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Cholesterol-Induced Caveolin Targeting to Lipid Droplets in Adipocytes: A Role for Caveolar Endocytosis

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We have investigated the targeting of caveolin to lipid bodies in adipocytes that express high levels of caveolins and contain well-developed lipid droplets. We observed that the lipid droplets isolated from adipocytes of caveolin-1 knock out mice contained dramatically reduced levels of cholesterol, indicating that caveolin is required for maintaining the cholesterol content of this organelle. Analysis of caveolin distribution by cell fractionation and fluorescent light microscopy in 3T3-L1 adipocytes indicated that addition of cholesterol rapidly stimulated translocation of caveolin to lipid droplets. The cholesterol-induced trafficking of caveolins to lipid droplets was shown to be dynamin- and protein kinase C (PKC)-dependent and modulated by src tyrosine kinase activation, suggesting a role for caveolar endocytosis in this novel trafficking pathway. Consistent with this, caveolae budding was stimulated by cholesterol addition. The present data identify lipid droplets as potential target organelles for caveolar endocytosis and demonstrate a role for caveolin-1 in the maintenance of free cholesterol levels in adipocyte lipid droplets.

Key words: adipocyte, caveolin, cholesterol, endocytosis, lipid droplets, protein traffic

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Lipid droplets are intracellular organelles specialized for the storage of lipids. Although their formation can be induced in any cell type, some tissues store fat for specialized functions. Among these are the steroidogenic cells which use cholesterol ester–rich lipid droplets as cholesterol reservoirs for steroid hormones synthesis. The cells in the retinal pigment epithelium contain retinol ester-rich droplets, which constitute a metabolic intermediate involved in the regeneration of the visual chromophore. Large triglyceride-rich droplets that occupy almost the entire cell volume are in adipocytes, the specialized cell type for the storage of energy as fat.

During recent years, significant progress has been made in the understanding of lipid droplet biology. It has been recognized that they consist of a neutral lipid core surrounded by a monolayer of phospholipids and cholesterol (1). PAT proteins (Perilipin, Adipophilin, TIP47) have been identified as building blocks of the lipid droplet coat, covering the ytoplasmic surface of this monolayer (2). Proteomic approaches have revealed the presence of a large set of proteins, presumably interacting with the lipid droplet protein coat (3–6). Remarkably, most of these proteins are not uniquely present in lipid droplets, but reside also in other cell compartments. Thus, the multiple functions related to these proteins suggest that lipid droplets can not only be considered as inert reservoirs but also be considered as dynamic structures involved in intracellular lipid trafficking.

Caveolins have recently been found to associate with lipid droplets (1,7,8). Caveolins are intramembrane cholesterol and fatty acid-binding proteins (9,10) which constitute the structural coat of flask-shaped invaginations called caveolae at the plasma membrane of the cell. These proteins have been proposed to play a role in cholesterol trafficking (11,12). Early studies identified caveolins at the surface of lipid droplets in cells overexpressing caveolin-2 (1,7), or a chimeric construct in which an endoplasmic reticulum (ER)-retrieval signal was linked to caveolin-1 (7), or cells treated with brefeldin A (1,7). Moreover, dominant-negative mutants of caveolin constitutively associate with lipid droplets (8). From these experiments, it was concluded that this localization resulted from mistargetting of caveolins due to their accumulation in the ER. However, recent studies have demonstrated that endogenous caveolins become associated with lipid droplets after fatty acid loading (5,13) or during liver regeneration (13).

How lipid loading influences movement of caveolins to lipid droplets is not known. In the present study, we used adipocyte cell systems in which prominent lipid droplets were developed as the result of *de novo* lipogenesis, and thus did not require exogenous fatty acids addition. These fat cells highly express caveolins (14) and constitute an interesting system to study the mechanism of caveolin targeting to lipid droplets. Studies of adipocytes obtained from cav-1 knock out (KO) animals revealed that caveolin is required to maintain normal cholesterol content of lipid droplets. Moreover, our data show that cholesterol

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stimulation, as well as oleic acid, induces caveolin translocation to lipid droplets. We demonstrate that this caveolar pathway between the fat cell plasma membrane and the lipid droplet involves a dynamin, protein kinase C (PKC) and *src*-dependent process. Together with the observation that cholesterol addition induces caveolar budding, these results suggest that caveolin translocation to lipid droplets involves caveolae endocytosis.

Results

Caveolin-1 is required to maintain the cholesterol content of lipid droplets

Triacylglycerols are by far the most abundant lipid species in adipocytes. Surprisingly, adipose tissue is also enriched in free, non-esterified cholesterol (15,16) stored in the lipid droplet (17), likely within the phospholipid monolayer at the lipid droplet surface (18). Since caveolin-1 is a cholesterol binding protein (9), particularly abundant in adipocytes (14), we postulated that caveolin might be involved in cholesterol trafficking in fat cells. By comparing the total cholesterol content of lipid droplets isolated from caveolin-1 KO mice and their wild type counterparts, we observed a dramatic (10-fold) reduction of cholesterol concentration in lipid droplets in caveolin-1 KO mice (Figure 1A). In some caveolin-1 knockout animals, cholesterol content of adipocyte lipid droplets was below the detection limit of the assay. Such a drastic reduction did not result from a general defect in lipid droplet formation in caveolin-1 KO mice, but was specific for cholesterol since (i) we selected male mice at a young age (12-week old) before any differences in adiposity could be observed [WT ($n = 5$): 30.38 g $\pm$ 3.08 – adiposity: 5.88% $\pm$ 0.67; KO ($n = 5$): 30.7 g $\pm$ 1.38 – adiposity: 5.63% $\pm$ 0.41]; (ii) a comparable amount of lipid droplet proteins was recovered from the adipose tissue of caveolin-1 KO and wild type controls, and (iii) the triglyceride content of lipid droplets did not differ between the two groups (Figure 1A).

Considering that these differences in lipid droplet cholesterol content could result from alterations in circulating lipid levels that might occur in caveolin-1 KO mice, we also studied mouse embryonic fibroblasts that had been differentiated into adipocytes *in vitro*. Figure 1B shows that filipin staining of free cholesterol (FC) decorated the plasma membrane and the lipid droplets of wild-type adipocytes. This pattern was strikingly different in cav-1-KO adipocytes, which exhibited a diffuse intracellular staining with no filipin accumulation around lipid droplets. In order to establish the direct role of caveolin in maintaining cholesterol content, we re-expressed caveolin-1 in cav-1 KO cells by the use of adenoviruses (see western-Blot on Figure 1B). We showed that caveolin-1 rescue led to the restoration of cholesterol distribution around lipid droplets in cav-1 KO mouse embryonic fibroblasts (MEFs) differentiated adipocytes (Figure 1B).

Taken together, these results demonstrate that caveolin-1 is required to maintain normal cholesterol content and distribution in adipocyte lipid droplets.

Exogenous cholesterol induces the translocation of caveolins to lipid droplets

Previous reports using immunofluorescence or isolated lipid bodies have shown that caveolins redistributed to lipid droplets upon oleate treatment in cultured cells (5,13). In agreement with previous data, we observed that oleate addition to 3T3-L1 adipocytes markedly increased caveolin-1 to perilipin ratios in the lipid droplet fraction (Figure 2A). This indicates that in adipocytes, lipid droplet caveolin content, but not lipid droplet formation, depends on the presence of exogenous fatty acids. Thus, it is likely that culturing adipocytes in the presence of foetal bovine serum, a highly variable and batch-dependent source of exogenous lipids, can therefore modulate the steady state caveolin content of lipid droplets.

We then investigated the effect of the addition of cholesterol to cultured 3T3-L1 adipocytes. We observed that exogenous cholesterol [10 μg/mL in an ethanol solution (19)] had a similar effect to fatty acids, leading to a 3-fold increase in the relative content of caveolin-1 in the lipid droplet fraction (Figure 2A). Equivalent results were observed when cholesterol was added in the form of a methyl-β-cyclodextrin complex (data not shown). Interestingly, the association of caveolin-1 with lipid droplets upon cholesterol addition was rapid, reaching maximal levels within 10 min at 37 °C with no further increase upon longer incubation times. Similar changes were also observed for caveolin-2 (Figure 2B).

The rapid action of exogenous cholesterol on lipid droplets caveolin content suggests that it does not imply changes in gene expression, but rather relies on the recruitment of caveolins from other compartments. Indeed, pretreatment of cells with 10 μM of the translation inhibitor, cycloheximide, for 1 h before cholesterol addition did not alter cholesterol-induced caveolin-1 recruitment to lipid droplets (data not shown), indicating that caveolin targeting to lipid droplets is independent of newly synthesized caveolin. Furthermore, in agreement with caveolin redistribution from other compartments, we observed that addition of cholesterol to cells decreased the caveolin signal by 17.5% $\pm$ 0.51 in purified plasma membrane and by 45.5% $\pm$ 16.3 in internal membrane preparations compared to unstimulated conditions (data from three independent experiments). We therefore used a 3T3-L1 stably expressing cav-1 EGFP to study the effect of cholesterol addition on caveolin distribution. Under unstimulated conditions, cav-1 EGFP is visible at the plasma membrane and in intracellular compartments, most likely the Golgi complex and endocytic compartments (Figure 2C). By fluorescence microscopy, we did not detect cav-1 EGFP redistribution to lipid droplets upon short-term exposure (10 min) of cells to cholesterol, although caveolin

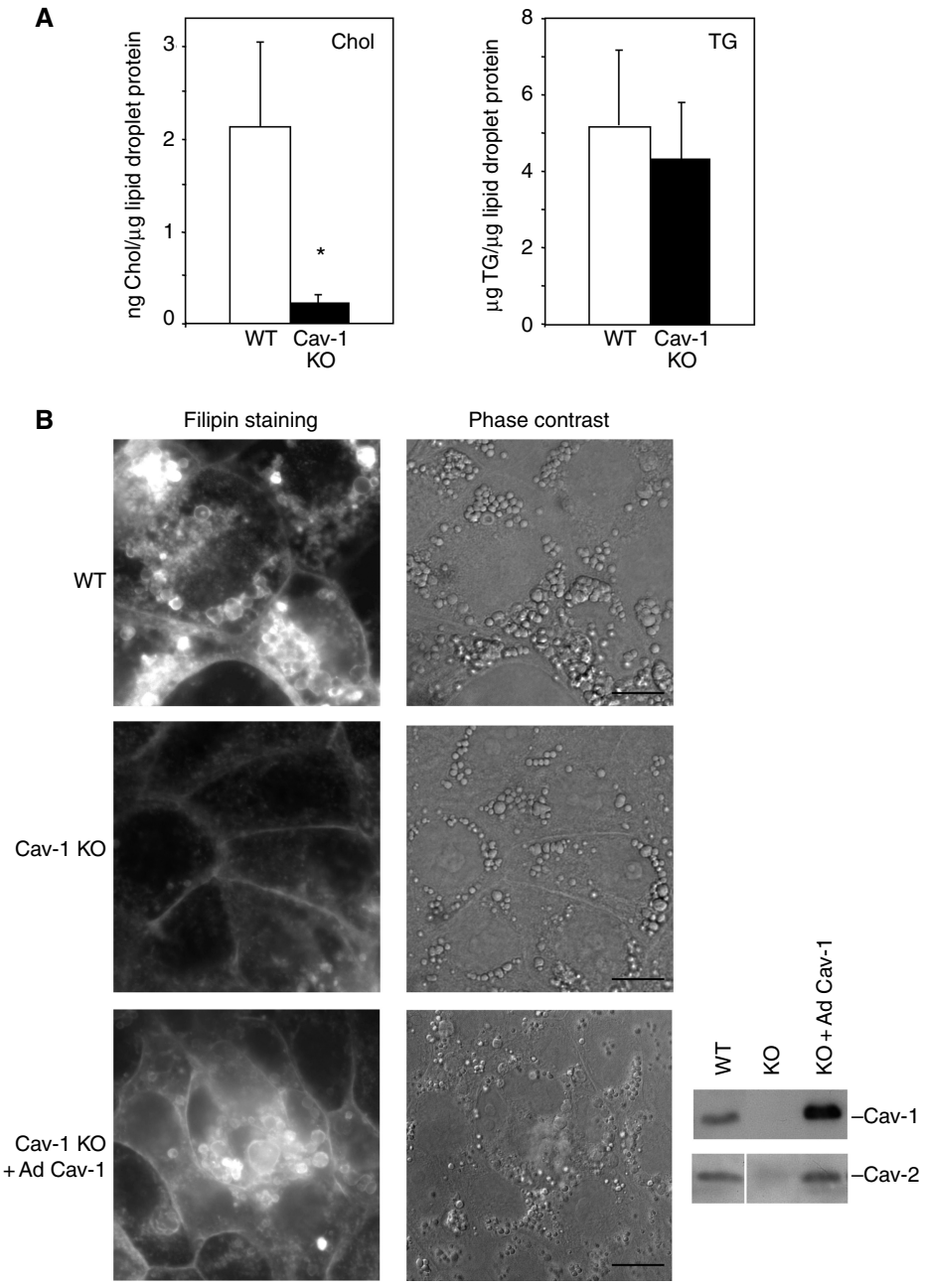

Figure 1: Cholesterol content of lipid droplets is reduced in adipocytes from caveolin-1 KO mice. (A) Lipid content of adipocyte lipid droplets in caveolin-1 KO mice. Adipocytes were isolated from epididimary adipose tissue by collagenase treatment, and lipid droplets were purified. Total cholesterol (Chol), Triglyceride (TG) and lipid droplets protein contents were measured as described in methods. All values are means ± sem obtained from five individual mice in each group. * indicates a statistically significant difference by Student's *t*-test ($p < 0.05$). (B) Filipin staining of differentiated adipocytes derived from MEFs obtained from wild-type (WT), Caveolin-1 KO (cav-1 KO) mice, and cav-1 KO rescued by adenoviral expression of cav-1 (cav-1 KO + Ad cav-1). Mouse embryonic fibroblasts (MEF)s were differentiated into adipocytes as described in methods. Lipid droplets are clearly identifiable under phase contrast. Note the absence of staining of lipid droplets in cav-1 KO adipocytes. Bar, 10 µm. Re-expression of both cav-1 and cav-2 in cav-1 KO MEFs is shown by Western blot.

translocation was clearly detectable at these early time points by Western blotting. Upon longer exposure to cholesterol (1–2 h), redistribution of cav-1 EGFP to lipid bodies was clearly observed in cells exposed to cholesterol stimulation (Figure 2C). This was associated with a decrease of fluorescence at the plasma membrane and intracellular compartments. Similar results were obtained when cholesterol stimulation was performed in the

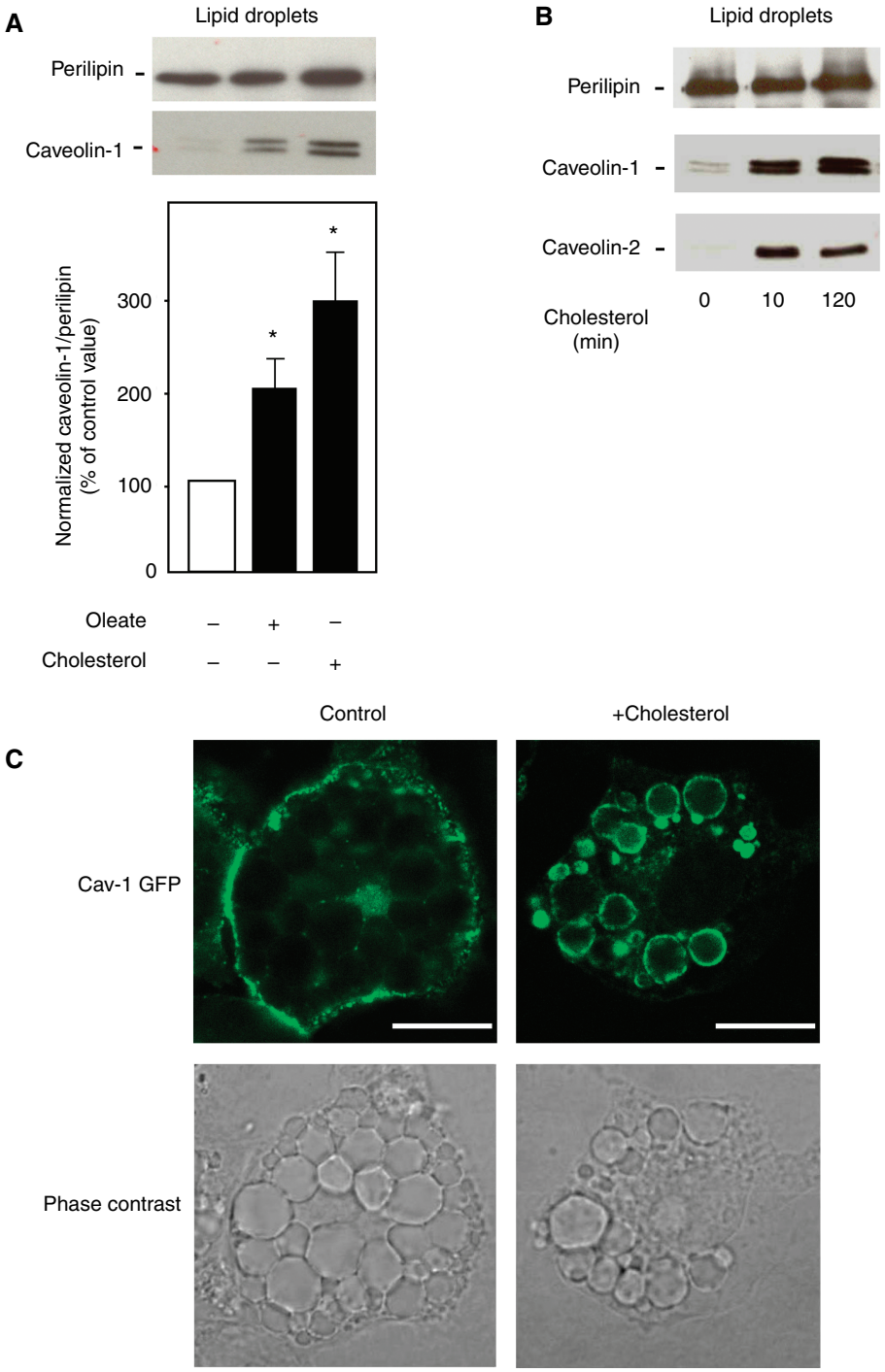

Figure 2: Effect of exogenous cholesterol on the intracellular distribution of caveolin. (A) Cholesterol and fatty acids increase caveolin concentration on lipid droplets. 3T3-L1 adipocytes were treated for 10 min with oleate (100 μM, in cyclodextrin complex) or cholesterol (10 μg/mL, in an ethanol solution). Then lipid droplets were isolated and used for western-blotting. Same results were obtained when cholesterol was added as cyclodextrin-complexes. Results are expressed as relative changes (%) in caveolin-1 after normalization to perilipin content of lipid droplets. Values are means ± sem obtained from 10 independent experiments. Differences between cholesterol or oleate treated cells and controls were statistically significant by Student's *t*-test, at the $p < 0.05$ level. (B) Time course of the effect of cholesterol on lipid droplet caveolin-1 and -2. The experimental conditions were the same as that of A except that cholesterol was maintained up to 120 min at 37 °C before lipid droplet purification. (C) 3T3-L1 adipocytes expressing stably cav-1 EGFP following retroviral infection and selection in hygromycin medium were cultured until day 10 of differentiation on coverslips as described in methods. Addition of cholesterol (10 μg/mL) during 120 min leads to the translocation of cav-1 EGFP onto lipid droplets. Bar, 10 μm.

presence of 10 μM cycloheximide. Altogether, these data indicate that upon cholesterol addition, caveolins translocate from the cell surface and intracellular compartments to the lipid droplets.

Caveolin-1 binds to cholesterol on lipid droplets

We next asked whether caveolin-1, when present on lipid droplets, was associated with cholesterol. To this aim, we used a photoactivable analogue of cholesterol, [^{3}H] photocholesterol (20). After labelling of 3T3-L1 adipocytes with [^{3}H]photocholesterol and subsequent irradiation of the cells, several proteins in the total cell lysate incorporated radioactivity (Figure 3, lane 1). After purification of lipid droplets from irradiated cells, two heavily labelled-bands were present on SDS-PAGE (see asterisks Figure 3, lane 2). Based on molecular weight, one of these cholesterol-binding proteins was likely to be caveolin-1. Its identity was confirmed by immunoprecipitation with a caveolin-1 antibody (see brackets Figure 3, lane 2–4) followed by Western blotting using the same antibody (Figure 3, lane 5–7). Specific labelling of caveolin-1 by [^{3}H]photocholesterol demonstrates a direct interaction between caveolin-1 and cholesterol on lipid droplets.

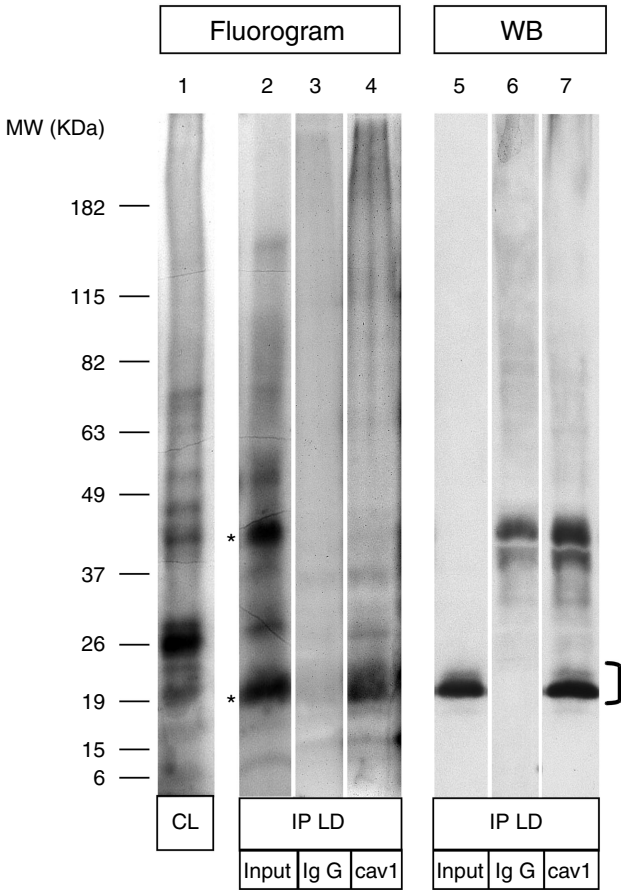

Figure 3: Interaction between caveolin-1 and cholesterol on lipid droplets. 3T3-L1 adipocytes were incubated for 16 h in the presence of [^{3}H]photocholesterol and then ultraviolet-irradiated. After lipid droplet purification, photolabelled proteins were resolved by SDS-PAGE (5–15%, Tris-Tricine) and visualized by fluorography. Lane 1: cell lysate (CL), lane 2: lipid droplet fraction (LD). Lipid droplet fraction was subjected to immunoprecipitation (IP) using antibodies against a caveolin-1 or nonimmune IgG. Samples were loaded on a SDS-PAGE and visualized by fluorography (Fluorogram, Lanes 2–4) or Western blotting (WB) using caveolin-1 antibody (Lanes 5–7).

Caveolin translocation to the lipid droplet is actin- and dynamin-dependent

To analyze the mechanism by which caveolins translocate to lipid droplets upon cholesterol treatment, we studied the effects of several inhibitors of cytosolic transport or endocytosis by quantifying the caveolin-1/perilipin content of lipid droplets.

We first tested the possibility that translocation could proceed in the form of cytoplasmic complexes containing caveolins, immunophilins and chaperone proteins as described previously for the transport of newly synthesized cholesterol from the endoplasmic reticulum through the cytoplasm to caveolae (21). However, we found no effect of the immunophilin binding agent cyclosporin A, which disrupts immunophilin-chaperone complexes, making the involvement of such cytosolic complexes unlikely (Figure 4A).

Another possibility was that the translocation of caveolins to lipid droplets proceeded via an endocytic pathway. Indeed, internalization of various plasma membrane molecules, bacterial toxins, viruses or proteins (such as albumin) has been found to occur *via* caveolar endocytosis (22,23). By testing different drugs impairing the cytoskeleton reorganization, we demonstrated that nocodazole had no effect on the cholesterol-dependent recruitment of caveolin to the lipid droplets, suggesting that functional microtubules were not required (Figure 4A). In contrast, cytochalasin D inhibited the cholesterol-dependent translocation of caveolin-1 to lipid droplets (Figure 4A), indicating the possible involvement of actin filaments in this process. Since endocytic processes, like caveolar endocytosis, have been shown to be dynamin-dependent (24), we infected 3T3-L1 adipocytes with adenoviral constructs directing tetracycline-dependent expression of dynK44A, a dominant-negative form of dynamin (25). After 16 h, the cells expressing the dynamin mutant exhibited no obvious morphological changes and were able to normally mobilize their lipid stores, indicating that the key adipocyte metabolic functions were maintained (data not shown). In contrast, DynK44A expression dramatically decreased cholesterol-induced recruitment of caveolin-1 to the lipid droplet fraction in fat cells (Figure 4B), suggesting that the targeting of caveolins to this organelle is a dynamin-dependent process.

Caveolin trafficking to lipid droplets requires PKC and src kinase activities

Caveolar endocytosis is known to be regulated by protein kinase C (PKC) (26–28). Therefore, we investigated the

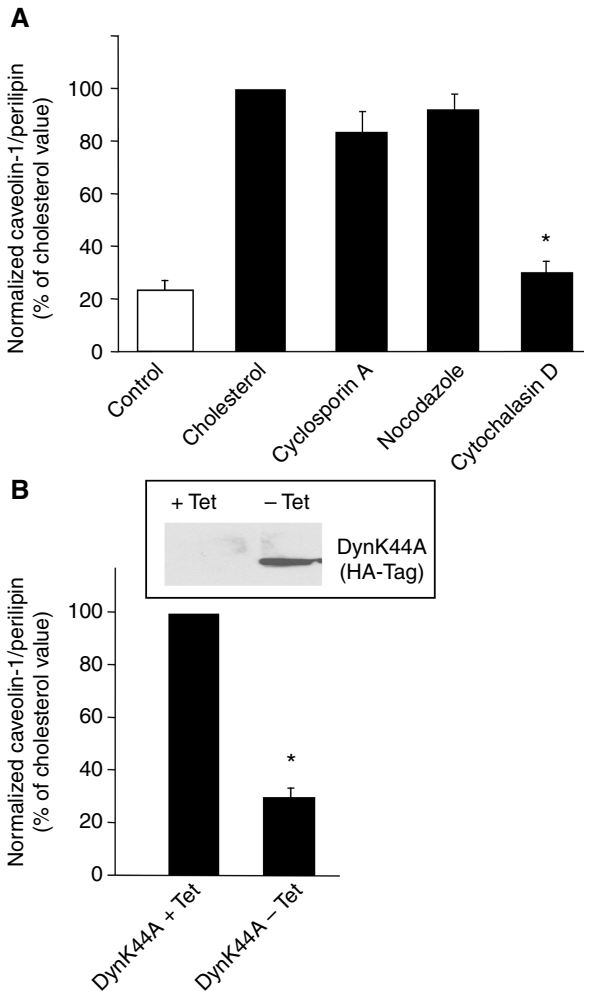

Figure 4: Modulation of cholesterol-induced accumulation of caveolin-1 on lipid droplets by drugs and dynamin K44A mutant. (A) Differentiated 3T3-L1 adipocytes were incubated for 10 min in the absence (open bar) or in the presence (black bars) of cholesterol. Cyclosporin A (10 μM) or nocodazole (2 μM) were added 1 h prior to cholesterol treatment. Cytochalasin D (2 μM) treatment was added for 2 h before cholesterol addition. Lipid droplets were then isolated and subjected to Western-blotting with caveolin-1 and perilipin antibodies. Results are expressed as relative changes (%) in caveolin-1 after normalization to perilipin content of lipid droplets. Values are means ± sem obtained from at least three independent experiments. (B) Infection of adipocytes with adenoviruses encoding a tetracycline-regulatable transcription activator and a dynamin dominant negative mutant (Dyn K44A) was performed as described in methods. 16 h post-infection, cholesterol was added. The presence of tetracycline (1 μg/mL) in the culture medium suppressed dynamin K44A expression as evaluated by immunoblotting cell lysates with antibodies against the hemagglutinin (HA)-tag of the dynamin mutant. Results were determined from three independent experiments.

effects of two different bisindolemaleimide compounds, Ro31.8220 and GF109203X, which inhibit all PKC classes (conventional, novel and atypical) when used at micromolar range, and only conventional and novel PKCs when applied at submicromolar range (29). Micromolar concentrations of both Ro31.8220 and GF109203X completely prevented the translocation of caveolins to lipid droplets upon cholesterol addition (Figure 5 A and quantification on Figure 5B). Moreover, a lower concentration of Ro31.8220 was equally potent, suggesting the involvement of conventional and/or novel PKCs in this process (Figure 5B).

Tyrosine kinases have also been described as key players in caveolar endocytosis (27,30). Accordingly, we found that cholesterol-induced accumulation of caveolin-1 on lipid droplets was prevented by genistein, a general tyrosine kinase inhibitor (Figure 5B). The involvement of *src* kinases was further suggested by the use of more specific inhibitors such as herbimycin A and PP2 and by the lack of effect of PP3, an inactive control for PP2 (Figure 5B). The effect of these tyrosine kinases inhibitors was additionally tested on 3T3-L1 stably expressing cav-1 EGFP by retroviral expression. Upon cholesterol addition, cav-1 EGFP is distributed from the plasma membrane and intracellular compartments to lipid droplets (Figure 5C). Indeed, 64 ± 14% lipid droplets are positive for cav-1 GFP upon cholesterol stimulation (Figure 5D). Treatment of 3T3-L1 adipocytes with PP2 and genistein prior cholesterol addition prevented significantly cholesterol-induced translocation of cav-1 EGFP to lipid droplets, whereas pretreatment with PP3 had no effect (Figure 5C and quantification on Figure 5D).

To directly demonstrate the involvement of c-*src* kinase in cholesterol-induced translocation of caveolin-1 to the lipid droplet, we expressed stably in 3T3-L1 adipocytes a constitutively active mutant form of c-*src*, Y527Fsrc, which exhibits a high kinase activity (31). Ectopic expression of this *src* mutant did not alter the differentiation of 3T3-L1, and induced no morphological or biochemical changes in lipid droplet formation and caveolin expression (data not shown). The expression of this constitutively active *src* mutant lead to high levels of caveolin-1 associated with lipid droplets in the absence of cholesterol stimulation (Figure 6). This demonstrates the importance of *src* activation in the translocation of caveolin-1 to lipid droplets, and suggests that the effect of cholesterol might be related to *src* activation.

Taken together, these studies suggest that cholesterol-induced redistribution of caveolin to lipid droplets may involve caveolae endocytosis. Biochemical studies have suggested that caveolae are relatively static cell surface structures in 3T3-L1 adipocytes (32). To investigate whether cholesterol stimulates caveolae internalization in this system, we used the ultrastructural method developed by Kirkham *et al.* (33) which enables to discriminate between surface-connected and internal structures due to the fact that the HRP reaction on an extracellular surface can be quenched by the use of the membrane-impermeable reducing agent, ascorbic acid. Indeed, CTB-HRP-positive caveolae (arrows) and surface CTB-HRP labelling are clearly evident in the absence of ascorbic acid (AA) (Figure 7A, -AA). In the presence of ascorbic acid (Figure

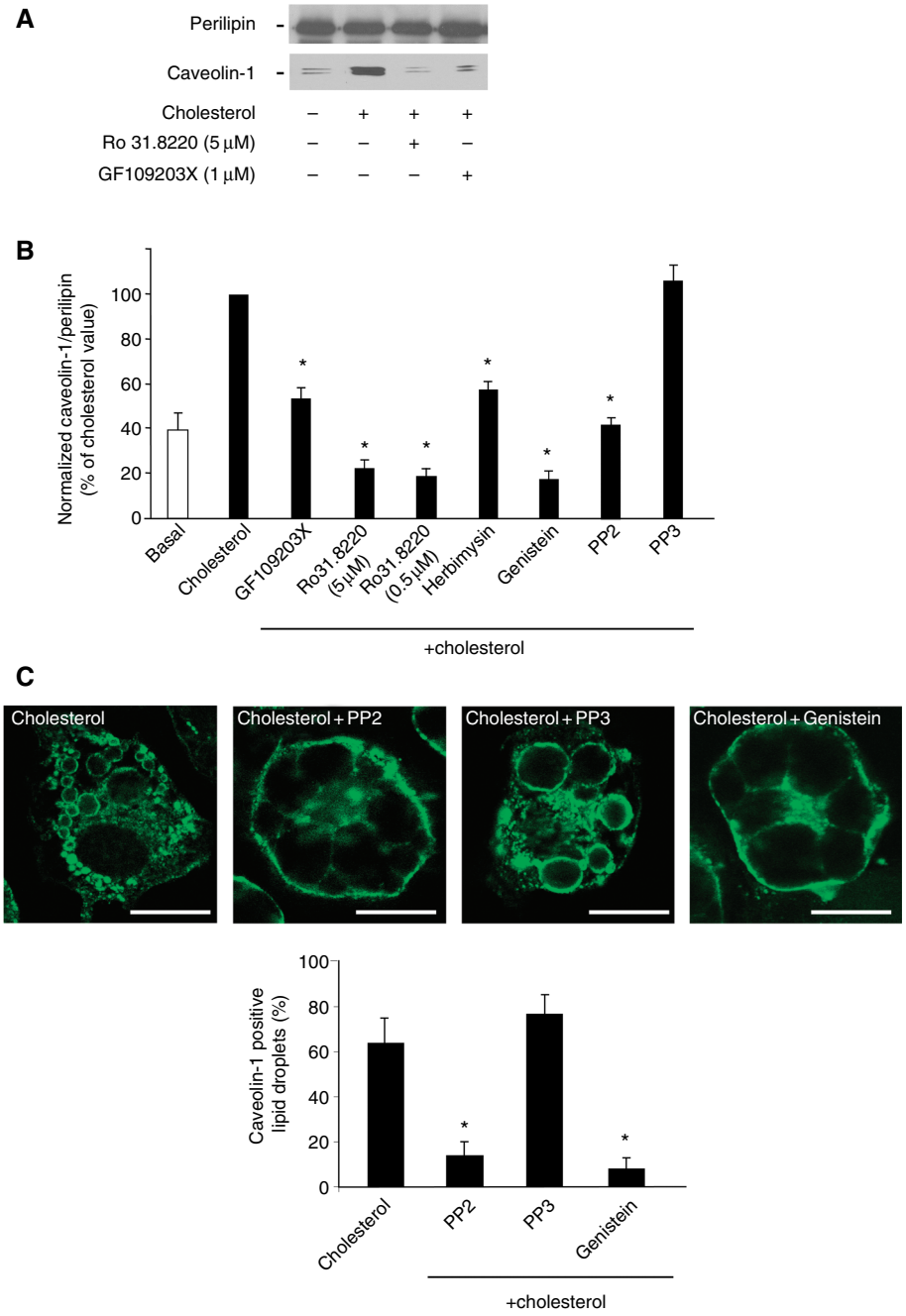

Figure 5: Cholesterol-induced translocation of caveolins onto lipid droplets is dependent on src and PKC kinases. (A) 3T3-L1 adipocytes were either treated or not with PKC inhibitors Ro31.8220 (5 μM) and GF 109203X (1 μM) for 1 h at 37 °C before cholesterol addition for 10 min at 37 °C and lipid droplet isolation was performed. Five microgram of lipid droplet proteins were loaded in each lane and perilipin was used as a positive control for equal loading. (B) Quantification of the effects of PKCs and tyrosine kinase inhibitors on caveolin association with lipids droplets. 3T3-L1 adipocytes were either treated or not with different inhibitors as described in A. Quantification of caveolin-associated to lipids droplets is expressed as relative changes (%) in caveolin-1 after normalization to perilipin content of lipid droplets. Values are means ± sem obtained from at least three independent experiments. Note that 0.5 μM Ro31.8220 1 h at 37 °C (submicromolar concentration inhibiting only conventional and novel PKCs) was equally potent for inhibiting cholesterol-induced translocation of caveolins onto lipid droplets as 5 μM Ro31.8220. Different *src* kinase inhibitors were tested: herbimycin (1 μM), genistein (50 μM) and PP2 (PP3 is a negative control for PP2, 10 μM for each). * indicates a statistically significant difference by Student's *t*-test ($p < 0.05$). (C) 3T3-L1 adipocytes expressing stably cav-1 EGFP were cultured on coverslips, and received cholesterol during 120 min before PFA fixation. The localization of cav 1-EGFP was examined in cells that had either been pre-treated or not with the indicated drugs. A quantification of caveolin-1 positive lipid droplets upon treatments with these inhibitors is presented. Bar, 10 μm.

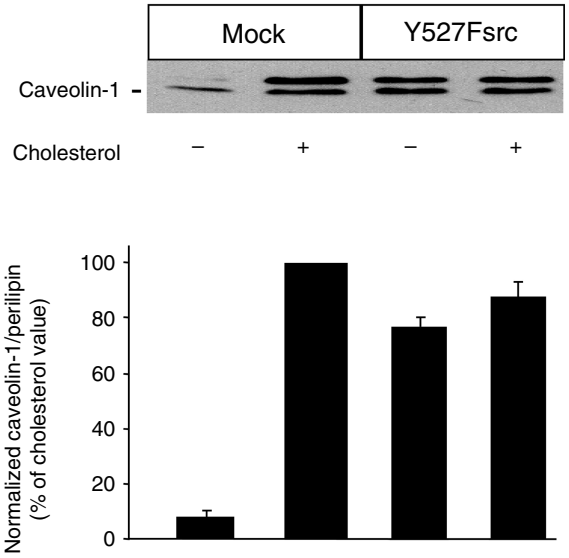

Figure 6: Effect of the constitutively active Y527Fsrc mutant on caveolin-1 content of lipid droplets. Western-blots of caveolin-1 were performed on isolated lipid droplet fractions obtained from 3T3-L1 adipocytes, either stably expressing or not the constitutively active Y527Fsrc mutant, were treated or not with cholesterol for 10 min. A representative blot is shown. Bars represent quantitative changes in caveolin-1 after normalization relative to perilipin obtained from three independent experiments. Values of cholesterol-treated mock-transfected cells were arbitrarily set to 100%.

7A, + AA), only internal (budded) caveolae (arrow heads) are labeled with CTB-HRP.

According to this method, we first studied the budding of caveolae in 3T3-L1 adipocytes as compared to the well-characterized MEFs studied previously. After warming CTB-HRP labelled cells to 37 °C for 1 min, we detected only 0.62 ± 0.02% of the total caveolae had detached from the cell surface, as compared to 2.1 ± 1.06% in MEFs under identical conditions. We next examined caveolar budding from the adipocyte cell surface following cholesterol stimulation, i.e. conditions where caveolin-1 redistributes to lipid droplets. For this purpose, CTB-HRP-labelled cells were warmed to 37 °C for 10 min in the presence or absence of cholesterol. Figure 7B shows that cholesterol caused a highly significant increase in budding of caveolae, with approximately 2–3 fold more caveolae budded after the 10 min incubation.

Therefore, although caveolae domains are relatively stable at the adipocyte plasma membrane, cholesterol induces caveolae budding from the fat cell surface.

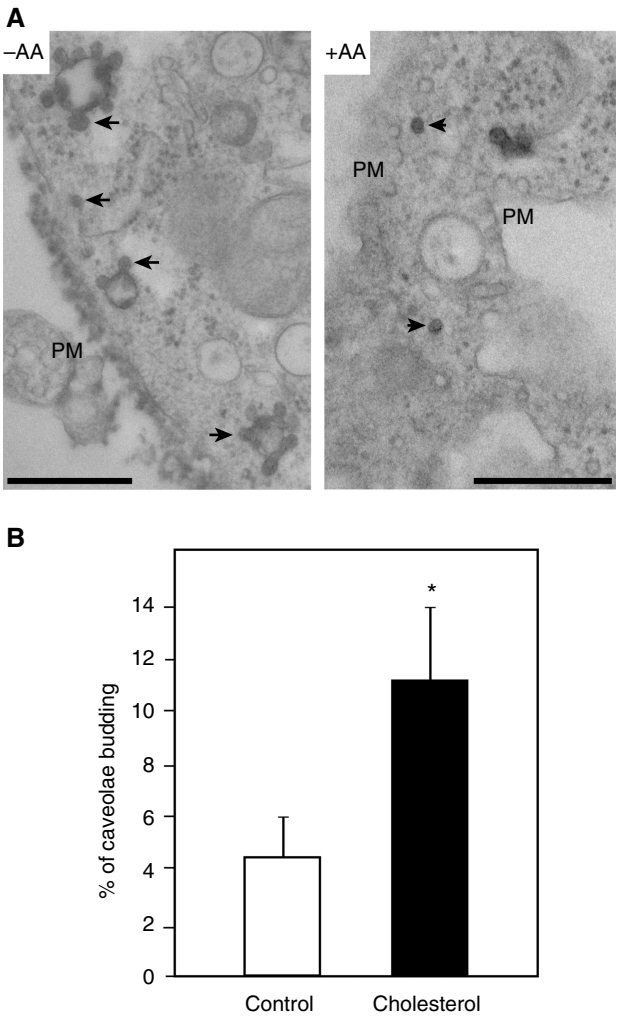

Figure 7: Ultrastructural analysis of caveolae budding in 3T3L1 adipocytes. 3T3-L1 adipocytes differentiated for 10 days were serum-starved for 16 h and then labelled with CTB-HRP for 40 min at 4 °C. CTB-HRP was internalized for 10 min at 37 °C in a medium containing ethanol or 10 μg/mL of cholesterol. Cells were further incubated at 4 °C for 40 min in freshly prepared DAB buffer to visualize the HRP-reaction product with or without 50 mM ascorbic acid (AA). Panel A shows representative electron microscopy images obtained in the presence or absence of AA. Labelled caveolae are indicated by arrowheads. The percentage budding was calculated by comparing the total number of CTB-HRP-positive caveolae (50–65 nm profiles labeled in the absence of AA) with those caveolae that are only intracellular (labelled in the presence of AA). Panel B showing quantitative analysis of cholesterol effect on caveolae budding is presented from a pooled result of five independent experiments.

Discussion

The present study provides evidence for a role of caveolin-1 in the regulation of FC levels in adipocytes. We show that caveolin-1 is required for association of FC with the lipid droplet surface. In addition, we provide evidence for a novel trafficking pathway to lipid droplets involving caveolae endocytosis. This pathway is stimulated by cholesterol addition which triggers dynamin-dependent caveolae budding through src activation.

We have shown for the first time that lipid droplets of adipocytes from caveolin-1 KO mice are low in cholesterol. Since caveolin was shown to be a cholesterol-binding protein (9), many studies have implicated caveolin-1 in cellular cholesterol homeostasis (11). Indeed, it has been proposed that caveolin can function in a number of cholesterol trafficking steps including selective cholesterol ester uptake (34), cholesterol efflux (8,35–39) and transport of newly synthesized cholesterol to the plasma membrane (21,40). Our results from caveolin-deficient cells provide the first evidence for a role of caveolin in regulating lipid droplet cholesterol content. This observation is consistent with previous reports showing that the expression of caveolins in cells lacking caveolins caused an enrichment of cholesterol in a low buoyancy putative lipid raft fraction (41). It is likely that the decrease in cholesterol concentration observed in caveolin-KO adipocytes would alter lipid droplet composition and/or functionality. Electron microscopy pictures have revealed dramatic defects in the architecture of lipid droplet cortex in adipocytes isolated from caveolin-1 KO mice (42).

Caveolins can be targeted to lipid bodies in nonadipose cell types upon exogenous addition of fatty acids (5,13). Here, we show that, in addition to oleate, cholesterol can induce translocation of caveolins from plasma membrane to adipocyte lipid droplets. Our results are consistent with the recently described dynamic trafficking of cav-3 GFP between cell surface and lipid bodies in nonadipose cells following fatty acids loading (43). Two different trafficking pathways of caveolin from the plasma membrane to lipid droplets can be envisaged: one involving caveolin-enriched membrane carriers and the other cytosolic complexes with immunophilins and chaperones (21,44). We showed that the caveolin-dependent pathway to the lipid droplet is unaffected by cyclosporin A, and is thus unlikely to involve cytoplasmic caveolin complexes. Our results rather suggest that an endocytic process is responsible for the translocation of caveolin to the lipid droplet. First, the use of a dynamin dominant-negative mutant that blocks both clathrin-dependent and -independent endocytosis (23) dramatically decreased the targeting of caveolin to the lipid droplets. Second, we present direct evidence that cholesterol stimulation led to increased release of caveolae from the fat cell surface. This is in agreement with findings in Hela cells where treatment with cholesterol or C8-LacCer stimulate the uptake of caveolar endocytic markers like bodipy-lactosylceramide and albumin (27). Third, targeting of caveolin to lipid droplets was modulated under conditions where caveolae endocytosis was affected. Taken together, this suggests that cells are able to monitor changes in the plasma membrane lipid composition by inducing caveolar endocytosis. One could speculate that the caveolae act as a 'sink' for cholesterol (12). How cholesterol trafficking to lipid bodies would occur is not clear. Direct binding of cholesterol to caveolin still remains questionable since stoichiometric binding to caveolin would probably not suffice to account for all the cholesterol transport (9). The redistribution of caveolins to lipid droplets could also sequester cholesterol from other intracellular pools. Such a trapping mechanism could explain the ability of adipocytes to accumulate large quantities of free cholesterol (15,17,45), a feature that makes adipose tissue the major pool of cholesterol in the body (15,45).

Caveolae, as portals for endocytosis, can mediate the entry of several proteins such as albumin, bacterial toxins, folate receptors and viruses (22,46). We found that cholesterol-stimulated trafficking of caveolins to lipid bodies is inhibited by cytochalasin D in line with previous data showing that internalization of caveolae is dependent on an intact actin network (26). In addition, cholesterol stimulated translocation to lipid droplets requires PKC and src kinases activities, consistent with the crucial role of these kinases in caveolae internalization (26,28), albumin uptake (27,30) as well as the internalization of the SV40 virus particle (47–49). Importantly, overexpression of a constitutively active form of *src* is sufficient to stimulate caveolin targeting to lipid droplets in the absence of exogenous cholesterol, suggesting that cholesterol triggers *src* activation as reported recently (27). Caveolin translocation to lipid droplets thus shares many features with the caveolar endocytic process from the plasma membrane described so far for different caveolar markers.

While our results suggest a role for caveolae endocytosis in targeting of caveolin to lipid droplets, the subsequent trafficking pathway is not yet clear. The use of CTBHRP allowed us to identify an effect on caveolae budding but further detailed studies will be required to identify the target of internalized caveolae and intermediates on the trafficking pathway to lipid droplets. Several itineraries subsequent to endocytosis are possible. For example, cholera toxin is targeted to the Golgi apparatus (50) whereas SV40 virus is directed to the ER *via* an intermediate compartment, the caveosome (51). Whether or not caveolins traffic via intermediate organelles before reaching lipid droplets remains to be addressed. Three routes could be postulated: (i) a direct route from the plasma membrane to lipid droplets (involving close contact between the caveolar bilayer and the surface of the lipid droplet); (ii) trafficking via the ER; (iii) a pathway involving the caveosome. A direct route can easily be envisaged because abundant caveolae in adipocytes are organized as surface connected-structures (caves) protruding deeply into the cell interior (52,53). This organization might facilitate transfer of caveolin from budded caveolae to the lipid droplet surface. Such a transport mechanism was suggested in a recent study showing that triacylglycerol can be synthesized at the site of fatty acid entry in a subclass of surface caveolae in primary adipocytes and then directly transferred to lipid droplets (54). However, we cannot exclude intermediate compartments in the lipid bodies-targeting pathway. Lipid droplets have been observed in close apposition to intracellular organelles, especially ER

(55). Proteomics of lipid droplets have described the presence of several members of the Rab family proteins involved in vesicular transport (3–6). In particular, recent papers show that recruitment of Rab18 to lipid bodies is dependent on the metabolic state of lipid droplets (56) and may have a role in ER recruitment around lipid droplets (57). This is consistent with the view that lipid bodies are targets for membrane traffic from other cellular compartments like caveosomes, endosomes or the ER. If translocation involves transfer from a membrane carrier, another issue which remains is, the transfer of caveolin from a bilayer to the fat droplet monolayer. Special contact-sites between lipid droplets and physically apposed organelles could therefore facilitate cholesterol transfer. Clearly, more work will be required to elucidate how this movement of caveolins to lipid droplets is organized in adipocytes.

Our results provide a possible mechanism for the lean phenotype observed for the caveolin-1 KO mice when they are subjected to a high fat diet which leads to obesity in wild-type mice (58,59). In the light of the present study, we propose that the presence of caveolin on lipid droplets links cholesterol and fatty acid storage. The consequences of a loss of lipid droplet cholesterol, if any, remain to be clarified. An exciting possibility is that cholesterol might serve as an intracellular signal for regulation of the size of adipocytes, linked to triacylglycerol stores (60). Indeed, the sensing of the replenishment of their lipid stores by adipocytes is of crucial importance for whole body energy homeostasis, as demonstrated by the dramatic metabolic changes that follow fat cell size enlargment primarily during obesity (61). Although the mechanisms involved remain completely unknown, there is some evidence to suggest that, as triglyceride storage increases, cholesterol is redistributed from the plasma membrane to the surface of the lipid droplet (62,63). Based on the fact that triacylglycerol and cholesterol storage are closely linked in adipocytes (16,64), it may be postulated that cholesterol might participate in the intracellular sensing for fat cell size and triacylglycerol content.

Thus, the caveolar regulation of cholesterol and triacylglycerol build-up in fat droplets of adipocytes deserves closer study. Possible connections open up new perspectives for the control of the storage of lipids from exogenous sources, a key feature of the metabolic disorders leading to obesity.

Materials and Methods

Animals and tissues

Caveolin-1 KO mice were described previously (65). Male mice homozygously KO for the caveolin-1 gene were compared to wild-type littermates at the age of 12-weeks old. At this age, adiposity of wild-type and cav-1 KO mice was similar. All animal experiments were approved by local authorities in accordance with criteria outlined by the American Physiological Society.

Plasmids, antibodies and reagents

Dog cDNA cav-1 EGFP (51) and a constitutively active mutant form of c-*src*, Y527Fsrc in pLNCX retroviral vector (31) were obtained from Dr Lucas Pelkmans (Max Planck Institute CBG, Dresden, Germany). pBabe hygro vector and BOSC23 cells were provided by Dr Frank Buchholz (Max Planck Institute CBG, Dresden, Germany). Cav-1 EGFP was cloned into retroviral expression vector pBabe hygro by using SalI/EcoRI sites. Adenovirus encoding the dynamin mutant (dynK44A) under the control of a tetracycline regulatable promoter and the chimeric transcription activator tTA adenovirus were kind gifts from Dr Sandra Schmid (Scripps Research Institute, La Jolla, california).

Antibodies used in this work were caveolin-1 (BD Biosciences, San Jose, USA and N20 from Santa Cruz, USA), caveolin−2 (BD Biosciences, San Jose, USA), perilipin (Progen, Germany), HA-tag(12CA5). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, USA).

Cholesterol, methyl-β-cyclodextrin, methyl-isobutylmethylxanthine (IBMX), dexamethasone (Dex), insulin, cholesterol and filipin were purchased from Sigma (Germany). 'Complete' protease inhibitor cocktail, hygromycin B, neomycin and cycloheximide were obtained from Invitrogen (Germany). Rosiglitazone, PKC inhibitors (Ro31–8220(3-(1-[3-(amidinothio)propyl]-1H-indol-3yl)-3-(1-methyl-1H-indol-3-yl)maleimide methane sulphate) and GF109203X {bisindolylmaleimide I; 2-[1-(3-dimethylaminopropyl)-1H-indol-3yl]-3-(1H-indol-3-yl)-maleimide)} and tyrosine kinase inhibitors (PP2, negative control PP3, herbimycin, genistein) were purchased from Calbiochem (San Diego, USA).

Cell culture

3T3-L1 cells (kind gift of Dr J. Pairault, Paris, France) were maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum. For experiments, 3T3-L1 cells were cultured in high-glucose DMEM, 10% fetal calf serum (FCS) until 2 days postconfluency (Day 0). Adipocyte differentiation was then induced by adding IBMX(100 μM), Dex(0,25 μM) and insulin(1 μg/μL) for 2 days, and then cultured only in high-glucose DMEM, 10% FCS and insulin alone for the rest of the differentiation process. Differentiated cells were usually harvested at day 10–12 following confluence. When indicated, cholesterol was added into the culture medium of cells that had been shifted to a serum-free medium containing DMEM and 0,2% bovine serum albumin (BSA) for 16 h. Cholesterol final concentration (10 μg/mL) (19) was applied as an ethanol solution or in the form of cyclodextrin complexes. Control cells received the ethanol vehicle alone that did not represent more than 0.1% vol.

Double infection of adenoviruses encoding the dominant-negative mutant form of dynamin (dynK44A) under the control of a tetracycline regulatable promoter, and the chimeric transcription activator tTA construct (66) were performed in 3T3-L1 adipocytes (day 10–12) as described in (67).

Retroviral constructs cav-1 EGFP and Y527Fsrc were ectopically expressed in 3T3-L1 cells using retroviral supernatants from Bosc23 packaging cells followed by selection for neomycin resistance (600 μg/mL) for pLNCX Y527Fsrc and hygromycin B resistance (200 μg/mL) for pBabe hygro Cav-1 EGFP. Populations of antibiotic-resistant 3T3-L1 fibroblasts were allowed to differentiate into adipocytes as described above.

MEFs were prepared from 13.5 p.c. embryos and cultured in DMEM supplemented with 10% FCS. Adipocyte differentiation was induced as described for 3T3-L1 cells except that Rosiglitazone (0.5 μM) was added into the differentiation inducer cocktail.

Adenovirus encoding canine caveolin-1 was constructed according to (68). cDNA of caveolin-1 was subcloned into the shuttle vector pAd Track CMV using NotI/XhoI sites. The adenoviral vectors were propagated in HEK293 cells, purified on Optiprep gradients and stored at − 80 °C. Infection of Cav-1 KO MEFs for 4 h in serum-free medium at day 3 of adipocyte differentiation, followed by 3 days of differentiation in DMEM/10% FCS

supplemented with insulin and rosiglitazone. Cells were further used for western-blotting anf filipin staining.

Isolation of lipid droplets

Lipid droplets were isolated as described previously (1). Briefly, 3T3-L1 adipocytes were washed twice with PBS and resuspended in 3 mL of disruption buffer (25 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, 5 mM EGTA, pH 7.4, 'complete' protease inhibitor cocktail-one tablet per 25 mL). Cells were disrupted by nitrogen cavitation at 800 psi for 10 min at 4 °C. The lysate was collected and mixed with an equal volume of disruption buffer containing 1.08 M sucrose. It was then overlaid sequentially with 2 mL each of 270 mM sucrose buffer, 135 mM sucrose buffer and Tris/EDTA/EGTA buffer (25 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, pH 7.4). Following centrifugation at 150 000 g for 90 min, fractions of 1.5 mL were collected from the top to the bottom of the gradient. The top fraction, containing the lipid droplets was then washed in four volumes of Tris/EDTA/EGTA buffer and centrifuged at 3500 g for 15 min before collecting the lipid droplets.

Total cell lysate

3T3-L1 adipocytes were incubated with appropriate effectors as described in the figure legends. Plates were washed with PBS, and then lysed in 500 mL of lysis buffer (50 mM Tris pH 7.4, 0.27 M sucrose, 1 mM Na-orthovanadate pH 10, 1 mM EDTA, 1 mM EGTA, 10 mM Na β-glycerophosphate, 50 mM NaF, 5 mM Na pyrophosphate, 1% (w/v) Triton X-100, 0.1% (v/v) 2β-mercaptoethanol and protease inhibitors). Whole cell lysate was centrifuged at 15 000 g for 10 min at 4 °C. Plasma and internal membranes from 3T3-L1 cells were prepared as described previously (69). Protein contents were determined using the Bradford assay (70).

SDS-PAGE and immunoblotting

Cell lysates and lipid droplets fractions were subjected to SDS-PAGE on 14% resolving gels and immunoblotted as previously reported (69). Nitrocellulose membranes were probed with antibodies against caveolin-1, caveolin−2, perilipin and HA-tag. Primary antibody detection was performed using an appropriate peroxidase-conjugated IgG, and visualized using enhanced chemiluminescence (Pierce-Perbio Biotechnology, Germany) on Kodak X-Omat film.

Photocholesterol labelling

3T3-L1 adipocytes were incubated with 0.5 mCi [^{3}H]photocholesterol/plate in delipidated FCS at 37 °C (20). After 16 h of incubation, the medium was removed and cells were washed with PBS. The cells were irradiated on the dish in PBS for 5 min on ice using a filtered ($\lambda > 310$ nm) beam of high-pressure mercury lamp. Lipid droplet fraction was then prepared; photolabelled proteins were resolved by SDS-PAGE (5–15%, Tris-Tricine) and visualized by fluorography. For caveolin-1 immunoprecipitation, the lipid droplet fraction was treated with 60 mM octylglucoside for 2 h at 4 °C with constant agitation. 5 μg of caveolin-1 (N20 antibody, Santa Cruz, USA) or non-specific mouse IgG were added and incubated overnight at 4 °C. 50 μL of protein G beads (Sigma, Germany) were then added and samples were incubated for 4 h. The supernatant with unbound proteins was collected and the beads were washed twice with lysis buffer and twice with PBS. Immunoprecipitated proteins were then eluted with SDS-PAGE loading buffer.

Lipid quantification

Lipid droplet total cholesterol content was determined after extraction of total lipids as described (71), using commercially available kits (Amplex red cholesterol assay kit from Molecular Probes or Cholesterol assay kit from Roche). Triacylglycerols were measured with a Glycerol assay kit (Roche, Germany).

Immunofluorescence and filipin labeling

3T3-L1 adipocytes on coverslips were washed with PBS, fixed with 3% paraformaldehyde (PFA, room temperature for 30 min) and permeabilized for 10 min with saponin 0,1% (wt/vol) at room temperature. The cells were then incubated with antibody against caveolin-1 (N20, Santa Cruz, USA) followed by incubation with antirabbit Cy3-conjugated secondary antibody (Jackson Immunoresearch), mounted in Mowiol and visualized by confocal laser fluorescence (Leica TCS SP2 or Zeiss LSM 510).

3T3-L1 cells expressing stably cav-1 EGFP were differentiated on coverslips, washed with PBS, fixed with PFA and mounted in Mowiol. To quantify caveolin-1 positive lipid droplets, images corresponding to the different treatments performed in parallel were captured at the same contrast and intensity. For each treatment, cav-1 EGFP staining around lipid droplets was determined and expressed as a ratio of cav-1 positive lipid droplets on total lipids droplets analyzed (lipid droplets were identified on DIC images captured simultaneously).

Filipin staining on MEFs was performed according to (72). Free cholesterol (FC) was stained with filipin used at 0.05 mg/mL in PBS/10% FCS for 2 h at room temperature in the dark and the coverslips were mounted in Mowiol. Filipin:FC fluorescence was imaged using an Olympus IX70 inverted microscope using a TILL-Photonics Monochromator for illumination and filipin was excited at 360 nm. The filter cube contained no excitation filter, a 395 LP dichroic mirror and a D445/40 BP emission filter. Images were acquired on a Micro-max 512 BFT CCD camera using Metamorph.

Quantitative EM analysis of caveolae budding

3T3-L1 adipocytes differentiated for 8–11 days were serum starved for 16 h, washed in prechilled CO_2-independent medium, and then incubated in CO_2-independent medium containing 20 μg/mL CTB-HRP for 40 min at 4 °C. The cells were then warmed for 10 min at 37 °C in medium contain 10 μg/mL cholesterol in ethanol or with the ethanol vehicle alone. Internalization was stopped by placing the cells on ice in pre-chilled CO_2-independent medium. Cells were further incubated at 4 °C for 40 min in freshly prepared DAB buffer with or without 50 mM ascorbic acid (± AA) and then processed for EM as per (33). 50 nm sections were viewed unstained using a Joel 1010 transmission electron microscope. To determine the percentage of internalized CTB-HRP-positive caveolae, 50 random areas were examined at a magnification of × 30 000. The percentage budding was calculated by comparing the total number of CTB-HRP-positive caveolae (50–65 nm profiles labeled in the absence of AA) with those caveolae that are only intracellular (labelled in the presence of AA). Five different independent experiments with 3T3-L1 adipocytes were examined and the results pooled. Over 2000 caveolar profiles were assessed. In some experiments, budding of caveolae in 3T3-L1 adipocytes was compared to mouse embryonic fibroblasts (MEFs). In these experiments, cells grown under standard cell culture conditions were incubated with CTB-HRP, warmed for 1min at 37 °C, and further processed as described previously (33). Two independent experiments were examined and the results pooled.

Statistical analysis

Statistical analysis was carried out using a Student's *t*-test. Data were considered statistically significant at p-values ≤ 0.05 and notified by *.

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