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Labeling of Adipocyte Membranes by Sulfo-*N*-Succinimidyl Derivatives of Long-Chain Fatty Acids: Inhibition of Fatty Acid Transport

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Summary. Sulfo-*N*-succinimidyl derivatives of the long-chain fatty acids, oleic and myristic, were synthesized and covalently reacted with isolated rat adipocytes. The plasma membrane proteins labeled by these compounds and the effect of labeling on the transport of long-chain fatty acids were investigated. Sulfo-*N*-succinimidyl oleate (SSO) and myristate (SSM) inhibited the transport of fatty acids (by about 70%). Inhibition of fatty acid transport was not a result of alterations in cell integrity, as intracellular water volume was not changed. It did not reflect effects on fatty acid metabolism, since it was observed under conditions where greater than 90% of the fatty acid taken up was recovered in the free form. The inhibitory effect was specific to the fatty acid transport system, as the transport of glucose and the permeation of retinoic acid, a substance with structural similarities to long-chain fatty acids, were unaffected. Sulfosuccinimidyl oleate reacted exclusively with a plasma membrane protein with an apparent size of 85 kDa while sulfosuccinimidyl myristate also labeled a 75-kDa protein. These proteins were among the ones labeled by diisothiocyanodisulfonic acid (DIDS) which also inhibits fatty acid transport irreversibly. The data suggest that the 85-kDa protein, which is the only one labeled by all three inhibitors is involved in facilitating membrane permeation of long-chain fatty acids.

Key Words fatty acids · transport · sulfo-*N*-succinimidyl fatty acid · plasma membrane protein · adipocyte · diisothiocyanodisulfonic acid

Introduction

Fat cells are the primary site for lipid storage and mobilization and, as such, one of their major roles is the uptake and release of long-chain fatty acids (FA). The permeation of long-chain fatty acids across the rat adipocyte plasma membrane has been reported to be mediated by a specific transport system which has the characteristics of facilitated diffusion (Abumrad et al., 1981; Abumrad, Park & Park, 1984; Schwieterman et al., 1988).

Abumrad et al. (1984) reported that 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), which potently inhibits the anion exchange protein (Band 3) in human erythrocytes (Cabantchik & Rothstein, 1974), also irreversibly inhibits long-chain FA transport in rat adipocytes. Gel electrophoresis of plasma membranes isolated from rat adipocytes which had been incubated with tritiated dihydro DIDS demonstrated a prominent peak of radioactivity associated with membrane component(s) migrating like a protein of M_r 80 to 90 kDa. On the other hand Schweiterman et al. (1988) implicated a 40-kDa membrane protein in the uptake of oleate by isolated rat adipocytes. This protein was isolated from detergent or salt-treated crude plasma membrane fractions by chromatography using an oleate-agarose affinity matrix as first described for serum albumin (Peters, Taniuchi & Anfinsen, 1972). An antibody raised against a M_r 40-kDa plasma membrane fatty acid binding protein from rat hepatocytes cross-reacted with the adipocyte counterpart and inhibited FA transport up to 60% (Stremmel et al., 1985; Schwieterman et al., 1988). The fatty acid binding protein was later shown to be similar in sequence and properties to the mitochondrial enzyme glutamate-oxaloacetate transaminase (mGOt) (Potter, Sorrentino & Berk, 1989).

In this report we have re-examined in detail the binding of DIDS to adipocyte membranes. In addition we have synthesized monofunctional nonpermeable sulfosuccinimidyl derivatives of long-chain fatty acids as affinity labels for membrane proteins which bind FA.

Sulfo-*N*-succinimidyl esters of short carboxylic acids have been previously employed as protein-modifying agents. These activated esters react in high yield with nucleophiles in proteins by formation of a stable covalent bond. Preferred nucleophiles include α and ε primary amino groups, the phenolic oxygen of tyrosine, and the thiol sulfur of cysteine (Anjaneyulu & Staros, 1987). Symmetric bis(sulfo-

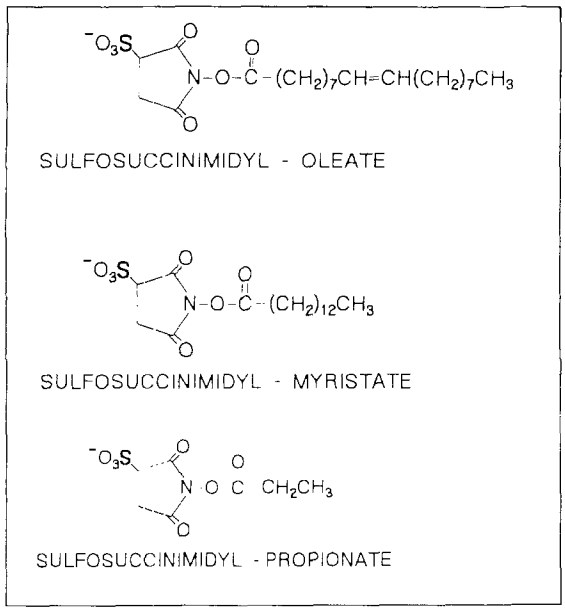

Fig. 1. The three sulfosuccinimidyl-FA synthesized: SS-oleate (SSO), SS-myristate (SSM), and SS-propionate (SSP). The acid was reacted with hydroxysulfosuccinimide in the presence of dicyclohexylcarbodiimide (DCC) under anhydrous conditions using dimethylformamide as solvent. Product identity was assessed by HPLC and mass spectrometry as detailed in the text

N-succinimidyl) esters have previously been synthesized and used as protein crosslinking agents to probe protomer-protomer interactions in protein complexes in solution (Giedroc et al., 1985; Richtand et al., 1985) and in membranes (Staros, 1982; Donovan & Jennings, 1986). In this study we describe the synthesis of monofunctional sulfosuccinimidyl esters of oleate, myristate and propionate using a modification of previous procedures. The effect that these active esters have on the uptake of long-chain fatty acids by rat adipocytes and the identification of membrane proteins and lipids with which they react are reported.

Materials and Methods

Synthesis of Sulfosuccinimidyl-Oleate, Myristate and Propionate

The syntheses of unlabeled and radiolabeled (Fig. 1) sulfosuccinimidyl derivatives of oleate (SSO), myristate (SSM) and propionate (SSP) were accomplished by a modification of the procedure described for the synthesis of carboxylic acid-$HOSu(SO_3)$ active esters by Staros (1982). The fatty acid to be derivatized (0.25 mmol, Sigma), $HOSu(SO_3)Na$ (0.25 mmol, Pierce Chemical), and dicyclohexylcarbodiimide (DCC) (0.275 mmol) were dissolved in 0.5 ml of dry *N,N*-dimethylformamide (DMF) and stirred at room temperature overnight. The precipitated dicyclohexylurea was removed by filtration (Whatman 1). The filtrate was transferred to 3°C for 4 hr. Eight volumes of ethyl acetate were added, and the precipitated product was collected by filtration (Ultipor $Nylon_{66}$, 45-μm pore size) under nitrogen in a Glove Bag (Instruments for Research and Industry) and then stored in a vacuumed desiccator over phosphorus pentoxide.

Characterization of Esters

Identity of SSO was confirmed by mass spectral analysis by observation of the molecular ion. Product purity was estimated for all compounds by high performance liquid chromatography (HPLC) to be greater than 90% on a Beckman 344 gradient system fitted with a 4.6 × 250 mm C_{18} reverse-phase column (Altech). Hydrolysis of the product in water into the original reactants could be observed by HPLC with a half-time of about 2 hr. The mobile phase consisted of methanol/water (70/30 vol/vol) delivered for 15 min and then followed by a 5-min linear gradient to a final phase of 100% methanol. Immediately after dissolving in water, ^{3}[H]-SSO constituted greater than 90% of the radioactivity recovered from the column with free ^{3}[H]-oleate accounting for about 10% of the counts. Separation between the two compounds was complete since free oleate could only be eluted using 100% methanol.

Synthesis of ^{3}[H]-DIDS

[^{3}H]-DIDS was synthesized from [^{3}H]-DADS (New England Nuclear) according to the procedure of Cabantchik and Rothstein (1974). The product was tested by its inhibition of fatty acid transport into adipocytes and by its labeling of red cell membranes, where it reacted with high specificity with Band 3.

Preparation of Adipocytes

Male Sprague-Dawley rats, 170–200 g, obtained from Harlan Industries, were sacrificed by decapitation. The epididymal fat pads were removed and adipocytes were isolated following methodology detailed previously (Abumrad, Park & Whitesell, 1986). The washed cells were suspended (30%, vol/vol) in Krebs-Ringer HEPES buffer (KRH) containing 0.2% fatty acid-free bovine serum albumin (BSA; Sigma, fraction V, fatty acid free) and glucose (2 mM) except where indicated.

Treatment with Sulfosuccinimidyl-Fatty Acid Derivatives

Stock solutions of SSO, SSM, or SSP were made by dissolving the compounds in dimethyl sulfoxide (DMSO). These stocks were used to add aliquots to the adipocyte cell suspension to the final concentrations indicated. DMSO concentration was kept below 0.05% and was added to control cells in all experiments. The cells were incubated in a metabolic shaker at 37°C for 25 min except where indicated. At the end of the incubation the cells were washed three times with KRH buffer (2 × *v*) containing 0.2% FA-free BSA in order to remove any unbound sulfosuccinimidyl-FA as well as any hydrolyzed FA. The cells were resuspended to a 30% vol/vol lipocrit and kept at room temperature for assay of FA transport.

FA Transport Assay

Membrane permeation of labeled FA was measured as described in detail previously (Abumrad et al., 1981, 1984). Medium (40 μl) containing labeled and unlabeled FA (typically 50–100 μM and 2000 cpm/μl), complexed to BSA, were pipetted into the bottom of polystyrene tubes. The assay was begun by the addition of an aliquot (usually 20–40 μl) of the fat cell suspension, and the mixture was swirled gently to ensure adequate mixing. At times ranging from 2 sec to 2 min the uptake process was stopped by rapid addition of 5 ml of an ice-cold phloretin-KRH stop solution, and the cells were immediately recovered by filtration on glass-fiber filters (Gelman Type A/E 61630). The filters were washed twice with 1 ml of cold stop solution, transferred to scintillation vials containing 5 ml counting fluid (ACS, Amerhsam) and radioactivity was determined on a Beckman LS 3801 counter. Zero-time radioactivity and filter blanks were determined as described previously (Abumrad et al., 1984). Blanks constituted about 20–40% of the 12-sec uptake value at FA : BSA ratios ranging from 1–3 and were routinely subtracted from uptake values.

Determination of FA Uptake Rates

Initial rates of long-chain FA uptake were determined from the best fit curve for time courses utilizing nonlinear regression analysis (Whitesell, Regen & Abumrad, 1989). The mathematical model used assumed two compartments for cellular FA uptake and is described by the following equation:

$$S_t = S_{1\infty}(1 - e^{(-\lambda t)}) + (kt).$$

In this model, S_t is the uptake of FA (nmol FA/ml packed cells) at time t, $S_{1\infty}$ is the amount of FA taken up at steady state in the first compartment, λ is the fractional rate of approach to steady-state uptake and k is the rate of FA entering the second compartment. The initial rate of FA uptake is given by $S\infty\lambda + k$. The first compartment reflects mainly the accumulation of free label. The second compartment reflects the rate of accumulation of FA metabolites. In the case of incubations without glucose where most of the FA remained in the free form inside the cell, a one compartment model (lacking the kt form) fit the time courses well.

In all experiments the cells took up about 10% of the added FA at the end of 1 min so changes in total FA were small and would not be expected to alter significantly the binding of FA : BSA or the rate of dissociation of the FA : BSA complex. Various estimates for this rate have been published (Svenson, Holmer & Anderson, 1974; Scheider, 1978; Weisiger & Ma, 1987). Under our experimental conditions, based on the most conservative estimate of 2.8×10^{-2} per sec, supply of unbound FA by the FA : BSA complex was not limiting and greatly exceeded (44-fold) the uptake rate.

Glucose-Transport Assay and Measurement of Intracellular Water Space

Aliquots of cell suspensions were assayed for stereospecific uptake of glucose employing L-[^{3}H]glucose and D-[^{14}C]glucose. The glucose-transport assay followed the procedure previously described by Whitesell and Abumrad (1985). In some experiments transport of [^{14}C]3-O-methylglucose was assayed simultaneously with that of [^{3}H]oleate using the procedure described for FA transport.

To determine cell water space, aliquots (30 μl) of cell suspension were pipetted into microfuge tubes containing silicone oil and corn oil (about 100 μl each) and 140 μl of ice-cold phloretin-KRH-stop solution containing [^{3}H]water and [^{14}C]2-deoxyglucose. Cells were pelleted (between the two oils) by centrifugation for 30 sec in a microfuge. The intracellular water space in the cell pellet was calculated as the difference between the [^{3}H]water space and the [^{14}C]2-deoxyglucose space (deoxyglucose marked the extracellular space since it is added to the cells in the presence of cold stop solution).

Determination of Radiolabeled Sulfosuccinimidyl-FA Binding to Adipocyte Membranes

Adipocytes from 6–8 rats were incubated with 200 μM [^{3}H]SSO or [^{3}H]SSM at 37°C for 25 min as described for transport studies. The adipocyte plasma membrane fraction was isolated according to a modification of the technique of Kono, Robinson and Sarver (1975), as described by Abumrad et al. (1984). This procedure yields a 15-fold enrichment in 5'nucleotidase of plasma membrane fractions. Membrane pellets were stored at 70°C until use.

To identify reaction sites of the SS-FA derivatives, membranes were subjected to SDS PAGE electrophoresis (7.5% acrylamide or 5–10% acrylamide gradient) according to Laemmli (1970). Gels were routinely stained with Coomassie blue or silver nitrate to identify protein bands or subjected to autoradiography to identify bands radiolabeled by the SS-FA compounds.

Studies using [^{3}H]-DIDS were done according to the same protocol outlined for SS-FA except that cell incubations were carried out for 45 min according to conditions shown previously to irreversibly inhibit oleate transport (Abumrad et al., 1984).

Results

Effect of Sulfosuccinimidyl-Fatty Acids on Uptake of Long-Chain Fatty Acids

Figure 2*A* illustrates the time course of fatty acid uptake into adipocytes treated with SSO as compared with control (untreated) cells. Adipocytes were incubated for 25 min at 37°C with the indicated concentrations of SSO added to the cells as 2–4 μl from a concentrated DMSO stock. Separate experiments showed that inhibition was apparent following a 5-min incubation and was optimal by 10 min. However, for consistency and convenience a 25-min incubation was routinely used. At the end of the incubation the cells were washed thoroughly with KRH containing 0.2% BSA to remove unbound SSO and then assayed for [^{3}H]oleate uptake. Changes in label-specific activity and binding to albumin were ruled out as complicating factors, as FA measured in media from washed and treated cell suspensions just before the assay were similar (75 and 78 nmol/nl packed cells for control and SSO-treated cells, respectively).

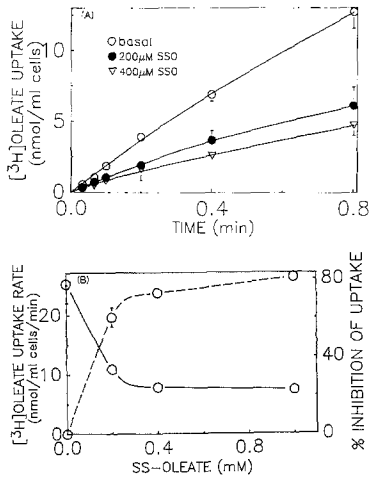

Fig. 2. (*A*) Effect of SSO on the time course of [^{3}H]oleate transport. Isolated adipocytes (30% vol/vol in KRH containing 0.2% BSA and 2 mM glucose) were incubated with SSO or oleate (200 μM) for 25 min at 37°C. The cells were washed three times to remove all unreacted SSO or residual oleate, and cell density was readjusted to 30% (vol/vol). [^{3}H]oleate uptake was studied at room temperature as described in Materials and Methods. Final concentrations of [^{3}H]oleate and BSA in the assay mixture were, respectively, 50 μM (3000 cpm/μl) and 90 μM. Preincubation with oleate had no effect on [^{3}H]oleate uptake, and the data was omitted for clarity. Time courses were curve fitted using nonlinear regression. (*B*) Inhibitory potency of various concentrations of SSO based on pooled data from 4–10 experiments (left ordinate). Percent inhibition by SSO of initial rates of oleate uptake, computed within each experiment and then averaged, is shown on the right ordinate.

As shown in Fig. 2*A*, SSO (200 μM) inhibited oleate uptake by about 65%. In parallel incubations, free oleate (200 μM) or free *N*-hydroxysulfosuccinimide (200 μM) were without effect (*data not shown*). The effects of several concentrations of SSO on initial rates of oleate uptake are shown in Fig. 2*B* for data pooled from 4–10 experiments. The averaged percent inhibition of uptake obtained by comparing treated and control cell preparations within each experiment is shown on the right axis. In separate experiments, treatment with SSO also inhibited the uptake of [^{14}C]linoleate and [^{14}C]stearate by about 65% (*data not shown*).

In order to determine the specificity of the effect of SSO on FA uptake several control studies were done. The possibility that SSO might directly or indirectly cause cell leaking or swelling was investigated by measuring intracellular water space (Table 1). No statistically significant effect of SSO was observed on intracellular water space at concentrations of 200 or 400 μM. Stereospecific uptake of glucose was determined and found to be largely unaffected. As shown in Table 1, a small nonstatistically significant inhibition was observed on basal or insulin-stimulated glucose uptake. Experiments were also conducted where the transport of [^{14}C]3-O-methylglucose was measured simultaneously with that of [^{3}H]oleate using the filtration assay. In these experiments, treatment with SSO did not alter glucose transport (measured over a period of 0.1–2 min) or sugar equilibrium space (measured at 60 min) under conditions where oleate uptake was inhibited by about 65% (*data not shown*).

Table 1. Effect of sulfosuccinimidyl oleate (SSO) on intracellular water space and on glucose transport[a]

SSO (μM)	Intracellular water space	Glucose transport	
		Basal	+ Insulin
	(% of control)		
200	113 ± 18	84 ± 8	—
400	85 ± 19	88 ± 11	81 ± 9

[a] Adipocytes were treated with the indicated concentrations of SSO for 25 min at 37°C. Cells were then washed to remove unreacted SSO and were assayed for glucose (2 mM) transport in the absence and presence of insulin. Intracellular water volume was assessed using double labeling with ^3H_2O and [^{14}C]2-Deoxyglucose added in the presence of cold buffer containing phloretin. Glucose transport in cells treated with SSO is shown as percent of transport in control cells (basal and insulin treated). Mean basal glucose transport rates were 0.5 ± 0.6 μl of medium cleared per μl cell water per min. Cell water averaged 1 μl per 5 × 10^5 cells. Insulin stimulated uptake about fivefold.

The effect of SSO treatment on the permeation of retionic was also investigated. Retinoic acid shares structural similarities with long-chain FA which include a 9-carbon-hydrocarbon chain and a carboxylic group. The presence of a trimethylated cyclohexene ring renders the molecule very lipophilic. The mechanism of its membrane uptake has not been well characterized, but it is generally thought to involve diffusion through the lipid bilayer. Permeation of retinoic acid (at 60 and 100 μM in the presence of 30 μM albumin) was unaffected by SSO treatment as shown in Table 2. This indicated that inhibition by SSO did not result in a general alteration of membrane properties or in a disruption of the diffusion of lipophilic substances. Further evidence to support this interpretation was the observation that inhibition of FA uptake in SSO-treated cells did not show a dependence on medium pH, i.e., on the proportion of nonionized FA in solution. Cells treated with SSO and then assayed at pH 6.0, 7.5 or 8.5 exhibited a similar inhibition of FA transport despite the large differences in the concentration of nonionized FA present at each pH (Table 3). This meant that the inhibitory effect did not reflect disruption of lipid diffusion of nonionized FA.

Sulfosuccinimidyl esters of carboxlic acids are

Table 2. Permeation of retinoic acid in adipocytes treated with SSO[a]

Treatment	Retinoic uptake (nmol/ml cells/min)		FA uptake (60 μM)
	(60 μM)	(100 μM)	
Control	170 ± 21	314 ± 35	22 ± 3
+SSO	162 ± 20	288 ± 33	5.8 ± 0.7

[a] Cells were incubated without or with SSO (440 μM) as described in the legend to Fig. 2, washed, resuspended (30% vol/vol) in KRH containing 30 μM BSA and assayed for FA or retinoic acid uptake using the same procedure described in Materials and Methods. Rates were derived from curve-fitted time courses (0.1–2 min) from two experiments.

Table 3. Effect of pH changes on oleate uptake and on its sensitivity to inhibition by SSO[a]

pH	Nonionized FA[b] (% of total)	FA uptake[c] relative to that at pH 7.5	SSO inhibition (%)
6.0	6.0	1.6	72
7.5	0.20	1.0	69
8.5	0.02	0.85	66

[a] Adipocytes treated with SSO according to the usual protocol were assayed for oleate transport at pH 6.0, 7.5 and 8.5. The inhibitory effect of SSO was computed by comparison of FA uptake rates in treated and untreated cells at each pH. Data for each pH are averages from two time courses (0.1–2 min).
[b] The percent of nonionized FA was calculated on the basis of a PK_a of FA in aqueous solutions of 4.8 (Small et al., 1984).
[c] Change in oleate uptake is shown as the ratio of the uptake at each pH over that at pH 7.5. The changes in oleate uptake observed when the pH of the assay medium was changed from 7.5 most likely reflect (but not proportionally) the effect of pH on FA-BSA binding (Spector, John & Fletcher, 1969) and a possible effect of pH on the transporter.

generally membrane impermeable (Donovan & Jennings, 1986), so effects on cell metabolism are unlikely. We determined that the same applied to SS esters of long-chain FA. As shown in Table 4, SSO treatment did not alter incorporation of oleate into lipids by adipocytes maintained in the presence of glucose. Furthermore SSO inhibition could be demonstrated in cells isolated and kept in the absence of glucose where greater than 86% of the FA inside the cell remained in the free unesterified form. As shown in Table 4 removal of glucose allowed recovery of 86% of the FA taken up at 1.0 min in the free unesterified form (*versus* 47% in the presence of glucose). However this did not alter the effect of SSO on the initial phase of oleate uptake which reflected initial influx of the FA (Fig. 3). Inhibition became less significant at later time points since uptake leveled off rapidly in the absence of glucose as a result of equilibration of free-FA label across the plasma membrane (Abumrad et al., 1984). The above data support the interpretation that the effect of SSO on FA uptake was exerted at the level of the plasma membrane.

Table 4. Effect of treatment with SSO on the distribution of intracellular oleate radioactivity into FA, diglycerides (DG), phospholipids (PL) and triglycerides (TG)[a]

Treatment	FA	DG	PL	TG
	(% of total cellular cpm)			
+Glc	48	20	3.0	29.0
+Glc, +SSO	47	21	3.5	28.5
−Glc	86	5.0	2.2	6.6
−Glc, +SSO	86	4.4	2.0	6.8

[a] Cells were isolated and maintained in buffer with or without glucose. Following treatment with SSO (400 μM, 25 min) the cells were washed, readjusted to a 30% vol/vol suspension and assayed for oleate incorporation into lipids (at 1.0 min). For this, Folch extracts of filtered cells were subjected to thin layer chromatography on silica G with petroleum ether : diethyl ether : acetic acid (80 : 20 : 1) as developing solvent (Abumrad et al., 1981). The data are averages from two experiments with duplicate determinations.

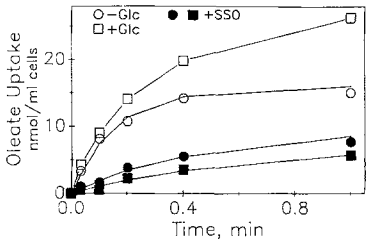

Fig. 3. Effect of glucose removal on inhibition of oleate uptake by SSO. Cells were isolated and maintained in the absence or presence of glucose (2 mM). Treatment with SSO and transport assay were as described under legend to Fig. 2 and in Materials and Methods. Distribution of intracellular [^{3}H]oleate in various lipids is shown in Table 3

The inhibitory effect of SSO decreased as the ratio of FA : BSA was increased. In two experiments oleate uptake was assayed at [^{3}H]oleate : BSA ratios of 0.5, 1.0 and 3.2. The percent inhibition of oleate uptake by SSO treatment (400 μM), estimated from two complete time courses, was 78, 69 and 50% at the respective ratios. This suggested that SSO did not interfere with the nonsaturable component of FA uptake which becomes more significant as the concentration of unbound FA is increased (Abumrad et al., 1981). The observation that, even at very low FA : BSA ratios (0.2), inhibition by SSO was not complete (79 ± 3%) might indicate the presence of more than one mediated process for FA uptake.

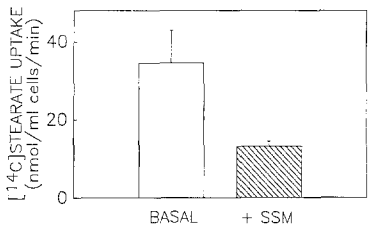

Fig. 4. Inhibition by succinimidyl myristate (SSM) of the uptake of [^{14}C]stearate. Cells were treated with SSM (200 μM) as described for SSO under the legend to Fig. 2 and in Materials and Methods. The data presented were pooled from three experiments where initial rates of uptake were derived from curve fitting. Final concentrations: [^{14}C]stearate, 60 μM, BSA = 29 μM

However, it more likely reflects some passive diffusion of FA at all ratios of FA : BSA, possibly in part as a result of SSO treatment. In many experiments treatment with SSO slightly increased (10–15%) the zero-time values consistent with this interpretation.

In order to study the effects of the length of the hydrocarbon chain on the inhibitory effect of sulfosuccinimidyl FA, two other derivatives, ^{3}[H]SS-myristate (14 carbons) and [^{14}C]SS-propianate (3 carbons), were synthesized and tested for their effect on oleate uptake. As shown in Fig. 4, [^{3}H]-SSM inhibited the uptake of [^{14}C]stearate. Similar data were obtained with [^{14}C]linoleate (*not shown*). The magnitude of the inhibitory effect of SSM was comparable to that seen with SSO. In contrast, SSP had little or no effect on [^{3}H]oleate uptake while cells treated with SSO in the same experiment demonstrated the typical inhibition (*data not shown*). These results were consistent with the described specificity of the FA transport system (Abumrad et al., 1984). The rest of the studies were conducted with SS-oleate and SS-myristate.

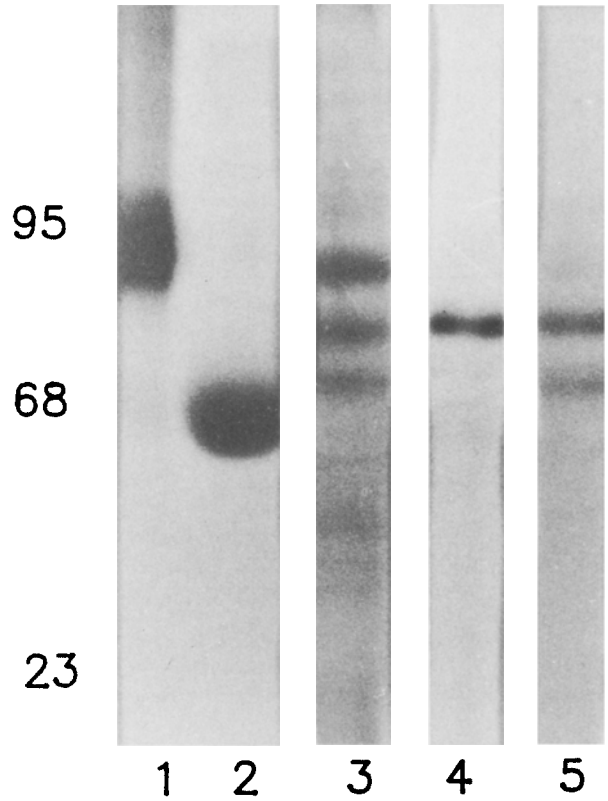

Fig. 5. Binding of [^{3}H]DIDS, [^{3}H]SSO and [^{3}H]SSM to adipocyte plasma membranes. Plasma membranes were prepared from cells treated with the various SS-derivatives or DIDS as described in Materials and Methods. All labeled derivatives were tested for their ability to inhibit oleate uptake. Laemmli gels (7.5%) loaded with 50–150 μg protein were processed for autoradiography as described in Materials and Methods. Labeled standards used were [^{3}H]DIDS-treated erythrocyte membranes (lane *1*) showing labeling of band 3 (95 kDa) and BSA (68 kDa) labeled with [^{3}H]SSM (lane *2*). Lanes *3–5* represent adipocyte membranes labeled with DIDS, SSO and SSM, respectively

Binding of Radiolabeled Sulfosuccinimidyl-FA and of DIDS to Adipocyte Membrane Proteins

Adipocytes treated as described with 200 μM radiolabeled SSO, SSM or DIDS were washed and used for preparation of membrane fractions. At least 95% of the radioactivity determined in the total cell membrane pellet was recovered in the plasma membrane fraction with less than 5% in the mitochondrial pellet. As shown in Fig. 5, all three compounds labeled a membrane protein of M_r 85–89 kDa (as determined on a 5–10% acrylamide gradient). In the case of SSO, this was the only protein band labeled. SSM (lane *5*) also labeled a 75-kDa protein. In addition to the two protein bands already mentioned, membranes from DIDS-treated cells (lane *3*) exhibited radioactivity in a protein which migrated parallel to the anion-binding Band 3 of erythrocytes (lane *1*) and weak labeling of two protein bands of molecular size smaller than 60 kDa and larger than 43 kDa. Labeling of membrane lipids (almost exclusively in phosphatidylethanolamine) was significant for SSO and DIDS and very low to undetectable with SSM. In the case of SSO, a maximum of 2 mmol of oleate were incorporated per mole of membrane phospholipid. This level of oleate binding would not be expected to alter membrane properties, which is consistent with the finding that retinoic acid permeation was not affected by SSO treatment.

Preliminary efforts to isolate the 85-kDa protein band were conducted by subjecting Triton-solubilized membranes to anion exchange (DEAE-Sephacel, Pharmacia). Protein radioactivity could be se-

lectively retained and eluted with a salt gradient in a single peak while lipid-associated radioactivity was recovered in the void volume. Radioactive protein eluates were concentrated by passage over a second short DEAE column and subjected to gradient gel electrophoresis (5–10%). Silver staining of radioactive protein eluates showed a major protein band with a molecular weight of 85–89 kDa (*data not shown*).

Discussion

Sulfo-*N*-succinimidyl esters of FA's of various chain lengths were synthesized and reacted with isolated adipocytes. Their reaction with the plasma membranes and their effect on FA transport were studied and correlated. FA transport was inhibited to a similar extent by the 14- and 18-carbon FA derivatives, SSM and SSO. On the other hand, the 3-carbon derivative, SSP, was not inhibitory. These results are consistent with the previously described specificity of the transport system for long-chain FA (Abumrad et al., 1984).

Inhibition of FA transport by SSM and SSO was not complete (70–80%). However it was specific to the FA transport system. Intracellular water space, glucose transport and its responsiveness to insulin, and retinoic acid permeation were not significantly altered. Inhibition was also shown not to be related to the metabolism of the FA, as it was not diminished by removal of glucose from the medium during cell isolation, maintenance and transport assay, conditions which greatly reduced the proportion of FA that was esterified (from 53 to 14% at 1 min). In line with this is the finding that the effect of SSO (or SSM) was most prominent during the very early period of the time course which represents initial uptake rates and is independent of FA metabolism (Abumrad et al., 1981). All of the above data indicated that sulfo-*N*-succinimidyl esters of long-chain FA do not penetrate the cell and their effects are mediated by interaction with the plasma membrane.

The SS-FAs and DIDS bound to a membrane protein which migrated with an apparent molecular weight of 85–89 kDa. In the case of SSO this was the only protein labeled. This strongly supports involvement of this protein in the binding or transport of long-chain FA. Other proteins which bound DIDS and SSM might be related to monocarboxylate and inorganic anions. DIDS inhibits both systems in adipocytes at concentrations similar to those required for inhibiting FA transport (*unpublished observations*). SS derivatives of FA might provide probes which are more specific than DIDS and more convenient to use for investigating anion transport systems in adipocytes.

Labeling by SSO of the 85-kDa protein labeled by DIDS rules out the possibility that this protein is the product of two 40-kDa proteins (Schwieterman et al., 1988) crosslinked by DIDS. None of the FA inhibitors we have tested bound to a protein in the 40-kDa molecular weight range similar to the membrane FA binding protein proposed by Berk and coworkers (Potter et al., 1989). However, since the inhibitory effect of FA derivatives was not complete, our results do not rule out the possibility that a 40-kDa protein is involved. Other characteristics differentiate the two proteins. The 85-kDa protein has to be solubilized from membrane by Triton while the 40-kDa protein can be isolated by increasing ionic strength. This would suggest that the 85-kDa protein is an intrinsic membrane protein while the 40-kDa is peripherally bound. The two proteins might serve distinct roles functioning in the binding or transport of long-chain FA in adipocytes. The data presented in this study suggest that the 85-kDa protein band from rat adipocytes is a strong condidate for further investigation as the long-chain FA transport protein. SSO, which binds with high specificity to this protein, should provide a useful tool towards its isolation and characterization.

On a more general level sulfosuccinimidyl esters of long-chain FA could provide interesting membrane labels to probe the endofacial lipid-embedded sides of membrane proteins which may not be accessible to hydrophilic probes. The length and chemical structure of the hydrocarbon chain could be modified to influence the specificity of binding.

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