SSO and other putative inhibitors of FA transport across membranes by CD36 disrupt intracellular metabolism, but do not affect FA translocation

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Abstract Membrane-bound proteins have been proposed to mediate the transport of long-chain FA (LCFA) transport through the plasma membrane (PM). These proposals are based largely on reports that PM transport of LCFA can be blocked by a number of enzymes and purported inhibitors of LCFA transport. Here, using the ratiometric pH indicator (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein and acrylodated intestinal FA-binding protein-based dual fluorescence assays, we investigated the effects of nine inhibitors of the putative FA transporter protein CD36 on the binding and transmembrane movement of LCFA. We particularly focused on sulfosuccinimidyl oleate (SSO), reported to be a competitive inhibitor of CD36-mediated LCFA transport. Using these assays in adipocytes and inhibitor-treated protein-free lipid vesicles, we demonstrate that rapid LCFA transport across model and biological membranes remains unchanged in the presence of these purported inhibitors. We have previously shown in live cells that CD36 does not accelerate the transport of unesterified LCFA across the PM. Our present experiments indicated disruption of LCFA metabolism inside the cell within minutes upon treatment with many of the “inhibitors” previously assumed to inhibit LCFA transport across the PM. Furthermore, using confocal microscopy and a specific anti-SSO antibody, we found that numerous intracellular and PM-bound proteins are SSO-modified in addition to CD36. Our results support the hypothesis that LCFA diffuse rapidly across biological membranes and do not require an active protein transporter for their transmembrane movement.—Jay, A. G., J. R. Simard, N. Huang, and J. A. Hamilton. SSO and other putative inhibitors of FA transport across membranes by CD36 disrupt intracellular metabolism, but do not affect FA translocation. J. Lipid Res. 2020. 61: 790–807.

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Glucose and long-chain FAs (LCFAs) are two key dietary nutrients available for energy in the body. Glucose serves as a fuel source especially utilized by the brain, while LCFA is preferred by key tissues such as cardiac muscle (1–4). LCFAs that are not immediately oxidized for energy are often delivered to adipose tissue to be stored as TGs for energy storage. While the mechanism by which glucose enters the cell is well established, the mechanism by which LCFAs cross the cell membrane is still widely debated (5–7).

Upon initial contact with cells, exogenous LCFAs are amphiphatic molecules that readily adsorb to the external leaflet of lipid bilayers and orient their carboxyl head group at the aqueous interface. About half of these LCFAs become un-ionized in this environment due to a shift in the pKa of the LCFA carboxyl from ~4 to ~7.5 upon insertion into the membrane environment. As uncharged molecules, un-ionized LCFAs can readily move down a
concentration gradient by rapidly flipping to the inner leaflet of the membrane where they again reach ionization equilibrium, resulting in the release of $\text{H}^+$ to the internal volume. This model has been consistently demonstrated and supported by studies of protein-free lipid vesicles (8–11), vesicles made from adipocyte plasma membranes (PMs) (12), isolated rat adipocytes (12), and various other cell types (13–18). LCFA amines (i.e., a LCFA in which the carboxyl group is swapped with an amine group) and a LCFA dimer probe also behave according to the predictions made with this model, and it is unlikely that specific proteins exist to catalyze the translocation of these unnatural ligands across the PM (8, 15, 19, 20).

Our laboratory previously developed a dual-fluorescence assay to establish that LCFA diffuses across model and biological membranes at a rate that is fast enough to support cellular metabolic needs and does not require active catalysis by protein transporter(s) (8–11, 19–22). This approach utilizes two different fluorescent probes, placed on the inside and outside of the membrane, to simultaneously monitor each step of LCFA diffusion through the lipid bilayer. The fluorescence of acrylodated intestinal FA-binding protein (ADIFAB) increases immediately as LCFA rapidly bind to and equilibrate with the outer membrane leaflet (adsorption) leaving only a small, but detectable, amount of unbound LCFA in the external buffer by ADIFAB. Simultaneously, 2′,7′-bis-(2-carboxyethyl)-5- (and-6)-carboxyfluorescein (BCECF) detects an immediate decrease in pH on the opposite side of the membrane (transmembrane movement), an event that has been attributed to the diffusion of un-ionized LCFA between membrane leaflets (flip-flop) and the release of protons at the inner leaflet (9).

Despite these findings, many previous studies have employed radiolabeled FA and/or perturbing techniques (e.g., BODIPY-FA) to measure LCFA “transport” into cells, often over long periods of time (minutes), and these studies propose a mechanism of protein-mediated LCFA transport (23–28). Also, the terms transport and “uptake” are often over long periods of time (minutes), and these studies propose a mechanism of protein-mediated LCFA transport (23–28). Also, the terms transport and “uptake” are used interchangeably to imply that FAs have crossed the PM. However, assays used in such studies: i) do not distinguish between the multiple steps of LCFA transport (binding and flip-flop); ii) do not reveal whether LCFA are bound to membrane proteins or lipids; iii) seldom separate LCFA transport at the membrane from the subsequent intracellular metabolism or binding to internal protein “sinks” (i.e., FA binding protein); and iv) often do not provide any information for the metabolic fate of added LCFA. Therefore, these types of assays do not measure protein-mediated LCFA transport directly; rather, they provide a measure of the total amount of radiolabeled FA associated with and/or internalized by cells after a period of time (i.e., LCFA uptake).

LCFA transport studies performed using only such assays that include limitations and perturbations often demonstrate saturable LCFA uptake into cells (29). Furthermore, the ability to diminish LCFA uptake by applying various chemicals and enzymes is often interpreted as saturation/inhibition of LCFA transport proteins (23, 30–36). Some studies have also shown that only LCFA “compete” for these transporters, which led to the conclusion that only LCFA (FAs with ≥12 carbons) require a protein transporter to enter cells (23, 27, 32). Further evidence for the role of these proteins in LCFA transport came from studies that positively correlated LCFA uptake (longer term) with changes in the expression levels of these putative transport proteins at the PM (28, 37–42). However, the effects of increased protein expression might have on LCFA metabolism are seldom considered, especially in the presence of purported LCFA transport inhibitors. Additionally, the kinetic rates of FA transport into cells are incompletely described, as are the mechanisms by which these transporters actually catalyze the movement of FA through the bilayer, leaving many unanswered questions, which the diffusion model can adequately address.

By contrast, the goal of this study was to use fluorescence methods to evaluate and test several chemicals, enzymes, and other conditions that have been used elsewhere as indirect evidence of inhibition of LCFA transport. In addition, we set out to address whether it is translocation or the subsequent metabolism of added LCFA that is actually inhibited and whether these chemicals and enzymes have any additional protein-independent effects on LCFA uptake into cells. Specifically, we investigated the ability of nonspecific proteases [trypsin (TRYP) (23, 32, 45–48) and pronase (PRON) (23, 45, 49)], nonspecific anion transport inhibitors [4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) (23, 32, 44, 49–52), phloretin (PHLO) (23, 32, 43–45, 53–60), quercetin (QUER) (45, 61, 62), and penta- chlorophenol (PCPL) (45, 63–65)], specific amino acid modifiers [phenylglyoxal (PG) (44, 60, 66–72) and diethylpyrocarbonate (DEPC) (60, 66, 70, 73, 74)], additional amino acid modifiers reported as “CD36-specific” inhibitors [sucinnimide oleate (SO) and sulfo-SO (SSO) (25, 27, 28, 32, 49, 51, 75–79)], and, finally, a medium-chain FA (MCFA) compared with a “competitive” LCFA [octanoate (C8:0) versus palmitate (C16:0) (23, 27, 32, 43, 52); Table 1]. All of these compounds/enzymes/conditions were used in order to inhibit or modify the function of putative membrane protein transporters in a diverse variety of ways.

Our new findings confirm that LCFA diffuse rapidly across biological membranes and do not require a protein transporter to catalyze their transmembrane movement. Our comprehensive investigation of purported LCFA uptake inhibitors demonstrates that these chemicals/enzymes either affect LCFA metabolism or disrupt measurements of LCFA uptake amount, while having no impact on LCFA entry rate. For the first time, we also present new aspects to consider for SSO use in these types of studies, including SSO hydrolysis, cellular entry, measurement perturbations via intracellular acidification, and diverse intra- and extracellular protein modifications.

**MATERIALS AND METHODS**

**Materials**

PC isolated from chicken egg (EggPC) dissolved in chloroform was purchased from Avanti Polar Lipids (Alabaster, AL). Olate...
Organic synthesis of SSO

For fluorescence and metabolic studies, SSO was synthesized as previously reported (51, 78). Briefly, oleate, HOSu(SO$_3$)Na, and 1,3-dicyclohexylcarbodiimide were placed inside a 100 ml wide-mouth round-bottom flask and covered with a septum. Distilled DMF was added to the mixture (0.5 ml DMF:0.25 mmol oleate) and allowed to stir at 20°C overnight. The precipitated dicyclohexylurea by-product was removed by filtration (Whatman nylon, 45 μm pore size) and eight volumes of anhydrous ethyl acetate were then added to the filtrate and stirred for 4 h at 4°C. The precipitated SSO was collected by filtration (Whatman nylon, 45 μm pore size) in a glove bag under N$_2$ gas, dried, and stored in a vacuumed desiccator over phosphorous pentoxide. For microscopy and hydrolysis studies, SSO was purchased from Santa Cruz Biotechnology (#208408; Dallas, TX), kept frozen under inert gas, and used within 1 month of purchase.
Buffers

Small unilamellar vesicle (SUV) buffer [50 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, and 5 mM glucose (pH 7.4)] was used to prepare and suspend lipid vesicles for fluorescence measurements, while M/K buffer [20 mM MOPS, 118 mM NaCl, 5 mM KCl, 1.1 mM MgSO₄, 1.1 mM KH₂PO₄, 2.5 mM CaCl₂, and 5.1 mM glucose (pH 7.4)] was used in all experiments involving isolated rat adipocytes. To inhibit only metabolism in adipocytes, glucose was substituted for an equivalent concentration of 2-deoxyglucose (2-DOG). 2-DOG was first presented to the cells in the buffer used to wash the isolated adipocytes following incubation and emission measurements. Stocks of PRON (20 mg/ml) and TRYP (50 mM) were prepared in recommended buffer solutions. PG (250 mM) was prepared in water and heated to 55°C in a water bath to facilitate dissolution. DIDS (50 mM), PHLO (100 mM), QUER (100 mM), PCPL (100 mM), SSO (200 mM), and SO (200 mM) were dissolved in DMSO. DEPC (500 mM) was prepared in absolute ethanol. Each stock solution was stored at −20°C between experiments, while PG and DEPC were prepared fresh before each experiment.

Fluorescence measurements

Fluorescence measurements were made using a Spex® Fluoromax-2 from Jobin Yvon (Edison, NJ). The ratiometric fluorescence of BCEO CF was measured using excitation wavelengths of 459 nm and 505 nm (R = Δ505/Δ459) with an emission of 535 nm. Due to an unexpected intrinsic excitation signal of DIDS, which interfered with the BCEO CF excitation signal at 439 nm, an adjusted BCEO CF ratio (R = Δ505/Δ405) was used in these samples. Titration of BCEO CF fluorescence (between pH 6 and pH 8) measured at this adjusted ratio was not significantly different from the control. The ratiometric fluorescence of ADIFAB was measured using an excitation wavelength of 386 nm and emission wavelengths of 432 nm and 505 nm (R = Δ505/Δ432).

SUVs and adipocytes in each cuvette were pretreated with each inhibitor for 15 min at 37°C, and inhibitors were present during the fluorescence measurements. The sample changer was warmed to 37°C by a flow of water pumped into the sample compartment from a temperature-controlled external water bath. The ratiometric fluorescence signals of each probe were measured alone or simultaneously as 10 μM OA (30 nmol oleate per cuvette) while stirring rapidly with a mini stir bar.

Control experiments

Effects of inhibitors on fluorescent probes. The effect of low temperature (4°C) and each inhibitor (at 37°C) on the fluorescence titration of ADIFAB and BCEO CF fluorescence was investigated to ensure that fluorescence artifacts would not lead to misinterpretation of our data. BCEO CF calibrations were performed in the presence of each inhibitor by titrating pH according to a previously described method (9). Briefly, SUVs containing 0.5 mM BCEO CF were permeabilized to H⁺ with nigericin (1 μg nigericin per 1 mg PC) such that pHin = pHout at all times. The pHout was measured with a pH mini-electrode (Microelectrodes, Bedford, NH) and was titrated with small volumes (1–2 μl) of KOH and H₂SO₄. The ratiometric fluorescence of BCEO CF was recorded at each pH. ADIFAB calibration was also performed as previously described (82). ADIFAB (0.2 μM) was placed in SUV buffer and its ratiometric emission was monitored in the presence of increasing amounts of oleate (0.2–1 μM).

In addition to monitoring ratiometric fluorescence, the excitation and emission spectra of each probe were measured to reveal whether any inhibitors had any intrinsic fluorescence signal overlap with either the emission of ADIFAB or the excitation of BCEO CF. Although ADIFAB was affected by nearly every inhibitor tested, we found that BCEO CF was only affected by DIDS, which introduces a higher excitation signal at 439 nm. However, we were able to correct for that problem by measuring the ratiometric excitation of BCEO CF at 505 nm and 405 nm in those samples.

Effects of inhibitors on FA translocation into SUVs. For SUVs, fluorescence measurements were made (in the presence or absence of each inhibitor) by placing SUV-containing entrapped BCEO CF acid in a polystyrene cuvette containing 3 ml of SUV buffer (∼730 μM PC). SUVs were incubated with inhibitor for 15 min and all samples were maintained at 37°C. With or without 3 min pretreatment of 100 μM octanoate or palmitate (which remained in the buffer upon OA addition), OA (10 μM; 1.4 mol% OA:PC) was delivered into the cuvette through the injection port on top of the Fluoromax-2 while monitoring the fluorescence of each probe. The effect of low temperature on LCFA translocation into SUVs.
was measured after chilling the SUV suspension to 4 °C in an ice bath. These experiments were designed to reveal whether inhibitors disrupted LCFA translocation in a protein-free membrane system and assist analysis of FA transport through biological membranes.

Effect of inhibitors on FA translocation into rat adipocytes

For adipocyte studies, 3 ml of suspended rat adipocytes [1–3 × 10^6 cells per cuvette; 0.6 g protein per milliliter (20)] were transferred into polystyrene cuvettes along with 0.2 μM ADIFAB or entrapped BCECF acid (described above) in the presence and absence of each inhibitor. Cells were incubated with inhibitor for 15 min or 100 μM octanoate or palmitate for 3 min, and all samples were carefully maintained at 37°C as OA (10 μM) was delivered into the cuvette. The relationship between intracellular metabolism and translocation was examined by replacing glucose in the buffer with 2-DOG or by cooling the cell suspension to 4°C in an ice bath for 5 min prior to adding OA. These experiments were designed to determine whether the movement of LCFA through biological membranes treated with inhibitors differs from that of model membranes, thereby revealing the role(s) of proteins in LCFA transport.

Design, development, and immunocytochemistry of a rabbit anti-SSO antibody

A rabbit polyclonal antibody was raised against SSO cross-linked to two lysines [epitope: (sulfosuccinimidyl oleoyl)QQYIKAN-SKFGITTEL-OH]. This was designed, developed, and injected into rabbits (Twentyfirst Century Biochemicals, Inc., Marlboro, MA). After peptide synthesis, HPLC purification of the peptide to >85% was performed, along with sequencing by collision-induced dissociation MS/MS, Nanospray MS, and HPLC analysis. The concluding affinity and immunodepletion for the final rabbit anti-SSO polyclonal antibody production were from the best one of three rabbits, accessed by dot blotting SSO-treated HeLa cell lysate prior to final antibody purifications (data not shown). The final concentration of antibody was 1.19 mg/ml. Additional dot blotting was done with the final anti-SSO antibody, including SSO-treated and untreated cells and cells expressing pcI-neo-CD36 or pcI-neo-empty control vector (Fig. 1; described below).

For SSO localization microscopy experiments, HeLa cells were grown in 8-well BD Falcon culture slides (Franklin Lakes, NJ) to ~60% confluence in low-glucose (5 mM) DMEM containing 10% FBS, within 3 days of DNA transfections. For DNA transfections, pcI-neo-CD36 and pcI-neo-empty vector control were added using Lipofectamine 2000 (Invitrogen) similar to our previous publications, where we showed CD36 was primarily PM localized (fractionation and microscopy) and was functionally active (18). In HeLa cells with or without CD36 expression, SSO was premixed to a final concentration of 100 μM or 200 μM into serum-free DMEM and added to the cells for 20 min. After SSO incubation, the cells were washed three times in DPBS + 0.1% Tween20 and 1% BSA and then fixed with 4% paraformaldehyde in DPBS at RT for 10 min. The cells were washed again with DPBS one time, and then cells were permeabilized with 0.1% TX100 in DPBS, where noted. Next, to reduce high background nuclear signal, Image-iT FX signal enhancer (Invitrogen) was added to cover the cells for 30 min. Next, cells were washed three times in DPBS and blocked in 10% BSA in DPBS for 1 h at RT. This blocking was followed by overnight 4°C incubation with monoclonal mouse anti-CD36 primary antibody (J63.1; Abcam, Cambridge, MA) at a dilution of 1:500 and rabbit anti-SSO antibody at 1:100. The cells were then washed three times for 5 min in DPBS buffer and then incubated with Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen; A-11034) and Alexa Fluor 594 goat anti-mouse IgG antibody (Invitrogen; A-11032) for 1 h at RT. After washing with DPBS three times for 5 min, the cells were mounted with ProLong Gold antifade reagent containing DAPI (Invitrogen; P-36951) and a #1 glass cover slide. The stained cells, including 3D Z-stack images, were photographed using an Olympus DSU spinning disk confocal microscope (Center Valley, PA) using CellSens Acquisition software, maintaining the gain and exposure settings between each channel for all images. Further image XY- and Z-stack 3D projects and colocalization colormapping were done using ImageJ (National Institutes of Health, Bethesda, MD), including the colocalization colormap plugin (83).

Dot blotting with anti-SSO antibody

For semi-quantitative detection of target protein interactions, recently transfected HeLa cells with either pcI-neo-empty vector control or pcI-neo-CD36 were grown on 10 cm dishes and select dishes were incubated in 200 μM SSO for 20 min. The cells were then washed in DPBS three times and harvested into 1.5 ml 4°C lysis buffer [1× RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TX100, 1% deoxycholate] + 1× HALT protease inhibitors]. Dot blotting was performed using harvested transfected HeLa cell lysates normalized to 3 μg/μl sample, using a BCA protein assay, and then 3 μl of normalized sample was directly pipetted onto a nitrocellulose paper. The membrane was blocked in StartingBlock T20 (Thermo Fisher Scientific, Wilmington, DE) for 1 h at RT, then the blots were incubated overnight at 4°C with 1:10,000 primary anti-SSO antibody in StartingBlock T20 or 1:1,000 monoclonal rabbit anti-CD36 primary antibody (ERP575; Abcam), rather than J63.1 antibody. Additionally, after film exposure with primary antibodies, the blots were stripped using Restore Western blot stripping buffer (Thermo Fisher Scientific) and probed with 1:1,000 monoclonal mouse β-actin primary antibody (#8226; Abcam) and 1:100,000 goat-anti rabbit HRP secondary antibody, and processed using film exposure as a loading control verification.

SSO hydrolysis assay

Using either M/K buffer, SUV buffer, or serum-free DMEM, SSO hydrolysis assays were performed by adding 200 μM SSO into each buffer and measuring intensity of light as a function of 280 nm wavelength on a PerkinElmer LAMBDA spectrophotometer (Waltham, MA) for various time points, using a 37°C water bath through each experiment. To find the half-life for each, equation 1 was used:

$$ t_{1/2} = \frac{t \ln(2)}{\ln \left( \frac{N_0}{N_t} \right)} $$

(Eq. 1)

where \( t = \) time; \( N_0 = \) amount of SSO at experimental start; and \( N_t = \) amount of SSO after a period of time. For SUV buffer, data were fitted using the least squared fit equation (asymmetric sigmoidal):

$$ \text{Denominator} = \{1 + 2^{-(1/S)} - 1\} \times [(EC50/X)^{\text{HillSlope}}]^S $$

$$ \text{Numerator} = \text{Top} - \text{Bottom} $$

$$ Y = \text{Bottom} + \left( \frac{\text{Numerator}}{\text{Denominator}} \right) $$

(Eq. 2)

For M/K buffer, data were fitted using the least squared fit equation (one phase association):

$$ Y = Y_0 + (\text{Plateau} - Y_0) \times [1 - \exp\left(-K \times \frac{X}{x}\right)] $$

(Eq. 3)
Analysis of the percentage of oleate transport

Several inhibitors interfered with ADIFAB fluorescence allowing for only qualitative measurement of LCFA binding with this probe. Alternatively, the titration of BCECF fluorescence with pH was unaffected, thereby allowing for quantitative analysis of LCFA diffusion through membranes. The magnitude of the observed pH drop was dependent on the amount of LCFA added and the amount of SUVs or cells present in each cuvette. Due to minor variations in each SUV or cell preparation, only samples prepared from the same cell preparation could be directly compared. The percent inhibition of LCFA transport, relative to control, was calculated by using the following equation:

\[
\% \text{ inhibition} = \left( \frac{\Delta \text{BCECF}_{\text{est}} - \Delta \text{BCECF}_{\text{inh}}}{\Delta \text{BCECF}_{\text{est}}} \right) \times 100
\]

(Eq. 4)

where \( \Delta \text{BCECF}_{\text{est}} \) and \( \Delta \text{BCECF}_{\text{inh}} \) represent the fluorescence change produced by 10 \( \mu \)M oleate in the absence (ctl) and presence (inh) of each agent/condition, respectively. The percent LCFA transport relative to control was then calculated using the following equation:

\[
\% \text{ LCFA transport} = (100\% - \% \text{ inhibition})
\]

(Eq. 5)

Values below and above 100\% transport represent decreased and increased transport, respectively. The effect of each inhibitor on transport was replicated three times in SUVs because these experiments were highly reproducible (low SD) and replicated five times using various adipocyte preparations (higher SDs due to variations in cell size, number, and integrity between experimental groups).

Measurement of intracellular oleate TG conversion

Cells were pretreated with each inhibitor and incubated in a still water bath for 15 min at 37°C. Next, [\(^{14}\)C]OA (10 \( \mu \)M) was added to 3 ml of cell suspension in a test tube, incubated for 20 min in a 37°C water bath gently shaking at 100–120 rpm. The suspension was then poured into 1 ml of “stop solution” (40:10:1 hexane:isopropanol:sulfuric acid) to immediately lyse the cells and extract the lipids. Samples were centrifuged (20 min at 70 \( g \)) after adding 3 ml deionized water and 2 ml hexane to wash and separate all lipids into the organic layer.

Approximately 20 \( \mu \)l of the organic hexane layer was placed on silica TLC plates along with 20 \( \mu \)l of lipid standard (monoglyceride, DG, TG, and OA), dried under N\(_2\) gas, and placed in a solvent tank containing ‘running solvent’ (80:20:1 hexane:ethanol:acetic acid). The plate was then removed from the tank, air-dried for 15 min, and placed into a second sealed tank to develop in the presence of iodine flakes. Different lipid bands were separated and scraped into scintillation vials. The proportion of [\(^{14}\)C]OA incorporated into total cellular lipid was calculated using the following equation:

\[
\text{CPM}_{\text{Lipid}} - \frac{\text{CPM}_{\text{Background}}}{\text{CPM}_{\text{Total}}}
\]

(Eq. 6)

where CPM\(_{\text{Lipid}}\) represents the total counts measured in a 20 \( \mu \)l aliquot from hexane layer and CPM\(_{\text{Lipid}}\) represents the total counts found in a particular lipid band separated by TLC. Background radioactivity (CPM\(_{\text{Background}}\)) was measured by scraping silica from lanes containing only lipid standard (no sample). The effect of each inhibitor on oleate metabolism into TG (at 20 min) was replicated five times. In previous studies, monoglyceride and DG have shown only slight changes compared with the large differences in TG measurements (18).

Statistical analysis

All statistical analyses were analyzed by Student’s t-test and performed using Usable \( \Sigma \) Stats (http://www.usablestats.com/) using the Welch-Satterthwaite procedure incorporating unequal variances. Data are presented as mean ± SD.

RESULTS

Inhibitors alter the ability of ADIFAB probe, but not BCECF, to reliably report concentrations of unbound FA

The effect of each purported LCFA transport inhibitor on BCECF calibration curves was tested to identify any fluorescence artifacts that might lead to incorrect interpretations of subsequent fluorescence data (Fig. 1A). BCECF fluorescence as a function of pH was unaffected by most inhibitors, with the exception of DIDS. In the presence of DIDS, the excitation profile of BCECF revealed an intense excitation signal from the DIDS molecule near 439 nm that was more intense than that of BCECF. Because BCECF is measured as an excitation ratio (\( R = 505 \text{ nm}/439 \text{ nm} \)), DIDS significantly reduced the observed BCECF ratio and produced more flat titration curves (data not shown), which would be expected to decrease the sensitivity of BCECF to the detection of LCFA diffusion. However, by adjusting the excitation wavelengths to 405 and 505 nm, we were able to correct for this artifact, and the resulting the BCECF calibration curve in the presence of DIDS did not vary significantly from the control at these wavelengths (Fig. 1A).

Contrary to BCECF, all of the inhibitors except the metabolic inhibitor 2-DOG affected the ability of ADIFAB to reliably measure unbound FA concentrations (Fig. 1B). Although the emission spectrum of ADIFAB, in the absence of OA, was not affected by most inhibitors (data not shown), the titration of ADIFAB fluorescence (i.e., sensitivity and/or linear range of detection) varied significantly in the presence of the uptake/transport inhibitors. Because ADIFAB is a fluorescently-labeled protein, these results were not surprising given that many of these inhibitors either digest or react covalently with proteins in the PM (except PHLO, QUER, and PCPL). While the response of ADIFAB to each inhibitor varied widely, ADIFAB was not affected by reducing the temperature to 4°C (data not shown). And at 37°C, ADIFAB fluorescence signal was either completely quenched (DEPC) or its sensitivity to OA was completely lost (DIDS, PCPL, SSO), suppressed (TRYP, PRON, SO, PG), or amplified (QUER, PHLO).

Inhibitors affect rapid FA metabolism or directly alter the intracellular pH, but have no effect on real-time FA movement into cells

Dual-fluorescence measurements have been used extensively by our group to monitor different steps of LCFA translocation in model and biological membranes in real-time [e.g., (16, 84)]. When used together, the fluorescence of ADIFAB and BCECF changes simultaneously
upon addition of LCFA, which indicates rapid LCFA incorporation into the outer PM leaflet (binding) followed by rapid transmembrane movement. These fast initial fluorescence changes then slowly recover to baseline levels in studies with rat adipocytes but not in HEK293 cells, which we have linked to the subsequent metabolism of the added LCFA into TG in the adipocyte cells (12, 18). Representative data showing the effects of each inhibitor, as well as lowering buffer temperature to 4°C, on FA translocation (pH drop) and FA metabolism (fluorescence recovery), were measured by changes in fluorescence (Fig. 2A–C).

As shown in Fig. 2A, SSO, PHLO, and PCPL significantly decreased the observed pH drop produced by a single dose of 10 μM OA added to a rapidly stirred suspension of adipocytes (Fig. 2A, lower panel), indicating either an alteration of intracellular pH when SSO, PHLO, and PCPL are present, or decreased FA translocation through the PM. ADIFAB fluorescence using SSO, PHLO, and PCPL, however, revealed rapid OA binding to the outer membrane (<2 s) followed by internalization/metabolism of OA at a rate similar to that observed in untreated cells (∼125 s). This suggests that intracellular pH is affected by these three inhibitors. This was further investigated using protein-free lipid vesicles with entrapped BCECF. Treatment of vesicles with each transport or metabolic inhibitor had no significant effect on the magnitude of the pH drop from a single dose of OA, with the exception of SSO and PCPL (Fig. 3A). This further indicates that the smaller pH decrease resulting from added LCFA is not directly linked to inhibition of a protein-mediated transport mechanism.

Conversely, these results suggest that the effects of PHLO on OA translocation into adipocytes could potentially be related to inhibition of protein transporters. However, important evidence exists showing that PHLO i) can permeate the lipid bilayer, ii) can intercalate between lipid chains, and iii) can float along the aqueous interface by interacting with the polar head groups of phospholipids (57). Considering that the phospholipid composition and curvature of the membranes of SUVs and adipocytes are different, it is possible that PHLO could have differential effects on LCFA translocation through the lipid phase. High concentrations of PHLO especially can also alter the membrane dipole potential (57). Thus, it is possible that PHLO alters LCFA diffusion through the lipid bilayer rather than inhibiting protein-mediated LCFA transport when used at the concentrations commonly employed in studies of FA transport/uptake. Changes in the surface charge of the membrane would change the LCFA ionization equilibrium in each membrane leaflet at pH 7.4. If PHLO increased the proportion of un-ionized LCFA in membranes, fewer H+ would be released at the inner leaflet and smaller pH changes would be observed per dose of added LCFA. More work is needed to fully understand what high concentrations of PHLO do to lipid membranes.

While TRYP, PRON, PG, and DEPC did not change the magnitude of the pH drop (indicating no change to intracellular FA entrance) when compared with the control, these inhibitors each affected the rate of fluorescence recovery in adipocytes (Fig. 2A, lower panel), suggesting that these compounds have effects on FA metabolism but not on transport. TRYP and PRON affected fluorescence recovery most significantly, suggesting that these enzymes may be cleaving extracellular domains of protein(s) that signal or initiate metabolic processes within the cell, such as CD36 (18).

While DIDS and QUER had no distinctive pH changing effects in protein-free vesicles (Fig. 3A), they both produced surprising results in cells. With the addition of 10 μM OA, a measurable pH increase was observed that was equivalent in magnitude to the pH decrease observed in untreated cells (Fig. 2B, lower panel). With DIDS, the similarities in the rate of BCECF fluorescence recovery suggests that OA metabolism is unaffected by DIDS. However, the reason for the observed increase in BCECF fluorescence upon addition of OA to either QUER- or DIDS-treated cells is not clear. Because these two inhibitors are the only bright yellow-emitting inhibitors employed in this study and, coincidentally, are the only two inhibitors that induce this inverted BCECF response, we believe that this may be the result of a fluorescent artifact. And while BCECF fluorescence in DIDS-treated cells recovered on a time scale similar...
The effects inhibitors have on fatty acid transport

Fig. 2. The effect of inhibitors on the binding, transmembrane movement, and metabolism of LCFA in rat adipocytes. Dual-fluorescence measurements of ADIFAB (top) and BCECF (bottom) fluorescence were made by adding 10 μM of exogenous OA (black arrowheads) to adipocytes pretreated with inhibitors (red arrows indicate and emphasize the response obtained in absence of any inhibitor). Because ADIFAB was significantly affected by most inhibitors (Fig. 1), data is only shown for a few select inhibitors, which all potentially interact with ADIFAB (except 2-DOG). In A–C, OA binding (ADIFAB fluorescence increase) occurred rapidly (<2 s) and simultaneously with transport to the inner leaflet (BCECF fluorescence decrease). A: OA was then internalized by the adipocyte cell for metabolism, as shown by ADIFAB and BCECF fluorescence recovery. Additionally, PHLO, SSO, and PCPL significantly reduced the size of the observed pH drop, while TRYP, PRON, PG, DEPC, and SO had no effect on the pH drop but had varying effects on the rate of BCECF fluorescence recovery. B: The addition of OA in the presence of DIDS and QUER caused an increase in BCECF fluorescence. The magnitude of the pH change and rate of BCECF fluorescence recovery is equivalent for DIDS and control samples while QUER blocked the recovery of ADIFAB and BCECF fluorescence. C:
to that of untreated cells, no recovery was observed in QUER-treated cells. ADIFAB fluorescence also did not recover for QUER-treated samples (DIIS was found to be incompatible for use with ADIFAB), suggesting that when QUER is present, FA may be: i) directly interacting with QUER, ii) not being internalized, or iii) retained by the cell (with H+ following their arrival at the inner membrane leaflet. We see direct QUER-FA interaction as a possibility due to the low solubility of QUER (potential aggregation formation, etc.), the scavenging abilities of QUER, and recent work showing inhibition of lipoxigenases by QUER when using FA as a lipoygenase substrate (85). Further work needs to be done on this question, especially with recent work revealing that QUER inhibits TG synthesis in rat hepatocytes (86).

In experiments designed to separate LCFA transport from intracellular metabolism, we employed two strategies that have previously provided arguments for a protein-mediated LCFA transport mechanism (1); substitution of extracellular glucose for 2-DOG and cooling cells to 4°C prior to adding exogenous FA. With our fluorescence assays, we found that neither condition had a significant effect on the magnitude of the pH drop produced by a single dose of OA (Fig. 2C). However, the pH did not recover to baseline during the measured time period, suggesting that OA metabolism is inhibited in both cases. Interestingly, ADIFAB fluorescence does recover as rapidly in control samples for both of these conditions, suggesting that LCFA are still internalized and trapped inside the cell through activation into long-chain acyl-CoA but may not be subsequently metabolized into TG in further metabolic steps, which explains the slow pH recovery. These results support the idea that LCFA can traverse the PM rapidly and independently of TG synthesis.

While the rate of LCFA transport showed no change, some inhibitors had an effect on quantity of OA transported across the membrane. Quantification of the cumulative results for the effects of each inhibitor on OA transport amount compared with control samples and also comparing protein-free versus biological live-cell membranes are shown in Fig. 2D. The percent OA uptake was determined by comparing the magnitude of BCECF fluorescence change in each sample with the pH change observed in nontreated cells. PCPL and SSO were found to most significantly reduce LCFA uptake amount in both cells and protein-free lipid vesicles compared with controls. PHLO treatment also reduced OA uptake drastically, but only in adipocytes. Many other tested conditions showed small but statistically significant differences in percent OA uptake between vesicles and cells. However, the same differences were also observed in treated cells and vesicle controls, suggesting that these differences are not protein dependent.

PCPL and SSO independently enter cells and potentially perturb cellular functions, and SSO modifies numerous proteins in addition to CD36

Initially, to investigate the effects of SSO observed in Fig. 2A, we employed the compound SO, which is a derivative of SSO that lacks the water-solubilizing aid of the sulfate group on the succinimidyl moiety. Addition of OA to vesicles and cells pretreated with SO did not affect the magnitude of the pH drop. However, a reduction in the magnitude of pH drop was observed with SSO (Fig. 2A, D). We hypothesized that the highly acidic sulfate group of SSO may titrate into membranes (similar to the FA carboxyl) and allow a percentage of this inhibitor (i.e., the un-ionized form) to flip-flop across the membrane and pre-acidify the internal volume prior to adding OA. Additionally, we hypothesized that the sulfate of SSO causes the compound to hydrolyze to some degree upon addition to aqueous buffer, thereby releasing OA to pre-acidify the internal volume (87). A combination of these two mechanisms was also considered.

Along with SSO, PCPL also drastically affected the magnitude of the pH drop upon OA additions (Fig. 3A). To determine whether this was a direct alteration of FA transport or an indirect effect of SSO and PCPL, these compounds were added directly to SUVs while monitoring the fluorescence of entrapped BCECF. The addition of equivalent doses of OA (“control”), SSO, and PCPL (10 μM) to SUVs each produced rapid pH drops (<2 s) of varying magnitudes (Fig. 3B). Subsequent addition of nigericin (a membrane-permeating ionophore) rectified the pH gradient, confirming that changes in BCECF fluorescence resulting from SSO or PCPL addition were due to intravesicular pH changes as opposed to fluorescence artifacts. Addition of 20-fold higher doses of SSO and PCPL, equivalent to the concentrations used to pretreat adipocytes in this study, produced 5- to 6-fold larger pH drops than those produced by 10 μM OA (Fig. 3C). In addition, the pH drop from SSO was significantly slower in cells (and M/K cell buffer) when compared with that observed in vesicles (SUV buffer) but appears complete within a few minutes. This is a key control experiment because neither SUVs nor cell buffer contained primary amines (i.e., Tris) that would react with SSO.

More than all other inhibitors combined, published studies have used SSO to probe mechanisms of cellular FA uptake (88-91) and often include the assertion that SSO is specific for CD36 (27, 92). Live mouse duodenal infusions have also been performed using SSO to probe CD36 function (93). With our new finding that SSO can rapidly affect intravesicular/intracellular pH, we further investigated the rate of SSO hydrolysis. In pH 7.0 buffer at 0°C, the half-life of SSO hydrolysis has been shown to be 4–5 h (94). In our

A similar BCECF fluorescence decrease and ADIFAB fluorescence increase was observed after cooling cells to 4°C or when using cells from fasted rats that were suspended in buffer containing 2-DOG. However, neither of these samples showed fluorescence recovery (indicating inhibition of LCFA metabolism), as seen in A. All fluorescence traces are representative of five separate measurements. D: Quantification of the cumulative effect of inhibitors on LCFA transport (A–C), comparing rat adipocytes and SUVs (raw data not shown). The percent OA transported was determined (see the Materials and Methods) for five and three replicates of adipocytes and SUVs, respectively, treated with each inhibitor or condition. Data are represented as mean ± SD. SUVs were compared with adipocytes by a Student’s t-test, *P<0.05 and **P<0.005.
experiments carried out at 37°C, we find a significantly shorter half-life for SSO hydrolysis into free OA. In our M/K cell buffer and SUV buffer, the SSO hydrolysis half-life was found to be 9.6 min (Fig. 3D) and 3.7 min (Fig. 3E), respectively. Although hydrolysis is significantly faster at 37°C, neither of these buffer conditions indicate that SSO hydrolysis is rapid enough to explain the immediate pH drop seen in Fig. 3B and C when SSO was added to SUVs.

With relatively slow hydrolysis of SSO (3–10 min), our BCECF studies raised additional questions about how SSO altered pH. SSO added to SUVs appears to fully equilibrate within seconds and equilibrate into cells in under 5 min (Fig. 3B, C), and we tested whether SSO could indiscriminately modify intra- and extracellular proteins. Hydrolysis of SSO will leave an inactive sulfo-NHS and free OA, which could acidify cytosol but not modify exposed lysines. Alternatively, if the entire SSO compound was able to passively diffuse by flip-flop through the PM, intracellular proteins could be modified and functionally hindered and would provide a possible explanation for the faster pH decreases observed in Fig. 3.

To probe this hypothesis, we developed a novel rabbit “anti-SSO” antibody including positive and negative controls. Using a dot blot, the antibody was observed to bind SSO-treated protein lysate (Fig. 4) and had essentially no binding when cells are not treated with SSO (Figs. 4B; only nuclear background binding was observed using microscopy). The antibody was also found to bind SSO-modified proteins in a dose-responsive manner (Fig. 5).

We performed confocal microscopy with this anti-SSO antibody on HeLa cells expressing CD36 (Fig. 5A–D) or control empty vector (Fig. 5E, F) after a 20 min SSO treatment on the living cells (prior to fixation). Initially, the cells were not permeabilized in order to investigate CD36 specificity of SSO and potential SSO-modified ectodomains of PM localized proteins, because we have previously shown that most of the CD36 in our cells is PM localized (18). We found that our SSO antibody was dose responsive and, at 100 µM SSO (Fig. 5A, B), there was a significant amount of CD36 that was not modified by SSO (red color), some SSO-modified CD36 (yellow color), and other membrane proteins that appeared to be modified by SSO that were not localized near CD36 (green color). At 200 µM SSO (Fig. 5C, D), the dose commonly used in many previous FA uptake experiments (51) [although 400 µM is now being used with more frequency (88, 92, 95)], most of the CD36 appears to be SSO-modified (yellow), although other SSO-modified proteins are still present (green color). This finding was also confirmed in cells that do not express CD36: substantially more SSO-modified proteins were stained on the PM as SSO treatment concentration was increased from 100 to 200 µM (Fig. 5E, F).
our observation of rapid acidification (seconds to minutes)

The ability of SSO to diffuse through the membrane supports its ability to lower pH and was found to modify numerous intracellular proteins. Interestingly, although SSO also entered cells and SUVs without permeabilization after fixation in order to probe for potential intracellular proteins modified by SSO. In cells that express CD36, significantly more SSO-modified protein was seen at both 100 and 200 μM SSO treatments with permeabilization (Fig. 5G–N) than without permeabilization (Fig. 5A–F). Once again, much of the CD36 was SSO modified, especially at 200 μM SSO (yellow color, Fig. 5J), but other proteins were clearly seen to be SSO-modified (green color, Fig. 5J, N). The partial but incomplete colocalization with CD36 was further verified using 3D confocal microscopy including 100 μM SSO treatment and PM permeabilization (Fig. 6A), which also showed significant intracellular localization of anti-SSO within the z-plane (Fig. 6Af–i).

These studies of SSO properties demonstrate for the first time that SSO not only alters intracellular pH but also nonspecifically modifies proteins other than CD36. In addition, at least some SSO also crosses the PM without hydrolyzing and modifies various intracellular proteins. The ability of SSO to diffuse through the membrane supports our observation of rapid acidification (seconds to minutes) of the internal volume of cells and vesicles. Also, depending on which proteins are being SSO-modified, these covalent changes may have side-effects in longer-term metabolism of FA, which we further investigated along with the other purported inhibitors of FA membrane transport.

Metabolic studies reveal that numerous compounds inhibit long-term FA conversion to TG

To better distinguish between LCFA transport at the PM, effects of short-term metabolism (Fig. 2) and subsequent long-term LCFA metabolism, conditions similar to our fluorescence assays, were used to measure intracellular [14C]OA incorporation into more complex lipids in the presence of each inhibitor (Fig. 7). For this, [14C]OA was added to untreated and inhibitor-treated cells, which were allowed to metabolize the FA for 20 min, after which cellular lipids were extracted to determine the percentage of incorporation into TG compared with untreated control cells. In a previous study, our group determined the metabolic fate of added [14C]OA at times 0 s, 10 s, 1 min, 5 min, and 20 min and determined that at 20 min, >80% of the total [14C]OA was converted to TG in rat adipocytes, with negligible changes in DG (12). In fact, even by 5 min, almost all unesterified OA had been metabolized by these cells. Therefore, in this study, our focus was on potential changes after 20 min in rat adipocyte TG storage in the presence of each inhibitor.

Similar to results in our FA transport studies (Fig. 2), we found that QUER drastically altered metabolism at 20 min. This affirms that the inverted BCECF signal response seen in fluorescence studies with QUER was indeed likely due to the yellow-emitting property of QUER, with the lack of BCECF fluorescence (i.e., pH) recovery (Fig. 2B) indicating that metabolism was in fact inhibited once OA entered the cell. Thus, we conclude that QUER inhibits TG synthesis specifically and rapidly, and it maintains inhibition over a long period of time, which is in agreement with other work (86).

The other yellow-emitting inhibitor, DIDS, did not appear to rapidly alter fluorescence recovery of BCECF (Fig. 2B), but after 20 min, a significant reduction in metabolism was in fact observed. PC and DEPC also exhibited a similar longer-term metabolic inhibition. PHLO and PCPL both had shown decreases in pH magnitude (Fig. 2A), indicative of decreased LCFA uptake amount, and they also inhibited TG synthesis over 20 min. As discussed before, PHLO may inhibit LCFA diffusion, at least at the concentrations that are used in these and other studies (Table 1) by permeating the lipid bilayer, intercalating between lipid chains, and interacting with the polar head groups of phospholipids, thereby altering their ionization properties. PCPL is also unique because it enters both adipocytes and protein-free SUVs and dramatically lowers pH levels (Fig. 3B, C), so it is not surprising that it significantly reduces LCFA metabolism within the cell over long periods of time.

Interestingly, although SSO also entered cells and SUVs to lower pH and was found to modify numerous intracellular proteins (Figs. 3, 5, 6), SSO treatment did not significantly alter intracellular [14C]OA conversion to TG at this
The effects inhibitors have on fatty acid transport

while it appears likely that SSO modifies many proteins, including those involved in FA metabolism within seconds of intracellular FA entry (as seen in Fig. 2), it is possible that the proteins that are unmodified by SSO are able to metabolize 10 μM of OA over 20 min to levels that are nearly comparable to control cells. Therefore, any reductions in FA uptake previously reported elsewhere are likely the result of intracellular acidification in the presence of high concentrations of SSO.

Palmitate acidifies the cells and decreases OA metabolism, while octanoate does not affect OA metabolism or acidification

Another argument in favor of protein-mediated LCFA transport has been the saturability and specificity of LCFA uptake into cells. For example, LCFA such as palmitate have been shown to reduce the uptake of other LCFA into cells, suggesting that purported transporters are specific for LCFA (23, 27, 32, 43, 52). We investigated these conclusions by preincubating cells with a 10-fold excess of MCFA octanoate [C8:0] or LCFA palmitate [C16:0] and then adding [14C]OA (Fig. 8A). Incorporation of [14C]OA into intracellular TG was then measured after 2.5 min and 20 min. Parallel experiments, in which [14C]OA was added after cooling the cells to 4°C, were used as negative metabolic controls. Additionally, the effects of preincubating adipocytes isolated from fasted rats with 2-DOG (instead of glucose) was used as an additional negative control. Both of these controls were also shown to inhibit pH recovery in fluorescence studies for minutes after OA addition (Fig. 2C).

Cooling cells to 4°C drastically decreased metabolism, as expected, as did treating glucose-starved cells with 2-DOG. Interestingly, palmitate at both 2.5 and 20 min also significantly decreased OA conversion into TG, but not as severely as cooling to 4°C or 2-DOG treatment. Also, the inhibition effect trends upward after 2.5 min, indicating that palmitate may be partially competing with OA as a metabolic substrate. Therefore, competition for metabolic enzymes might explain the “inhibitory” effect of palmitate on OA, especially in the commonly used OA transport (uptake) assay that uses radioactive-labeled OA added to cells modulation (yellow) and without SSO-modification (red). D: An enlarged inset from the white box in C, displaying significantly more SSO-modified PM-localized protein (green) and SSO-modified CD36 (yellow). G–N: Live HeLa cells expressing CD36 were treated with 200 μM of SSO (G–J) or 100 μM of SSO (K–N), then washed, fixed with paraformaldehyde, and permeabilized using 0.1% Triton X-100. After incubations with primary mouse anti-CD36 and rabbit anti-SSO antibodies, the cells were washed and stained with anti-mouse Alexa fluor 594, anti-rabbit Alexa fluor 488, and nuclear stain DAPI. Only merged images are shown. B: An enlarged inset from the white box in A, illustrating PM-localized CD36 with SSO-modification (yellow) and without SSO-modification (red).

Fig. 5. SSO modifies numerous PM and intracellular localized proteins, including CD36. HeLa cells expressing CD36 (A–D) or control empty vector (E, F) were washed and fixed with paraformaldehyde after a 20 min incubation in serum-free DMEM containing either 100 μM of SSO (A, B, E) or 200 μM of SSO (C, D, F). These cells were not permeabilized in order to only stain PM-localized proteins. After incubations with primary mouse anti-CD36 and rabbit anti-SSO antibodies, all cells were washed and stained with anti-mouse Alexa fluor 594, anti-rabbit Alexa fluor 488, and nuclear stain DAPI. Only merged images are shown. B: An enlarged inset from the white box in A, illustrating PM-localized CD36 with SSO-modification (yellow) and without SSO-modification (red).
Fig. 6. 3D confocal microscopy of SSO-modified protein shows intracellular modifications and negative control shows almost no binding of anti-SSO without SSO treatment. A: HeLa cells expressing CD36 were incubated for 20 min in serum-free DMEM containing 100 μM of SSO, then washed, fixed with paraformaldehyde, and permeabilized. After incubations with primary mouse anti-CD36 and rabbit anti-SSO antibodies, the cells were washed and stained with anti-mouse Alexa fluor 594, anti-rabbit Alexa fluor 488, and nuclear stain DAPI. A representative cell is shown as a merged image, including the XY-plane through the cell (a–d) and a full rotation of the cell through the Z-plane (f–i). SSO-modified proteins that are not CD36 (green) are seen throughout the cell, including near the nucleus (a–c, g, i). CD36 not modified by SSO is also present, especially on the outer PM of the cell (d–g). SSO-modified CD36 is seen as yellow in each merged image, and further illustrated by a colocalization colormap (e) where red indicates areas of highest colocalization, blue indicates lowest, and the nuclear background colocalization signal from DAPI has been masked. B: Negative antibody control showing CD36-expressing HeLa cells probed with rabbit anti-SSO and anti-CD36 antibodies without any SSO treatment, along with DAPI nuclei staining.

that are quenched at different time points, with end-point radioactivity being measured. Conversely, MCFA octanoate had no effect on \( ^{14} \text{C} \) OA metabolism at any time point when compared with the control, which is in agreement with previous studies (96).

To further determine whether the effect of palmitate on \( ^{14} \text{C} \) OA metabolism is specific to inhibition of metabolic processes or whether it is also an indirect effect resulting from disrupted transport of \( ^{14} \text{C} \) OA across the PM, we performed experiments in protein-free lipid vesicles (Fig. 8B). This was done by monitoring BCECF fluorescence in response to the pretreatment of 100 μM of octanoate or palmitate, followed by a subsequent dose of 10 μM of OA. When 10 μM of OA were added, the magnitude of the resulting pH change decreased in SUVs pretreated with LCFA palmitate, and this effect was several-fold weaker for MCFAs such as octanoate, possibly due to the increased partitioning of LCFA over MCFAs (97). Interestingly, when the pH change produced by octanoate and palmitate alone was examined, the pre-acidification of SUVs was much more pronounced following palmitate addition (light gray bars, Fig. 8B). Because competition for LCFA transporters cannot occur in this protein-free membrane system, our results suggest that lower intravesicular pH induced by high concentrations of palmitate is unfavorable to the inward diffusion of subsequently dosed OA according to our model (9). We believe that this phenomenon also explains the initial lag in \( ^{14} \text{C} \) OA metabolism observed in cells pretreated with palmitate, and the gradual loss of this inhibition is likely the result of dissipating the pH gradient with time.

**DISCUSSION**

**Three possible mechanisms of LCFA translocation with inhibitors point to the diffusion model**

The goal of this study was to: i) further clarify the predominant mechanism of LCFA uptake (diffusion or protein-mediated transport) in the presence of various purported inhibitors; ii) clarify the mechanism of protein-mediated transport should this process demonstrate specific inhibition by individual inhibitors, with a special focus on the widely used SSO; and iii) investigate the role of metabolism on the net uptake of LCFA into cells and how these processes may be impacted by these inhibitors.

The inhibitors selected for this study are chemicals and enzymes that have been commonly used in other assays to support a hypothetical protein-mediated transport mechanism for LCFA (Table 1). We selected multiple inhibitors and assay conditions in an attempt to inhibit LCFA transport directly or indirectly by several possible mechanisms. Most are nonspecific chemicals and proteases that are believed to inhibit one or more of the proteins of interest in this study. The high concentrations employed in our study were similar to those used in other studies.
Because our dual-fluorescence assays provide a direct and real-time measure of LCFA binding and transmembrane movement, we developed several predictive models to better understand how various mechanisms of LCFA transport would impact the direction or magnitude of detected pH changes (Fig. 9). Because ADIFAB fluorescence was altered by most inhibitors (Fig. 1B), our main focus was on the BCECF response. Although ADIFAB did not reliably report titrated concentrations of exogenous OA, it did show a fast (milliseconds to seconds) response when LCFA was added that was comparable to BCECF (Fig. 2). This rapid initial response has also been observed with several other cell types (12–18).

As shown in our model, protein-mediated transport of only LCFA anions to the inner leaflet (Fig. 9B) would have produced a pH increase as measured by intracellular BCECF probe, because transported LCFA would insert into the inner membrane and become 50% protonated according to their physical chemical properties, resulting in loss of protons from the cytosol of cells. No such BCECF pH increase was observed in the absence of inhibitor in this study. Passive diffusion or cotransport (Fig. 9A, C) of LCFA anions with H⁺ would be expected to produce an intracellular pH drop detected by intracellular BCECF, as we observe in our experiments. However, in the case of protein-mediated cotransport, application of high concentrations of LCFA transport protein inhibitors would significantly ablate the pH drop, which we did not observe in this study. Therefore, we propose diffusion (Fig. 9C) to be the predominant mechanism of LCFA translocation because the observed pH drop rate and magnitude was found to be independent of any protein-mediated transport process. This was also true in parallel experiments with protein-free lipid vesicles (Fig. 2D), further confirming this diffusion model.

**Inhibitors do not alter the rate of LCFA uptake but some reduce LCFA diffusion by pre-acidification of the inner volume**

In both adipocytes and protein-free SUVs, we observed that SSO and PCPL decreased the magnitude of pH drop size produced by a subsequent dose of OA (but not the rate of fluorescence change). When independently tested in adipocytes and SUVs, addition of SSO and PCPL alone caused a large pH drop, which indicates that these inhibitors are crossing the bilayer (or additionally hydrolyzing into free OA, in the case of SSO) and acidifying the intracellular/intravesicular space. When LCFA was then added to these pre-acidified SUVs or cells treated with SSO or PCPL, a pH drop size decrease would therefore be expected, according to the diffusion model.

**SSO alters intracellular and PM-localized proteins and is nonspecific for CD36**

SSO is often claimed to be a specific inhibitor of CD36 [for review see (27)] and has been shown to specifically bind K164 of CD36 (88). CD36 is at the center of the LCFA transport debate and because SSO is the most widely used inhibitor targeting LCFA uptake, it was the most closely investigated of the nine other compounds used in this
study. Additionally, because we found that SSO independently acidifies protein-free SUVs as well as cells (Fig. 3), we aimed to determine whether SSO was merely generating free OA by hydrolysis while still outside the cell/SUV (leaving a free nonreactive NHS in buffer and a free FA that would acidify the cell) or whether SSO was also flip-flopping through the PM by a mechanism similar to OA (Fig. 10). The translocation of intact unhydrolyzed SSO could disrupt cell function because this molecule is highly reactive and could covalently react with charged Lys side chains on the surface of any number of intracellular proteins. Alternatively, SSO could also be specific to the extracellular PM domain of CD36 [i.e., the FA-binding pocket as often suggested (29, 88, 98)] or may also react with other PM-localized proteins.

To address these important issues, we first determined the half-life of SSO hydrolysis at 37°C in buffers commonly used in our studies (Fig. 3D, E). Although significant hydrolysis occurred within minutes, complete hydrolysis of SSO was not observed in any of the buffers or media tested. Additionally, the hydrolysis did not occur on a millisecond to second timescale that would explain the rapid pH drops observed when SSO was added to SUVs and cells. Hydrolysis occurred on a timescale of seconds to minutes and would be expected to cause a slower rather than an instantaneous pH drop, which was not observed (Fig. 3B). Therefore, we investigated to determine whether SSO may actually enter the cell without the prerequisite of hydrolysis into free OA. We developed a novel antibody against SSO-modified lysines and demonstrated that it was highly
A wide array of inhibitors alter metabolism of LCFA

While our real-time assays demonstrated that purported inhibitors of LCFA transport did not in fact inhibit LCFA from entering cells and SUVs, a large percentage of them did inhibit OA conversion into TG inside rat adipocytes. Within 5 min post addition, QUER was the only compound to drastically slow BCECF and ADIFAB fluorescence recovery in adipocytes (Fig. 2B), which is suggestive of metabolic inhibition. Known conditions used to block FA metabolism in adipocytes (4°C and 2-DOG in place of glucose) produced a similar response (Fig. 2C). At 20 min, however, even more of the tested compounds were found to have a significant effect on FA metabolism. The percent of metabolic inhibition for compounds with statistically significant inhibition were: DIDS ~56%, PHIL0 ~40%, PCPL ~70%, QUER ~58%, PG ~35%, DEPC ~10% (Fig. 7), and 4°C ~67% (Fig. 8). Note that within the range of error, PCPL and QUER both inhibited metabolism as severely as the 4°C-cooled cells. Additionally, with QUER, esterification of only ~10% of the added [14C]OA (data not shown) suggests that this compound may directly inhibit the initial step of LCFA activation into LCFA-CoA, which represents a previously unreported mechanism of action for this compound. Furthermore, using 100-fold excesses of another LCFA (palmitate), the resulting “inhibitory” effect on LCFA transport can be explained by: i) competition for the same LCFA metabolic enzymes (Fig. 8A) and ii) a reduction of OA diffusion and the associated release of more H+ across the membranes according to our model. Because MCFAs such as octanoate have higher aqueous solubility, there is significantly less pre-acidification upon pretreatment (Fig. 8B).

Limitations and Conclusions

A widely used assay to determine LCFA transport mechanisms in cells in other studies has been performed by adding radioactive exogenous LCFA, quenching the cells at various time points, and measuring radioactivity at each endpoint. The present work included some of these methods as a point of comparison. The major issue with the commonly used uptake measurements quantified by endpoints is that they do not identify which specific step is being inhibited (i.e., transport, CoA esterification, or metabolism), if any, nor do they report on the metabolic fate of added LCFA. Strategies to quantify real-time uptake, therefore, are valuable. Potential issues arise when using fluorescence indicators in live cell experiments, but we believe that these indicators are useful in this context despite these limitations. The authors thank Michael T. Kirber for his technical confocal microscopy assistance and Philip N. Collier for his organic chemistry expertise.

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