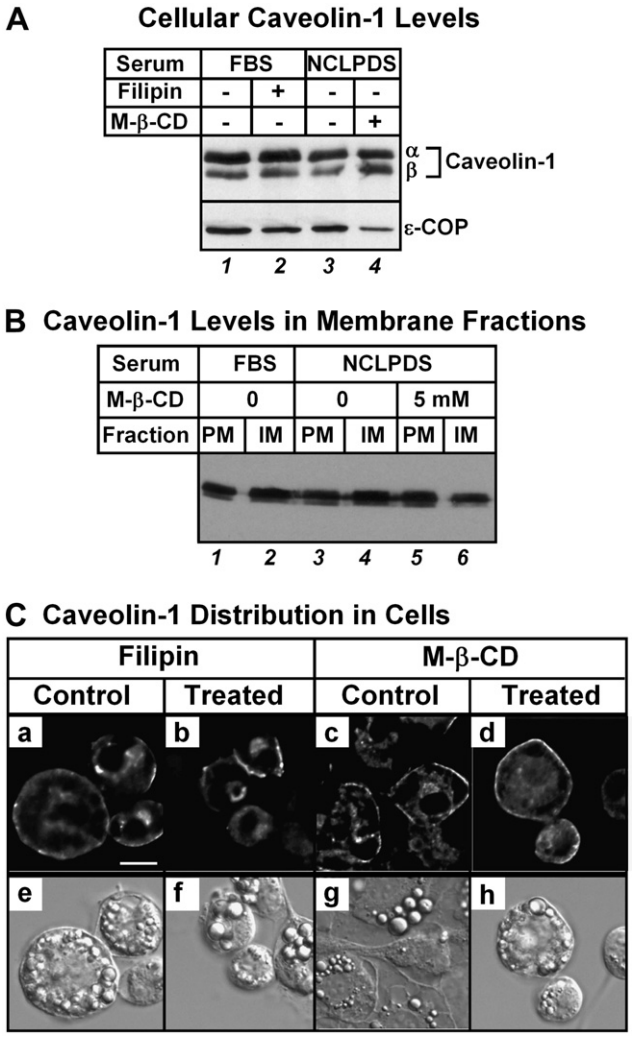

Fig. 3. Cholesterol depletion does not alter levels or cell surface expression of caveolin-1. Adipocytes cultured for the last 2 days of differentiation in the presence of FBS or NCLPDS, were treated ±5 μg/ml filipin or 5 mM M-β-CD. (A) Analysis of caveolin-1 (top) or ε-COP (loading control, bottom) by immunoblotting of whole cell lysates. (B) PM and IM-enriched fractions were prepared and caveolin-1 was analyzed by immunoblotting. Note that the α and β isoforms of caveolin-1 were not well resolved in the blot shown in (B). (C) Adipocytes were treated ±5 μg/ml filipin (a,b,e,f) or 5 mM M-β-CD (c,d,g,h) and immunostained for caveolin-1. Representative fluorescence (a–d) and DIC images (e–h) at the same magnification (bar = 10 μm).

by flow cytometry (Fig. 4B). Similar cell surface expression of CD36 was detected in untreated and M-β-CD-treated cells. Collectively these data demonstrate that depletion of cell surface cholesterol reversibly inhibits adipocyte LCFA uptake without altering the cell surface distribution of either caveolin-1 or CD36.

The findings that cellular cholesterol can control fatty acid uptake and influence insulin stimulated glucose uptake [23] point to an important role for cellular cholesterol in the acute regulation of energy metabolism and homeostasis, beyond effects on gene expression [25]. How cholesterol exerts these effects remains unknown. Although CD36 and caveolin-1 participate in LCFA uptake, are localized in

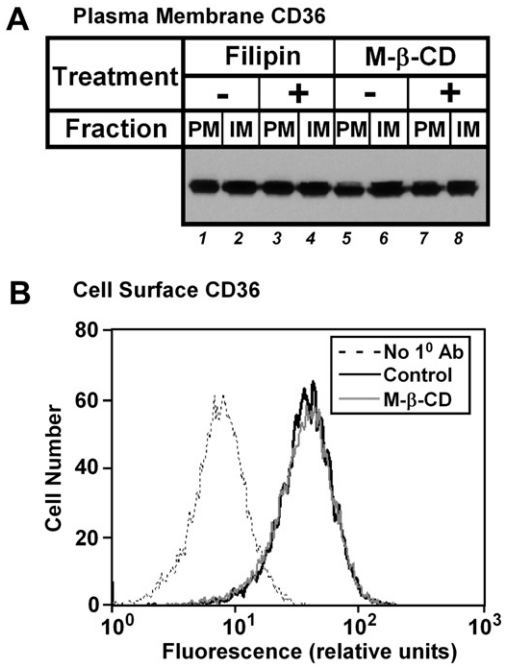

Fig. 4. Cholesterol depletion does not alter levels or cell surface expression of CD36. (A) 3T3-F442A adipocytes were treated ±5 μg/ml filipin or 5 mM M-β-CD and PM and IM fractions were immunoblotted for CD36. (B) 3T3-L1 adipocytes either treated with (grey) or without (black; solid and dashed) 5 mM M-β-CD were stained with (solid black and grey) or without (dashed) anti-CD36 antibody. All samples were stained with FITC-conjugated anti-mouse IgA secondary antibody. The fluorescence of 10^4 cells per sample was analyzed by flow cytometry. Triplicate samples were analyzed and representative histograms are shown.

caveolae/lipid rafts and have the potential to translocate between the PM and intracellular compartments [16], our results demonstrate that cholesterol-depletion mediated inhibition of LCFA uptake in adipocytes can occur without redistribution of these proteins from the cell surface. It remains possible that the activity of one or more mediators of LCFA uptake, including CD36, caveolin-1 or others such as FATP or $FABP_{PM}$, is directly affected by cell surface free cholesterol. Further studies will be required to resolve these issues.

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