Cell Volume Regulation and Signaling in 3T3-L1 Pre-adipocytes and Adipocytes: On the Possible Roles of Caveolae, Insulin Receptors, FAK and ERK1/2

Kathrine Eduardsen*, Susanne L. Larsen*, Ivana Novak, Ian H. Lambert, Else K. Hoffmann and Stine F. Pedersen

Department of Biology, University of Copenhagen, Copenhagen, *Contributed equally to this work

Key Words

RVD • RVI • ERK1/2 • Insulin • Osmotic stress

Abstract

Caveolae have been implicated in sensing of cell volume perturbations, yet evidence is still limited and findings contradictory. Here, we investigated the possible role of caveolae in cell volume regulation and volume sensitive signaling in an adipocyte system with high (3T3-L1 adipocytes); intermediate (3T3-L1 pre-adipocytes); and low (cholesterol-depleted 3T3-L1 pre-adipocytes) caveolae levels. Using large-angle light scattering, we show that compared to pre-adipocytes, differentiated adipocytes exhibit several-fold increased rates of volume restoration following osmotic cell swelling (RVD) and osmotic cell shrinkage (RVI), accompanied by increased swelling-activated taurine efflux. However, caveolin-1 distribution was not detectably altered after osmotic swelling or shrinkage, and caveolae integrity, as studied by cholesterol depletion or expression of dominant negative Cav-1, was not required for either RVD or

RVI in pre-adipocytes. The insulin receptor (InsR) localizes to caveolae and its expression dramatically increases upon adipocyte differentiation. In pre-adipocytes, InsR and its effectors focal adhesion kinase (FAK) and extracellular signal regulated kinase (ERK1/2) localized to focal adhesions and were activated by a 5 min exposure to insulin (100 nM). Osmotic shrinkage transiently inhibited InsR Y¹⁴⁶-phosphorylation, followed by an increase at t=15 min; a similar pattern was seen for ERK1/2 and FAK, in a manner unaffected by cholesterol depletion. In contrast, cell swelling had no detectable effect on InsR, yet increased ERK1/2 phosphorylation. In conclusion, differentiated 3T3-L1 adipocytes exhibit greatly accelerated RVD and RVI responses and increased swelling-activated taurine efflux compared to pre-adipocytes. Furthermore, in pre-adipocytes, Cav-1/caveolae integrity is not required for volume regulation. Given the relationship between hyperosmotic stress and insulin signaling, the finding that cell volume regulation is dramatically altered upon adipocyte differentiation may be relevant for the understanding of insulin resistance and metabolic syndrome.

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Stine F. Pedersen
Dept. of Biology, University of Copenhagen
13, Universitetsparken, DK-2100 Copenhagen (Denmark)
E-Mail sfpedersen@bio.ku.dk

Introduction

Cellular hydration status has fundamental impact on cell function, hence, volume sensing and regulation are essential for maintaining normal cell function (see [1]). Insulin resistance, a central component of metabolic syndrome and diabetes, is associated with altered hydration status, so much that in fact rehydration of diabetic patients has been reported to be sufficient to attenuate insulin resistance [2, 3]. In accordance with this, a series of studies in hepatocytes have demonstrated that hyperosmotic tissue dehydration induces insulin resistance, at least in part by impairing insulin-mediated activation of protein kinase B (PKB)/Akt (see [4]). Hyperosmotic stress, similar to insulin exposure, stimulates translocation of the glucose transporter, GLUT4, to the plasma membrane in 3T3-L1 adipocytes, resulting in increased glucose transport [5, 6]. However, while hyperosmotic stress in itself stimulates glucose transport and as such is insulinomimetic, hyperosmotic pretreatment of adipocytes induces cellular insulin resistance, abolishing further activation of glucose transport by insulin [7]. Thus, the relationship between osmotic stress and insulin signalling is far from clear. A possible link is suggested by the fact that insulin receptor (InsR) localizes to caveolae [8], flask-shaped membrane invaginations organized by the membrane protein caveolin-1 (Cav-1; [9]). InsR interacts directly with Cav-1, and activation of InsR results in phosphorylation of Cav-1 on Tyr¹⁴ [10].

The processes of volume recovery after osmotic swelling and shrinkage are denoted, respectively, Regulatory Volume Decrease (RVD) and Regulatory Volume Increase (RVI). RVD is accomplished by activation of pathways for K⁺, Cl⁻ and organic osmolyte efflux, whereas RVI generally entails the activation of Na⁺,K⁺,2 Cl⁻ cotransport via NKCC1 and/or Na⁺/H⁺ exchange via NHE1 [1]. The mechanisms by which cells sense volume changes and transmit them to activate the volume sensitive ion transport pathways are incompletely understood. Candidate mechanisms include ligand-independent clustering of integrins and/or growth factor receptors, or other mechanisms sensitive to the organization of the plasma membrane [1]. A role for caveolae and specifically Cav-3 in attenuating hyposmolarity-induced cell swelling in rat ventricular myocytes was recently suggested [11]. However, this study did

not address the RVD process *per se*, and there are to our knowledge no direct studies of the role of caveolae in the RVI process. A role for caveolae in mechanosensing is, however, well established [12-14], and their possible role in sensing of cell volume perturbations has been suggested by several lines of evidence. Firstly, upon osmotic cell swelling and shrinkage, plasma membrane folding is substantially decreased and increased, respectively, conditions that might conceivably impact on caveolar structure (see [1]). Accordingly, a reversible decrease of the number of caveolae in the plasma membrane upon osmotic swelling was recently demonstrated and shown to play a role in relief of membrane tension during Mechanical stress conditions [15]. Second, the actin-based cortical cytoskeleton, which contributes to the organization of caveolae, undergoes extensive remodeling upon cell volume perturbations (see [16, 17]). Third, regulation of swelling-activated Cl⁻ [11, 18, 19] and taurine [20] efflux by caveolae and/or lipid rafts has been proposed. Fourth, the shrinkage-activated transporter Na⁺/H⁺ exchanger 1 (NHE1) localizes at least in part to caveolae [21]. Finally, FAK and ERK1/2, major signaling molecules controlled by caveolae following mechanical stress, are central in the cellular response to osmotic stress (see [1]).

Adipocyte differentiation is associated with a dramatic increase in the expression of Cav-1 and the number of caveolae in the plasma membrane, as well as increased expression of InsR [8]. We therefore hypothesized that caveolae could be early sites of sensing of cell volume perturbations and play a particularly important role in control of volume sensitive signaling in adipocytes, at least in part through modulation of InsR/FAK/ERK1/2 signaling, and that a pre-adipocyte-adipocyte system would be an excellent model for addressing this issue. Volume regulation and volume regulatory ion transport after osmotic swelling and shrinkage were assessed in undifferentiated 3T3-L1 pre-adipocytes (low Cav-1 levels and few caveolae), differentiated 3T3-L1 adipocytes (high Cav-1 levels and abundant caveolae) and after methyl- β -cyclodextran (m β CD)-induced disruption of lipid domains including caveolae and specific dominant-negative Cav-1 mediated disruption of caveolae. We report here that fully differentiated 3T3-L1 adipocytes exhibit increased RVD and RVI responses compared to pre-adipocytes, however, caveolae integrity is not required for RVD and RVI in pre-adipocytes.

Materials and Methods

Reagents and solutions

Unless otherwise specified, reagents were analytical grade and from Sigma-Aldrich or Mallinckrodt Baker B.V. (Deventer, NL). Calcein-AM and rhodamine-phalloidin were from Invitrogen. Primary antibodies were from Sigma (Vinculin), Cell Signaling Technology (total ERK1/2 and ERK1/2 phosphorylated on Thr²⁰² and Tyr²⁰⁴ and InsR β phosphorylated on Tyr¹¹⁴⁶), Transduction Laboratories (total InsR β), BioSource (FAK phosphorylated on Tyr³⁹⁷), and Santa Cruz Biotech (Cav-1). Mouse monoclonal antibody against β -actin was from Sigma-Aldrich. A paraformaldehyde stock solution (20% w/v in ddH₂O) was prepared fresh regularly and stored at 4°C. The isotonic Ringer used for large angle light scattering experiments and confocal imaging of cell volume contained (in mM): 137 NaCl, 5.4 KCl, 1 MgSO₄, 1 Na₂HPO₄, 0.5 NaH₂PO₄, 1.2 CaCl₂, 10 HEPES, 5 glucose (pH 7.4, 300 mOsm). In hypotonic Ringer, NaCl was reduced to 60 mM, resulting in an osmolality of 150 mOsm. In Ringer for Coulter counter experiments, 25 mM NaCl was replaced by NaHCO₃, added immediately before use followed by adjustment of pH to 7.4. Phosphate-buffered saline (PBS) contained (in mM) 137 NaCl, 3 KCl, 8 Na₂HPO₄, and 1.5 mM KH₂HPO₄. TRIS buffered saline (TBS) contained (in mM): 150 NaCl, 10 Tris-HCl, 1 MgCl₂, 1 EGTA, pH 7.3. Unless otherwise indicated, experiments were carried out at 37°C.

Culture and differentiation of 3T3-L1 cells

3T3-L1 cells were maintained in DMEM supplemented with 10% Fetal Calf Serum (FCS), 1% Penicillin/Streptomycin (both GIBCO) at 37°C, 5% CO₂, 100% humidity. Cells were passaged twice a week by gentle trypsinization, and only passages 12–30 were used for experiments. Differentiation to adipocytes was initiated 48 h after confluence, by supplementing the growth medium with 1 μ M dexamethasone, 0.5 mM IBMX and 10 μ g/ml insulin (both Sigma-Aldrich). After another 48 h, IBMX and dexamethasone were removed, and the insulin-supplemented medium was changed every 2–3 days. The first adipocytes were observed on day 4–5 after adipogenic treatment, and 80–90% of the cells were fully differentiated after approximately 2 weeks.

Depletion and measurement of cellular cholesterol

Cells were grown in 10 cm Petri dishes to a confluence of 80%, washed 3 x in serum-free DMEM, incubated for 2 h at 37°C and 5% CO₂ in this medium plus 5 mM methyl- β -cyclodextran (m β CD, Sigma), and finally washed 3 x in PBS. Cellular cholesterol content was measured using the CHOD-PAP colorimetric assay (Roche) according to the manufacturer's instructions. Briefly, the cells were incubated in 8 ml isopropanol for 30 min on a shaking table to extract the lipids. The isopropanol was collected and evaporated to 1 ml. Remaining hydrophilic components were dissolved in 1 ml 1 M NaOH on a shaking table for 10 min. Proteins were scraped off using a rubber policeman, and homogenized by 10–12 strokes through a 27 G syringe. Protein content was determined using the DC Protein Assay (BioRad), with protein standards prepared in 1 M NaOH. 10 μ l isopropanol with the dissolved lipids was added

to 1 ml CHOD-PAP reagent, rapidly mixed, incubated for 10 min, and absorbance at 500 nm measured relative to an isopropanol blank. Cholesterol concentrations were calculated using the equation: $c = 575 \cdot A_{\text{sample}}$, where c is the cholesterol concentration in the 10 μ l sample added to the CHOD-PAP reagent, 575 is a proportionality factor provided by the manufacturer and A_{sample} is the measured absorbance. To obtain the cellular cholesterol content in μ g/mg protein, c is multiplied by the volume (in dl) of the evaporated sample (V_{sample}) and divided by the total protein quantity in mg ($m_{\text{protein, total}}$), i.e.: $N_{\text{cholesterol}} = (V_{\text{sample}} \cdot c \cdot 1000) / m_{\text{protein, total}}$.

Constructs and transfection

The bicistronic pCINeo/IRES-GFP vectors containing MDCK Cav-1b and dominant negative (DN) Cav-1 (Δ 1–81), respectively, were a kind gift from Dr. Jan Eggermont, Catholic University of Leuven, Belgium). Cav-1 Δ 1–81 was generated by PCR amplification of the open reading frame encoding amino acids 82 to 178 of MDCK caveolin-1. Pre-adipocytes were seeded on round 25 mm glass coverslips to a confluence of 70% on the day of transfection. Cells were transfected using 2 μ g DNA and 4 μ l Lipofectamine 2000 in a total of 2 ml OptiMEM medium (both Invitrogen), according to the manufacturer's instructions, and used for experiments 24 h after transfection. Transfection efficiency was assessed as GFP fluorescence using the 40X/1.25 NA objective and the 488 nm Ar/Kr laser line of a Leica IRB/E microscope with a Leica TSC NT confocal laser scanning unit (Leica Microsystems, Heidelberg, Germany).

Immunocytochemistry and confocal laser scanning microscopy

Cells grown on glass coverslips were stimulated as indicated, and fixed by addition of a final concentration of 2% paraformaldehyde, followed by incubation for 15 min at room temperature and 30 min on ice. Cells were washed in TBS, permeabilized for 10 min in TBS containing 0.2% Triton-X-100, and incubated for 30 min in blocking buffer (TBS + 5% BSA). Cells were incubated overnight at 4°C with primary antibody diluted in TBS + 1% BSA, washed extensively, and incubated for 2 h with the relevant secondary antibodies in TBS + 1% BSA. Where indicated, cells were exposed to rhodamine-conjugated phalloidin (2 U/ml) together with the secondary antibody (1:600 in TBS + 1% BSA) for 5 min after the incubation with secondary antibodies. Finally, cells were washed extensively in TBS + 1% BSA, and mounted using N-propyl-gallate antifade mounting medium.

Fluorescence was visualized using a 40 X/1.25 NA plan apochromat objective on a Leica TSC NT confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany). The 488 and 568 nm Ar/Kr laser lines and 364 nm UV laser lines were employed as relevant, and settings optimized to minimize fluorescence bleedthrough. Optical slice thickness was 1 μ m, and pinhole size 1 airy disc. Images were frame averaged, and processed using Adobe Acrobat software. No image manipulation was carried out beyond preparation of overlays and brightness/contrast adjustments. No or negligible labeling was detectable in the absence of primary antibody.

Cell volume measurements

Large angle light scattering. Relative volume changes in cell populations were measured using large-angle light scattering, essentially as in [23]. Near-confluent cells on rectangular glass coverslips were placed in a cuvette at a 50° angle relative to the excitation beam, in a RatioMaster spectrophotometer (Photon Technology International) equipped with a 75 W Xenon lamp. The cells were continually perfused and solutions and cuvette chamber were temperature controlled at 37°C. Excitation- and emission wavelengths were 577 and 580 nm, respectively. Data was collected and analyzed using the software program FeliX™ version 1.2. An inverse linear relation between osmolarity and emitted signal was obtained in the relevant range (not shown), as described in [24]. Data are shown as the inverse of the relative intensity of the emitted light (since a reduction in cell volume results in an increase in light scattering, and vice versa). I.e., I_0/I , where (I_0) is the emitted light (in arbitrary units) under initial, isotonic conditions (I_0). RVD and RVI rates were calculated from the light scatter traces, as the slope of the initial, linear part of the recovery curve (the first 50 s) after peak swelling or shrinkage. In differentiated adipocytes, „peak shrinkage or swelling“ was taken as the minimal/maximal volume obtained, respectively, although the cells exhibited no or minimal osmotic volume changes. See the grey dashed lines in Figure 2 for illustration of the slopes calculated.

Electronic cell sizing. Cells were grown in 150 cm² culture flasks and were 80% confluent on the day of the experiment. Where indicated, cholesterol was depleted as described above. Cells were washed once in warm PBS, detached by gentle trypsinization, resuspended in 18 ml DMEM, pelleted at 700 rpm for 30 s, and resuspended in 2.5 ml 37°C isotonic (control and RVD experiments) or hypotonic (RVD/RVI experiments) Ringer's solution. After 30 min preincubation with gently shaking, experiments were initiated by diluting a 2.5 ml aliquot of the cell suspension in 50 ml iso- or hypotonic Ringer's solution in the preheated beaker of a Coulter® Multisizer II with a 100 µm aperture tube. Signals from the Multisizer were collected and analyzed using Multisizer AccuComp software. Cell volumes were calculated from the collected distribution curves after calibration with 14,1 µm Coulter® calibration standard latex beads (Beckman Coulter).

Relative cell volume measurements of individual cells. Cells were seeded on round 25 mm #1 glass coverslips and transfected with DN-Cav-1 24 h prior to experiments as described above. Immediately prior to experiments, cells were loaded with 5 µM CellTrace calcein red-orange-AM (Invitrogen) for 10 min, followed by two washes in isotonic Ringer to remove extracellular dye. The cells were mounted in isotonic Ringer on the stage of a Leica DM IRB/E microscope with a Leica TSC NT confocal laser scanning unit (Leica Microsystems A/S, Heidelberg, Germany). The cell chamber was thermostatted to maintain a constant temperature of 37°C. Hypotonic conditions were induced by diluting the Ringer 1:1 with buffered ddH₂O. The cells were monitored using a 40X/1.25 NA objective, the 488 and 568 nm Arg/Kr laser lines to visualize GFP and calcein fluorescence, respectively, and backscattered light to visualize cell structure. An x-y image was acquired every 2 s

over a 3-5 min period, and cell volume was monitored as dilution of calcein fluorescence [25]. DN-Cav-1 transfected cells were easily identified by their GFP fluorescence, allowing separate analysis of volume recovery in transfected cells and control cells in each experiment. ROIs in individual cells were analyzed using Leica software, and relative cell volume was calculated by normalization to the initial isotonic cell volume.

Immunoblotting. Cells grown to 90% confluence were washed twice in isotonic Ringer's solution, pre-equilibrated for 30 min, stimulated as indicated, and washed twice in 5 ml ice cold PBS. Cells were lysed (lysis buffer: 10% SDS, 1% bromophenol blue, 10% glycerol, 5 mM Tris-Cl pH 6.8, 1 mM Na₃VO₄), scraped off using a rubber policeman, homogenized through a 27 g syringe, reheated to 95°C, and centrifuged at 5°C (20.000 x g, 5 min) to pellet debris. Protein content was measured using the colorimetric DC Protein assay (BioRad) and BSA (Pierce) protein standards, and equal amounts of protein were diluted in NuPAGE sample buffer containing 50 mM dithiothreitol (DTT). Proteins were analysed by SDS-PAGE and western blot analysis using the Novex XCell (E19001) and XCell II blot module systems (Novex, San Diego, CA), essentially as in [26].

Taurine and ⁸⁶Rb⁺ efflux measurements

The ¹⁴C-taurine and ⁸⁶Rb⁺ efflux measurements were performed at room temperature, essentially as previously described [23]. Cells, grown to 80% confluence in 6 well polyethylene dishes, were loaded in growth medium with ¹⁴C-taurine or ⁸⁶Rb⁺ (2h, 37 °C). At the end of the loading period, the cells were washed four times in isotonic NaCl solution to remove extracellular isotope. The efflux experiments were performed by removal/addition of 1 ml aliquots of experimental solution at 2 min intervals. When indicated the isotonic medium was exchanged with hypotonic NaCl medium. All aliquots were transferred to scintillation vials for estimation of ¹⁴C and ⁸⁶Rb⁺ activity (scintillation fluid: Ultima Gold). At the end of the efflux experiment, the cells were lysed (0.5 M NaOH, 2 h), and the lysate plus two washes (distilled H₂O) were transferred to scintillation vials for estimation of the ¹⁴C and ⁸⁶Rb⁺ activity. ⁸⁶Rb⁺ activity was corrected for decay and the total radioactivity in each experimental setup was calculated as the sum of radioactivity in all samples plus the activity remaining in the cells at the end of the experiment (lysates plus washes). The natural logarithm of the fraction of activity remaining in the cells at each experimental time point was plotted as a function of time. The fractional rate constants for ¹⁴C and ⁸⁶Rb⁺ release were calculated for each time point as the negative slope of the curve between the time point and the previous time point. Taurine and Rb⁺ release was estimated at room temperature, while all other experiments were performed at 37°C.

Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M.) of at least three independent experiments, or as individual experiments representative of at least three independent experiments, unless otherwise indicated. Student's

Fig. 1. Caveolin-1 organization in 3T3-L1 pre-adipocytes and adipocytes: effect of osmotic volume perturbations and cholesterol depletion. **A.** 3T3-L1 pre-adipocytes (top row) and differentiated adipocytes (lower row) were exposed to 1 min of osmotic swelling in hyposmotic Ringer solution (150 mOsm, RVD) or 7 min in 150 mOsm followed by 1 min of isotonicity (RVI-after-RVD, here denoted RVI). Cells were subsequently paraformaldehyde-fixed, permeabilized, and labeled using a rabbit polyclonal caveolin-1 antibody (1:100) followed by FITC-conjugated secondary antibody. Fluorescence was visualized by confocal imaging. Arrows indicate the location of lipid droplets, visible as dark areas. **B.** 3T3-L1 pre-adipocytes were incubated in 5 mM methyl- β -cyclodextrin (m β CD) for 2 h to deplete cellular cholesterol, and the cholesterol content (μ g per mg protein) measured as described in Materials and Methods. **C.** Control and cholesterol-depleted 3T3-L1 pre-adipocytes were labeled for Cav-1 and exposed to osmotic swelling and shrinkage as described for Panel A. Data are single experiments representative of at least 3 independent experiments (A,C), or mean of 3 experiments with S.E.M. error bars (B). *): The value differs significantly from that in control pre-adipocytes, $p < 0.05$. Scale bar in A and C: 20 μ m, scale bar in insert: 10 μ m.

t-test was used for statistical evaluation, and P values < 0.05 were taken to indicate a significant difference.

Results

Cav-1 localization in 3T3-L1 cells: Effects of osmotic volume perturbations and cholesterol depletion

The first question to be addressed was whether osmotic stress is associated with detectable changes in

caveolae organization in 3T3-L1 pre-adipocytes and adipocytes that are rapid enough to play a role in the acute RVD and RVI processes. As seen in Fig. 1A (top panel), under isotonic conditions, pre-adipocytes have a fibroblast-like appearance and a patchy distribution of Cav-1. Cav-1 is seen to localize mainly to the plasma membrane but also intracellularly, the latter predominantly in the perinuclear region (Fig. 1A). In addition to the previously demonstrated increase in Cav-1 expression upon differentiation of these cells [27], differentiation to

Fig. 2. Cell volume regulation in adherent 3T3-L1 cells measured using large-angle light scattering. Cells were grown on rectangular cover slips to a confluency of 90%. Where indicated, pre-adipocytes were differentiated to adipocytes or cholesterol depleted, as described in Materials and Methods. The relative cell volume was assessed spectrophotometrically by large angle light scattering. **A:** Pre-adipocytes and adipocytes were briefly exposed to Ringer solutions of the indicated osmolarity, long enough to induce osmotic changes but not long enough to induce cell volume regulation. **B-D.** The cells were perfused first with isotonic Ringer, then with hypotonic Ringer (150 mOsm) for 7 min as indicated by the bar, resulting in osmotic swelling and volume recovery (RVD) and subsequently returned to isotonic Ringer, resulting in osmotic shrinkage and recovery (RVI after RVD, here denoted RVI). The relative cell volume is given as I_0/I where I_0 is the mean intensity of the scattered light under isosmotic control conditions. **D-E.** The RVD (**A**) and RVI (**B**) rates were calculated as the slope of the light scatter curve the first 50 s after peak swelling or shrinkage, as shown by the dotted lines in Panel A. Data are mean with SEM error bars, of 6 (pre-adipocytes, white box), 6 (cholesterol-depleted preadipocytes, grey box) or 4 (adipocytes, black box) experiments. *): Significantly different from the value in control pre-adipocytes, $p < 0.05$.

adipocytes was associated, more rounded morphology, and formation of prominent cytoplasmic lipid droplets, visible as black spots in the cytoplasm (Fig. 1A, lower panel, arrows). To assess the possible effect of cell volume perturbations on caveolae organization, the 3T3-L1 cells were swollen by exposure to a hypotonic Ringer (150 mOsm compared to 300 mOsm under isotonic conditions), or osmotically shrunk using the so-called “RVI-after-RVD” protocol. In this protocol, the cells are hypotonically challenged (150 mOsm), allowed to volume regulate, and returned to isosmotic Ringer. Since RVD reflects osmolyte efflux, intracellular osmolarity in the new steady state is now lower than that of the isotonic Ringer,

and the cells will shrink and carry out RVI (see also below). As seen in Fig. 1A (middle and right panels), 1 min after osmotic cell swelling, a weakening of the Cav-1 signal is evident in the differentiated adipocytes, whereas 1 min after osmotic shrinkage, the Cav-1 labelling appears more intense. However, these changes are likely a consequence of the dilution and concentration, respectively, of the signal upon volume perturbation and concurrent changes membrane folding/unfolding, and no other changes in Cav-1 distribution were detectable in either cell type.

Next, the pre-adipocytes were cholesterol depleted to further reduce the number of caveolae at the

Fig. 3. Cell volume regulation in 3T3-L1 pre-adipocytes measured by electronic cell sizing or by calcein imaging. A-D. Electronic cell sizing. Where indicated, pre-adipocytes were cholesterol depleted prior to the experiment as described in Materials and Methods. Cells were mildly trypsinized, and pre-incubated for 20 min in isotonic (300 mOsm), HCO_3^- -containing Ringer's solution for RVD and isotonic control experiments (A,B), or in hypotonic (150 mOsm) HCO_3^- -containing Ringer's solution for RVI and hypotonic control experiments (C,D). For measurements, 25×10^5 cells were transferred to 50 ml of 37°C Ringer's solution in the Coulter counter. A-B. Representative time traces. C-D. Rates of RVD and RVI were calculated from the Coulter Counter time traces in panels A-B, using the slope of the curves within the first 2 min after peak cell swelling or shrinkage. Data are summarized as means with SEM error bars, of 3 independent experiments for each condition. E-G. Effect of dominant negative caveolin-1 (DN-Cav-1) on RVD in 3T3-L1 preadipocytes. E-F. Representative time traces for single cells, where shift in tonicity is indicated by an arrow. G. Summary data. Cells were seeded on glass coverslips and transfected with DN-Cav-1 (Cav-1 Δ 1-81) in pCINeo/IRES/GFP, 24 h prior to experiments, as described in Materials and Methods. Cells were loaded with 5 μM CellTrace calcein red-orange, and immediately mounted in isotonic Ringer in a preheated chamber on the stage of the confocal microscope. Hypotonicity was induced by diluting the saline 1:1 with buffered ddH₂O, at the time indicated by the arrow. Cells were monitored using a 40X/1.25 NA objective, the 488 nm Arg/Kr laser line to visualize GFP fluorescence, and the 568 nm line to visualize calcein fluorescence, and backscattered light to visualize cell structure, respectively. ROI's were placed over individual cells, and recovery rates were calculated as the slope of the curve the first 10 s after peak in swelling, in GFP-labelled (DN-Cav-1) or control cells. Data are shown as means with SEM error bars, $n=3$ independent experiments for each condition.

plasma membrane compared to that in control pre-adipocytes. Cholesterol depletion was carried out by m β CD preincubation, a procedure which disrupts

both caveolae and lipid rafts. A 1 h preincubation with 5 mM m β CD resulted in an about 50% decrease in the cholesterol content of the pre-adipocytes (Fig. 1B),

Fig. 4. Swelling-activated $^{86}\text{Rb}^+$ efflux from 3T3-L1 pre-adipocytes and adipocytes. *A.* effect of adipocyte differentiation on swelling-activated K^+ efflux. 3T3-L1 pre-adipocytes and adipocytes were preloaded with $^{86}\text{Rb}^+$ for 2 h, followed by efflux measurements as described in Materials and Methods. At time 6 min (arrow), extracellular osmolarity was reduced by 50%, from 325 to 163 mOsm. Rb release is shown as a fractional rate constant estimated with 2 min intervals. Data are means with S.E.M. error bars, of 5 independent experiments per cell type. *B.* The relative swelling-induced increase in $^{86}\text{Rb}^+$ efflux was calculated from the data in *A*, as the swelling-induced increase in rate constant after 6 min of hypotonic exposure, relative to that under isotonic conditions. *) Significantly different from the value in undifferentiated pre-adipocytes. *C.* 3T3-L1 pre-adipocytes (control and cholesterol depleted as described) were exposed to isotonic conditions or hypototically swollen as in *A*, and rate constants calculated as in *A*. Data are means with S.E.M. error bars, of 3-6 independent experiments per cell type. *D-E.* Pre-adipocytes (*D*) or adipocytes (*E*) were cholesterol-depleted or not as indicated. The cells were exposed to isotonic conditions or hypototically swollen as in *A*, and rate constants calculated as in *A*, at time 6 min after swelling. Data are means with S.E.M. error bars, of 3-5 independent experiments per cell type.

a reduction previously shown to render the plasma membrane of 3T3-L1 cells essentially devoid of caveolae-like structures [8]. In congruence with previous reports [28], cholesterol depletion altered the patchy distribution of Cav-1 in the pre-adipocytes to a more diffuse pattern, yet, the majority of Cav-1 appeared to remain in the plasma membrane (Fig. 1C). This phenomenon has previously been demonstrated after cholesterol depletion of 3T3-L1 cells and was suggested to reflect the association of Cav-1 with non-invaginated caveolar patches

[8]. A similar pattern was seen in the differentiated adipocytes (data not shown).

Similar to the results obtained in the control pre-adipocytes (Fig. 1A), osmotic swelling and shrinkage did not exert detectable additional changes in Cav-1 organization in the cholesterol-depleted pre-adipocytes (Fig. 1C). It is emphasized that this does not exclude more subtle changes in Cav-1 organization not detectable by the visualization method employed.

Cell volume regulation in 3T3-L1 cells: effects of differentiation and cholesterol depletion

We next asked whether the capacity for volume regulation differed between adipocytes, pre-adipocytes, and cholesterol-depleted pre-adipocytes (i.e. high, medium, and low caveolae content, respectively). Figure 2A shows the osmometer curves for pre-adipocytes and adipocytes, obtained by exposing the cells as briefly as possible to the desired osmolarity, allowing osmotic changes to happen, but minimizing volume-regulatory recovery. As seen, in both cell types, extracellular osmolarity and hence presumably cell volume, correlates relatively well with the normalized large-angle light scattering signal, supporting the notion that the method is suitable for assessing cell volume changes in both cell types. The pattern of osmotically induced cell volume changes and subsequent volume recovery monitored using large angle light scattering is illustrated for (B) pre-adipocytes, (C) differentiated adipocytes, and (D) mβCD-treated pre-adipocytes, and summarized in panels E and F. When pre-adipocytes were exposed to hypotonic Ringer (150 mOsm compared to 300 mOsm under isotonic conditions), they rapidly swelled, followed by a RVD which was largely completed within 3-5 min of the initiation of hypotonic conditions (Fig. 2B). When the pre-adipocytes were returned to isosmotic Ringer they carried out RVI-after-RVD - for simplicity denoted RVI. RVD and RVI were relatively fast at early time points in these cells, yet remained incomplete for the duration of the experiment. In marked contrast, in the differentiated adipocytes, the initial volume regulation was so rapid that essentially no osmotic volume perturbation was detectable prior to volume down-regulation, and an apparent “overshoot” of regulation (representing loss of osmolytes) was evident for the RVD process (Fig. 2C). Cholesterol depletion of the pre-adipocytes was associated with an increased rate of RVD, whereas the rate of RVI was unaffected (Fig. 2D). Figures 2E and 2F summarize the rates of RVD and RVI, respectively as measured by large-angle light scattering, in the differentiated adipocytes, and in control and cholesterol-depleted pre-adipocytes.

Large angle light scattering has the advantage of allowing non-invasive measurements on adherent cells, but does not permit absolute volume measurements. This is possible by electronic cell sizing employing the Coulter counter technique, which, however, requires an amount of material which renders it unfeasible for use with differentiated cells, and which necessitates cell detachment. These experiments were furthermore carried out in the presence of 25 mM HCO₃⁻, to take into account

Fig. 5. Effect of adipocyte differentiation on swelling-activated taurine efflux. 3T3-L1 pre-adipocytes and adipocytes were preloaded with ¹⁴C-taurine for 2 h, followed by efflux measurements as described in Materials and Methods. At time 6 min (arrow), extracellular osmolarity was reduced by 50%, from 325 to 163 mOsm. Taurine release is shown as a fractional rate constant estimated with 2 min intervals. Data are means with S.E.M. error bars, of 5 independent experiments per cell type. B. 3T3-L1 pre-adipocytes (control and cholesterol depleted as described) were exposed to isotonic conditions or hyposmotically swollen as in A, and rate constants calculated as in A. Data are means with S.E.M. error bars, of 5 independent experiments per cell type. *) Significantly different from the value in undifferentiated/control pre-adipocytes.

the possible contribution of anion exchange to cell volume regulation (see [1]). Electronic cell sizing showed that RVD (Fig. 3A-B) and RVI (Fig. 3C-D) were unaffected by cholesterol depletion. It should be noted, however, that cholesterol depletion was, in itself, associated with cell

Fig. 6. Insulin- and osmotic stress-induced InsR, FAK and ERK signaling in 3T3-L1 pre-adipocytes. A. 3T3-L1 pre-adipocytes under isotonic control conditions or exposed to 100 nM recombinant human insulin for 5 min as shown, were paraformaldehyde-fixed, permeabilized, and labeled for pY¹¹⁴⁶-InsR, pY³⁹⁷-FAK, pT²⁰²/Y²⁰⁴-ERK1/2 as indicated (green) and costained for vinculin (red) to indicate the localization of focal adhesions. Arrows on inserts from the merged images indicate colocalization. B. 3T3-L1 pre-adipocytes were incubated under isotonic conditions (Isotonic) or exposed to hypotonic Ringer for 15 min (RVD), or to hypotonic Ringer for 7 min followed by 15 min in isotonic Ringer (RVI-after-RVD, here denoted RVI). B. Fluorescence was visualized by confocal imaging, and images are representative of at least 3 independent experiments per condition. Scale bars 20 μ m, insert scale bar 2.5 μ m.

shrinkage of about 7% (in congruence with findings in Ehrlich Lettré Ascites (ELA) cells [29]).

As cholesterol depletion targets not only caveolae but also e.g. lipid rafts, we next transfected the cells with

GFP-tagged dominant-negative (DN) Cav-1. This is known to disrupt caveolar structure [22, 30], a finding confirmed in 3T3-L1 preadipocytes (Data not shown). The cells were subsequently loaded with a red-shifted

Fig. 7. Phosphorylation levels of caveolin-1, InsR and ERK1/2 upon cell shrinkage and swelling in 3T3-L1 preadipocytes. Cells were grown to near-confluence in 10 cm Petri dishes, cholesterol depleted where indicated, and exposed to hypotonic Ringer for 1, 15 or 30 min (RVD), or to hypotonic Ringer for 7 min followed by 1, 15 or 30 min in isotonic Ringer (RVI-after-RVD, here denoted RVI). Cells were lysed, separated by SDS-PAGE, and analysed by Western blotting using antibodies against p Tyr1146InsR and p Thr202/Tyr204 ERK1/2. Blots were analysed by densitometric scanning and data summarized as mean with SEM error bars, of 3 (pInsR), 5 (pERK1/2, pre-adipocytes) and 2 (pERK1/2, adipocytes) experiments per condition. Note that in the representative blots in B and C, the order of lanes differs from that in the quantification bar chart. Given the low number of experiments, these data are not statistically significant and should be seen as supporting the immunofluorescence data in Figure 6.

calcein-AM, and cell volume recovery after hypotonic challenge was monitored by confocal imaging of calcein fluorescence intensity (Fig. 3E-G). As seen, there was no difference in the rates of RVD between control cells and cells expressing DN-Cav-1, substantiating the notion that there is no correlation between the number of caveolae in the plasma membrane and the capacity for volume regulation in 3T3-L1 cells.

Collectively, these results indicate that caveolar integrity is not required for the RVD and RVI responses at least in 3T3-L1 pre-adipocytes.

Osmolyte efflux pathways in RVD in 3T3-L1 cells: effects of differentiation and cholesterol depletion

In order to determine which osmolyte efflux pathways might underlie the dramatic differences in RVD rates between pre-adipocytes and adipocytes, we next determined the swelling-activated efflux of $^{86}\text{Rb}^+$ (as a measure of K^+) and ^{14}C -taurine in both cell types. An increase in K^+ efflux is generally rate-limiting for RVD

[1]. As seen in Fig. 4A-B, compared to the pre-adipocytes, adipocytes exhibited a lower basal $^{86}\text{Rb}^+$ efflux, yet a similar relative swelling-induced increase in $^{86}\text{Rb}^+$ efflux after osmotic swelling. After cholesterol depletion, the swelling-induced K^+ efflux at time 6 min was slightly attenuated in both pre-adipocytes and differentiated adipocytes (only statistically significant in the latter) (Fig. 4D-E). Efflux of the organic osmolyte taurine contributes to especially the later phases of RVD in many cell types, and a role for caveolae in regulation of taurine flux has been proposed [20]. From time 8 min of swelling onwards, the rate constants for swelling-activated taurine efflux were significantly greater in differentiated adipocytes compared to pre-adipocytes (Fig. 5A). Further, the swelling-induced efflux of taurine from pre-adipocytes was strongly reduced by cholesterol depletion (Fig. 5B).

Taken together, these findings demonstrate that in contrast to the initial rate of RVD, the later swelling-induced efflux of taurine does appear to correlate positively with caveolae number/integrity, whereas K^+ efflux does not.

Possible role of caveolae and Cav-1 in volume-sensitive signaling events

To further evaluate the possible contribution of caveolae-regulated signaling events to cell volume sensing and volume-sensitive signaling, we next assessed the volume-sensitivity of the InsR1 and its effectors FAK and ERK1/2 in the 3T3-L1 cells. Upon differentiation of the 3T3-L1 cells to adipocytes, InsR1 expression increased approximately 20-fold ($n=3$, data not shown) in agreement with previous reports [31]. In the differentiated adipocytes, activated ERK1/2 was essentially absent ($n=2$, not shown), in congruence with the known inhibitory effect of caveolae on ERK signaling [32]. The subsequent experiments were, therefore, carried out in pre-adipocytes only. It was first verified that InsR1 stimulation elicited ERK1/2 activation under the experimental conditions employed. The cells were treated with 100 nM insulin for 5 min under isotonic conditions, fixed, and labelled for the phosphorylated, active forms of InsR (pY¹¹⁴⁶), ERK1/2 (pT²⁰²/Y²⁰⁴), and FAK (pY³⁹⁷) (Fig. 6A). Cells were co-stained with vinculin as a marker for focal adhesions. As expected, insulin treatment strongly increased pInsR staining, predominantly in focal adhesions (FAs). pFAK labelling was also strongly increased and essentially confined to FAs. Finally, pERK1/2 labeling was greatly increased upon insulin treatment, in both the nucleus and in FAs (arrows).

We next determined the effects of cell volume perturbations on InsR, FAK, and ERK1/2 activity in the pre-adipocytes by immunofluorescence analysis. 15 min of osmotic swelling modestly decreased InsR phosphorylation and slightly increased nuclear ERK1/2 activity, with no detectable effect on FAK activity. Conversely, 15 min of osmotic shrinkage using the RVI-after-RVD protocol was associated with marked increases in pInsR and pFAK labelling, especially in FAs, and if anything a modest decrease in nuclear pERK labelling (Fig. 6B). A similar pattern was seen in Western blots of pInsR and pERK (Fig. 7A-B). Finally, to address whether the volume-sensitivity of ERK1/2 was dependent on caveolae integrity, we repeated these experiments in cholesterol-depleted pre-adipocytes (Fig. 7C). As seen, although basal ERK1/2 activity was increased after cholesterol depletion of pre-adipocytes, the pattern of volume sensitivity of ERK1/2 was similar to that seen under control conditions. Thus, in 3T3-L1 pre-adipocytes, InsR activity in the absence of added ligand was slightly decreased by osmotic swelling, ERK activity was increased, and FAK activity was not detectably affected. In contrast, after osmotic shrinkage, InsR and FAK

activity was increased, whereas ERK activity was unaffected or modestly decreased.

Discussion

A central unsolved question in the field of cell volume regulation is the nature of the volume sensor(s). Caveolae have been proposed as candidate volume sensors, however, very few studies have directly addressed this hypothesis. Here, we employed the 3T3-L1 pre-adipocyte - adipocyte system in conjunction with cholesterol depletion and DN-Cav-1 expression to address the potential involvement of caveolae/lipid rafts in volume regulation and the impact of osmotic stress on caveolae-regulated signaling events. A pertinent finding of the present study is that fully differentiated 3T3-L1 adipocytes exhibit greatly increased RVD and RVI responses compared to 3T3-L1 preadipocytes. However, our findings suggest that this cannot be ascribed to the increased level of Cav-1/caveolae in the adipocytes, and that in the 3T3-L1 system, the capacity for cell volume regulation is not dependent on caveolar integrity.

RVD and RVI rates are increased by adipocyte differentiation, yet caveolae integrity is not required for RVD in pre-adipocytes

Osmotic volume perturbations were not associated with detectable changes in caveolae structure in either pre-adipocytes or adipocytes that occurred early enough to be relevant for the rapid processes of RVD and RVI. Such changes may well occur at later time points, thus, Kang et al. [33] showed that in NIH3T3 cells, caveolae were internalized after 30 min of hypertonicity. However, as volume regulatory responses are usually initiated less than 1 min after volume perturbation, changes occurring after this time are not of relevance for initiation of the volume regulatory response. Obviously, we cannot exclude the existence of osmotically induced changes in caveolar organization that would go undetected at the limited resolution of fluorescence confocal imaging, and this possibility should be investigated in future studies using e.g. electron microscopy. We next asked whether differentiation of pre-adipocytes to adipocytes altered the volume regulatory properties of 3T3-L1 cells, and if so, whether this could be attributed to the increased number of caveolae in the differentiated adipocytes. Three approaches were employed: large angle light scattering, electronic cell sizing, and calcein-based live confocal imaging of cell volume changes. Caveola levels in the

plasma membrane were manipulated by adipocyte differentiation, cholesterol depletion, and transient expression of DN-Cav-1. First of all, the light-scattering experiments showed that compared to pre-adipocytes, adipocytes appear to exhibit about three-fold increase in the rates of RVD and RVI. Since a prime characteristic of adipocyte differentiation is a greatly increased number of caveolae, this would be in line with a role for caveolae in volume regulation. However, neither cholesterol depletion nor caveola down-regulation (DN Cav-1 expression) reduced the rates of RVD or RVI in pre-adipocytes. In fact, cholesterol depletion significantly increased the rate of RVD as measured by large-angle light scattering. While this effect was not further addressed here, it is tempting to speculate that this may reflect the cholesterol depletion-induced stimulation of the volume-regulated anion channel, VRAC, as previously demonstrated by us and others in other cell types [18, 19, 34]. Rather than involving caveolae, this effect on VRAC is dependent on changes in the biophysical properties of the plasma membrane and cytoskeletal rearrangement induced by cholesterol depletion [19, 34]. In congruence with this notion [19], the increase in RVD rate in pre-adipocytes after cholesterol depletion was not seen using the Coulter counter technique, in which the cells are trypsinized resulting in F-actin disruption. As noted above, cholesterol depletion does not specifically target caveolae, but also disrupts lipid rafts, and alters membrane biophysical properties and F-actin organization [19, 29, 35]. We therefore also specifically disrupted caveolae by transiently transfecting the pre-adipocytes with DN Cav-1, which has been shown to effectively disrupt caveolae structure [22, 30]. While caveolae organization was clearly disrupted, this treatment had no effect on RVD as monitored by confocal imaging of calcein-loaded cells. An interesting observation was the different time courses of volume recovery obtained with the different measurements, although the relative differences between the different treatments were similar. While it is not surprising that the recovery rates obtained using the Coulter counter method were the slowest given the requirement for cell trypsinization for this type of measurements, we have no immediate explanation for the apparently faster rate of recovery obtained using the calcein dilution method compared to the light scattering approach. Possibilities include individual cell versus population measurements, slight differences in temperature in the two setups and perfusion vs non-perfusion, conditions. We cannot fully exclude that light-scattering properties are altered in swollen adipocytes

compared to swollen pre-adipocytes, which would skew the conclusion. However, the osmometer curves indicate that the light scattering properties of adipocytes follow rather well those of pre-adipocytes, hence it seems unlikely that such an artifact should dominate the effect. It is also interesting to note that we previously demonstrated a similar accelerating effect, with almost no actual swelling prior to volume regulation, in NIH3T3 cells expressing constitutively active RhoA [23]. In this case, the increased rate of recovery could be attributed at least in part to increased volume-sensitivity of VRAC [23].

Taken together, our findings indicate that caveolae integrity is not a requirement for either RVD or RVI in 3T3-L1 pre-adipocytes. Thus, the findings do not support the notion of caveolae as an essential site of cell volume sensing. In this regard, it is interesting to note that MCF-7 cells which have no detectable Cav-1 expression [36] nonetheless exhibit RVD [37]. Also human erythrocytes, which do not express detectable amounts of caveolin-1, -2, or -3 [38], are able to volume regulate, although in human erythrocytes, the RVD response requires additional purinergic stimulation to open Ca^{2+} -activated channels [39].

Swelling-induced taurine efflux is increased by adipocyte differentiation and decreased by cholesterol depletion

A large number of ion channels, including several involved in RVD, are affected by the cellular cholesterol content (for a review see [35]). While the initial RVD rate is mainly dependent on efflux of K^+ and Cl^- , efflux of organic osmolytes such as taurine becomes important at later time points (see [1]). The efflux of K^+ was reduced by adipocyte differentiation, and the fractional swelling-induced K^+ efflux was unaffected. Further, the swelling-induced K^+ efflux was slightly attenuated by cholesterol depletion in both pre-adipocytes and differentiated adipocytes. Hence, although K^+ efflux is most commonly rate-limiting for RVD, changes in K^+ efflux do not seem to explain the difference in RVD rates between 3T3-L1 adipocytes and pre-adipocytes.

Adipocyte differentiation was furthermore associated with a significant increase in the rate of swelling-induced taurine efflux and, conversely, cholesterol depletion strongly reduced swelling-induced taurine efflux. These data are in accordance with previous studies in Caco-2 and ELA cells demonstrating that swelling-induced efflux of taurine is dependent on caveolae [20, 29]. Interestingly, in ELA cells, the net efflux of taurine

was reported to be not decreased but increased by cholesterol depletion because while TauT-mediated taurine uptake and volume sensitive organic anion channel (VSOAC) activities were decreased, the release of organic osmolytes via a leak pathway different from the volume-sensitive pathways for amino acids and anions was increased [29]. The fact that the RVD rate was slightly increased by cholesterol depletion, while taurine efflux was reduced shows that taurine efflux is not limiting for the early RVD process in 3T3-L1 pre-adipocytes. This role may be played by Cl⁻ efflux, which, although not studied here, is likely to be increased, based on the previously demonstrated increase in VRAC after cholesterol depletion in other cell types [18, 19, 34].

Thus, collectively, these data indicate that the RVD rate in adherent, yet not in suspended, pre-adipocytes is increased by cholesterol depletion, at least in part due to increased Cl⁻ efflux under these conditions, while taurine efflux is decreased, yet is not rate-limiting for early RVD (possibly because of low taurine content in these cells). In contrast, there was no detectable effect of DN Cav-1 on RVD in these cells. Thus, these findings do not support a role for caveolae in cell volume sensing in the 3T3-L1 adipocyte system, at least not in pre-adipocytes. The question then arises as to what is the cause of the increased rate of RVD in adipocytes compared to pre-adipocytes? Firstly it should be noted that we cannot exclude a threshold effect, i.e. that although caveolae integrity is clearly not required for RVD in pre-adipocytes the robust increase in the number of caveolae in differentiated cells might somehow contribute to the increased volume perturbation responsiveness in these cells. It is however also possible that the increased capacity for volume regulation reflects other aspects of adipocyte differentiation not addressed here. While at this point this is speculation, increased or accelerated VRAC activity is in our view a likely mechanism. Further, the data also indicate that increased taurine efflux in the adipocytes may contribute somewhat also at earlier time points than where a significant difference is detectable. This would require that K⁺ flux is not rate limiting, which is possible, but for which we have not been able to find documentation in the literature. Finally, an interesting possibility is that not only ion fluxes but also differences in glycerol- and water fluxes between the pre-adipocytes and adipocytes contribute to the differences in apparent RVD rates. Thus, adipocyte differentiation is associated with a major upregulation of the aquaglyceroporin AQP7, and increased glycerol efflux and presumably greater

water permeability [40,41]. We speculate that the altered glycerol and water flux in the differentiated adipocytes may in fact be very important for the observed effect.

Cell volume perturbations alter InsR, ERK- and FAK signaling, yet swelling-induced ERK activation is unaffected by cholesterol depletion

We next turned to the possible role of caveolae in volume-sensitive signaling. Insulin is well known to stimulate the activity of shrinkage-activated transporters such as NHE1 and NKCC1 (see [4]. Further, InsR is greatly upregulated upon adipocyte differentiation, and localizes at least partially to caveolae [8]. Finally, both InsR and two important InsR effectors, FAK and ERK1/2, have previously been implicated in cell volume sensing (see [1, 42]). The phosphorylated InsR localized prominently to focal adhesions, and stimulation with insulin activated both ERK1/2 and FAK locally in focal adhesions. Most commonly, cell swelling is associated with increased, and shrinkage with transiently decreased, ERK1/2 activity [43-45], whereas FAK has been reported to be activated by osmotic shrinkage [42]. In the present study, we add an interesting spatial component to these studies by demonstrating that osmotic shrinkage activated InsR in focal adhesions, and this was associated with activation of FAK, and an initial decrease, followed by an increase, in ERK activity. While the mechanism remains to be demonstrated directly it is notable in this regard that InsR signalling has been reported to either phosphorylate or dephosphorylate FAK in a manner that appears to be dependent on cellular architecture and InsR density [46]. Cholesterol depletion increased basal ERK activity, which was, conversely, decreased by adipocyte differentiation. Importantly, however, the cell volume dependent regulation of ERK was largely unaffected by cholesterol depletion. In apparent agreement with these findings, studies in portal veins from Cav-1 knockout mice showed that Cav-1 contributed to maintaining basal ERK1/2 activity low, but was not essential for trophic effects of stretch (pressure) in the vascular wall [47]. Given the localization of activated InsR, ERK1/2 and FAK to focal adhesions after InsR activation, which was increased by osmotic cell shrinkage, it is interesting that focal adhesions have been proposed as major sites of sensing of mechanical and osmotic stress [12]. Whether these findings point to a role for focal adhesion mediated InsR-ERK/FAK signalling in osmotic volume sensing remains to be directly investigated in future studies.

In conclusion, we show here that differentiated 3T3-L1 adipocytes exhibit robustly increased RVD and RVI responses as measured by large-angle light scattering, as well as increased swelling-induced taurine efflux. However, at least in 3T3-L1 preadipocytes our studies do not support a central role for caveolae in cell volume sensing. Given the relationship between hyperosmotic stress and insulin signaling, the finding that cell volume regulation is dramatically altered upon adipocyte differentiation may be relevant for the understanding of insulin resistance and metabolic syndrome.

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