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Effects of sterculic acid on stearoyl-CoA desaturase in differentiating 3T3-L1 adipocytes☆

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Abstract

The effects of sterculic acid on cell size, adiposity, and fatty acid composition of differentiating 3T3-L1 adipocytes are correlated with stearoyl-CoA desaturase (SCD) expression (mRNA and protein levels) and enzyme activity. Fluorescence-activated cell scanning (FACS) analysis showed that adipocytes differentiated with methylisobutylxanthine, dexamethasone, and insulin (MDI) plus 100 μM sterculic acid comprised a population of predominantly large cells with reduced adiposity compared to MDI-treated cells. Although both groups had similar amounts of total fat, their fatty acid profiles were strikingly different: MDI-treated cells had high levels of the unsaturated palmitoleic (Δ^9-16:1) and oleic (Δ^9-18:1) acids, whereas the cells cultured with MDI plus sterculic acid accumulated palmitic (16:0) and stearic (18:0) acids together with a marked reduction in Δ^9-16:1. Although the cells treated with MDI plus sterculic acid had similar levels of *scd1* and *scd2* mRNAs and antibody-detectable SCD protein as the MDI-treated cells, the SCD enzyme activity was inhibited more than 90%. The accumulation of 16:0 and 18:0, together with normal levels of fatty acid synthase (FAS) and aP2 mRNAs, shows that de novo synthesis and elongation of fatty acids, as well as cell differentiation, were not affected by sterculic acid. Because of the increase in cell size in the sterculic acid-treated cells, the insulin-stimulated 2-deoxyglucose (2-DOG) uptake was determined. Compared to MDI-treated cells, the 2-DOG uptake in the cells treated with sterculic acid was not affected. These results indicate that sterculic acid directly inhibits SCD activity, possibly by a turnover-dependent reaction, without affecting the processes required for adipocyte differentiation, *scd* gene expression or SCD protein translation.

Keywords: Cyclopropenoic fatty acids; Sterculic acid; Stearoyl-CoA desaturase; 3T3-L1 adipocytes; Fatty acid composition

Sterculic acid [8-(2-octyl-1-cyclopropenyl) octanoic acid (Fig. 1A)] and malvalic acid [7-(2-octyl-1-cyclopropenyl) heptanoic acid (Fig. 1B)] are plant lipids that contain a highly strained propene ring in their carbon chains. Cyclopropenoic fatty acids (CPFA) are the major seed oil components of plants from the order *Malvales* [1] where sterculate and malvalate account for 57.4% and 14.5% of the total fatty acid levels, respectively [2]. Other seed oils containing variable amounts of CPFA include cotton oil (0.5–1.0% of the total lipid, ~65% malvalic acid and ~35% sterculic acid) and baobab oil (6.8% sterculic acid, 6.9% malvalic acid, and 1.8% of the reduced isomer dihydrosterculic acid) [3].

It is known that sterculic acid is a potent inhibitor of stearoyl-CoA desaturase (SCD) in vivo and in vitro

☆ *Abbreviations:* Δ^9-16:1, palmitoleic acid; 16:0, palmitic acid; Δ^9-18:1, oleic acid;18:0, stearic acid; 16:0-CoA, palmitoyl-CoA; 18:0-CoA, stearoyl-CoA; 2-DOG, [1-^{3}H]2-deoxyglucose; ACS, acyl-CoA synthase; CPFA, cyclopropenoic fatty acids; cyclo-7,8-18:0, *trans*-7,8-methyleneoctadecanoic acid; cyclo-9,10-18:0, *trans*-9, 10-methyleneoctadecanoic acid; cyclo-11,12-18:0, *trans*-11,12-methyleneoctadecanoic acid; DHEA, dehydroepiandrosterone; MDI, differentiation cocktail containing methylisobutylxanthine, dexamethasone and insulin; SCD, stearoyl-CoA desaturase; TRO, troglitazone.

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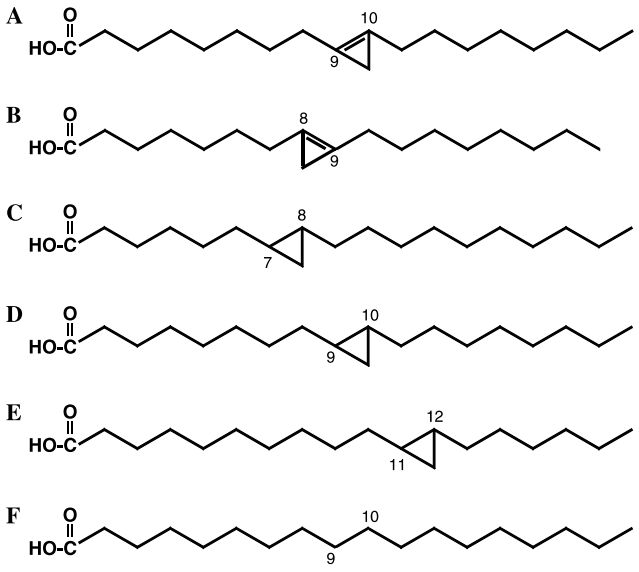

Fig. 1. Chemical structures of: (A) sterculic acid or 8-(2-octyl-cyclopropenyl) octanoic acid; (B) malvalic acid or 7-(2-octyl-1-cyclopropenyl) heptanoic acid; (C) cyclo-7,8-18:0 or *trans*-7,8-methyleneoctadecanoic acid; (D) cyclo-9,10-18:0 or *trans*-9,10-methyleneoctadecanoic acid; (E) cyclo-11,12-18:0 or *trans*-11,12-methyleneoctadecanoic acid; and (F) stearic acid or 18:0.

[4–9] and that heat destroys its inhibitory activity [3,10]. Several other isomers of sterculic acid, including alcohols (hydroxy sterculate and 1,2-dihydroxy sterculate), methyl esters, methyl ethers, and hydrocarbons, also inhibit SCD with different potencies [6,11].

SCD catalyzes the NADH- and O_2-dependent desaturation of palmitate (16:0) and stearate (18:0) at carbon 9 to produce palmitoleate (Δ^9-16:1) and oleate (Δ^9-18:1), respectively. In higher vertebrates, these reactions occur predominantly in liver and adipose tissues and represent the first step in the formation of long-chain unsaturated fatty acids [12]. In the mouse, three isoforms of the *scd* gene have been identified, namely *scd1* [13], *scd2* [14], and *scd3* [15]. Although these isoforms share 85–88% identity of amino acid sequence, their tissue distribution is strikingly different. Most organs of different mouse strains express SCD1 and SCD2 with exception of the liver, which expresses mainly SCD1. SCD3 is expressed in the skin [15] and in the Harderian [16] and the preputial glands [17]. B-lymphocytes express SCD2, whereas mature T-lymphocytes express none of the three isoforms [18,19]. The physiological significance for this tissue distribution or the role of these isoforms in the maintenance of lipid composition has not been clarified.

Many developmental, dietary, hormonal, and environmental factors regulate SCD expression. Fat-free diets with high-carbohydrate content, as well as diets rich in saturated fatty acids, induce hepatic SCD1 mRNA expression [13,20,21]. Insulin [22], glucose, and fructose [23–25], cholesterol [26,27], cold temperatures [28,29], light [30], and some drugs (fibrates, peroxisome proliferators) [31,32] induce SCD expression.

On the other hand, polyunsaturated fatty acids (PUFA) especially of the $n-6$ and $n-3$ families [27,33–35], glucagon [22], conjugated linoleic acid (CLA) [36,37], tumor necrosis factor-α, and interleukin-11 [38] inhibit SCD1 mRNA expression in the liver. CLA [39] and some sulfur-substituted (thia) fatty acids [40,41] inhibit SCD catalytic activity without directly altering gene expression.

The mouse 3T3-L1 preadipose cell line has been used to study cellular differentiation, maturation, and development [12]. Under the appropriate hormonal stimuli, these cells exhibit a dramatic increase in gene transcription, leading to elevated levels of glycolytic, lipogenic, and lipolytic enzymes as well as other adipocyte-specific proteins [13,42]. After differentiation, the cells acquire the morphological and biochemical characteristics typical of mature adipocytes and their total lipid content increases up to 600% mainly by the predominance of Δ^9-16:1 [43,44]. The increase of Δ^9-16:1 is primarily due to the increase in *scd1* mRNA levels and SCD specific activity [13]. We have shown that SCD expression and activity can be modulated by the steroid hormone dehydroepiandrosterone (DHEA), 7-oxo-DHEA, and troglitazone (TRO), during the differentiation of these cells. DHEA [44] and TRO [43,45] reduced the SCD1 mRNA levels and SCD specific activity, while 7-oxo-DHEA induced SCD1 mRNA expression and increased SCD specific activity [44].

Our previous studies focused on determining correlations between changes in lipid composition, SCD mRNA levels, protein expression, and catalytic activity to more thoroughly characterize the differentiation process of 3T3-L1 cells [43,44]. This approach has now been used to study the effects of sterculic acid and three cyclopropanoic fatty acids on *scd1* and *scd2* gene expression, on SCD specific activity, as well as in the morphology and selected physiological responses (2-deoxyglucose uptake) of differentiating 3T3-L1 preadipocytes.

We show here that sterculic acid-treated 3T3-L1 adipocytes resulted in a population of bigger cells with reduced adiposity, without any effect on 2-DOG uptake. Our results also show that sterculic acid directly inhibits SCD specific activity without affecting *scd* gene expression or SCD protein expression.

Materials and methods

Materials. FBS, various preparations of Dulbecco's modified Eagle's medium (DMEM), TRIzol reagent, penicillin–streptomycin mixture and 0.25% trypsin–EDTA were from Gibco-BRL (Gaithersburg, MD). Adenosine 5′-triphosphate (ATP), β-nicotinamide adenine dinucleotide, reduced form (NADH), coenzyme A (disodium salt), dexamethasone, [1-^{3}H]2-deoxyglucose (specific activity 5 Ci/mmol),

cytochalasin B, Nile Red, stearoyl-CoA (18:0-CoA), the *trans*-monoenoic fatty acids, and fatty acid methyl ester standards were from Sigma (St. Louis, MO). Calf serum was from BioWhittaker (Walkersville, MD), insulin was from Eli Lilly (Indianapolis, IN), DMSO, perchloric acid and methylisobutylxanthine were from Aldrich (Milwaukee, WI), and nylon and PVDF membranes (0.22 μm pore size) were from Millipore (Bedford, MA). [9,10-^{3}H]Stearoyl-CoA (specific activity 60 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). [α-^{32}P]dCTP (specific activity 3000 Ci/mmol) was from New England Nuclear (Boston, MA). TRO was a gift from Parke-Davis (Ann Arbor, MI).

Sterculic acid. Sterculic acid was prepared by saponification of the oil from *Sterculia foetida* seeds with 5% KOH in 90% methanol for 30 min at 37 °C, followed by acidification to pH 2.0 and extraction with hexane. No further purification of sterculic acid was pursued. The composition of the saponified oil was determined by LC-MS, with sterculic acid comprising 65% of the total content, similar to previous reports [2]. The final product was dissolved in 100% ethanol, adjusted to 0.5 M (based on the percentage of sterculic acid content) and stored at −30 °C under N_2 gas in glass screw–cap tubes.

Synthesis of cyclopropanoic fatty acids. Three positional cyclopropanoic fatty acids isomers were synthesized by the Simmons–Smith reaction [46] using diiodomethane (CH_2I_2) and the following *trans*-monoenoic fatty acids: *trans*-7-octadecenoic acid (Δ7*t*-18:1) was used to produce *trans*-7,8-methyleneoctadecanoic acid (cyclo-7,8-18:0) (Fig. 1C); *trans*-9-octadecenoic acid (Δ9*t*-18:1, elaidic acid) was used to produce *trans*-9,10-methyleneoctadecanoic acid (cyclo-9,10-18:0) (Fig. 1D); and *trans*-11-octadecenoic acid (Δ11*t*-18:1, *trans*-vaccenic acid) was used to produce *trans*-11,12-methyleneoctadecanoic acid (cyclo-11,12-18:0) (Fig. 1E).

Culture and differentiation of 3T3-L1 cells. The 3T3-L1 preadipocyte cell line was cultured in high glucose (HG)-DMEM with 10% calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) in 10-cm plastic petri dishes until they were 100% confluent. Control cells were cultured in HG-DMEM with 10% FBS and antibiotics. For differentiation, 2-day post-confluent cells (referred to as day 0) were incubated for 48 h in HG-DMEM with 10% FBS, antibiotics and a differentiation cocktail consisting of methylisobutylxanthine (115 μg/mL), dexamethasone (390 ng/mL), and insulin (10 μg/mL), hereafter termed MDI. After 48 h, the cells were maintained in HG-DMEM with 10% FBS, antibiotics, and insulin only. This medium was changed every 2 days until the cells were collected for analysis. For sterculic acid and cyclopropanoic fatty acids treatments, 100 mM stock solutions were prepared in 2 mM fatty acid-free (FAF) BSA, filter sterilized with a 0.22 μm pore membrane, and stored at 4 °C. For TRO treatments, a 10 mM stock solution was prepared in DMSO and added to give a final concentration of 10 μM. Sterculic acid, the cyclopropanoic fatty acids, and TRO were replenished with every medium change.

Fluorescence-activated cell scanning. The size and the adiposity of the cells were determined by staining with the fluorescent dye Nile Red [47,48] and FACS analysis was performed as previously described [44].

Lipid analyses. The fatty acid composition of the cells was determined by extraction with organic solvents and gas chromatography of the methyl ester derivatives as previously described [44].

mRNA analyses. Northern blot analyses were performed with total RNA obtained with the TRIzol reagent [43]. After agarose–formaldehyde gel electrophoresis and transfer to nylon membranes, the RNA was hybridized with ^{32}P-labeled cDNA-specific probes for *scd1*, *scd2*, FAS, aP2, and pAL15 (as control for loading) as previously described [44].

Western blot of SCD protein. 3T3-L1 whole cell extracts were obtained from 10-day cultures by detergent-mediated lysis in the presence of protease inhibitors. The protein content was determined by dye binding [49] using BSA as standard. Cell proteins (20 μg) were separated in a 10% PAGE under reducing conditions, transferred to PVDF membranes, and incubated with a polyclonal (rabbit) antibody that recognized the last 20 amino acids of rat SCD [50] followed by incubation with an anti-rabbit IgG-HRP conjugate. The reaction was developed by ECL as previously described [44].

Preparation of microsomes and SCD assay. Microsomes were prepared from 3T3-L1 adipocytes cultured for different days by differential centrifugation and the SCD activity was determined by the the production of $^{3}H_2O$ using [9,10-^{3}H]stearoyl-CoA as substrate and liquid scintillation counting [44].

2-DOG uptake measurement. Adipocytes obtained after 12 days of differentiation were used for this assay [51]. For this experiment, the cells were grown in 6-well plates and differentiated with MDI or with MDI plus sterculic acid as described above, with the exception that from day 6 to day 12, insulin was omitted from the replacement medium. Non-specific, basal, and insulin-stimulated 2-DOG uptake assays (50 μM, 0.33 μCi/well) were performed as previously described [44]. TRO (10 μM) was included as an experimental control since it is known to increase insulin-stimulated glucose uptake [52].

Statistical analyses. Results were analyzed using Student's two-tailed *t* test and the functions provided in Excel 2001 (Microsoft, Redmond, WA). A p value less than 0.05 was considered to be statistically significant.

Results

Morphology of 3T3-L1 adipocytes

Fig. 2A shows non-differentiated post-confluent 3T3-L1 preadipocytes cells cultured for 10 days without the MDI differentiating cocktail. The cells cultured with 100 μM of either sterculic acid or the three cyclopropanoic fatty acids alone had a similar morphology to control cultures (photos not shown). Fig. 2B shows the characteristic morphology of fully differentiated mature adipocytes after culture with MDI for 10 days. These cells were big, round, and filled with abundant fat droplets. Figs. 2C and D show the morphology of the adipocytes cultured with MDI plus 100 μM sterculic acid and with MDI plus 10 μM TRO, respectively. These cells also showed the characteristic morphology of differentiated adipocytes, but the inclusion of sterculic acid (Fig. 2C) caused a qualitative increase in cell size together with a reduction in the abundance and the size of lipid droplets. In the case of the cells treated with MDI plus TRO (Fig. 2D), the cells appeared smaller and with decreased-sized fat droplets.

FACS analysis of 3T3-L1 adipocytes

The observed changes in cellular morphology were quantitated by FACS (insets of Fig. 2) as previously described [44]. Region R1 was defined to exclude cellular debris, region R2 (forward scatter or FSC, from 200 to 500) and region R3 (FSC from 500 to 1000) were defined to accommodate the heterogeneity observed in cell size. Table 1 shows the results from the R2 (small cells) and R3 (large cells) regions as the percentage of the total cell count, the arithmetic mean of the FSC (cell size of the population), and the arithmetic mean of the side scatter (SSC, intensity of Nile Red fluorescence, i.e., the

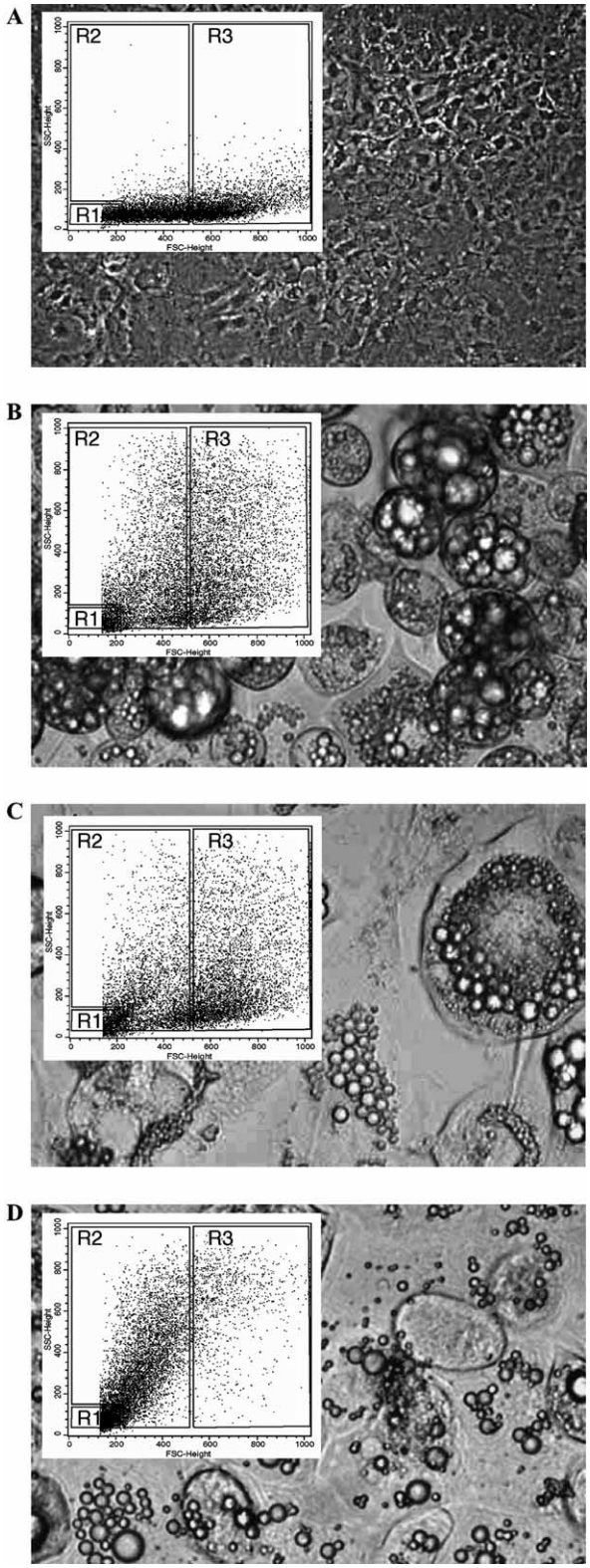

Fig. 2. Morphology of 3T3-L1 cells revealed by phase contrast microscopy after 10 days in culture. (A) Non-differentiated control cells (magnification 100×). (B) Fully differentiated adipocytes treated with MDI (magnification 400×). (C) Cells treated with MDI plus 100 μM sterculic acid (magnification 400×). (D) Cells treated with MDI plus 10 μM TRO (magnification 400×). The insets in A–D show the results of FACS analysis.

adiposity). The SSC measurements were normalized relative to the non-differentiated cell cultures for comparison.

Control cells (inset to Fig. 2A) consist of almost equal proportions of small and large cells (53.7% and 46.3%, respectively), both with low Nile Red fluorescence, consistent with the non-differentiated state. Upon differentiation with MDI there was a 6.8- and a 2.6-fold increase in adiposity due to the accumulation of lipids in the small and large cells, whose proportions remained around 50%. However, the cell size distribution and adiposity differed depending on the presence of sterculic acid or TRO. As shown previously, the treatment of differentiating 3T3-L1 adipocytes with MDI plus 10 μM TRO resulted in smaller cells with lower total lipid accumulation [44]. In contrast, treatment of cells with MDI plus 100 μM sterculic acid caused a shift in the cell size distribution toward larger cells (61.5%) with lower adiposity (normalized adiposity of 1.4, Table 1).

Differences in fatty acid composition after differentiation

Table 2 shows the lipid composition of the control group as well as the composition of the cells treated with MDI and with MDI plus either sterculic acid or the three cyclopropanoic fatty acids tested.

In non-differentiating preadipocyte control cultures, ~120 ng of total fatty acids (16:0, Δ^9-16:1, 18:0, and Δ^9-18:1) was detected. The most abundant fatty acid was 18:0 while the least abundant was Δ^9-16:1. The desaturation index, defined as the sum of monounsaturated fatty acids divided by the sum of saturated fatty acids, reflects changes in the lipid composition of the cell as the desaturation activity changes [37]. For the control culture, the desaturation index was 0.09, consistent with low desaturase activity. The addition of sterculic acid to control cultures produced a slight increase in the total fatty acid levels (166 ng) due to the increase in the levels of saturated fatty acids. Interestingly, the levels of Δ^9-16:1 and Δ^9-18:1 were even lower than those of the control cultures, with a lower desaturation index of 0.04, indicating a further reduction in desaturase activity. The fatty acid composition of the cells cultured in the presence of any of the three cyclopropanoic fatty acids was identical to the control cultures (data not shown).

The cells treated for 10 days with the MDI differentiation cocktail consisted of fully differentiated adipocytes and their total lipid content was 4.8 times greater than the non-differentiated preadipocytes (583 vs. 121 ng, respectively). In these cells, increased levels of Δ^9-16:1 (220 ng) and Δ^9-18:1 (61 ng) accounted for almost half of the total fatty acid content (48.2%), together with a higher desaturation index (1.76), consistent with increased desaturase activity.

Overall, the addition of the cyclopropanoic fatty acids to MDI-treated 3T3-L1 cells produced minor,

Table 1
Fluorescence-activated cell scanning[a] of differentiating 3T3-L1 adipocytes stained with Nile Red

Cell treatment	R2 region (small cells)				R3 region (large cells)			
	Percentage of total[b]	Cell size[c]	Nile Red fluorescence[d]	Normalized adiposity[e]	Percentage of total[b]	Cell size[c]	Nile Red fluorescence[d]	Normalized adiposity[e]
Control[f]	53.7	337	2.8	1.0	46.3	664	3.2	1.0
MDI	44.0	334	19.3	6.8	56.0	668	8.3	2.6
+Sterculic acid	38.5	**407**[g]	**6.0**	**2.1**	61.5	677	**4.6**	**1.4**
+TRO[h]	**81.9**	**300**	**10.8**	**3.8**	**18.1**	645	**13.4**	**4.1**

[a] FACS results are the average of $n = 3$ replicate experiments, where 10,000 cells were counted. The relative standard error for the replicates was less than 5%.

[b] Percentage of cells detected in either the R2 of the R3 regions, corresponding to either small or large cells, respectively.

[c] Cell size deduced from forward scatter intensity (FSC-height).

[d] Fluorescence intensity measured as side scatter intensity (SSC-height) after staining intracellular lipid with Nile Red.

[e] Adiposity indicated by changes in Nile Red fluorescence intensity after normalization to the fluorescence observed from control cells.

[f] The control cells were confluent preadipocytes harvested at day 10.

[g] Statistically significant differences vs. MDI-treated cells within either the R2 or R3 regions are shown in bold ($p < 0.05$, Student's two-tailed t test).

[h] TRO (troglitazone, 10 μM) was included as an experimental control since this compound is known to reduce cell size and should thus increase the number of cells detected in the R2 region.

Table 2
Effect of sterculic acid and the cyclopropanoic-18:0 derivatives on the fatty acid composition of differentiating 3T3-L1 adipocytes

Fatty acid[a]	Control[b]	Sterculic acid[c]	MDI	MDI-treated cells plus			
				Sterculic acid[c]	Cyclo-7,8-18:0	Cyclo-9,10-18:0	Cyclo-11,12-18:0
Δ^9-16:1	1.5 (1.2)	0.4 (0.2)[d]	220 (37.5)	**20**[e] (3.5)	**265** (37.1)	197 (34.6)	234 (36.7)
16:0	36 (30.0)	39 (23.5)	122 (20.9)	**268** (46.8)	144 (20.2)	118 (20.8)	133 (20.9)
Δ^9-18:1	6.9 (5.7)	4.2 (2.5)	61 (10.4)	**51** (8.9)	73 (10.2)	57 (9.9)	68 (10.6)
18:0	54 (44.5)	65 (39.6)	37 (6.2)	**97** (16.9)	36 (5.0)	36 (6.4)	36 (5.6)
Sterculic acid		2.0 (1.2)		6.8 (1.2)			
Cyclo-18:0 FA					6.9 (0.9)	6.2 (1.1)	7.7 (1.2)
Total fatty acids	121	166	583	573	**713**	570	639
∑Unsaturated FA	8.4 (6.9)	4.6 (2.7)	281 (48.2)	**71** (12.3)	**338** (47.4)	254 (44.5)	302 (47.2)
∑Saturated FA	90 (74.3)	104 (62.6)	159 (27.2)	**365** (63.7)	180 (25.2)	154 (27.0)	169 (26.4)
Desaturation index[f]	0.09	0.04	1.76	**0.19**	1.87	1.64	1.78

[a] Individual fatty acids isolated from 10-day cultures reported as ng per culture. Numbers in parenthesis indicate the percentage contribution to total fatty acids reported. Values shown are means of $n = 3$ experiments with individual fatty acid analyses repeated in duplicate. In all cases, variances were less than 10%.

[b] The control cells were confluent preadipocytes harvested at day 10.

[c] Sterculic acid and the cyclopropanoic fatty acids were added to a final concentration of 100 μM.

[d] Statistically significant differences vs. the control group are underlined.

[e] Statistically significant differences vs. the MDI-treated culture are shown in bold ($p < 0.05$, Student's two-tailed t test).

[f] Desaturation index is defined as (∑unsaturated FA/∑saturated FA).

non-significant changes in their fatty acid composition and their desaturation index (Table 2). However, in the cells treated with cyclo-7,8-18:0, there was a significant increase in total fatty acid levels, primarily due to an increase in the level of Δ^9-16:1.

The cells differentiated with MDI plus 100 μM sterculic acid showed a dramatic change in their fatty acid composition (Table 2). The levels of the monounsaturated fatty acids were reduced about 75% compared to the MDI-treated cells (71 vs. 281 ng, respectively) mainly due to the dramatic decrease of about 90% in the levels of Δ^9-16:1 (20 vs. 220 ng). In contrast, the sum of the saturated 16:0 and 18:0 fatty acids accounted for more than half (63.7%) of the total fatty acid content. Taken together, the fatty acid profile of the cells treated with MDI plus sterculic acid shows that in these cells, desaturase activity was almost completely inhibited with a desaturation index of 0.19 that was most similar to that of the non-differentiated control group (0.09). Fig. 3 shows representative gas chromatographs obtained from 10-day cultures of MDI and MDI plus sterculic acid-treated cells. The marked reduction in the peak of Δ^9-16:1, as well as the accumulation of 16:0 and 18:0 in the sterculic acid-treated cells, is clearly evident.

A time-course experiment showed that the changes in the fatty acid composition and the percentages of fatty acids described above for the cells treated with sterculic acid could be noted as early as day 4 of the differentiation protocol (data not shown).

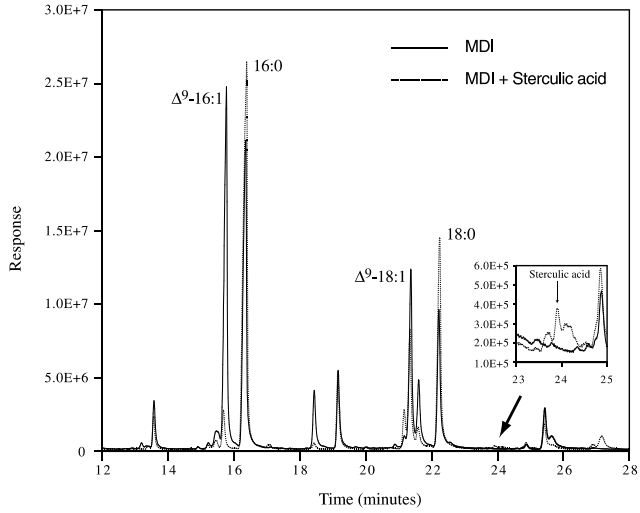

Fig. 3. Representative gas chromatographs of cells cultured in media supplemented with MDI or with MDI plus 100 μM sterculic acid for 10 days. The peaks for palmitoleic (Δ^9-16:1), palmitic (16:0), oleic (Δ^9-18:1), and stearic (18:0) acids are shown. The inset is a blow-up between 23 and 25 min to show the peak of sterculic acid.

Effect of sterculic acid on SCD activity in differentiating adipocytes

Table 3 shows the SCD specific activity measured during the differentiation of 3T3-L1 adipocytes. Depending on the day of culture, the SCD activity in MDI-treated cells was around 0.6 nmol/min mg protein and was maximal at day 8. The addition of 100 μM sterculic acid to the culture medium produced a reduction of more than 90% in the SCD specific activity in all days tested. None of the three cyclopropanoic fatty acids had any effect on the SCD specific activity.

Northern blot analysis of differentiating 3T3-L1 adipocytes

Differentiation of 3T3-L1 preadipocytes is associated with a general increase in gene transcription, leading to elevated levels of glycolytic, lipogenic, and lipolytic enzymes and other adipocyte-specific proteins [42]. During the process of differentiation the level of the SCD1 mRNA increases, while almost no change in SCD2 mRNA is observed [43]. To examine if the observed decreased in SCD activity was due to changes in *scd* mRNA levels, we determined the level of expression of *scd1*, *scd2*, and other genes associated with fatty acid synthesis (FAS) and differentiation (aP2). Fig. 4 shows that the cells treated with MDI plus sterculic acid had the same mRNA levels of *scd1*, *scd2*, FAS, and aP2 as the MDI-treated adipocytes.

Table 3
Effect of sterculic acid on stearoyl-CoA desaturase (SCD) activity[a] in differentiating 3T3-L1 adipocytes

Cell culture	SCD activity[b]		
	Day 6	Day 8	Day 10
MDI	0.603 ± 0.01	0.622 ± 0.02	0.591 ± 0.03
+Sterculic acid	$\mathbf{0.038 \pm 0.01}$[c]	$\mathbf{0.063 \pm 0.01}$	$\mathbf{0.060 \pm 0.02}$

[a] Activities are expressed as nmol/min mg protein. Values are shown as means ± SD of $n = 3$ determinations.
[b] Microsomes were isolated from cultures on the days indicated.
[c] Statistically significant differences vs. MDI are shown in bold ($p < 0.05$, Student's two-tailed t test).

Western blot analysis of SCD expression

Fig. 5 shows that sterculic acid alone did not induce any SCD. Likewise, none of the three cyclopropanoic fatty acids induced SCD either (data not shown). The addition of 100 μM sterculic acid or the cyclopropanoic fatty acids to MDI-treated adipocytes resulted in similar levels of SCD. However, there were slightly higher levels of SCD in the cells treated with cyclo-7,8-18:0, which could partially explain the observed increase in total fatty acids and in Δ^9-16:1 levels (Table 2).

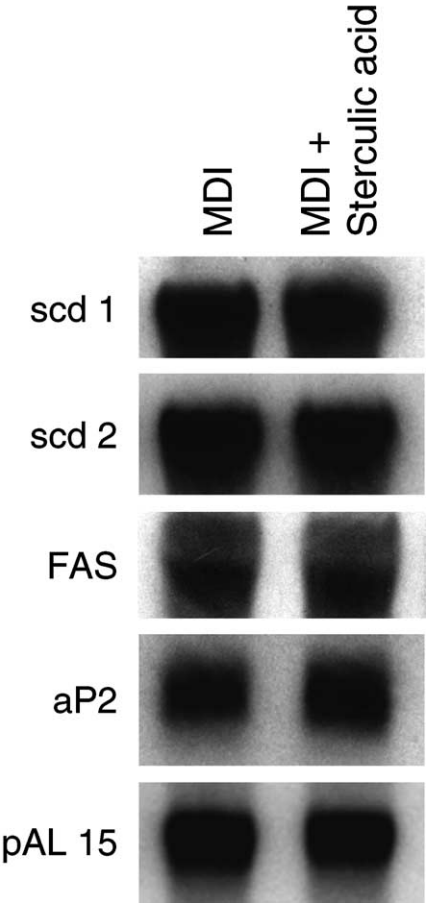

Fig. 4. Northern blot analysis of the effect of 100 μM sterculic acid on *scd1*, *scd2*, FAS, and aP2 mRNAs levels in MDI-differentiating 3T3-L1 adipocytes. pAL 15 was included as control for loading.

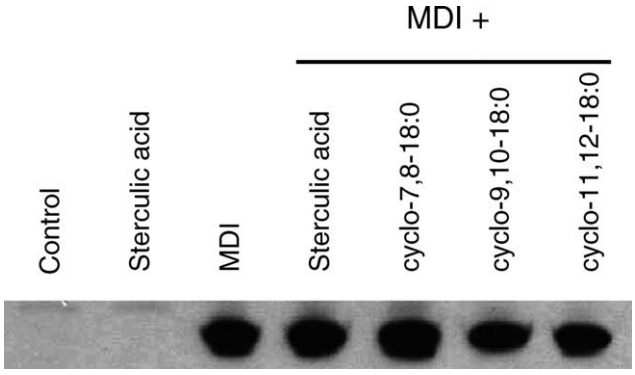

Fig. 5. Effect of sterculic acid and the three cyclopropanoic fatty acids on SCD protein expression. SCD was detected by Western blot in 10-day cultures of control and MDI-differentiating 3T3-L1 adipocytes where the culture medium was supplemented with 100 μM of either sterculic acid, cyclo-7,8-18:0, cyclo-9,10-18:0 or cyclo-11, 12-18:0.

Insulin-stimulated 2-deoxyglucose uptake

Increased fat cell size is highly associated with insulin resistance and the development of diabetes [53]. Enlarged adipocytes correlate better with insulin resistance than any other measure of adiposity [54,55]. Since the addition of sterculic acid gave an enlarged population of adipocytes, similar to what was obtained after DHEA treatment [44], we determined their rate of 2-DOG uptake (Table 4). Cytochalasin B, a non-specific inhibitor of glucose uptake [56], was used as a control to correct for non-specific uptake occurring independent of insulin stimulation. As previously shown [44] the addition of 10 μM TRO to cells treated with MDI was also used as a positive control to increase insulin-dependent 2-DOG uptake, due to the known effect of TRO to increase insulin sensitivity.

In the control cells, the 2-DOG uptake was low even at the highest insulin concentration tested. This result is compatible with the non-differentiated state of the control cells. When TRO was added to the MDI differentiation cocktail, there was a significant increase of ~1.6-fold in the basal 2-DOG uptake and of 3.7- and 3.1-fold at 1 and 100 nM insulin, respectively. This control result demonstrates the cells were responding to appropriate stimuli as previously observed [44]. In the MDI-treated cells and in the MDI plus sterculic acid-treated cells, there was no significant difference in the basal 2-DOG uptake at any insulin concentration tested.

Discussion

Sterculic acid is a cyclopropenoic fatty acid that occurs naturally in *S. foetida* seeds oil, accounting for more than half (65%) of the total fatty acid composition. After saponification of the *S. foetida* oil, free sterculic acid (as well as the other fatty acids present) was extracted and conjugated to BSA. As seen in Fig. 3, this sterculic acid-BSA complex was then taken up by the 3T3-L1 cells from the culture medium [57] and the enzyme acyl-CoA synthase (ACS) produced sterculoyl-CoA intracellularly, which is the inhibitor of SCD [5,9].

Besides its long recognized activity as a potent inhibitor of Δ9-desaturase [4,5], Δ5- and Δ6-desaturases are not affected. In the Morris hepatoma 7288C cell line, sterculic acid did not inhibit the Δ5- or Δ6-desaturase activities, as evidenced by the normal levels of polyunsaturated fatty acids [58]. This lack of inhibition in the Δ6-desaturase activity was also found in the microsomes obtained from the liver [9] and the brain [59] from *S. foetida* oil-fed rats.

Even though sterculic acid was present during the entire 10-day culture protocol, the intracellular levels, as well as those of the other three cylopropanoic fatty acids tested, were very low, only ~1.2% of the total fatty acids (Table 2 and Fig. 3). These levels were significantly lower than those previously reported for the Morris hepatoma 7288C cell line, in which despite being at only

Table 4
Effect of sterculic acid on insulin-stimulated 2-deoxyglucose uptake in MDI-differentiating 3T3-L1 adipocytes

Culture	2-Deoxyglucose uptake rate[a]			
	Cytochalasin B[b]	Insulin (nM)		
		0	1	100
Control[c]	2.3 ± 0.1	16 ± 1.9	23 ± 8.3	28 ± 3.8
MDI	8.2 ± 1.6	81 ± 18	131 ± 27	187 ± 12
+Sterculic acid	10.0 ± 1.9	98 ± 24	162 ± 21	204 ± 26
+TRO[d]	9.3 ± 0.5	**131 ± 20**[e]	**486 ± 21**	**583 ± 13**

[a] The [1-^{3}H]2-deoxyglucose uptake rate from 12-day cultures is reported as pmoles/min. Results are the means ± SD of $n = 3$ experiments, with individual experiments performed in triplicate.

[b] Cytochalasin B, a non-specific inhibitor of glucose uptake, was added to a final concentration of 50 μM.

[c] The control cells were confluent preadipocytes harvested at day 12.

[d] Troglitazone (TRO, 10 μM) was included as an experimental control since it is known to increase insulin-stimulated glucose uptake.

[e] Statistically significant differences vs. MDI are shown in bold ($p < 0.05$, Student's two-tailed t test).

39 μM in the culture media, sterculic acid accumulated to 32% of the total fatty acid mass, the majority in the triglyceride fraction [2,58]. These disparate results between the 3T3-L1 adipocytes and the Morris hepatoma cells are likely due to differences in the origin and physiology of these cell lines.

Cell morphology. The population of MDI plus sterculic acid-treated 3T3-L1 adipocytes consisted of predominantly large cells with reduced adiposity, when compared to normally differentiated MDI-cells (Table 1 and Fig. 2). Our previous studies showed that inhibition of SCD transcription by DHEA produced similar changes in the morphology of the cells [44]. It is unclear whether the reduction in the levels of unsaturated fatty acids—particularly Δ^9-16:1—may exert an undefined effect on the plasma membrane or in its partitioning to other intracellular membranes, thus affecting the overall size of the resultant cells.

Lipid composition. The addition of sterculic acid to the differentiating 3T3-L1 adipocytes produced dramatic differences in their fatty acid composition (Table 2 and Fig. 3). Although their total fatty acid content was similar to that in MDI-treated cells, this was due to the accumulation of the saturated fatty acid fraction (63.7% of the total fatty acids). Furthermore, the accumulation of 16:0 and 18:0 together with normal levels of fatty acid synthase (FAS) mRNA shows that de novo synthesis and elongation of fatty acids during the differentiation of the adipocytes were not affected by sterculic acid. It is clear that the synthesis of the unsaturated fatty acids, particularly Δ^9-16:1, was inhibited by the treatment with sterculic acid which was also reflected by the low desaturation index of 0.19, similar to the non-differentiated control cells of 0.09.

Insulin-stimulated 2-DOG uptake. It is widely accepted that increased adiposity is associated with insulin resistance and increased risk of type 2 diabetes [53]. Several studies have found an association between the size of the adipose cells and glucose uptake. In rats fed with a high fructose diet, the epididymal adipose tissue was increased due to a hypertrophy of the adipocytes, together with an impairment in glucose homeostasis and increased insulin resistance [60]. Our previous studies with 3T3-L1 cells treated with DHEA showed an increase in 2-DOG uptake, regardless of the increased size of the cells and their reduced adiposity [44], which was attributed to the thermogenic effects of DHEA. On the other hand, a reduction in the adipocyte's cell size is associated with increased insulin sensitivity and glucose uptake. Treatment of 3T3-L1 cells with TRO resulted in smaller cells with reduced adiposity compared to normally differentiated cells, together with an increase in 2-DOG uptake [44,61].

In order to determine if the observed changes on the size and morphology of the 3T3-L1 adipocytes treated with sterculic acid had any effect on their physiological responses, the rate of 2-DOG uptake was measured. The addition of sterculic acid to MDI-treated cells did not affect their ability to incorporate glucose at the basal level or after insulin stimulation (Table 4). Therefore, it seems that the degree of adiposity, rather than the size of the cell, could have a more profound impact on the ability of the cells to take up glucose. In this study, the cells treated with sterculic acid resulted in big cells, a morphology congruent with reduced glucose uptake capacity. However, the reduction in adiposity seems to compensate for the cell size and the final outcome would be the combined effects of size (big cells) with adiposity (small droplets), with normal 2-DOG uptake.

It is also known that the insulin-stimulated uptake of 2-DOG requires downstream signaling from the cell membrane and depends on the metabolic state and the degree of differentiation of the cells [44,62]. This result suggests that regardless of the altered fatty acid composition and increased size of the cells, sterculic acid did not interfere with metabolic responses that depend on the functionality of the plasma membrane as is the uptake of 2-DOG.

mRNA levels. Our previous studies have shown that different substances can modulate the expression of the SCD genes in differentiating 3T3-L1 cells and in cultures of primary adipocytes. TRO [43] and DHEA [44] specifically reduced the level of *scd1* mRNA while 7-oxo-DHEA increased it. With all these compounds, the levels of *scd2* mRNA remained unchanged. In the present study, we show that sterculic acid did not have any effect on either *scd1* or *scd2* gene expression (Fig. 4) or in the differentiation process as evidenced by the aP2 mRNAs levels.

SCD protein levels and catalytic activity. It is known that the sterculoyl-CoA, but not free sterculic acid, is the actual inhibitor of SCD [5,9]. After sterculic acid was complexed to BSA, the cells were able to incorporate it from the culture medium and to synthesize the CoA derivative intracellularly. Since the observed SCD specific activity did not correlate with the *scd1* mRNA or the SCD protein levels (Figs. 4 and 5) it is reasonable to assume that sterculic acid, in the form of sterculoyl-CoA, directly inhibits SCD, possibly by a turnover-dependent reaction. A possible mechanism of how this inhibition might occur is discussed later.

Relationship between structure and SCD inhibition. The cyclopropane fatty acids tested in this study provided more clues about the structure(s) involved in the inhibition of SCD. It seems clear that a cyclopropane ring, regardless of the position in the fatty acid chain, is not responsible for the observed inhibition of SCD. Thus our results show that none of the cyclopropane isomers tested gave significant changes on the cells' morphology, their fatty acid composition (Table 2), SCD protein levels, or the SCD specific activity (Table 3). This is particularly significant in the case of

cyclo-9,10-18:0, whose overall structure is the most similar to sterculic acid, except for the single bond between carbons 9 and 10. In this regard, sterculic acid possesses two major features that might contribute to its inhibitory activity. First, no hydrogen atoms are available at C-9 and C-10 for abstraction and further desaturation, and second, it contains a highly reactive and strained cyclopropene ring (Fig. 1).

The enzymes that contain a diiron center in their active site have been classified into three classes based on the structural motifs that bind these iron atoms [63,64]. Although the fatty acid desaturases of class II and class III do not share primary sequence similarity, it is believed that they share a common reactivity. Based on kinetic isotope data [65] one hypothesis for the desaturation reaction is that the enzyme removes hydrogen atoms sequentially, starting with the one at the C-9 position followed by the removal of the second hydrogen atom from the C-10 position. This stepwise mechanism is highly specific in the position at which the double bond is introduced (between carbons 9 and 10) and implies that the C-9 and C-10 bond is accurately positioned with respect to the diiron center [63,66].

After the diiron center in SCD is activated by NADH and O_2, the double bond between carbons 9 and 10 in sterculic acid might produce an unknown (or yet unidentified) SCD–sterculoyl-CoA inactive complex that can no longer dissociate to proceed with the normal desaturation of the natural substrate(s). A retardation in SCD electrophoretic mobility was recently observed in liver microsomes incubated with sterculoyl-CoA (F.E. Gomez, J.M. Ntambi, B.G. Fox, data to be published) which strongly suggests a covalent modification, possibly by a turnover-dependent reaction, of the SCD protein by sterculoyl-CoA.

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