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Original Article

The effect of caveolin-1 (Cav-1) on fatty acid uptake and CD36 localization and lipotoxicity in vascular smooth muscle (VSM) cells

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Abstract: The purpose of this study was to determine whether caveolin-1 (Cav-1) is involved in lipotoxicity in vascular smooth muscle (VSM) cells by altering CD36 membrane localization. Normal A7r5 cells (cultured rat aortic smooth muscle cells), Cav-1 overexpressing cells, and cells treated with 10 mM cyclodextrin for 30 minutes were immunolabeled with Cav-1 and CD36. The peripheral to central ratio of CD36 in Cav-1 overexpressing cells (1.52±0.19) was significantly higher than in control cells (1.05±0.16, p=0.035) and cyclodextrin-treated cells (0.861±0.279, p=0.035). Fatty acid uptake at 5, 10, and 15 seconds was quantified with fluorescence of C1-BODIPY 500/510 C12, a long-chain fatty acid analog. A7r5 VSM cells overexpressing Cav-1 had decrease a in the rate of fatty acid uptake compared to control cells. Cells treated with cyclodextrin also had a decrease in fatty acid uptake compared to control. Cav-1 overexpressing cells incubated in 0.05 mM palmitate had 31.4±8.8% apoptosis, where only 3.9±1.0% of Cav-1 overexpressing cells incubated in palmitate were apoptotic (p=0.044). Cyclodextrin treatment resulted in a decrease in apoptosis in cells incubated in 0.1 mM palmitate (69.7±2.1%) compared to control cells incubated in palmitate (85.6±2.7%) (p=0.003). These data suggest that in cells overexpressing Cav-1, CD36 is relocated to the plasma membrane of VSM cells, where it may play an increased role in fatty acid uptake and possibly lipotoxicity.

Key Words: Caveolin-1, CD36, vascular smooth muscle, atherosclerosis, lipid metabolism, lipid transport

Introduction

Lipid rafts are rigid areas of the plasma membrane that confine the movement of phospholipids. They result from the coalescence of cholesterol, glycosphingolipids, and sphingomyelin [1]. Caveolae are flask-shaped invaginations of 50-100 nm that represent a subdomain of lipid rafts and are enriched in cholesterol, sphingolipids, and a family of 21-24 kDa integral membrane proteins called caveolins (see [2-4] for a review). There are three different isoforms: caveolin-1 (Cav-1), caveolin-2 (Cav-2), and caveolin-3 (Cav-3) and the three main Cav-1 containing compartments are plasma membrane caveolae, Cav-1 positive vesicles or "cavicles", and large pericentrosomal caveosomes [5-7].

Caveolae are involved in a wide variety of cellular processes [8] and have been implicated in the uptake of a variety of compounds such as folates, albumin, and alkaline phosphatase [8-11]. Caveolae and caveolins are also involved in endocytosis, lipid homeostasis, signal transduction, and tumorigenesis [2-4]. Smooth muscle cells contain abundant caveolae that are organized in distinct patches on the cell membrane [12]. Cav-1 is the major structural caveolin isoform in smooth muscle and all three isoforms of caveolin are found in smooth muscle cells

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[13]. Cav-2 and Cav-3 may play roles other than formation of caveolae, such as regulation of cell signaling, Cav-1 expression, and metabolism [3].

There are several proposed mechanisms for fatty acid uptake in cells. Fatty acids may be taken up by cells via a nonsaturable mechanism representing passive flip-flop [14]. However, fatty acids may also be transported across the cell membrane by facilitated transport proteins, such as plasma membrane fatty acid binding protein (FABPpm) [15], fatty acid transport protein (FATP) [16], fatty acid translocase (FAT/CD36) [17], and Cav-1 [18-22]. CD36 is an 88 kDa protein that is abundantly expressed in endothelial cells, smooth muscle cells, and macrophages [23]. CD36 increases saturable, high-affinity fatty acid uptake when overexpressed [24] and decreasing CD36 by knocking it out decreases fatty acid uptake [25].

It is possible that caveolae may play a role in CD36 mediated fatty acid uptake. Several studies demonstrate an association between CD36 and Cav-1. For example, CD36 localizes to caveolae and interacts with Cav-1 [26-29]. Cav-1 and FAT/CD36 were shown to be enriched in detergent-resistant membranes isolated from cholesterol-loaded alveolar type II cells, suggesting that CD36 is possibly located in caveolae or lipid rafts at the plasma membrane level [30]. Not only is it possible that Cav-1 and CD36 are associated with each other, but Cav-1 may be responsible for the localization of CD36 in the cell [26, 31, 32]. This suggests that caveolae may be associated with the process of transporting fatty acids across the cell membrane and in the normal functioning, transport, and expression of CD36.

Lipotoxicity occurs when excess lipids accumulate in non-adipose tissues and results in cell dysfunction or apoptosis [33]. Lipotoxicity has been shown to occur in a variety of tissue types [34-40]. We have previously demonstrated that VSM has a limited capacity to store fatty acids as triglycerides and that it may be susceptible to lipotoxicity [41]. Since caveolae and lipid rafts may contain CD36, then it is possible that modulating Cav-1 expression will have an effect on the amount of fatty acid taken up into the cell and thus play a role in lipotoxicity in VSM. Therefore, we hypothesize that overexpression of Cav-1 will redistribute CD36 to the cell membrane. This will result in an increase in fatty acid uptake and thus, an increase in fatty acid-induced apoptosis.

Materials and methods

Cell Culture

A7r5 VSM cells from rat aorta (American Type Culture Collection, Manassas, VA) were grown in 75-cm2 culture flasks and on Lab-Tek II chambered coverglass (Nalge Nunc International, Rochester, NY) in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) that contained 5.5 mM D-glucose, 26.2 mM NaHCO3, 1 mM C3H3O3Na, and 4 mM L-glutamine. DMEM was supplemented with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY) and 1% antibiotic/antimycotic solution (Sigma). Cells were incubated in a 5% CO2/humidified chamber at 37°C. The media was changed every two days to avoid microbial contamination.

Transfection with caveolin-1 cDNA

A7r5 cells grown to about 80% confluency in T-75 flasks were transfected with PCI-NEO vector containing the coding sequence for human Cav-1 (generously provided by Dr. Eric Smart, University of Kentucky) using a Tfx delivery solution. Tubes containing 7.5 ml serum free culture media (no antibiotic/antimycotic solution) and 19.95 µg of plasmid were prepared (control cells were transfected with the PCI-NEO vector without the coding sequence for human Cav-1). A tube containing only 7.5 ml serum free culture media was used for mock transfection. The tubes were vortexed and 89.7 µl of Tfx reagent (Promega, Madison, WI) was added to each tube. The tube was vortexed again and incubated at 25°C for 10-15 minutes. The media from the T-75 flasks was discarded and the Tfx/DNA mixture was added. After 1 hour of incubation at 37°C, the Tfx/DNA mixture was overlayed with 16.5 ml DMEM containing serum and incubated for 48 hours at 37°C.

Selection for stably expressing A7r5 cells

The antibiotic G-418 Sulfate (Promega) was added to DMEM to select for transfected cells. Two days after cells were transfected, the media was changed to media containing G-

418 (500 μg/ml in DMEM). When the cells from the mock transfection were all dead, the G-418 in the media was reduced to 200 μg/ml in DMEM.

Cell lysate preparation

Cells were harvested from flasks with trypsin-EDTA (Sigma) and centrifuged at 1000 x g for 5 minutes. Cells were resuspended in 200 μl ice-cold lysis buffer (pH 6.4) containing 0.12 M octyl β-D-glucopyranoside, 2% (v/v) Triton X-100, 0.3 M NaCl, and 49.7 mM 2N-morpholino ethanesulfonic acid. The cells plus lysis buffer were incubated at 4ºC for 30 minutes and then sonicated (ELMA sonicator Transsonic Digital Sonicator) for 10 minutes at maximum power. The lysate was centrifuged at 15,000 x g for 10 minutes at 4ºC. The supernatant was transferred to a centrifuge tube and stored at -20ºC.

SDS Page and Western Blot

The protein concentration of each sample was determined by Bradford Assay (Bio-Rad, Hercules, CA). Lysates each containing equal amounts of protein were separated by SDS-PAGE using a 4-12% Bis-Tris Criterion XT precast gel (Bio-Rad) and then electrotransferred to a 0.45 μm nitrocellulose membrane for Western blot analysis. The nitrocellulose paper was immunolabeled with purified rabbit anti-Cav-1 primary antibody (1:1000) (BD Transduction Laboratories, San Jose, CA) followed by alkaline phosphatase conjugated to anti-rabbit IgG secondary antibody (Rockland Immunochemicals for Research, Gilbertsville, PA). The nitrocellulose was developed in alkaline phosphatase developing buffer (Bio-Rad) to visualize protein bands.

Immunofluorescence labeling

A7r5 cells grown in chambered coverglass were fixed with 2% paraformaldehyde solution containing 350 mM NaCl, 160 mM HEPES, and 10 mM CaCl2. Fixed cells were then incubated in a permeabilization solution (500 μM β-escin, 150 mM NaCl, and 15 mM Na-citrate) containing 1% normal donkey serum (Sigma, St. Louis, MO). Cells were then incubated overnight in permeabilization solution with 1% normal donkey serum (Sigma), goat polyclonal CD36 IgG (1:100) (Santa Cruz Biotechnologies, Santa Cruz, CA) and mouse anti-Cav-1 IgG (1:100) (BD Transduction Laboratories). After incubation with both primary antibodies, cells were incubated for 3 hours in permeabilization solution containing 1% bovine serum albumin (BSA), donkey anti-mouse IgG conjugated to Alexa-488 (green) (1:100) (Molecular Probes, Eugene, OR) and donkey anti-goat IgG conjugated to Alexa 568 (red) (1:100) (Molecular Probes). Cells were rinsed with a solution containing 150 mM NaCl, 15 mM Na-citrate, and 2% BSA. 300 μl of Mowiol 4-88, an anti-fade medium used for immunofluorescence, was added to the chambers.

Confocal microscopy and image analysis

Laser scanning confocal microscopy was performed by using the Bio-Rad Radiance 2000 (Bio-Rad) on an IX-70 inverted microscope (Olympus, Tokyo, Japan). The images were captured using a 60X water immersion objective and transmitted to a computer with the LaserSharp 2000 program (Bio-Rad). All fluorescence images were acquired using the excitation lasers (BioRad) of ArKr/Ar 488 (for green) and Kr/Ar 568 (for red) and the emission filters (BioRad) of 515±30 nm (for green) and 600±40 nm (for red). The transmitted light images were acquired with Nomarski using a 637-nm red diode laser. Image acquisition was performed in the x, y, and z dimensions with x-y resolution of 0.09 μm and with z steps of 0.30 μm for all fluorescence images. The magnification (zoom), laser iris, gain, and offset parameters were optimized for the laser and were kept constant for each image. MetaMorph software (Universal Imagine, Chesterfield, PA) was used for image processing after acquisition. For each image, the central z plane was taken and background noise was removed by the median filter. The ratio of overlapping signals from the green-fluorescence and red-fluorescence images from each sample was reviewed by creating an overlay image. Line scans to determine pixel intensity across the cell and colocalization analysis was performed using the MetaMorph software. The peripheral to central ratio was calculated by taking the average of the pixel intensity from the line scan in the first and last 25% of the cell (line drawn through the perinuclear region) divided by the pixel intensity from the line scan from the middle 25% of the cell.

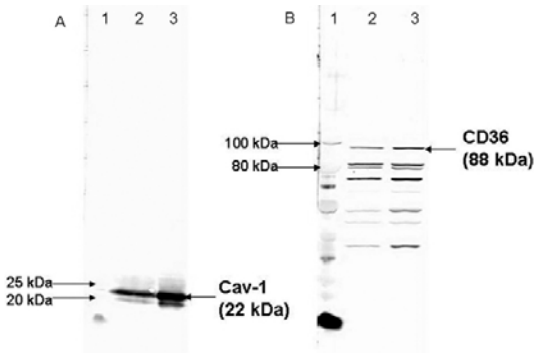

Figure 1: Transfection of A7r5 cells with a plasmid encoding a gene for Cav-1 resulted in an increase in A) Cav-1 protein (about 22 kDa) and B) CD36 protein (about 88-90 kDa). A molecular weight marker (lane 1), protein from control cells (lane 2), and protein from Cav-1 overexpressing cells (lane 3) were electrophoresed and subjected to Western blot. The blot was labeled with Cav-1 primary antibody in A) and CD36 primary antibody in B). Extra bands in B) are due to non-specific binding of the CD36 antibody.

Fatty acid uptake

A7r5 cells grown to about 70% confluency in T-25 flasks were rinsed with PBS (phosphate buffered saline), harvested with 1X trypsin-EDTA solution (Sigma), and then centrifuged. Cells were resuspended in 2 μM of C1-BODIPY 500/510-C12 (Molecular Probes, Carlsbad, CA), a fluorescent long-chain fatty acid analong, and incubated for 5, 10, and 15 seconds. Fatty acid uptake was stopped by adding ice-cold 4% paraformaldehyde in PBS (pH 7.4) and incubated for one hour on ice in the dark. For the zero time point, C1-BODIPY-C12 and paraformaldehyde were pre-mixed and added to the cells at the same time. At the end of the one hour incubation, cells were centrifuged and resuspended in PBS. A FACScan flow cytometer (Becton Dickensen, San Jose, CA) with CellQuest software was used to determine fatty acid uptake. Fluorescence was collected and gated on size.

Apoptosis determination

A7r5 cells grown to about 70% confluency in T-75 flasks were incubated in DMEM (serum-free) containing 0.05 or 0.1 mM palmitate conjugated to albumin (6.8:1) for 24 hours. After the incubation, the media was taken off and detached cells in the media were centrifuged. Cells were harvested with 1X trypsin-EDTA solution (Sigma). The trypsinized cells were centrifuged and added to the collected detached cells. The cells were rinsed in PBS that contained 0.5% (w/v) BSA. The cells were then incubated in 1% (w/v) paraformaldehyde in PBS (pH 7.4) for 1 hour on ice in the dark. The cells were then centrifuged, rinsed in PBS with 0.5% (w/v) BSA, collected, and incubated in a permeabilization solution that contained 0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate in water for two minutes on ice. The cells were then centrifuged and rinsed in wash buffer from an APO-BrdU TUNEL apoptosis kit (Phoenix Flow Systems, San Diego, CA). The cells were incubated in a DNA labeling solution from the kit for 1 hour in a 37°C water bath. The cells were rinsed in a rinse buffer from the apoptosis kit, centrifuged, and resuspended in Fluorescein-PRB-1 from the kit in the dark for 30 minutes at 25°C. Propidium iodide (PI) with RNase was added to the cells and they were incubated for another 30 minutes at 25°C in the dark. A FACScan flow cytometer (Becton Dickensen) with CellQuest software was used to determine the percent apoptosis. Fluorescence was collected and gated on size. Instrument settings according to the apoptosis kit from Phoenix Flow Systems were used.

Statistical analysis

Statistics were calculated using Microsoft Excel using a one-tailed Student's t test for all data except for fatty acid uptake experiments. Statistics for fatty acid uptake results were calculated using SigmaStat using one way ANOVA with post hoc Student-Newman-Keuls test. p values ≤ 0.05 were considered significant. All values are expressed as mean ± standard error of the mean (SEM).

Results

To see if Cav-1 was overexpressed in A7r5 VSM cells, cells were transfected, and subjected to Western blot. **Figure 1a** shows that Cav-1 was successfully overexpressed in A7r5 cells. There was 65% more Cav-1 protein in cells overexpressing Cav-1 (lane 3) than control cells (lane 2). Overexpression of Cav-1 also resulted in an 88% increase in CD36 protein (lane 3) compared to control cells (lane 2) (**Figure 1b**).

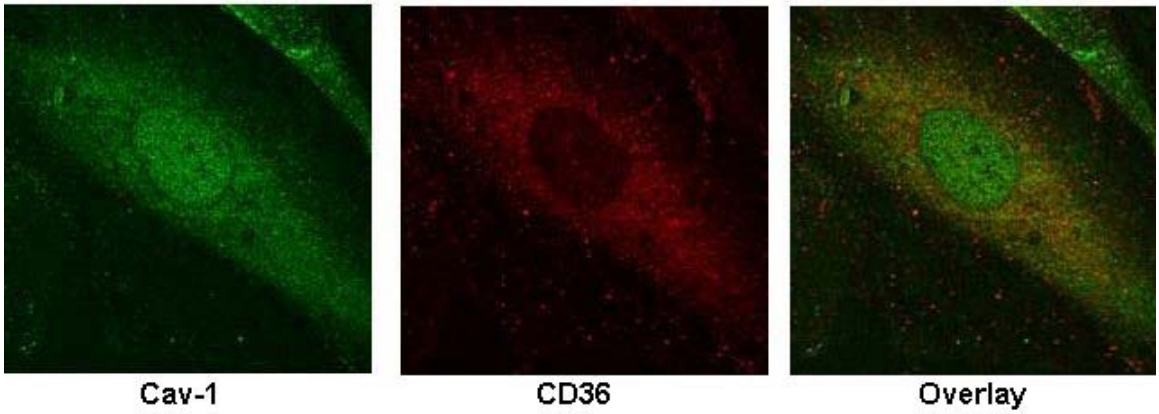

Figure 2: Normal A7r5 cells. Images were taken using confocal microscopy. Cells were immunolabeled with antibodies staining for Cav-1 (green) and CD36 (red). The central z plane in shown. Colocalization is indicated in yellow.

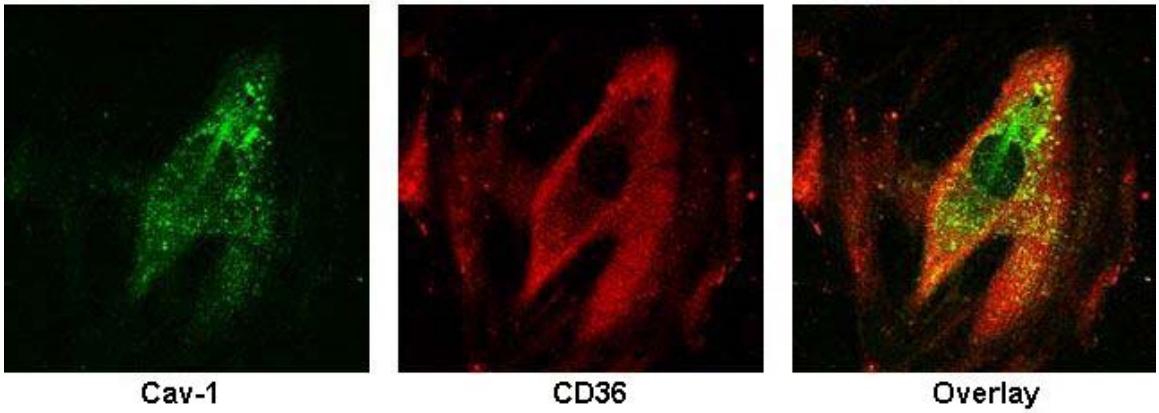

Figure 3: Overexpression of A7r5 cells with Cav-1. Images were taken using confocal microscopy. Cells were immunolabeled with antibodies staining for Cav-1 (green) and CD36 (red). The central z plane is shown. Colocalization is indicated in yellow.

Several lines of evidence demonstrate that CD36 is associated with caveolae and lipid rafts [26-30] and may play a role in the distribution of CD36 inside the cell [26, 31, 32], suggesting that caveolae may play a role in CD36-mediated fatty acid uptake in other cell types. To see if overexpression of Cav-1 caused a redistribution of CD36 to the membrane in vascular smooth muscle cells, confocal microscopy was performed on control A7r5 cells, A7r5 cells overexpressing Cav-1, and A7r5 cells that had lipid rafts disrupted. As shown in **Figure 2**, control A7r5 cells had fairly uniform distribution of CD36 throughout the cytoplasm. Cav-1 in control cells, on the other hand, had a punctate appearance throughout the cell. A7r5 cells were transfected with a plasmid overexpressing Cav-1 to see if more Cav-1 caused a change in CD36 distribution throughout the cell. According to **Figure 3**, Cav-1 overexpression resulted in movement of CD36 towards the cell membrane. Cav-1, however, retained its punctate distribution throughout the cell and appeared to relocate to internal compartments.

Cyclodextrin treatment results in the disruption of lipid rafts in cells. To see if disrupting lipid rafts and caveolae had the opposite results of overexpression of Cav-1 in VSM cells, we incubated A7r5 cells in 10 mM cyclodextrin for 30 minutes. Cells that were treated with

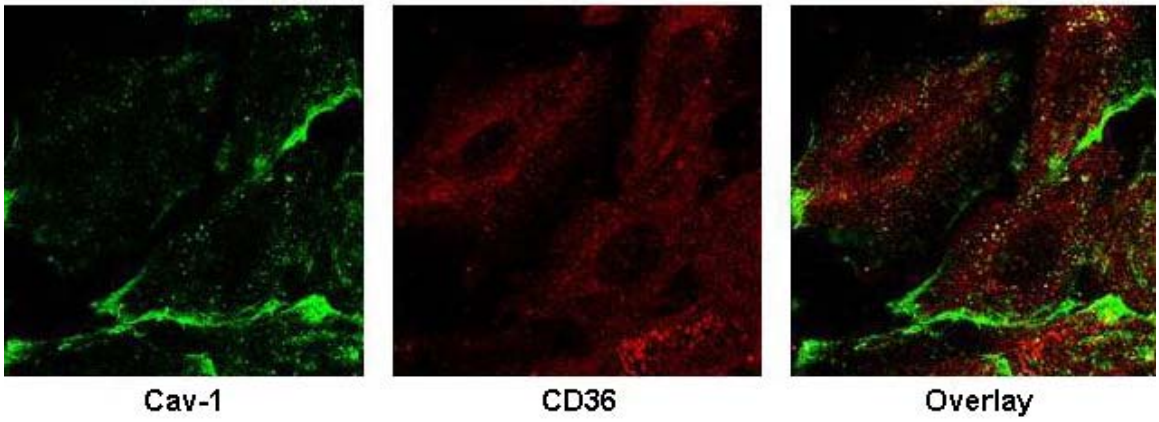

Figure 4: Disruption of caveolae and lipid rafts with 10 mM cyclodextrin for 30 minutes. Images were taken using confocal microscopy. Cells were immunolabeled with antibodies staining for Cav-1 (green) and CD36 (red). The central z plane is shown. Colocalization is indicated in yellow.

cyclodextrin had movement of CD36 towards the nucleus (**Figure 4**) compared to control cells (**Figure 2**). Cav-1 distribution in cyclodextrin treated cells no longer had the punctate distribution that was observed in control cells. Instead, Cav-1 appeared to be localized to different parts of the cell membrane. Quantification of the amount of overlap in both Cav-1 overexpressing and cyclodextrin-treated cells showed that cyclodextrin treatment resulted in less Cav-1 overlapping CD36 (20.61±6.06%, n=4) than control cells (48.17±7.96%, n=4) and Cav-1 overexpressing cells (32.34±8.40, n=4) (**Figure 5**).

The distribution of CD36 in the images was quantified by performing intensity line scans in the perinuclear region of the cell. The average of the intensity of fluorescence of CD36 in the first and last 25% of the line scan was divided by intensity of fluorescence of CD36 in the middle 25% of the line scan to get the peripheral to central intensity ratio. There was

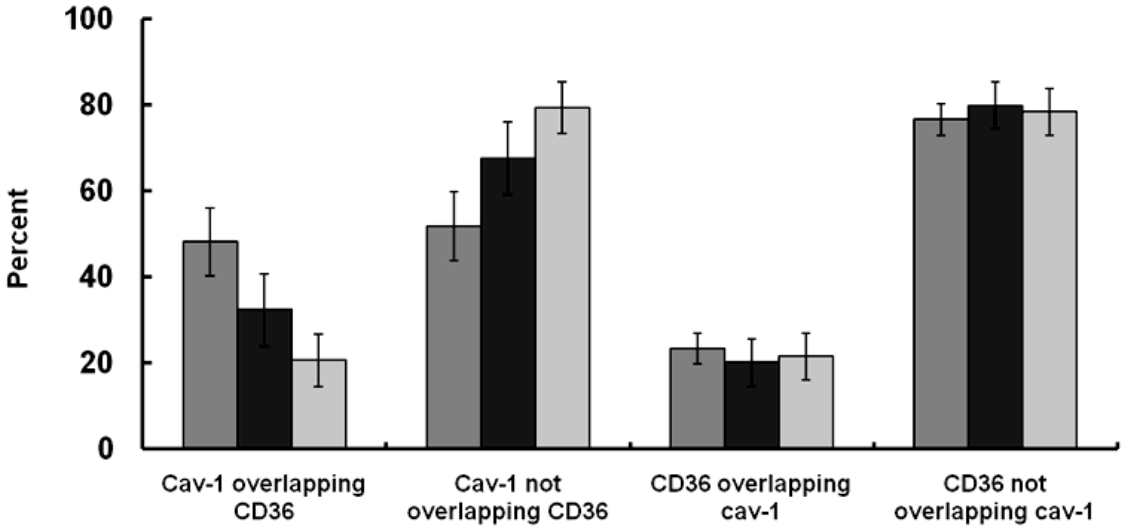

Figure 5: Colocalization data for normal A7r5 cells (dark gray bars), A7r5 cells overexpressing Cav-1 (black bars), and A7r5 cells treated with 10 mM cyclodextrin for 30 minutes (light gray bars). Results are expressed as the mean±SEM. n=4 for each group.

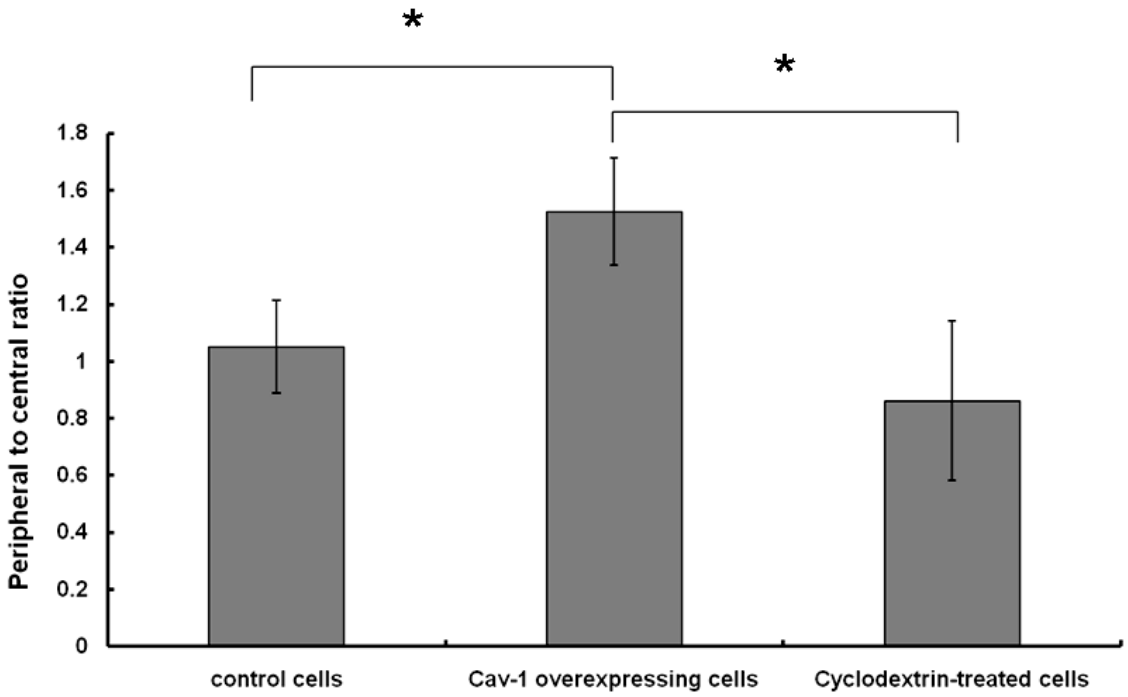

Figure 6: Overexpression of Cav-1 in A7r5 cells significantly (p=0.035) increased the peripheral to central ratio of CD36 compared to control. Results are expressed as the mean±SEM. n=4 for each group. The symbol * denotes that p<0.05.

a significant increase in CD36 in the periphery of cells overexpressing Cav-1 compared to control cells (p=0.035) (**Figure 6**). However, there was not a significant decrease in the peripheral to central ratio in cyclodextrin treated cells compared to control cells (**Figure 6**). Thus, overexpression of Cav-1 in VSM cells results in a movement of CD36 to the cell membrane, but the disruption of the lipid rafts did not alter the distribution.

CD36 is a membrane protein that is involved in fatty acid uptake [17]. If overexpression of Cav-1 results in the movement of CD36 from the interior of the cell to the plasma membrane, then it is possible there may be an increase in fatty acid uptake due to increased CD36 in the membrane. A fluorescent long-chain fatty acid analogue, C1-BODIPY-C12, has been used to demonstrate fatty acid uptake in Saccharomyces cerevisiae [42]. The length of this analog is approximately the length of a 16 carbon fatty acid and can be used to represent long chain fatty acids [42]. To see if overexpression of Cav-1 resulted in an increase in the rate of fatty acid uptake, control cells, Cav-1 overexpressing cells, and cells treated with 10 mM cyclodextrin for 30 minutes, were incubated in 2 μM C1-BODIPY-C12 for 0, 5, 10, and 15 seconds. The rate of fatty acid uptake was calculated by subtracting the amount of fluorescence at 5, 10, and 15 seconds by the amount of fluorescence at 0 seconds. According to **Figure 7**, control A7r5 cells seemed to have a higher rate of fatty acid uptake compared to cells overexpressing Cav-1. As expected, cyclodextrin treatment appeared to have the lowest rate of fatty acid uptake compared to control cells and cells overexpressing Cav-1. Therefore, overexpression of Cav-1 may result in a decrease in the rate of fatty acid uptake in VSM cells.

We have previously shown that excess fatty acid given to VSM cells does not get stored as triglycerides and results in lipoapoptosis in these cells [41]. An increase in fatty acid uptake, should, therefore, cause an increase in apoptosis. We used control cells and cells transfected with Cav-1 and incubated the cells in albumin or albumin conjugated to 0.05 mM

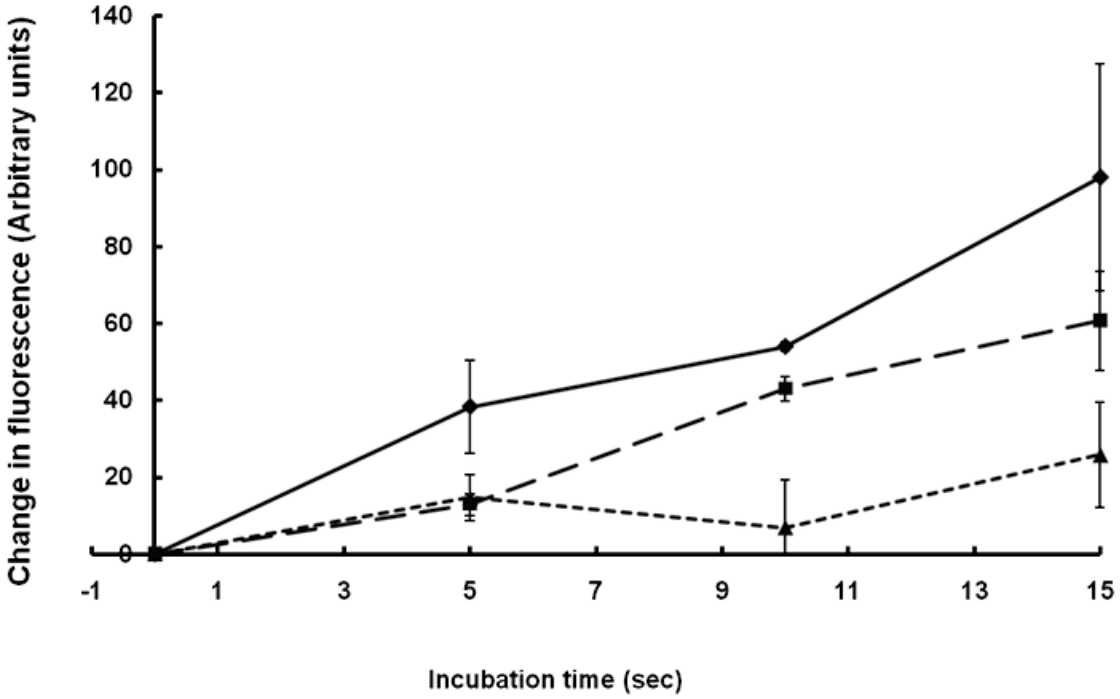

Figure 7: Rate of fatty acid uptake in control A7r5 cells (solid line), A7r5 cells overexpressing Cav-1 (line with long dashes), and A7r5 cells treated with 10 mM cyclodextrin for 30 mintes (line with short dashes). Fatty acid uptake was determined with flow cytometry. Results are expressed as the mean±SEM. n=3 for each group.

palmitate (6.8:1 ratio) for 24 hours. Cells overexpressing Cav-1 incubated in palmitate had a significant increase in apoptosis compared to control cells incubated in palmitate (p=0.044) (**Figure 8**). Thus, overexpressing Cav-1, which results in a redistribution of CD36 to the membrane, also results in an increase in lipoapoptosis.

To see if disrupting lipid rafts with cyclodextrin had the opposite effects on apoptosis, control cells and cells treated with 10 mM cyclodextrin for 30 minutes were incubated in albumin and 0.1 mM palmitate (conjugated to albumin in a 6.8 to 1 ratio) for 24 hours. Disruption of lipid rafts with cyclodextrin resulted in a significant decrease in the amount of apoptosis (p=0.003) (**Figure 9**). Therefore, disruption of CD36 in lipid rafts causes a decrease in fatty acid uptake and a decrease in lipoapoptosis.

Discussion

Several pieces of evidence demonstrate that CD36, a protein involved in fatty acid uptake, may be associated with caveolae and lipid rafts [26-30]. However, this has yet to be elucidated in VSM cells. We have previously shown for the first time that VSM cells are susceptible to lipotoxicity [41]. In this study we hypothesized that modulation of CD36 expression in response to changes of Cav-1 expression or disruption of lipid rafts with cyclodextrin would alter lipid uptake and lipotoxicity in VSM cells.

We demonstrated that overexpressing Cav-1 in cultured VSM cells results in a redistribution of CD36 to the periphery of the cell and an increase in the amount of CD36 protein in the cells. Our data in VSM cells supports work done in other cell types that also demonstrate both a redistribution of CD36 and an increase in CD36. For example, Frank et al. showed that Cav-1 overexpression causes translocation of CD36 to the cell membrane and increased CD36 expression several-fold in HEK-293T cells and that cotransfection of Cos-7 cells

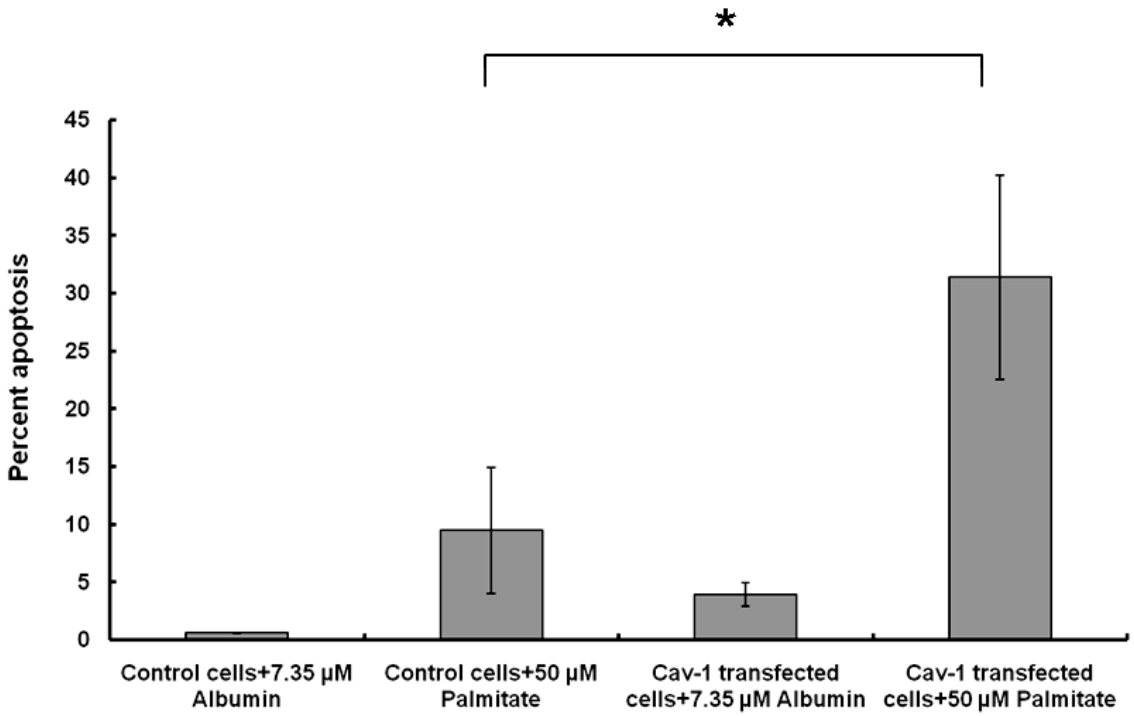

Figure 8: A7r5 cells overexpressing Cav-1 and incubated in 0.05 mM palmitate had significantly (p=0.044) more apoptosis than control cells incubated in 0.05 mM palmitate. Apoptosis was determined with flow cytometry. Results are expressed as the mean±SEM. n=4 for each group. The symbol * denotes that p≤0.05.

with Cav-1 and CD36 resulted in the targeting of CD36 to the plasma membrane [26]. In skeletal muscle cells, CD36 is located in an intracellular pool, from which it can translocate to the sarcolemma [31, 32], suggesting that there is a translocation mechanism for increasing fatty acid uptake by CD36 in muscle cells [32, 43]. In our experiments, disrupting caveolae and lipid rafts with cyclodextrin resulted in a trend of CD36 moving towards the nuclear region of the cell. This supports data in COS-7 cells where, in the absence of Cav-1, CD36 was retained intracellularly in a perinuclear compartment [26]. How Cav-1 targets CD36 to the plasma membrane is not known, especially since there was not substantial colocalization of CD36 and Cav-1 observed in our study. In skeletal muscle cells, CD36 colocalized with the muscle-specific caveolae marker Cav-3 in the sarcolemma, indicating that caveolae may regulate cellular fatty acid uptake by CD36 in skeletal muscle [44]. Thus, it is possible that Cav-3 or perhaps Cav-2 may play a more direct role in the translocation of CD36 to the plasma membrane. While the effects of Cav-1 on CD36 distribution may be indirect, they nevertheless occur and any condition in vivo that alters Cav-1 expression would be expected to also alter CD36 distribution.

In both control cells and Cav-1 overexpressing cells, Cav-1 had a punctate and apparently random distribution throughout the cytoplasm. This was also shown in Chinese hamster ovary (CHO) cells where confocal microscopy revealed a punctate pattern for Cav-1 and a smooth, homogenous pattern for CD36, with little overlap between the two proteins [45]. In Cav-1 overexpressing cells, there was a decrease in the percentage of Cav-1 overlapping CD36. This is possibly due to an increase in the amount of Cav-1 in the cell from transfection and because more CD36 is located in the plasma membrane than inside of the cell and is consistent with an indirect effect of Cav-1 on CD36 distribution.

CD36, which has been associated with caveolae and lipid rafts [26-30], is a protein involved in fatty acid uptake [17]. In our study, we observed an increase in palmitate-induced

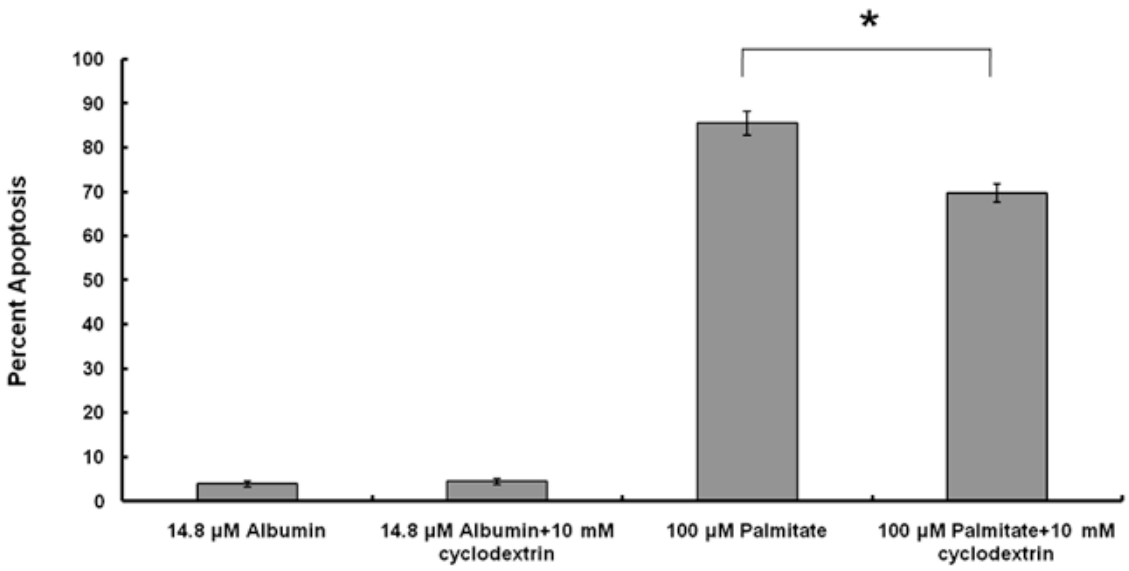

Figure 9: Cyclodextrin treatment (10 mM for 30 minutes) resulted in a significant (p=0.003) decrease in palmitate-induced apoptosis in A7r5 cells overexpressing Cav-1. Apoptosis was determined with flow cytometry. Results are expressed as the mean±SEM. n=4 for each group. The symbol * denotes that p≤0.05.

apoptosis with Cav-1 overexpression, which may be due in part to the redistribution of CD36 to the cell membrane. Theoretically, if more CD36 were in the plasma membrane, then there would be an increase in fatty acid uptake in the cells. However, we found that there was a decrease in the rate of fatty acid uptake in Cav-1 overexpressing cells compared to control cells. This contradicts data in other cell types that demonstrate that an increase in CD36 results in an increase in fatty acid uptake [24] and knocking down CD36 results in a decrease in fatty acid uptake [25]. There are several possible explanations why there would be a decrease in the rate of fatty acid uptake in cells overexpressing Cav-1 compared to control cells. It has been shown that caveoale are also involved in the export of lipid from cells [46]. One possible explanation is that during Cav-1 overexpression there actually is an increase in unidirectional fatty acid uptake, but at the same time there is also an even greater increase in fatty acid unidirectional export due to the increase in caveolae and CD36 in the membrane, thus making the net rate of fatty acid uptake lower.

The second possible explanation for the decrease in the rate of fatty acid uptake in Cav-1 overexpression cells is that the increased caveolae which results from increasing Cav-1 expression results in a decrease in the amount of space available in the plasma membrane for lipid rafts. We observed a decrease in colocalization between CD36 and Cav-1 in Cav-1 overexpressing cells, but yet there was still movement of CD36 towards the plasma membrane. Thus, it is possible that CD36 is targeted mainly to non-caveolar lipid rafts. If there are less lipid rafts, then there are less places for CD36 to be inserted in the plasma membrane and thus less fatty acid uptake into the cell. However, because there is an increase in caveolae, which is also involved in fatty acid uptake [18-22], there would still be an increase in fatty acid uptake during longer time periods, which would explain the increase in lipoapoptosis that we observed at 24 hours.

Along the same lines of space constraints, it is possible that overexpressing Cav-1 will increase the amount of all lipid-raft containing compartments, not just in the plasma membrane. This would cause an increase in the number of intracellular caveosomes and may explain the more punctate appearance of Cav-1 labeling in the confocal images of Cav-1 overexpressing cells. If this is the case, then the increase in Cav-1 containing

compartments would result in less room for CD36 in the perinuclear region of the cell, pushing it towards the periphery. However, this does not imply that CD36 is actually inserted in the plasma membrane. If it is not, and is only located towards the periphery of the cell, then this could be another explanation for the decrease in fatty acid uptake in Cav-1 overexpressing cells.

We found that disrupting lipid rafts with cyclodextrin decreased the rate of fatty acid uptake. This supports other studies which show that dissociation of caveolae with by removing cholesterol with a sterol-binding agent significantly reduce fatty acid uptake [19, 47] and removal of cholesterol from the membrane by cyclodextrin and knocking down caveolae with a dominant negative mutant results in disassembly of caveolae structures and a time-dependent reduction of oleate and stearate incorporation [28].

We have previously shown that VSM cells are susceptible to lipotoxicity [41]. In this study, we found that overexpressing Cav-1 in VSM cells results in an increase in palmitate-induced apoptosis and disrupting caveolae and lipid rafts with cyclodextrin decreases the amount of apoptosis after 24 hours of incubation. This increase in lipotoxicity with Cav-1 overexpression is probably due to either an increase in the number of caveolae in the cell or to the increase in CD36 being relocated to the plasma membrane. In the long term, an increase in CD36 or caveolae would result in an increase in fatty acid uptake into the cell. If the increase in fatty acid uptake exceeds the amount of fatty acids that can be oxidized, then lipotoxicity can occur.

The results found in these experiments may have implications for diseases such as atherosclerosis and the metabolic syndrome. It has been previously shown that Cav-1 null mice have high fatty acids and triglycerides, decreased leptin, reduced glucose uptake, and reduced insulin receptor protein levels [48]. In young Cav-1 knockout (KO) mice there were elevated levels of triglycerides and free fatty acids [49]. In ApoE KO mice, knocking out CD36 expression protects against the development of atherosclerosis by reducing atherosclerotic lesions [50, 51] and CD36 KO mice have increased serum fasting levels of nonesterified FFA and show decreased uptake of oleate in isolated adipocytes [52]. These studies suggest that regulating Cav-1 and/or CD36 may modulate the progression of atherosclerosis and the metabolic syndrome.

VSM cells can exist in several different phenotypes [53, 54]. VSM cells that are in the proliferative phenotype have decreased caveolae [46]. Since VSM cells in the contractile phenotype have more caveolae and Cav-1, then, based on the conclusions from this study, contractile VSM cells may be more susceptible to lipotoxicity because of more uptake of lipid into the cell. Thus, during atherosclerosis, smooth muscle cells in the in the contractile phenotype in the medial layer, which is the dominant phenotype in a normal conductance artery [53, 54], would undergo lipotoxicity. This would theoretically increase the percentage of VSM cells in the proliferative phenotype, which could contribute to the proliferation of the arterial smooth muscle layer during atherosclerotic lesions. In ApoE/Cav-1 double KO mice, there was a drastic reduction in the area of atherosclerotic lesions [51]. Thus, mutations in Cav-1 that would result in a lack of function or absence of Cav-1 could possibly protect against proliferation of VSM cells in lesions and may be one of the reasons why there was a decrease in lesion size seen in the ApoE/Cav-1 double KO mice. Our data partially support this hypothesis by showing that cells overexpressing Cav-1 had an increase in lipotoxicity. However, our fatty acid uptake experiments do not support this hypothesis because we observed a decrease in fatty acid uptake. Further research needs to be done to elucidate whether or not there truly is an increase or decrease in fatty acid uptake, particularly at longer time points.

In conclusion, we have demonstrated, for the first time in VSM, that overexpressing Cav-1 results in a redistribution of CD36 to the plasma membrane and we have strong evidence that Cav-1 or CD36 or both may play a role in fatty acid uptake and lipotoxicity and may have implications for health and disease.

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