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Caveolae and lipid trafficking in adipocytes

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Abstract

The abundance of caveolae in adipocytes suggests a possible cell-specific role for these structures, and because these cells take up and release fatty acids as their quantitatively most robust activity, modulation of fatty acid movement is one such role that is supported by substantial *in vitro* and *in vivo* data. In addition, caveolae are particularly rich in cholesterol and sphingolipids, and indeed, fat cells harbor more cholesterol than any other tissue. In this article, we review the role of adipocyte caveolae with regard to these important lipid classes.

Keywords

adipocyte; caveolin; cavin; cholesterol; fatty acid; lipid droplet

Caveolae

Caveolae, or little caves, which are small (50–100 nm) invaginations of the cell surface membrane, were first described in electron micrographs of several cell types that were taken in the early 1950s [1,2]. Their cellular distribution is not ubiquitous but they are abundant in endothelial cells and various epithelial cell types [3], and of particular relevance to this article, they are abundant in adipocytes [4–7]. Their vesicle-like shape and plasma membrane (PM) localization suggest a possible role in endocytosis, transcytosis and/or vesicle trafficking, and indeed, there is evidence that they can play such a role, which might be relatively robust in endothelial cells where caveolae seem poised for endocytosis/transcytosis by virtue of their abundance and proximity to both the blood and tissue sides of the cell [8]. However, in most cases, caveolae are quite stable [9] and participate to a significantly less quantitative extent than other mechanisms for these processes [3,10]. Another function of caveolae that attracted considerable attention is the possibility that these structures could serve as platforms for organizing a large number of somewhat diverse cell surface signaling pathways [11]. However, mice and humans lacking caveolae (see later) survive despite a number of pathological conditions, indicating that gross developmental defects do not result from their absence. Thus, confirmation of caveolar involvement for a

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specific signaling pathway will require several lines of independent evidence, as has recently been noted [12].

Caveolae composition: caveolins

The study of caveolae was restricted to morphological techniques until the discovery of its first component protein, caveolin (now caveolin-1 or Cav1), a small (178-amino acid) integral membrane protein of the PM inner leaflet with a hydrophobic intramembranous loop of approximately 32 residues, which is U-shaped and does not reach the cell exterior [13]. This unusual topography is dependent on proline 110, which when mutated to alanine results in a transmembrane orientation, as detected by an amino terminal FLAG tag that becomes accessible to the extracellular milieu only in the mutant [14]. Rothberg *et al.* performed freeze-etch electron microscopy (EM) on cells, which revealed that caveolae have a striated coat [13], possibly analogous to clathrin and its adaptor proteins, which comprise the coat for clathrin-coated pits and vesicles [15]. For caveolae, the striated coat is thought to be comprised of the recently named cavin proteins, which are described in detail in the next section (reviewed in [16]). Early experiments demonstrating that ectopic expression of Cav1 in lymphocytes, which do not have caveolae, resulted in the morphological appearance of these structures [17] and led to the erroneous conclusion that caveolins were necessary and sufficient for the formation of caveolae, probably because these cells express cavin proteins. Two additional caveolin isoforms, Cav2 and -3, which have sequences and membrane topography similar to Cav1, were subsequently described, with Cav2 requiring Cav1 for function [18] and Cav3 being a muscle-specific isoform [19]. The caveolins form homo- and hetero-oligomers that are thought to comprise their functional units in caveolae [20–22].

Caveolae composition: cavins

Recently, a number of laboratories have provided experimental evidence that a family of four proteins, now termed cavins-1–4, is present in caveolae, where they may serve different biochemical and cellular functions [23–28] that most likely includes forming the striated caveolar coat structure that was observed in freeze-etch electron micrographs [13]. Thus, cavin-1, also known as polymerase transcription releasing factor (PTRF), was shown to be required for caveolae formation *in vitro* and *in vivo*. Knocking down cavin-1 by RNA interference in cells [23,24], in zebra fish by antisense morpholino-RNAs [24] and in mice by gene deletion techniques [25], causes loss or abolishment of morphologically detectable caveolae. Similarly, *in vitro* studies of cavin-2, previously known as serum deprivation protein response (SDPR), demonstrated that its expression regulates caveolae curvature and its knockdown reduces caveolae number [27] and the amount of the other caveolae resident proteins [28]. A role for cavin-3, or SDR-related gene product that binds to C kinase (SRBC) in caveolar endocytosis has also been documented, whereas cavin-4 or MURC has been described only with regard to its muscle-specific expression [29] and its sequence similarity to the other cavins, as well as its presence in caveolae [28]. Cavins-1–3 form a complex as determined by co-immunoprecipitation of the endogenous proteins in adipocytes and by pull downs of ectopically expressed proteins [28]. The tissue expression patterns of Cav1 and cavin-1 appear identical or nearly so, and Cav3, which is necessary for muscle caveolae formation [25], also requires cavin-1 expression for this to happen [28]. However, the pattern of cavin-2 and -3 expression [28] varies from tissue to tissue, suggesting the likelihood that these proteins may have cell- and/or tissue-specific functions. Some of these may be distinct from their role in caveolae, particularly for cavin-3, which is abundantly expressed in the brain, where Cav1 expression may be restricted to endothelial cells [30], and it is not as readily detected in whole-brain western blots compared with cavin-3 [28]. Moreover, while Cav3 is expressed to an approximately equal extent in skeletal and cardiac

muscle, cavin-4 is expressed at much higher levels in the former tissue [28], raising the possibility that caveolae composition differs in these tissues.

A number of studies identified the cavins (PTRF, SDPR and SRBC) as possible caveolae-associated proteins prior to the more recent studies cited in the previous paragraph that established some of their functional roles. Mineo *et al.* used an interaction screen to identify SDPR (cavin-2) as the component of caveolae that localized PKC isoforms to these structures [31]. Vinten and colleagues used an immunological screen to raise a monoclonal antibody to an adipocyte protein they termed p-60 cavin, and they showed in immunogold EM studies that their antibody labeled adipocyte caveolae to the same extent as an anti-Cav1 antibody [32]. The name 'cavin' was first used in this study and it has been suggested that 'cavin' be adopted as the family name [16]. Later, Vinten and colleagues identified the sequence of their antigen as PTRF (now termed cavin-1), confirmed its presence on the cytoplasmic face of caveolae and showed that it was absent from the nuclei of adipocytes by cell fractionation and immunofluorescence [33]. Aboulaich *et al.* identified cavins-1–3 (PTRF, SDPR and SRBC) by proteomic analysis of enriched caveolae from human adipocytes and showed that these proteins resided on the cytoplasmic face of the PM [34]. Despite these early observations, functional studies were relatively slow to develop probably for two reasons: namely, the primary sequences of the cavins are relatively nondescript, with leucine zipper and PEST domains being their main biochemical features; and the history of their initial descriptions was unrelated to caveolae, as PTRF was identified as a possible nuclear protein [35], and SDPR [36] and SRBC [37] as cell growth-related proteins. How the cavins and caveolins interact to form a mature caveola structure in the PM is under active investigation, with data supporting the notion that generation of caveolin oligomers occurs in the Golgi, followed by their trafficking to the PM along with cholesterol [38]. Subsequently, cavin-1 is recruited to caveolae; Bastiani *et al.* also showed recruitment of cavin-1 to cell surface caveolin [28], but Hansen *et al.* showed a role for cavin-2 in recruiting cavin-1 to the PM [27]. Further work is needed to resolve how caveolae are assembled.

Caveolae & adipocytes

What turned out to be adipocyte caveolae were first described as pinosomes in transmission [39] and freeze-fracture [40] EM studies of primary rat adipocytes, and it was evident from this early work that they are relatively abundant structures in these cells. When antibody reagents became available, Cav1 was confirmed to be an especially abundant adipocyte protein [4,5], and its robust presence in fat cell caveolae was verified by an immuno-EM study of PM sheets, a study that also confirmed the large cell surface area occupied by these structures [6]. It was estimated from a more recent EM study that caveolae comprise 50% of the fat cell surface area [7]. This caveolar abundance plus the importance of adipocytes in metabolic regulation (reviewed in [41]) prompted numerous studies of insulin signaling and Glut4 trafficking in this cell type, resulting in conflicting conclusions both in support of and against a role for caveolae in these processes. We reviewed these data as of 2007 and discussed these apparent discrepancies, which we interpreted to be due to technical differences in experimental procedures [42], and this still remains the most likely explanation.

Caveolae composition: cholesterol & other lipids

An early finding about the requirements for caveolae formation was that they required cholesterol for their characteristic shape, as treatment of cells with the cholesterol-binding antibiotic, nystatin, caused them to flatten [13]. Indeed, Cav1 was shown to be a stoichiometric cholesterol-binding protein in reconstitution assays [43], and free cholesterol

was shown to regulate Cav1 mRNA expression in fibroblasts [44]. The levels of cholesterol in purified caveolar preparations from primary rat fat cells were directly measured and found to be enriched by threefold compared with the surrounding surface membranes, and twofold enrichments of sphingomyelin in caveolae were also observed [45], as might be expected from the known association properties of these two lipid species [46]. It is surprising that the study by Ortegren *et al.* appears to be the only analysis of caveolar lipid composition performed on purified caveolae [45]. Moreover, while the role of cholesterol in caveolar function has been considered in some detail [47], the role of sphingomyelin in this regard has been studied to a significantly lesser degree (however, see [48]). It is of note that all three cavins have been reported to be phosphatidylserine-binding proteins [24,37,49], and although this membrane lipid was not specifically analyzed in adipocyte caveolae, glycerol lipids were found to be the same in caveolae compared with the bulk PM [45]. Figure 1 summarizes the current information available for the composition of caveolae and how their proteins and lipids might be organized. It remains unclear whether the cavins bind caveolins, as disruption of the caveolar structure in adipocytes with octylglucoside [50] does not result in co-immunoprecipitation of Cav1 or Cav2 with the cavins [28], and thus it may be a combination of lipid–protein and protein–protein interactions that is needed for caveolae formation and caveolin–cavin interaction. Also depicted in Figure 1 is that there are membrane regions other than caveolae that are rich in cholesterol and sphingomyelin, so-called lipid rafts, which are a heterogeneous class of domains [51], the exact nature of which remains unclear, but they are often functionally characterized in cells by their resistance to solubilization in nonionic detergents [52]. As illustrated in Figure 1, flotillin-1 (flotillin-2 is another isoform and they are both also known as Reggie-1 and -2) is a caveolin-like protein that is associated with lipid rafts [53] but not caveolae [54], and it may also form caveolae-like invaginations [55].

Caveolar & cholesterol dynamics

The regulation of cholesterol at multiple levels and its inter- and intra-cellular trafficking are some of the most studied areas of cell biology [56–58], and this article focuses on cholesterol in relation to caveolae, with a further focus on adipocytes. The interplay of caveolins and cholesterol has also been the subject of considerable study (reviewed in [47,59]), but numerous questions remain, particularly in the context of the role of cavins in caveolae structure/function. Although Cav1 can bind cholesterol in a stoichiometric fashion, the relationship of caveolae and cholesterol concentration is not a linear one. Hailstones *et al.* demonstrated that caveolae require a threshold level of cholesterol for their formation, with both cholesterol depletion (mediated by cellular statin exposure or by extraction with β -methylcyclodextrin) and repletion causing a respective loss and gain of caveolae over a very small cholesterol concentration range [60]. This switch-like behavior is similar or identical to that of cholesterol sensing in the endoplasmic reticulum by Scap–SREBP, the enzyme complex that is responsible for turning on and off numerous genes for cholesterol metabolism [61]. We also showed that the relationship of caveolin expression and cholesterol has a threshold in the absence of caveolae formation [62]. We created a series of HEK293 cells expressing different levels of Cav1 and with increasing levels of expression, there was no corresponding increase in cholesterol until a certain threshold of Cav1, was reached, which we concluded represented the level at which caveolin oligomers could form lipid rafts [62]. HEK cells do not express cavin proteins, so no caveolae are formed under these conditions. The question remains as to how caveolae sense cholesterol levels and it would not be surprising if this is achieved by cavins and/or by caveolin–cavin complexes.

In humans, adipose cells are a major site of cholesterol storage, with as much as half the cholesterol in the body in obese individuals being found mainly (>90%) as free cholesterol in adipocytes [63]. Cholesterol entry into adipocytes occurs mainly by the classical LDL–

LDL receptor endocytosis-mediated pathway [64], but may also occur via lipoprotein scavenger receptors, such as CD36 [65], which are located in caveolae in adipocytes [54]. The cholesterol-dependent movement of Cav1 and caveolae to adipocyte lipid droplets has also been reported, but it is unclear if this represents a quantitatively important route for cholesterol deposition [66]. The site of free cholesterol storage in fat cells is the lipid droplet [67], in which the regulation of lipid storage and release and droplet dynamics have led to its relatively recent recognition as an organelle in its own right [68]. *De novo* cholesterol biosynthesis appears to be a minor pathway in these cells [63]. Given the role of adipocytes in storing increasing cholesterol amounts with the amount of adiposity [63], it would seem possible that obesity could alter caveolae protein expression and numbers in adipocytes, but in rats fed a high-fat diet there was variability, in that Cav1 and Cav2 were not found to be uniformly upregulated and their expression patterns differed from one another with duration of high-fat diet and in different adipocyte depots [69]. On the other hand, Kozak *et al.* found a correlation between early nutritional status, adipose expansion and caveolar gene expression in mice fed a high-fat diet [70]. In this study of mice fed for 8 weeks on various diets then subjected to a high-fat diet for a further 8 weeks, Cav1, Cav2 and cavin-1 mRNA all showed a significant increase in expression from 8 to 16 weeks, although protein levels were not measured nor were actual caveolae assessed morphologically in either study.

Given the large amount of cholesterol stored in adipocytes, it seems plausible that these cells could serve as a cholesterol reservoir for other tissues, although the teleological need for such a reservoir seems obscure given the capacity of most cells to make their own, as well as the cholesterol availability from dietary sources. Nevertheless, fat cells express the ATP-binding cassette cholesterol pump ABCA1 [71], which is considered to be responsible for cholesterol efflux from cells [72]. Moreover, caveolae have been implicated to be a site for cholesterol efflux [44,73] and ABCA1 has been reported to interact with Cav1 [74]. However, proteomic analysis of caveolae from several sources, including adipocytes, has failed to reveal the presence of ABCA1 [34,42,75,76], and if adipocyte-stored cholesterol is provided to other tissues via ABCA1, it seems unlikely that caveolae play a role in this process.

Caveolar lipid dynamics: fatty acids & triglycerides

Adipocytes are the major site for energy storage in the body in the form of triglycerides (TGs), which are released as calorie-rich fatty acids (FAs) for use in other tissues, primarily heart and skeletal muscle [77]. Humans derive their TGs almost entirely from dietary intake, and following their lipolysis in the digestive tract and reassembly into lipoprotein particles in the form of cholesterol esters, FAs are released from lipoprotein particles by lipoprotein lipase in the capillary endothelium [78], and they also circulate at an effective concentration of 0.1–0.5 mM as FAs bound to albumin [79]. How FAs cross the adipocyte PM, or indeed any cell surface membrane, has been a highly controversial subject, with a number of membrane proteins being described as putative FA transporters, including the FA transport proteins (FATPs), which are CoA ligases, and the lipoprotein receptor CD36 (reviewed in [80]). There is evidence supporting a simple diffusion mechanism for transmembrane FA movement, [81] and an unknown membrane pump has been implicated in this process [82]. The role of metabolism as a driving force for FA transport has also been considered, which is a possibility that is compatible with diffusion-mediated transmembrane movement as the first step in the process [83] and is consistent with ability of FATPs to enhance cellular FA uptake [84]. In other words, the rate of transmembrane FA movement would be enhanced by mass action following their conversion to CoA derivatives by FATP, and CoA derivatives cannot recross the PM.

We considered that Cav1 and/or caveolae might play a role in transmembrane FA movement because Cav1 is one of the most abundant cell surface molecules of the fat cell (indeed, probably the most abundant), and its expression is markedly induced during the differentiation of cultured fat cells when they acquire their lipid-filled appearance [4,85]. Cav1 has been shown to be a FA-binding protein by affinity labeling procedures [86]. Common dietary FAs such as palmitate, stearate and oleate are hydrophobic molecules that readily partition into artificial phospholipid bilayers, where their pKs are altered such that they become protonated and flip across to the inner leaflet with millisecond kinetics [87]. However, in aqueous media, FAs are anions, which are mild detergents at physiological pH levels, and they are toxic to cells at concentrations that can be achieved *in vivo* [88,89]. Considering that caveolae are detergent-resistant domains, and for all the reasons described earlier, we [62,88] and others [90] have postulated that caveolins/caveolae serve to modulate transmembrane FA movement and protect cells from FA-mediated toxicity by buffering their levels in the PM.

Perhaps the first evidence that caveolae are important for lipid storage and movement *in vivo* comes from the phenotype of the Cav1-null mouse, which lacks adipocyte caveolae, is hyperlipidemic and has very small adipocytes, indicating an inability of these cells to store fat properly [91]. Recently, it has been shown that cavin-1-deficient mice have essentially the same lipodystrophic and insulin-resistant phenotype [25]. Moreover, humans that are functionally deficient for either Cav1 [92,93] or cavin-1 [94–97] also have a highly similar, if not identical, lipodystrophic phenotype, as well as other physiological abnormalities. It has recently been shown that Cav1-deficient murine adipocytes, unlike wild-type adipocytes, undergo autophagy [98]. Whether or not this is secondary to lipotoxicity remains to be determined, and *in vitro* mechanistic studies comparing FA traffic and accumulation in cells expressing caveolae or not may further illuminate the mechanism underlying the inability of caveolae-null mice and humans to adequately store fat.

The rapidity with which adipocytes take up and metabolize FAs [99] complicates a mechanistic dissection of the individual steps in the FA uptake process in this cell type. Thus, we utilized HEK293 cells, which lack many of the putative FATPs and also lack a robust lipogenic capacity to make TGs, and we created stable cell lines expressing varying amounts of Cav1 [62]. These cells also lack cavin expression and hence do not make caveolae. We found that a threshold level of Cav1 expression enhanced PM cholesterol content and enhanced the partitioning of FAs to the inner leaflet of the bilayer where caveolins are localized, and this partitioning reached equilibrium in approximately 10 min. The effect of caveolin in this regard was independent of FA type, with unsaturated and cis and trans monounsaturated FAs all showing identical properties. Similarly, the extent of conversion of FAs to mono-, di- and tri-glycerides over a time course of 10–15 min was the same in cells whether or not Cav1 was expressed, and we concluded that caveolin-enriched lipid rafts had the capacity to buffer FA anions by virtue of their interaction with the numerous positive charges present in the juxtamembrane sequences of Cav1's C-terminus (Figure 2; also refer to Figure 10 in [62]).

To further determine the effects of caveolins on FA movement in cells, we conducted studies in HEK293 cells expressing Cav3 and we used fatty amines as additional probes whose spectroscopic behavior with pH-sensitive probes would be opposite to that of FAs. We found that Cav3 essentially behaved identically to Cav1 in modulating transmembrane FA movement, that fatty amines exhibited the same kinetic behavior as FAs and that both caveolin isoforms protected cells from prolonged (48 h) exposure to 0.75–1.5 mM oleate bound to albumin [88]. Moreover, prolonged exposure to lower concentrations (80 μ M) of exogenous oleate resulted in enhanced TG accumulation in both Cav1- and Cav3-expressing cells [88]. Taken together, these *in vitro* studies are entirely consistent with the results from

the knockout mice and in caveolae-deficient humans. In other words, reduced lipid accumulation and/or enhanced FA-induced adipocyte toxicity due to lack of caveolae results in lipodystrophy. The role of caveolae in modulating the effects of FA movement can also be determined upon stimulation of lipolysis. In the absence of extracellular albumin, prolonged lipolytic stimulation of primary adipocytes results in reduced cell viability, as determined by enhanced release of lactic dehydrogenase [89]. We have compared hormonally stimulated lipolysis in cultured fat cells expressing caveolae or not and found that the lactic dehydrogenase release is greater in the absence of caveolae, which is consistent with all of the previous data and conclusions about the role of caveolae [MESHULAM ET AL., UNPUBLISHED DATA].

An additional role for caveolins and caveolae in lipid storage stems from the observation that Cav1 has been observed to associate with lipid droplets [100–103]. Regarding the cited studies of adipocytes, it is important to note that caveolin is such an abundant protein in these cells that it is often detected by western blot or by mass spectrometry in unlikely subcellular localizations (e.g., Glut4 storage vesicles [see Supplementary Table 3 of [104]]). The detection of caveolins on lipid droplets by immunofluorescence is similarly confounded by the proximity of the endoplasmic reticulum to the droplet, to which Cav1 can traffic under certain circumstances [105,106]. The lipid droplets *per se* of primary adipocytes from Cav1- [91] and cavin-1-knockout mice [DING ET AL., UNPUBLISHED DATA] look essentially normal except for their small size, and therefore, we attribute the dysfunctional nature of the caveolae-null cells in part to alterations in their cell surface properties. Finally, there has been a report that adipocyte caveolae are functionally heterogeneous [107] and that TG synthesis can take place in a subpopulation of caveolae [90]. The mechanistic basis for these interesting observations is not known and further work will be necessary in order to verify these findings.

Conclusion

The abundance of caveolae in adipocytes and their biophysical properties suggest the hypothesis that they play a protective role in modulating lipid (FA) trafficking in and out of these cells and for proper lipid (TG) storage. This hypothesis is supported by a significant amount of *in vivo* and *in vitro* data.

Future perspective

Although caveolae have been extensively studied, particularly since 1992 when Cav1 was discovered, it is only in the last 2 years that the role of cavin proteins in caveolae formation and function has been recognized. Determining specific functions for the individual cavins, inside and outside of caveolae, is likely to be an exciting and highly competitive enterprise in the coming years. This will include determining whether the cavins themselves can also modulate FA movement and storage. Moreover, it is possible that naturally occurring mutations in human cavin-2 and -3 will be found, with the former, similarly to cavin-1, having a lipodystrophic phenotype. Finally, the question remains to be answered as to whether or not lipid rafts other than caveolae can modulate lipid metabolism.

Executive summary

Caveolae are small (50–100 nM) invaginations of the cell surface

- These structures are abundant in endothelial and fat cells, but their general functions in these and other cells remain unknown.

Caveolins are required for caveolae formation

- Caveolins-1 and -2 in cells other than muscle, and Cav3 in cardiac and skeletal muscle, comprise a family of unusual integral membrane proteins whose oligomers form membrane rafts upon which caveolae are assembled.

Cavins are peripheral membrane proteins that comprise the striated coat of caveolae

- Cavins-1–4 have been documented to colocalize with caveolae, and cavin-1 and -2 isoforms, along with caveolins, are required for the characteristic morphological appearance of these structures.

Adipocytes express particularly abundant levels of caveolae

- Morphological and protein expression studies have shown that fat cells express the highest levels of caveolins and caveolae of any cell type.

Caveolae are rich in cholesterol & sphingomyelin

- Along with the aforementioned caveolin and cavin proteins, caveolae require cholesterol and sphingomyelin for their formation.

Caveolae modulate fatty acid traffic in & out of adipocytes

- *In vitro* and *in vivo* comparisons of cells expressing caveolins and caveolae with those lacking these proteins and structures reveals dramatic differences in fatty acid flux and storage, including in adipocytes.

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Figure 1. Caveola composition

The lipid and protein species characteristic of caveolae are depicted in an individual caveola. Cavin-1–3 form a complex with one another, but it is not yet clear what governs their role in caveola formation or what protein–protein and protein–lipid interactions are necessary for this (see main text for details). In addition, noncaveolar detergent-resistant membrane domains also exist (lipid rafts), shown here with flotillin-1 protein, which complicates analysis of plasma membranes, in which two or more such domains can occur.

Figure 2. Fatty acid partitioning in caveolae

The positive charges on the C-termini of caveolins stabilize fatty acid anions and allow their high concentration on the inner membrane leaflet of caveolae.