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CD36 Enhances Fatty Acid Uptake by Increasing the Rate of Intracellular Esterification but Not Transport across the Plasma Membrane

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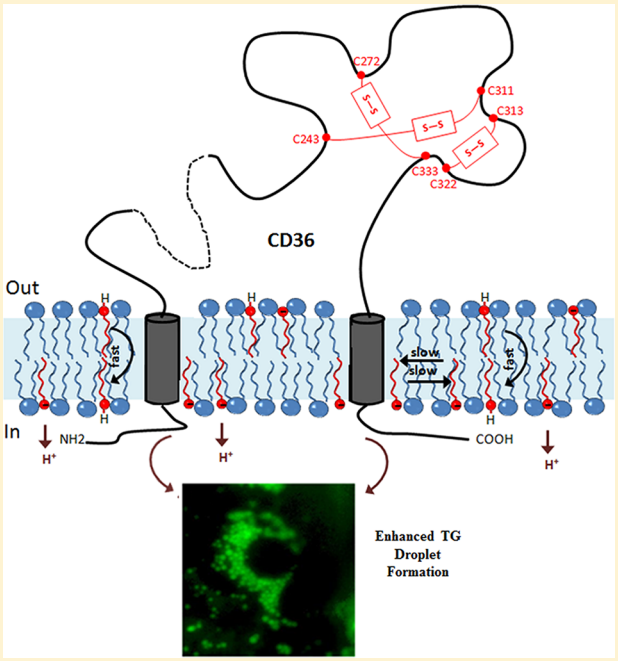
Supporting Information

ABSTRACT: CD36 is a multifunctional protein that enhances cellular fatty acid (FA) uptake, a key step in energy metabolism, and its dysregulation in multiple tissue sites is central to obesity-linked diabetes, a risk factor for atherosclerosis. Although CD36 has been implicated in FA uptake in a correlative way, the molecular mechanisms are not known. Their elucidation in cells is confounded by receptor-mediated uptake of low-density lipoprotein by CD36 and the competitive and/or contributive effects of other proteins involved in FA transport and metabolism, which include caveolin(s), fatty acid transport protein (FATP), intracellular fatty acid binding protein, and enzymes involved in the conversion of FAs to esters. Here we utilized a simpler cellular system (HEK cells), which lack caveolin-1, CD36, and FATP and metabolize FAs slowly compared to the time frame of transmembrane FA movement. Our previous studies of HEK cells showed that caveolin-1 affects FA binding and translocation across the plasma membrane and but not FA esterification [Simard, J. R., et al. (2010) *J. Lipid Res. 51* (5), 914−922]. Our key new finding is that CD36 accelerates FA uptake and extensive incorporation into triglycerides, a process that is slower (minutes) than transmembrane movement (seconds). Real-time fluorescence measurements showed that the rates of binding and transport of oleic acid into cells with and without CD36 were not different. Thus, CD36 enhances intracellular metabolism, i.e., esterification, and thereby increases the rate of FA uptake without catalyzing the translocation of FA across the plasma membrane, suggesting that CD36 is central to FA uptake via its effects on intracellular metabolism.



Fatty acids (FAs) play important roles in a variety of cellular functions, such as the production and storage of energy, synthesis of phospholipids, and regulation of gene expression in the nucleus. Because most cells, except for adipocytes and muscle cells, have a very limited capacity for storing FAs as triglycerides (or for *de novo* synthesis of FAs), FA supplied from the plasma is the most important means of fat intake. Therefore, deciphering the mechanisms by which FAs are taken up by cells is crucial for our understanding of the roles of FAs in regulating cellular homeostasis.

As an essential nutrient, FAs enter cells for the utilization by several pathways, which include transfer from plasma albumin, receptor-mediated uptake of low-density lipoprotein (LDL) [mainly oxidized LDL (oxLDL)], and hydrolysis of triglycerides in triglyceride-rich lipoproteins at capillary endothelial cells. The multifunctional protein CD36 has been implicated in all three of these mechanisms.[2,3]

Mechanisms for CD36 catalysis or direct involvement in the transfer of unesterified FA across the plasma membrane have been studied for decades but have remained elusive. Although CD36 has been linked to enhanced uptake of FA into several cell types, especially adipocytes,[4−7] it is not clear whether this occurs by accelerated flux of FA across the plasma membrane, enhancement of FA metabolism, cell signaling, or a combination of these mechanisms. By contrast, the more recently discovered family of transporters, the FATPs, were shown to activate FA and hence drive uptake by catalyzing formation of acyl-CoA, the first metabolic precursor for the utilization of FA inside cells.[8−10] This activation provides both a metabolic and a biophysical explanation, because the acyl-CoA is very impermeable to the lipid bilayer of membranes[11−13] and

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hence is trapped on the cytosolic leaflet where it is formed. In contrast, the unesterified FAs are able to move rapidly and bidirectionally across cell membranes.[14]

We sought to gain new insights into how CD36 enhances the uptake of unesterified FAs into cells by separating contributions of transport of FA in the plasma membrane from intracellular utilization. Our strategy for achieving this encompassed both the design of the cell system and the methods applied. We previously developed an experimental cell system for studying the involvement of caveolins in the transport of FA in the plasma membrane of cells that have relatively limited FA metabolism. With HEK 293 cell lines containing virtually no endogenous CD36 or caveolin-1, we demonstrated enhanced binding of FA to the cytosolic leaflet with an increased level of expression of caveolin-1.[1] In this study, we expressed CD36 in HEK cells without expression of caveolin-1. We used fluorescence methods to monitor binding of oleic acid to the extracellular membrane leaflet and its movement to the cytosolic compartment in real time. Metabolism was monitored over time by quantifying esterification products of [^{14}C]oleic acid. We then used the fluorescent FA analogue BODIPY 500/510 to visualize lipid droplet formation in HEK293 cells with and without expression of CD36.

EXPERIMENTAL PROCEDURES

Materials. All reagents for buffer solutions were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Oleic acid (99% pure) was purchased from Sigma-Aldrich and [^{14}C]oleic acid from Perkin-Elmer (Boston, MA). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dii-oxLDL) was purchased from Biomedical Technologies Inc. (Stoughton, MA) and was used fresh within 2 weeks of purchase according to the supplier's recommendations. All cell culture reagents and fluorescent probes were purchased from Invitrogen (Carlsbad, CA) unless otherwise stated and restriction enzymes from New England Biolabs (Ipswich, MA). The rabbit anti-CD36 polyclonal antibody was purchased from Abcam (Cambridge, MA), the goat anti-rabbit Cy3 red fluorescent secondary antibody from Jackson ImmunoResearch (West Grove, PA), and the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) from Sigma-Aldrich, and fluorescent chemiluminescent reagents were purchased from Perkin-Elmer. Thin layer chromatography (TLC) plates (250 μm, 20 cm × 20 cm) with silica gel G were purchased from Fisher Scientific (05-719-800), and TLC bands were counted in UltimaGold cocktail (Perkin-Elmer, Shelton, CT) in a liquid scintillation counter.

Preparation of Buffers, Stock Solutions of Fatty Acids, and Fluorescent Probes. Fresh Mops-Krebs buffer was prepared (20 mM Mops, 1.1 mM $MgSO_4$, 5.1 mM glucose, 1.1 mM KH_2PO_4, 5 mM KCl, 118 mM NaCl, and 2.5 mM $CaCl_2$, with the final pH adjusted to 7.4). The following stock solutions were prepared in DMSO: 10 mM oleic acid, 1 mM BCECF-AM, 1.8 mM FPE, and 20 mM BODIPY 500/510 [4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid (D-3823)] (10 mM oleic acid and BODIPY 500/510 in a 5:1 ratio). A fresh 200 μg/mL Dii-oxLDL stock containing 0.3 mM EDTA in PBS (pH 7.4) was added directly to cell culture medium containing DMEM with 10% FBS and 1% Pen-Strep.

Generation of a HEK293 Stable Cell Line Expressing CD36. A pCMV-Sport 6.1 vector from Invitrogen and mouse CD36 cDNA were subcloned into an empty pCI-neo mammalian expression vector. For transfection, pCI-neo-CD36 DNA mixed with a 1:3 ratio of DNA to Lipofectamine 2000 (Life Technologies) was added dropwise to HEK293 cells. Forty-eight hours after transfection, cells were passaged, and 800 μg/mL G418 was added to the medium the following day. DMEM containing G418 was refreshed every 2 days until selection had been achieved. Cells that survived were grown under 800 μg/mL G418 for 2 weeks and expanded into multiple plates for maintenance. CD36 protein expression was assessed using Western blotting.

Subcellular Fractionation of HEK293-CD36 Cells Using a Sucrose Gradient. HEK293 cells cultured in 10 cm dishes were resuspended in TES buffer [20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 250 mM sucrose] containing 1× Halt protease inhibitors (Thermo Scientific) and scraped off the plates. The cell suspension was homogenized using Pyrex potter-Elvehjem tissue grinders followed by centrifugation at 16000*g* for 1 h. The supernatant was further centrifuged at 46000*g* for 1 h, yielding pellets containing high-density microsomes (HDM). The supernatant was centrifuged again at 46000*g*, yielding low-density microsomes (LDM) in the pellets. Pellets collected from these two steps were each dissolved in Tris-HCl buffer (pH 7.4) for future analysis. Pellets obtained from the first step of centrifugation were dissolved in TES buffer and layered in 41% sucrose followed by centrifugation at 86000*g* for 1 h. Pellets containing nuclei and mitochondria were collected and redissolved in Tris-HCl buffer. A clear visually detectable band at the interface was collected, diluted in TES buffer, and centrifuged at 86000*g* for 30 min. Pellets containing the plasma membrane were collected and redissolved in Tris-HCl buffer. Total protein concentrations of each fraction were determined using modified Lowry methods.

Culture and Differentiation of 3T3-L1 Adipocyte Cells. 3T3-L1 cells were maintained in preadipocyte growth medium containing DMEM supplemented with 10% calf serum and 1% Pen-Strep. Differentiation was induced 48 h after cells had reached confluence by replacing calf serum with FBS as well as by addition of an adipogenic cocktail containing 0.86 mM insulin, 51.7 mM isobutylmethylxanthine, and 1 mM dexamethasone. After 2 days, the medium was replaced with adipocyte growth medium using DMEM containing 10% FBS and 0.43 mM insulin, as described previously.[15]

Western Blotting. Total protein collected from HEK293 cells with or without subcellular fractionation was separated on a 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis gel and transferred to the PVDF membrane under 100 V for 1 h. The membrane was blocked in PBST buffer containing 5% BSA for 1 h at room temperature and washed with PBS buffer. The membrane was incubated with the primary rabbit polyclonal anti-CD36 antibody at a 1:3000 dilution overnight at 4 °C followed by washing and incubation for 1 h with the goat anti-rabbit HRP-conjugated secondary antibody at a dilution of 1:16000. The membrane was thoroughly washed with PBST buffer, briefly incubated with fluorescent chemiluminescent reagents, and processed by being exposed to film.

Flow Cytometry. Cells cultured in 10 cm dish monolayers were grown to ∼80% confluence in low-glucose DMEM containing 10% FBS and 1% Pen-Strep. To this serum-containing medium was added 10 μg/mL Dii-oxLDL for 30 min at 37 °C. The cells were then removed from the dishes using enzyme-free cell dissociation reagent and spun down at 300*g* for 60 s. The cells were then fixed by resuspension in PBS containing 4% paraformaldehyde for 5 min, pelleted, and then

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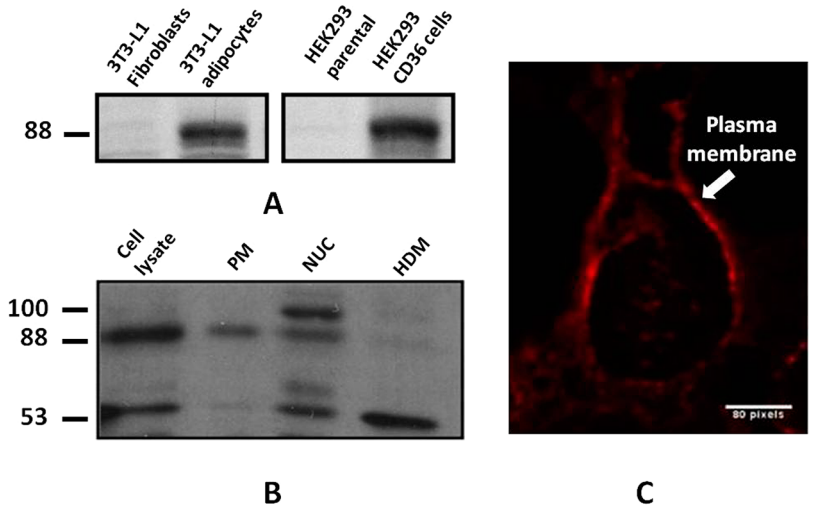

Figure 1. Western blot showing CD36 expression and localization. (A) In HEK293 cells, CD36 expression was comparable to that of 3T3-L1 adipocyte cells. (B) Cellular fractionation shows CD36 is expressed at molecular masses of ~53 and ~88 kDa in nuclei and the high-density membrane fraction at comparable levels. In the plasma membrane, CD36 is detected mainly at a molecular mass of ~88 kDa. (C) HEK293-CD36 cells growing on a culture were labeled with goat anti-CD36 and Cy3 conjugated secondary antibodies. Cells were photographed under a Nikon deconvolution wide-field epifluorescence system, and images were processed using ImageJ.

permeabilized for 5 min by being resuspended in 0.1% Triton X-100. A final wash was performed using PBS, and the cells were resuspended in PBS containing 1× Halt Protease Inhibitor Cocktail containing EDTA and counted using trypan blue exclusion, to normalize the cell numbers. A Benton Dickinson (Franklin Lakes, NJ) FACScan flow cytometer was used to quantify Dii-oxLDL fluorescence (excitation at 514 nm and emission at 550 nm), and data analysis was performed using BD Cellquest Pro version 5.2.

Immunofluorescence Staining. HEK293 cells were grown in poly-D-lysine-coated p35 glass-bottom culture dishes from MatTek (Ashland, MA). Cells were washed with ice-cold PBS buffer and then fixed with 4% paraformaldehyde at room temperature for 15 min. Cells were washed again with PBS buffer three times and blocked in 10% rabbit serum in TBST for 1 h followed by a 1 h incubation with the rabbit anti-CD36 primary antibody at a dilution of 1:500. Sera containing the primary antibody were washed with PBS buffer and incubated three times with the Cy3 fluorescent anti-rabbit secondary antibody in rabbit serum at a dilution of 1:1000. After being washed with PBS buffer three times, cells were mounted with ProLong Gold antifade reagent (Life Technologies) and covered with a cover slide. The stained cells were photographed using the Nikon (Melville, NY) deconvolution wide-field epifluorescence system, and images were processed using ImageJ.

Fluorescence Measurement of the Transport of FA across the Plasma Membrane. HEK cells cultured in 10 cm dish monolayers in DMEM-containing serum were incubated with 2 μM BCECF-AM [2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein] in DMSO for 30 min or with 10 μM FPE [*N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine] in DMSO for 1 h at 37 °C and then detached from the bottom by adding enzyme-free cell dissociation buffer, including 1× Halt protease inhibitors for 5 min, in the dark. The detached cells were spun down at 300*g* for 60 s and washed three times with 37 °C DPBS buffer containing 1× Halt protease inhibitors. Cells were then resuspended in Mops-Krebs buffer (pH 7.4) containing 1× Halt protease inhibitors in a final volume of 3 mL at a density of 5×10^5 cells/cuvette and placed in a Spex Fluoromax-2 fluorescence spectrometer from Jobin Yvon (Edison, NJ). To each 3 mL of cell suspension was added oleic acid in DMSO at a rate of 6 μL per addition from 10, 20, and 40 mM stocks to give concentrations 20, 40, and 80 μM, respectively. In a typical measurement, real-time monitoring of ratiometric BCECF fluorescence was conducted using dual excitation wavelengths (λ_1 = 490 nm, and λ_2 = 440 nm) at a fixed emission wavelength (535 nm). Real-time monitoring of FPE fluorescence was recorded using excitation and emission wavelengths of 496 and 519 nm, respectively.

Measurement of Fatty Acid Esterification. Cell suspensions were prepared following the same procedure that is described above for measuring the transport of FA across the plasma membrane. To quantify esterification products, 20 μM [^{14}C]oleic acid was added to suspended cells at 37 °C for individual time periods of 0.5, 30, 45, and 60 min, which were being shaken constantly. Metabolism was terminated by adding 1 mL of a stop solution (40:10:1 hexane/2-propanol/sulfuric acid) to the cells. Lipid components were extracted by adding 2 mL of hexane and 3 mL of H_2O and mixed by vigorous shaking. Samples were then centrifuged at 1000 rpm for 15 min; 50 μL of the total lipids lying on the top layer was taken for total liquid scintillation counting. To separate different lipid components, 25 μL of the lipid mixture was loaded onto a silica gel plate and thin layer chromatography was performed in a neutral tank containing a hexane/ethyl ether/acetic acid mixture (80:20:1) until the solvent was 1 cm from the top of the plate. The TLC plate was air-dried for 15 min and then stained with iodine vapor to identify the bands. After the evaporation of iodine, each individual lipid component was scraped off the silica gel plate and collected in a counting vial. Methanol was added to each vial via addition of 10 mL of Ecoscint solution for liquid scintillation counting. In parallel dishes, the amounts of total protein and phospholipids were determined by modified Lowry and Bartlett methods, respectively.

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Visualization of Lipid Droplet Formation in HEK293 Cells using BODIPY 500/510 and Oleic Acid. HEK293 cells grown in poly-D-lysine-coated p35 glass-bottom culture dishes from MatTek were incubated for 24 h with 20 μM BODIPY 500/510 and oleic acid (5:1 ratio), followed by three washes using PBS buffer, and then the cells were kept in serum-free DMEM. Cells were photographed using the Nikon deconvolution wide-field epifluorescence system and processed using ImageJ.

RESULTS

Characterization of the HEK293 Stable Cell Line Expressing CD36. We first characterized our HEK293 cell line stably expressing CD36 using Western blotting and immunofluorescent staining. As shown in Figure 1A, CD36 was not detected in HEK293 parental cells but in HEK293-CD36 transfected cells at a molecular mass of ~88 kDa, indicating that CD36 was overexpressed in HEK293 cells in the mature, glycosylated form. To determine whether the level of expression of CD36 in HEK293 cells was comparable to its level of endogenous expression in adipocytes, we also examined CD36 expression in 3T3-L1 fibroblasts and adipocytes. As expected, CD36 was not detected in the fibroblasts but only in the induced, differentiated adipocytes (Figure 1A). The intensity of the CD36 band detected from the HEK293-CD36 cells was comparable to that of 3T3-L1 adipocytes, normalized to β-actin (data not shown), indicating that CD36 was expressed in HEK293 cells at or near physiological levels.

We further examined localization of CD36 in HEK293-CD36 stable cells using subcellular fractionation and immunofluorescent staining. As shown in Figure 1B, CD36 was detected in all fractions. In the nucleus, CD36 migrated at molecular masses of ~53 and ~88 kDa, corresponding to the unglycosylated and glycosylated forms, respectively, while in the plasma membrane fraction, CD36 was detected mainly in the glycosylated form. In the high-density microsomal fraction, CD36 was detected only in the deglycosylated form. This was also expected, as high-density microsomes contain mainly the endoplasmic reticulum (ER) where CD36 was first synthesized. Post-translational modification occurred during translocation of CD36 from the ER to the plasma membrane. To confirm this finding, we used immunofluorescent staining to visualize CD36 expression. As shown in Figure 1C, by using an anti-CD36 antibody combined with a secondary antibody conjugated with a Cy3 fluorescent probe, we detected the red fluorescent signal mainly on the plasma membrane, further confirming that CD36 was expressed on the plasma membrane.

We then established that CD36 in our cells functioned to take up oxLDL, because this is one of the well-established roles of mature, glycosylated membrane-localized CD36 protein.[16,17] An assay was designed to test the functionality of CD36 expressed in the HEK293 cell line by the ability CD36 expressed in these cells to take up fluorescent Dii-oxLDL, as compared to control HEK293 cells. As shown in Figure 2, the same number of cells expressing CD36 incorporated more fluorescent Dii-oxLDL (red) than control cells (green) that did not express CD36. Because numerous other oxLDL scavenger proteins exist,[18] the low level of Dii-oxLDL uptake in the control HEK293 cells is likely due to low-level expression of one or more of these other scavenger receptors, with some cells probably expressing almost no level of scavenger receptors, as seen by the lowest signal spike in the control cells. Alternatively, the control cells may represent the background

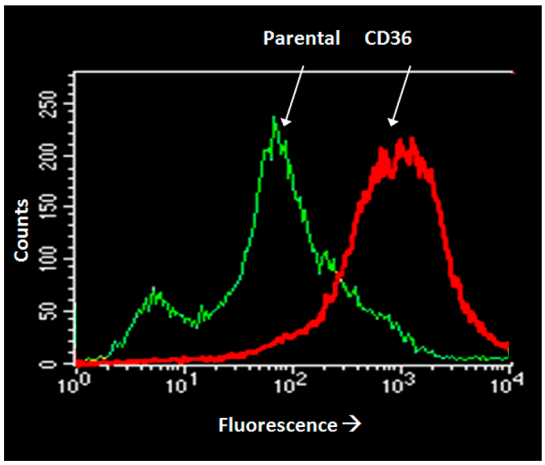

Figure 2. Flow cytometry measurement of fluorescence uptake of Dii-oxLDL. Using 10 μg/mL Dii-oxLDL (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), HEK293 parental cells and HEK293 cells that express CD36 were incubated for 30 min in serum-containing medium. Data for HEK293 control cells (green) and HEK293 cells that express CD36 (red) are shown.

level of fluorescence, while the cells with CD36 give a significantly stronger signal.

Kinetic Measurement of the Transport of Fatty Acid across the Plasma Membrane of the Cells Using Multiple Fluorescence Probes. Adsorption of FA to the plasma membrane was measured by using the fluorescent probe FPE, which is incorporated into the outer leaflet of the phospholipid bilayer and reports changes in membrane surface potential. As characterized in model membranes and cells, the arrival of FA causes a decrease in FPE fluorescence.[19] Our results (Figure 3A,B) showed that FPE fluorescence decreased rapidly and immediately upon addition of exogenous oleic acid to both HEK293 parental and HEK293-CD36 cells, indicating that adsorption of oleic acid to the cells was fast and independent of CD36 expression.

After the initial decrease, FPE fluorescence remained constant over the next few minutes in HEK293 parental cells. In contrast, after the initial fast decrease in FPE fluorescence, HEK293-CD36 cells exhibited a small but consistent slow increase as the FPE fluorescence returned toward the baseline after the initial decrease. This indicates a net decrease in the amount of FA anions in the phospholipid headgroup region where they are detected by the fluorescein group of FPE.[19]

To follow the temporal transport of the added oleic acid after its rapid binding had been established, we further monitored the arrival of oleic acid at the cytosolic leaflet by using the fluorescent intracellular pH indicator BCECF. As previously described, BCECF detects protons released into the cells after movement of FA in its uncharged form across the lipid bilayer.[20−22] As shown in panels A and B of Figure 4, BCECF fluorescence decreased rapidly after addition of 40 μM oleic acid in both HEK293 parental and HEK293-CD36 cells.

With lower concentrations of added OA, the same rapid decrease was observed but with a smaller total decrease in fluorescence, as expected (Supporting Information). These results suggested that the overall delivery of oleic acid into the cells was rapid and independent of overexpression of CD36 on the plasma membrane. After the initial rapid decrease, BCECF fluorescence remained steady within the same time frame of monitoring in both types of cells, suggesting that by

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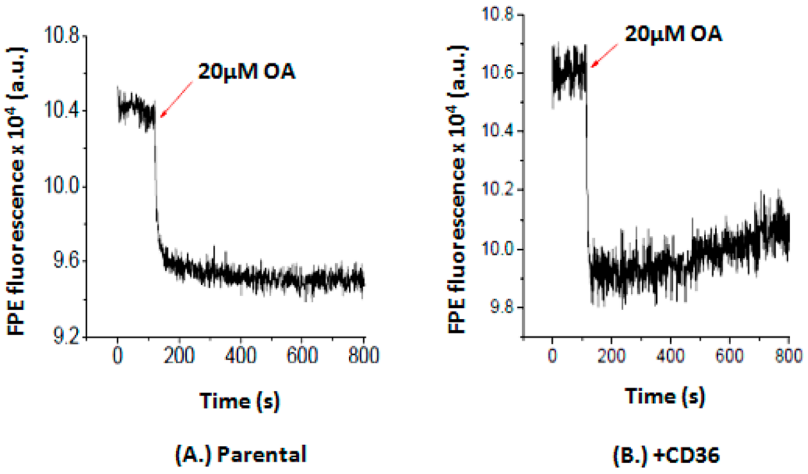

Figure 3. Detection of binding of oleic acid to the plasma membrane using FPE. FPE fluorescence was recorded before and after addition of 20 μM oleic acid to (A) HEK293 parental cells and (B) HEK293-CD36 stable cells. The fast decrease in FPE fluorescence (within seconds) was typical for all concentrations studied (10–40 μM) and was quantitatively similar for both cell lines. The fluorescence intensity is an arbitrary unit and is subject to many experimental variations that are difficult to control. Therefore, for our study, the extent of the decrease was compared qualitatively.

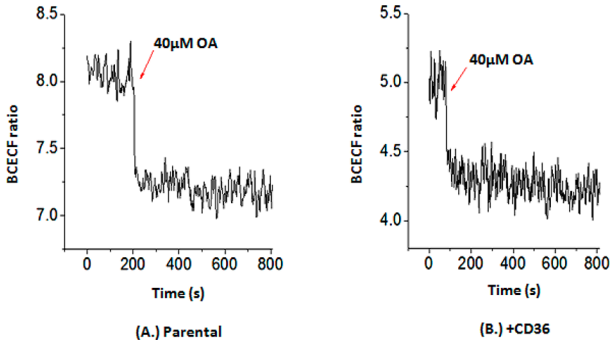

Figure 4. Measurement of the transport of oleic acid across the plasma membrane using BCECF-AM. Oleic acid (40 μM) was added to (A) HEK293 parental cells and (B) HEK293CD36 stable cells, and the ratio of the BCECF fluorescent signal at dual wavelengths was monitored before and after the addition of oleic acids. See the Supporting Information for dose–response data.

comparison to rat adipocytes,[23] intracellular utilization of the added oleic acid was not extensive in either type of cell within the short time period monitored by fluorescence. Note that the low signal-to-noise ratio in these BCECF fluorescence traces made BCECF insensitive to small changes such as those suggested by FPE.

Quantification of Fatty Acid Esterification Products Using Thin Layer Chromatography. The metabolic fate of exogenous FA added to the cells was quantified directly over longer time periods by incubating the cells with [^{14}C]oleic acid. The quantities of [^{14}C]oleic acid esterification products at 0.5, 30, 45, and 60 min are plotted in Figure 5A–D (note the differences in the *Y*-axis scales). The effects of overexpression of CD36 in HEK cells on triglyceride formation were very significant, and dramatic differences were evident at 30 min. In contrast, within the same time frame, in cells with CD36 the DG level was very low (<1.5 pmol/μg of protein) and similar in parental and CD36 cells. Levels of phospholipids and monoglycerides remained low at all time points (<2.5 pmol/μg of protein), although there was a significant increase in HEK293-CD36 cells compared to that in HEK293 parental cells after 0.5 min.

Visualization of Triglyceride Droplet Formation in HEK293 Cells Using BODIPY 500/510 and Oleic Acid. To further investigate whether the triglycerides synthesized from the exogenous oleic acid formed lipid droplets, HEK293 and HEK293-CD36 cells were incubated with a mixture of BODIPY 500/510 and oleic acid for 24 h. The BODIPY 500/510 is not metabolized in a manner identical to that of oleic acid (rate and extent of esterification to triglyceride), and this design utilized BODIPY 500/510 as a probe for oleic acid esterification.[24] As shown in single-cell images (Figure 6), fluorescent lipid droplets accumulated in both types of HEK293 cells; however, HEK293 cells overexpressing CD36 accumulated a greater number of droplets and larger droplets.

DISCUSSION

CD36 was originally characterized as a fatty acid transport protein in the plasma membrane of adipocytes by classical biochemical measurements of uptake,[25] a major finding supported subsequently by newer molecular biology methods.[26] The adipocyte plasma membrane has multiple other proteins with postulated roles in modulating the transmembrane flux of FAs, which include caveolin-1 and FATP.[27] Studying the molecular mechanisms of any of these proteins in membranes is challenging because FAs enter fat cells[23] and HepG2 cells[28] *in vitro* on a time scale of seconds or less.[29] The exogenous FA is metabolized within a few minutes, and the intracellular pH, as monitored by BCECF fluorescence, increases to its initial value in this time frame.[1,23] To overcome the confounding influence of metabolism on biophysical transport mechanisms in the plasma membrane, and in an extension of our previous studies focusing on caveolin-1, we utilized a simpler cellular system (HEK cells) for studying FA transmembrane movement because HEK cells lack caveolin-1, CD36, and FATP and metabolizing FAs is not their primary function.

If we focus first on transport in the plasma membrane, our application of two fluorescent probes, FPE and BCECF, permitted individual monitoring of adsorption to the outer leaflet (binding) and translocation of FA across the plasma membrane in real time, respectively (Figure 7). Binding of FA was complete within ~10 s and independent of CD36 overexpression. Notably, our FPE probe could have detected

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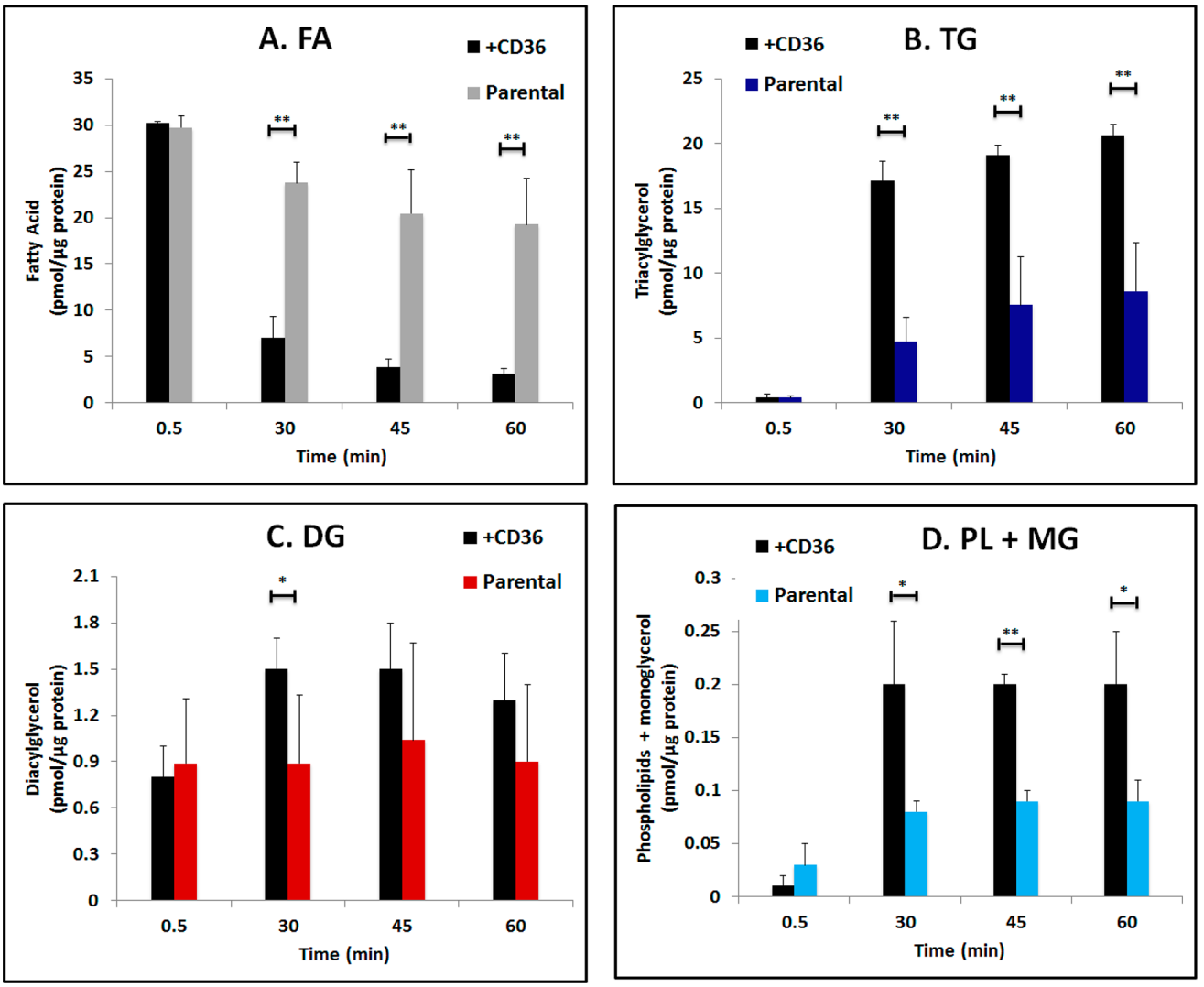

Figure 5. Quantitative analysis of oleic acid esterification in HEK293 cells with or without CD36 overexpression. [^{14}C]Oleic acid (20 μM, 30 pmol/μg of protein) was added to the cell suspension of HEK293 parental and HEK293-CD36 stable cell lines. Lipid components were separated by thin layer chromatography and quantified by radioactivity counting. The quantities of [^{14}C]oleic acid esterification products at 0.5, 30, 45, and 60 min are plotted in panels A–D (note the expanded vertical scales of panels C and D). Data are representative of four individual experiments and are represented as means ± the standard deviation. By a Student's t test, *p < 0.05 and **p < 0.001.

alternative outcomes. For example, if the majority of the FAs were initially bound in the hydrophobic extracellular domain of CD36, a potential binding site predicted from its amino acid sequence,[30] these FAs would not have appeared in the lipid bilayer until they had been released by CD36. If the desorption of FA into the lipid bilayer occurred slowly (minutes), a slow initial decrease in FPE fluorescence would have been observed, contrary to our observation of a single fast phase. In our previous analogous experiment with HEK cells overexpressing only caveolin-1, we observed a similar fast component with FPE. Note that our experimental design of adding oleic acid in DMSO rather than using albumin as a FA donor presented conditions favorable for observing interactions of the added oleic acid with CD36 by not having albumin in the medium to compete for FA binding.[31]

The BCECF probe, located in the cytoplasm (Figure 7), measures the combined steps of adsorption and transmembrane movement. The fast decrease in BCECF shows fast adsorption, and a rapid arrival of FA at the cytoplasmic leaflet. A slower BCECF fluorescence decrease might be expected in cells overexpressing CD36 if the transport of FA across the plasma membrane is mainly controlled or heavily mediated by CD36, as hypothesized from some previous studies.[4,8,25,32] These results taken together suggest that the transport of FA across the membrane is mainly through diffusion that does not normally require CD36.

Our observation of very fast binding of oleic acid, together with the expected slow metabolism in HEK293 cells (Figure 5), permitted observation of smaller and slower fluorescence changes in the first few minutes after the addition of FA with the FPE signal, which has a high sensitivity. In HEK293 cells overexpressing CD36, a small linear FPE fluorescence recovery was observed over 10 min. This result indicates a continuous loss of a small fraction of ionized FA from the outer leaflet of the plasma membrane and most likely reflects the onset of esterification of oleic acid, which was faster than that for the parental cells.

The effects of overexpression of CD36 in HEK cells on triglyceride formation were very significant as shown in Figure 5. A very significant increase in the level of incorporation of

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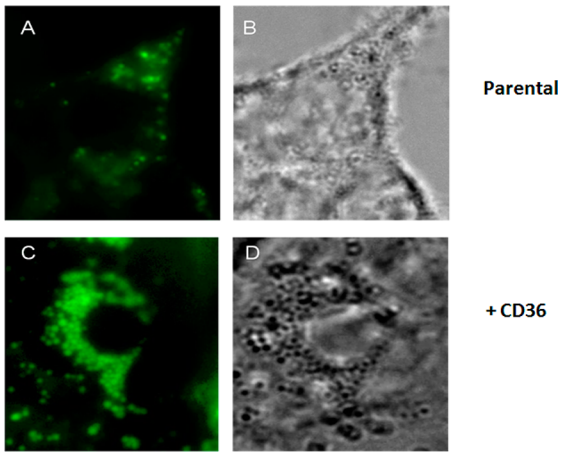

Figure 6. Detection of lipid droplet formation in HEK293 cells with or without CD36 overexpression using single-cell imaging. A BODIPY 500/510/oleic acid mixture was added to HEK293 parental cells (A and B) and HEK293-CD36 cells (C and D) separately, and the cells were incubated for 24 h and washed three times using PBS. Cells were then imaged under a Nikon deconvolution wide-field epifluorescence system and processed using ImageJ.

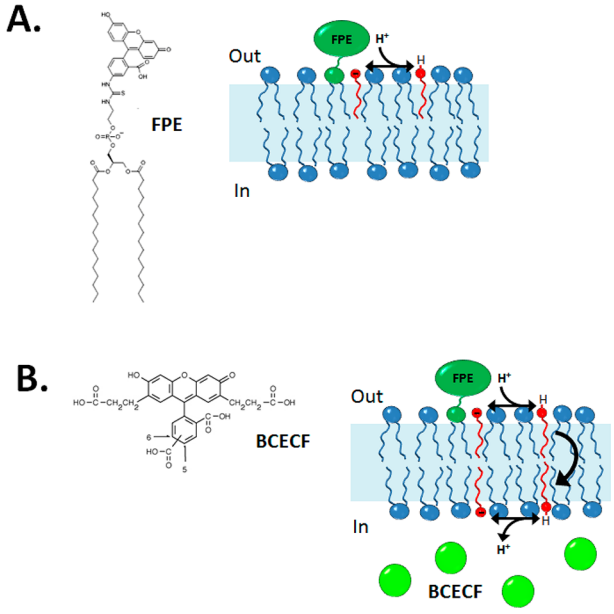

Figure 7. Schematic depiction of (A) FPE in the outer membrane leaflet and (B) BCECF inside the cytosol and the involvement of the fatty acid with each.

exogenously added oleic was seen in these cells compared to that in parental cells. CD36 stimulation of TG accumulation greatly exceeded that found in our parallel experiments with overexpression of caveolin-1 and -3 in HEK293 cells.[1,33] Our data show that CD36 significantly promoted synthesis of TG and MG and PL but not DG. In a separate experiment to determine if the total enhanced synthesis of MG and PL originated from MG, PL, or both, a double TLC that allows quantification of MG and PL separately was performed. The majority of the total enhanced MG and PL content was due to an increased level of synthesis of MG (results not shown). This further supports our conclusion that CD36 accelerates FA esterification, especially synthesis of TG. It should be noted that in parallel studies with adipocytes in vitro, fatty acids are esterified to products (mainly triglycerides) within 5 min, and the BCECF fluorescence recovery is complete within this short time interval.[23] In contrast to the rat adipocytes, FA esterification in HEK293 cells overexpressing CD36 is significantly slower.

The structure of lipid droplets is composed of a hydrophobic core containing mainly triglycerides surrounded by a phospholipid monolayer with multiple attached proteins that regulate lipolysis and storage.[34] After a 1 h incubation of the cells with BODIPY 500/510 and oleic acid, faint lipid droplets were visualized in HEK293 cells overexpressing CD36 (data not shown), and these became more observable at longer incubation times (e.g., 24 h), presumably because small droplets fused over time. Although fluorescent droplets were observed inside both types of cells (Figure 6), they were larger in size and amount in HEK293 cells overexpressing CD36 than in HEK293 parental cells. This finding is in harmony with the recent finding that increased heart CD36 levels lead to intramyocardial lipid accumulation.[35]

Taken together, these new findings suggested that CD36 does not play a major role in blocking or accelerating FA plasma membrane transport. CD36 expression affected the overall uptake by enhancing FA esterification, especially synthesis of triglycerides and lipid droplet formation. Our studies suggest a focus on mechanisms that enhance esterification such as signaling mechanisms and interactions of CD36 with enzymes such as acyl-CoA synthases.

LIMITATIONS AND FUTURE DIRECTIONS

The biophysical methods used in our study are not sensitive enough to detect binding of a single FA molecule in the extracellular domain, as proposed by several studies, although it could be sensitive to binding of multiple FA molecules, as in our study of HEK cells with caveolin. FA binding could also occur when FAs diffuse laterally in the lipid bilayer to the transmembrane domains of CD36. Future studies will explore biophysics strategies for exploring these binding interactions, which could be important for signaling functions and effects on metabolism. However, these studies strongly suggest that if they occur they do not affect mass transport or enhance the kinetics of FA translocation in the plasma membrane. The methods applied permitted us to distinguish biophysical transport from metabolism as contributors to the overall uptake of FAs but did not unravel the mechanisms of stimulation of triglyceride synthesis. As with all studies of overexpression of a protein, levels of other proteins can be increased; this will be one focus of our future work.

ASSOCIATED CONTENT

Supporting Information

Dose-dependent measurement of transport of oleic acid across the plasma membrane using BCECF-AM (Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

FA, fatty acid; FABP, fatty acid binding protein; FATP, fatty acid transport protein; TLC, thin layer chromatography.

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