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# Dihydrosterculic acid from cottonseed oil suppresses desaturase activity and improves liver metabolomic profiles of high-fat–fed mice

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ABSTRACT

Polyunsaturated fatty acid (PUFA)–rich diets are thought to provide beneficial effects toward metabolic health in part through their bioactive properties. We hypothesized that increasing PUFA intake in mice would increase peroxisome proliferator activated receptor delta (PPARδ) expression and activity, and we sought to examine the effect of different PUFA-enriched oils on muscle PPARδ expression. One of the oils we tested was cottonseed oil (CSO) which is primarily linoleic acid (53%) and palmitic acid (24%). Mice fed a CSO-enriched diet (50% energy from fat) displayed no change in muscle PPARδ expression; however, in the liver, it was consistently elevated along with its transcriptional coactivator Pgc-1. Male mice were fed chow or CSO-, saturated fat (SFA)–, or linoleic acid (18:2)–enriched diets that were matched for macronutrient content for 4 weeks. There were no differences in food intake, body weight, fasting glucose, glucose tolerance, or energy expenditure between chow- and CSO-fed mice, whereas SFA-fed mice had increased fat mass and 18:2-fed mice were less glucose tolerant. Metabolomic analyses revealed that the livers of CSO-fed mice closely matched those of chow-fed but significantly differed from SFA- and 18:2-enriched groups. Fatty acid composition of the diets and livers revealed an impairment in desaturase activity and the presence of dihydrosterculic acid (DHSA) in the CSO-fed mice. The effect of DHSA on PPARδ and stearoyl-CoA desaturase-1 expression mimicked that of the CSO-fed mice. Taken together, these data suggest that DHSA from CSO may

*Abbreviations:* 18:2, linoleic acid–rich diet; 18:2n6, linoleic acid; CHO, carbohydrate; CPFA, cyclopropyl fatty acid; CSO, cottonseed oil; DHSA, dihydrosterculic acid; FFA, free fatty acid; GC, gas chromatography; GTT, glucose tolerance test; HCVLF, high carbohydrate, very low fat; HFD, high-fat diet; IP, intraperitoneal; LDHa/b, lactate dehydrogenase a or b isoform; MUFA, monounsaturated fatty acid; MS, mass spectrometry; NAFLD, nonalcoholic fatty liver disease; NMR, nuclear magnetic resonance; PPARα, peroxisome proliferator activated receptor alpha; PPARδ, peroxisome proliferator activated receptor delta; PUFA, polyunsaturated fatty acid; RER, respiratory exchange ratio; SCD1, stearoyl-CoA desaturase-1; SFA, saturated fat; TEE, total energy expenditure; TG, triglyceride.

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be an effective means to increase PPARδ expression with concomitant suppression of liver stearoyl-CoA desaturase-1 activity.



## 1. Introduction

Dietary fatty acids play a vital role in the development and prevention of several diseases including obesity and diabetes. Polyunsaturated fatty acids (PUFAs) have been identified as potential bioactive lipids that can improve metabolism and prevent the detrimental adaptations to a high-fat diet (HFD). Mechanistically, PUFAs have been shown to enhance the expression of genes that control the rate of fat oxidation in highly metabolically active tissues, such as liver and skeletal muscle [1]. Despite this observation, previous investigations linking dietary fats to altered metabolism have focused on supplementation of omega-3 PUFAs and have largely ignored omega-6 PUFAs.

Over the last 2 to 3 decades, there has been a common theme portrayed within nutrition and metabolism research that omega-6 PUFAs are proinflammatory. This notion has been promoted due largely to the fact that n-6 PUFAs are precursors to the synthesis of more complex proinflammatory molecules such as prostaglandins and leukotrienes [2]. Although the latter is technically sound, the reality is that merely consuming n-6 PUFAs does not cause them to become proinflammatory without some form of activation. Instead, consumption of linoleic acid (18:2n6), which is the most abundant n-6 PUFA in the American diet, can lead to increased arachidonic acid (20:4n6) without any increase in inflammation, coagulation, or detrimental effects [3]. What is more likely following 18:2n6 consumption is that n-6 PUFAs *can* serve as proinflammatory precursors *but* are only activated when a stimulus for inflammation is present, such as with adipose tissue remodeling.

Evidence of the beneficial effects of 18:2n6 was seen in our previous studies where we were able to increase metabolic activity in a transgenic mouse model by indirectly increasing peroxisome proliferator activated receptor delta (PPARδ) activity in muscle [4]. From those results, we began to examine whether consumption of an 18:2n6-rich diet could improve metabolism in mouse models of HFDs. Three specific oils were chosen, one rich in 18:2n6 but low in saturated and monounsaturated (MUFA) fatty acids (safflower oil), one rich in saturated fatty acids (cocoa butter), and another rich in 18:2n6 and relatively high in saturated fatty acids (cottonseed oil, CSO). We hypothesized that increasing PUFA intake in mice would increase PPARδ expression and activity, and we sought to examine the effect of different PUFA-enriched oils on muscle PPARδ expression. To begin our investigation on the metabolic effects of CSO, we examined food intake, body weight, and metabolite changes in livers of mice after 4 weeks on the diets. Next, we assessed metabolomic profiles and then followed with a more detailed assessment of cyclopropene fatty acid content of the diets.

## 2. Methods and materials

### 2.1. Animal and diets

Male C57BL/6 mice (age 8 weeks) from Jackson Laboratory (Bar Harbor, ME, USA) were divided into 4 groups (n = 5 per group) and fed a standard chow diet, a CSO-enriched diet (22% wt/wt), safflower oil–enriched diet (18:2: 18% wt/wt), or high cocoa butter–enriched diet (saturated fat, SFA: 18% wt/wt). The diets were matched in all other macro- and micronutrients and were used to compare the effects of CSO to those of 18:2n6-enriched and SFA-enriched oils (Table 1). Ingredients of the test diets are listed in Table 2. Animals were then euthanized by isoflurane overdose followed by cardiac exsanguination, and tissues were recovered and stored at −80°C for further analysis. All procedures were approved by the University Animal Care and Use Committee at Texas Tech University.

### 2.2. Tissue analysis

To measure the effects of dietary fat on metabolic protein expression, liver and red gastrocnemius tissues were homogenized in RIPA buffer (10 mmol/L Tris-Cl [pH 8.0], 1 mmol/L EDTA, 0.5 mmol/L EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 140 mmol/L NaCl) supplemented with protease inhibitor cocktail (Cell Signaling Technology, Beverly, MA, USA), and the proteins were separated and blotted using standard immunoblotting procedures. All primary antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA) (sc-13067, sc-74440, sc-20670, sc-133731, sc-27230, sc-1616), and secondary antibodies were from Cell Signaling Technology.

### 2.3. Fatty acid composition

Each freeze-dried meal was extracted to recover the oil. Meals (10-15 g) were ground with a motor and pestle and then Soxhlet extracted with petroleum ether (75 mL) for 6 hours. The solvent was separated by rotary evaporation until the samples reached a constant weight. Oil yield was determined gravimetrically. Oil samples were then transmethylated to form esters for chromatographic analysis. Each sample (30 mg) was weighted into a 15-mL test tube along with 1 mL of hexane containing a known amount of tridecanoic acid as an internal standard. Methanolic base (200 μL) (Supelco, Bellefonte, PA, USA) was added, and each solution was vortex mixed and allowed to react at 70°C for 10 minutes. After cooling, 1 mL of brine was added, and the solutions were remixed and then allowed to settle. The upper organic phase containing the esters was transferred into gas chromatography (GC) autosampler vials.

GC was conducted with an Agilent 7890A chromatograph fitted with a Phenomenex (Torrance, CA, USA) ZB-WAX column (30-m × 0.25-mm i.d., 0.25-μm film thickness). Hydrogen was used as the carrier gas flowing at a linear velocity of 30 cm/s. The split inlet and Flame ionization detector were operated at 300°C. One-microliter injections were used, and the inlet split ratio was 1:50. The oven was programmed to start at 160°C, which was held for 2.5 minutes and then ramped at 1.0°C/min to 182°C, then ramped at 5°C/

**Table 1 – Macronutrient and principle fatty acid composition of the experimental diets fed to mice**

| | Chow (8604) | CSO (TD.140228) | SFA (TD.130051) | 18:2 (TD.130049) | High fat (TD.06414) |
|---|---|---|---|---|---|
| Protein | 243 | 177 | 177 | 177 | 235 |
| CHO | 402 | 330 | 330 | 330 | 273 |
| Total fat | 47 | 222 | 222 | 222 | 343 |
| SFA | 8 | 280 | 530 | 100 | 370 |
| MUFA | 9 | 180 | 330 | 150 | 470 |
| 18:2n6 | 19 | 27 | 27 | 164 | 160 |
| 18:3n3 | 2 | >5 | 10 | 10 | 10 |
| kcal/g | 3.0 | 4.0 | 4.0 | 4.0 | 5.1 |

Male C57Bl/6 mice (age 8 weeks) were fed chow, CSO-enriched, cocoa butter (SFA)–enriched, or safflower oil (18:2)–enriched diets for 4 weeks. Fat-enriched diets differed in the type composition of major lipids but were matched for total macronutrient content. HFD is listed for comparison only. Values are g/kg of each diet. Abbreviations: CHO, carbohydrate; SFA, saturated fatty acid; 18:1, oleic acid; 18:2n6, linoleic acid; 18:3n3, alpha linolenic acid. Note: 1 kcal = 4.184 kJ.

min to 210°C, which was held for 17.5 minutes, then ramped a final time at 20°C/min to 245°C, which was held for 2 minutes. Most fatty acid ester peaks were identified by comparison with known standards. In addition, a freshly extracted CSO sample was used to identify the elution times for the cyclopropyl fatty acids, that is, malvalic, sterculic, and dihydrosterculic (DHSA) acids. Fatty acids were quantified by internal standardization using response factors generated between tridecanoic acid and the individual fatty acids as previously described [5]. The Supelco 37-component standard plus an added authentic standard of DHSA was used for this purpose. Weight distribution was determined from the sum of the weights of each detected fatty acid.

Because of their small size, a slightly different protocol was used to determine the fatty acid distribution of the mouse livers. Each liver sample was freeze-dried in a 2-mL microcentrifuge tube. One milliliter of hexane and two chrome steel balls were added to each tube, and the samples were wet ground with a Biospex (Bartlesville, OK, USA) beadbeater-8 mill operated at 90% power for 5 minutes. Peaks were identified from known standard plus expected peaks based on components previously reported in mouse livers. The fatty acid distributions were determined from the relative peak areas corrected for response factor differences based on their molecular weight and carbon number.

**Table 2 – Ingredient composition of the diets fed to mice (g/kg)**

| Ingredient | Test diet | | |
|---|---|---|---|
| | CSO | SFA | 18:2 |
| Casein | 200 | 200 | 200 |
| L-Cystine | 3.0 | 3.0 | 3.0 |
| Corn starch | 109.5 | 109.5 | 109.5 |
| Maltodextrin | 120 | 120 | 120 |
| Sucrose | 100 | 100 | 100 |
| Cellulose | 200 | 200 | 200 |
| Mineral mix[a] | 35 | 35 | 35 |
| Vitamin mix[b] | 10 | 10 | 10 |
| Choline bitartrate | 2.5 | 2.5 | 2.5 |
| Oil | 220[c] | 220[d] | 220[e] |
| TBHQ | 0.04 | 0.04 | 0.04 |

Values as reported by the manufacturer for the 3 test diets. Abbreviation: TBHQ, tertiary butylhydroquinone as antioxidant.
[a] Mineral mix, AIN-93G-MX (94046).
[b] Vitamin mix, AIN-93G-VX (94047).
[c] CSO oil: 220 g/kg CSO.
[d] High-SFA diet: 40 g/kg soybean oil plus 180 g/kg cocoa butter.
[e] High-18:2 diet: 40 g/kg soybean oil plus 180 g/kg linoleic safflower oil.

### 2.4. *Glucose tolerance test*

After 4 weeks on the respective diets, animals were fasted for 4 hours after the start of the light cycle and intraperitoneally (IP) injected with 0.75 g/kg glucose. Blood samples (50-100 $\mu$L) were collected via tail vein nick before injection (0 minute) and at 20, 40, 80, and 120 minutes following IP injection. Glucose levels were determined using the glucose oxidase-peroxidase method as previously described [4].

### 2.5. *Macronutrient metabolism and energy expenditure*

Mice were placed in metabolic chambers at the start of the dark cycle to collect respiratory gas exchange measurements. Oxygen consumption, carbon dioxide production, respiratory exchange ratio (RER = carbon dioxide production/oxygen consumption), and total energy expenditure (TEE) were collected at 12-second intervals for 48 hours using the Accuscan Metabolic Cage System (AccuScan Instruments, Columbus, OH, USA). Food intake was measured at the end of the light cycle after days 1 and 2 [4].

### 2.6. *Nuclear magnetic resonance tissue sample preparation*

Liver tissue samples were thawed on ice. Individual 50-mg tissue samples were placed on ice in 10 mmol/L phosphate buffer followed by homogenization by an Omni Bead Ruptor Homogenizer (Omni International Inc, Waterbury, CT, USA) for 3 minutes. Homogenized tissues were transferred to 2-mL Eppendorf tubes and were centrifuged for 10 minutes at 5000*g*. The supernatant was then transferred to a new 2-mL tube, ice-cold methanol (2:1, v/v) was quickly added to aliquots of the supernatants, and the tubes were then vortexed for 30 seconds to enhance protein precipitation followed by cooling to –20°C for 30 minutes. After a precipitation period, the tubes were vortexed once more for 10 seconds and centrifuged at 5000*g* for 10 minutes. The supernatant was dried in a speed vacuum overnight. The dried supernatant was then reconstituted in the nuclear magnetic resonance (NMR) buffer (10% D2O, containing

1 mmol/L formate and 0.5 mmol/L 4,4-dimethyl-4-silapentane-1-sulfonic acid) and adjusted to pH 7.4 ± 0.05 [6].

### 2.7. NMR data collection, metabolite detection, and quantification

All 1-dimensional (1D) $^{1}$H NMR spectra were collected at 25°C on a 600-MHz Varian VNMRS spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a cryogenic probe according to our previously published method [7]. Each 1D spectrum was accumulated for 1028 scans with an acquisition time of ~2.5 seconds (25 000 complex points) and a 3-second repetition delay for a total collection time of ~2 hours. 1D 1H NMR spectra were referenced to 0.5 mmol/L 4,4-dimethyl-4-silapentane-1-sulfonic acid. NMR signals arising from small metabolites (<1000 Da) were identified and quantified relative to formate (1 mmol/L) as the internal reference by Chenomx software version 6 (http://www.chenomx.com). All metabolite concentrations are reported as values relative to formate.

### 2.8. In vitro studies

Mouse FL83-B liver-like cells were obtained from ATCC (Manassas, VA, USA) and grown under standard conditions in Dulbecco modified Eagle medium + 10% fetal bovine serum with 1% penicillin/streptomycin. Cells were grown to confluence; then serum-free media supplemented with 100 μmol/L palmitate or linoleate (sodium salt) conjugated to bovine serum albumin was added for 18 hours. DHSA (as its methyl ester) (Matreya LLC, State College, PA, USA) dissolved in dimetyl sulfoxide was added to a 1-μmol/L final concentration. After 18 hours, total cellular RNA was collected and used to measure target gene expression using quantitative reverse transcriptase polymerase chain reaction.

### 2.9. Statistical analyses

Statistical analyses were performed using R (version 3.0.3). Results are described as means ± SEM. Differences between groups were analyzed via Student *t* test or pairwise differences when comparing control groups vs treatment groups. Differences between groups were considered significant at $P < .05$. Power analysis was based on data from our previous work [4].

## 3. Results

### 3.1. A CSO-enriched diet does not affect body weight gain and improves glucose tolerance

CSO is an unusual food oil in that it is composed of high levels of 18:2n6 and palmitic acid (16:0), which are generally believed to be obesigenic and proinflammatory. However, our previous studies have disproved this theory [3], and we intended to assess the effect of feeding a CSO-enriched diet to mice. We first assessed the dietary triglyceride (TG) composition among the 4 diets, and no unexpected differences existed (Fig. 1A-B). The CSO and SFA diets were high in SFA, predominantly 16:0. Desaturation indices revealed that the SFA diet was relatively enriched in 18:0 and that CSO had elevated levels of

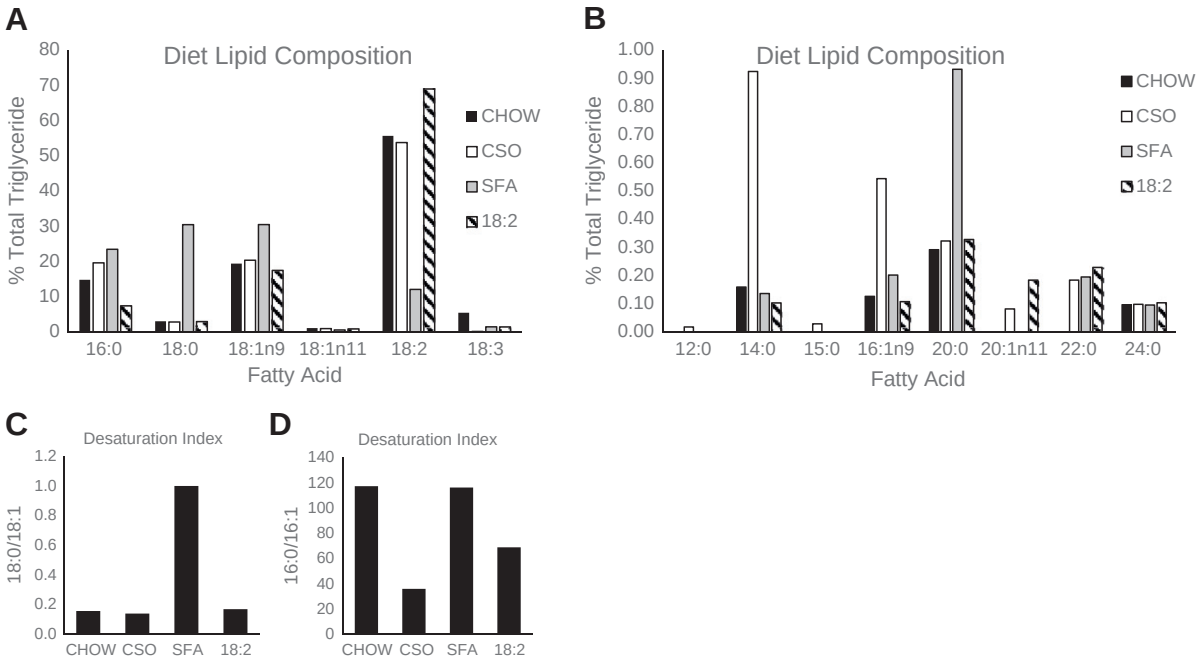


**Fig. 1 – Fatty acid composition of test diets. Total neutral lipids were extracted from the test diets, and fatty acid composition was determined using GC-MS. Expected differences were noted between the various diets based on the experimental design among major (A) and minor (B) lipid species. Desaturation indices revealed expected differences in the amount of SFA/MUFA of 18-carbon (C) and 16-carbon (D).**

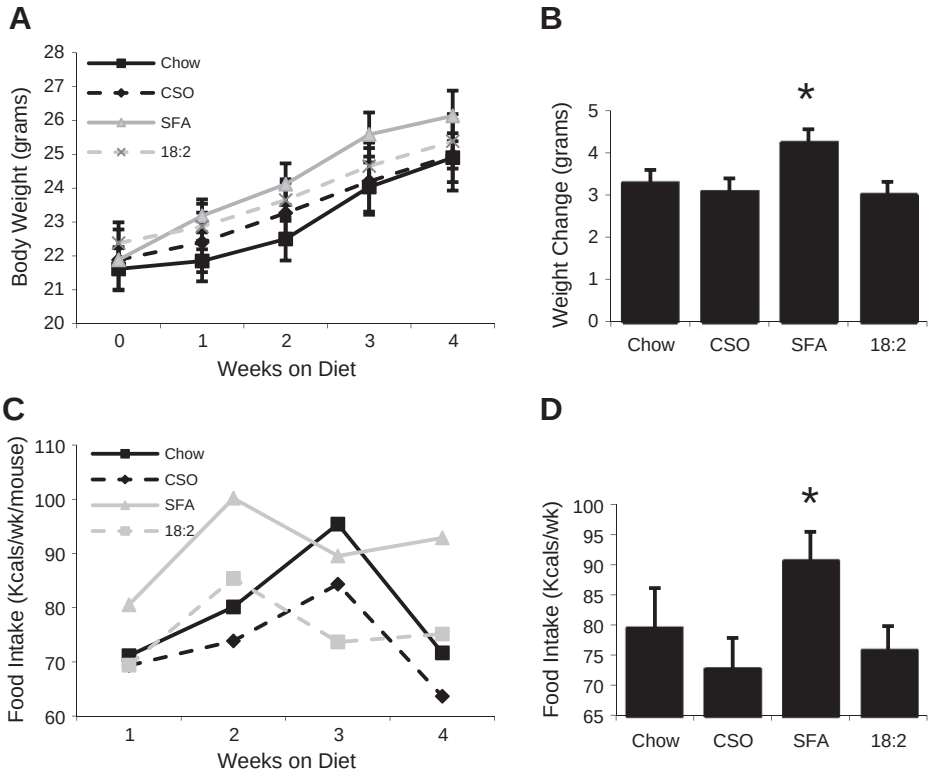


**Fig. 2 – Body weight and food intake. A, Body weight and change in body weight (B) expressed as difference in mouse body weight from the initiation of the feeding study in Δg/wk. C, Food intake (g/wk) and energy consumption (D) (kcal) per wk/g of body weight over the entire feeding study. n = 6 animals per group; *P < .05 vs chow-fed group. Values are presented as means ± SEM. Note: 1 kcal = 4.184 kJ.**

palmitoleic acid (16:1n9) (Fig. 1C-D). The animals were given ad libitum access to chow, CSO-enriched, SFA-enriched, or 18:2-enriched diets for 4 weeks. Body weight was measured weekly, and although all animals increased body weight over the course of the study, no groups significantly differed from chow-fed mice at the end of 4 weeks (Fig. 2A-B). After 1 week on the diet, CSO-fed mice gained more weight than chow-fed mice (0.6 g; $P = .03$), but this difference disappeared from weeks 2 to 4. SFA-fed mice gained more weight at weeks 1 (1.3 g; $P = .001$), 2 (1.6 g; $P = .006$), and 3 (1.6 g; $P = .01$), but they were not different by week 4 (1.2 g; $P = .1$). Food intake did not differ between any of the diet groups (Fig. 2C-D). Although there was a faster increase in body weight among the SFA-fed mice, the change in body weight among all groups was relatively similar by week 4 (eg, 12 weeks of age) largely reflecting "normal" weight gain with age. Fasting glucose was unchanged in the CSO- and SFA-fed groups and higher in 18:2-fed mice vs the chow-fed mice ($P = .02$) (Fig. 3A). Post IP glucose tolerance test (GTT) values were lower among CSO- and SFA-fed groups (Fig. 3B), with the area under the curve significantly reduced (Fig. 3C). From these data, it

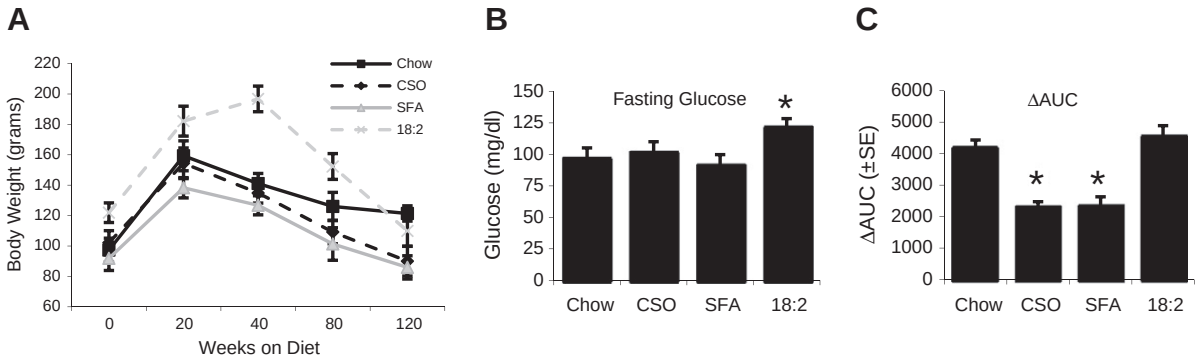


**Fig. 3 – Fasting and post IP injection glucose tolerance tests. A, Mice were injected IP with 0.75 g/kg body weight glucose, and plasma glucose was measured at the indicated time points. B, The 18:2-fed groups displayed higher fasting glucose and (C) greater glucose area under the curve. n = 6 animals per group; *P < .05 vs chow-fed group. Values are presented as means ± SEM.**

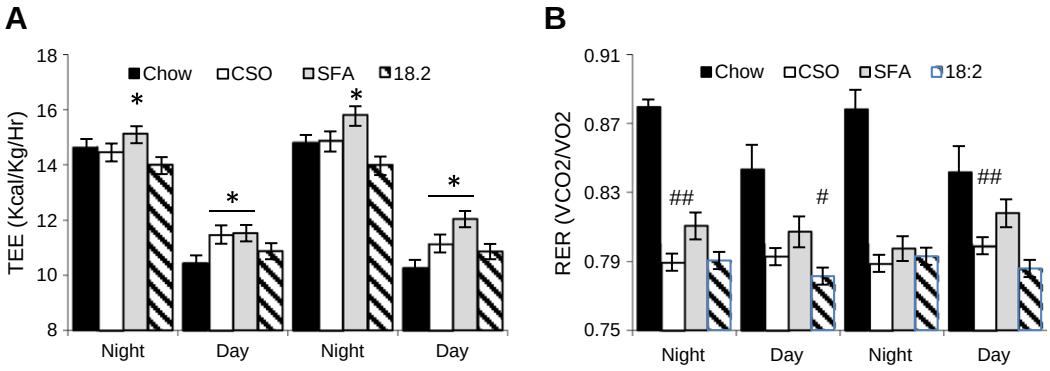


**Fig. 4 – Continuous measurement of total energy expenditure and RER. A, TEE expressed as kcal/h from 2 days of metabolic testing of mice following 4 weeks of feeding with either chow, CSO, 18:2, or SFA. B, Average RER from each light/dark cycles over the 2-day period. n = 6 animals per group; *P < .05 vs chow-fed group; #P < .05 vs. SFA-fed group. Values are presented as means ± SEM. Note: 1 kcal = 4.184 kJ.**

appears that an HFD enriched in CSO does not increase body weight and it improves glucose tolerance relative to the chow-fed and 18:2-enriched diets.

### 3.2. *CSO enhances energy expenditure and fat oxidation*

In addition to glucose tolerance, we assessed TEE and RER continuously over 48 hours. To investigate the effects of CSO consumption on energy expenditure, we measured heat production and metabolic gas exchange of mice fed the chow, CSO, SFA, or 18:2 diets for 4 weeks. SFA mice tended to weigh more than other mice; therefore, TEE was normalized to body weight. Despite normalizing, the mice consuming SFA-rich diets had higher TEE, which may be partly explained by their higher food intake (ie, thermic effect of food) (Fig. 4A). During light and dark cycles, mice consuming 18:2-rich diets had lower TEE compared with both the CSO- and SFA-fed groups. RER, which reflects gross macronutrient oxidation, indicated that the CSO- and 18:2-fed animals were oxidizing more fat for fuel and the SFA-fed animals used more carbohydrate (CHO) as fuel. Taken together with the GTT and fasting glucose data, it appears that the SFA-rich HFD increases body weight, food intake, and glucose oxidation, whereas 18:2-rich HFD leads to impaired glucose tolerance and reduced TEE. Mice fed CSO-rich HFD diets seem to display improved GTT and no appreciable defects in TEE or CHO oxidation.

### 3.3. *CSO increases PPARδ protein expression in liver and skeletal muscle*

Next, to investigate the effect of CSO consumption on the expression of proteins that regulate substrate handling/oxidation and metabolism in metabolically active tissues, we performed immunoblotting with lysates from both skeletal muscle and liver tissues. CSO-fed mice displayed elevated PPARδ expression in skeletal muscle, whereas 18:2- and SFA-enriched diets showed little or no PPARδ expression (Fig. 5A). In addition to muscle, the livers of mice fed CSO-enriched diets displayed a large increase in the PPARδ coactivator PGC-1α, along with PPARδ itself, and lactate dehydrogenase a or b isoform (LDHa/b) expression compared with all other diets (Fig. 5B). The protein expression changes in the liver strongly suggest that CSO provides metabolic adaptations that favor enhanced fatty acid oxidation and utilization.

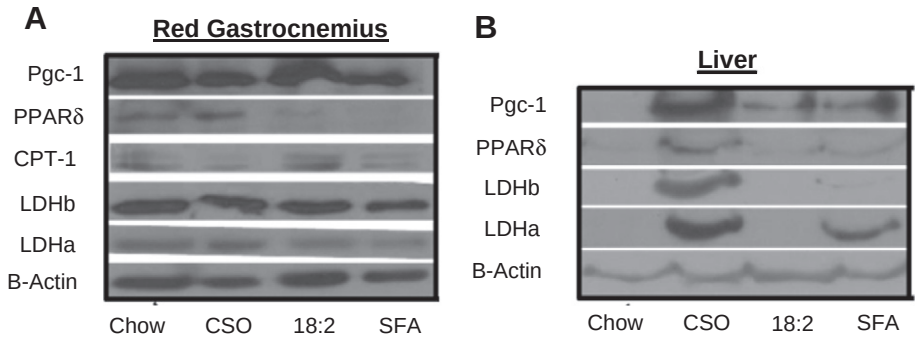


**Fig. 5 – Liver and muscle metabolic protein expression. A, Red gastrocnemius muscle and (B) liver were homogenized, and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and probed using Western blotting. CSO-fed mice had higher Pgc-1 and PPARδ protein expression in liver vs chow-, SFA-, and 18:2-fed groups. Each sample is pooled from 6 animals per group.**

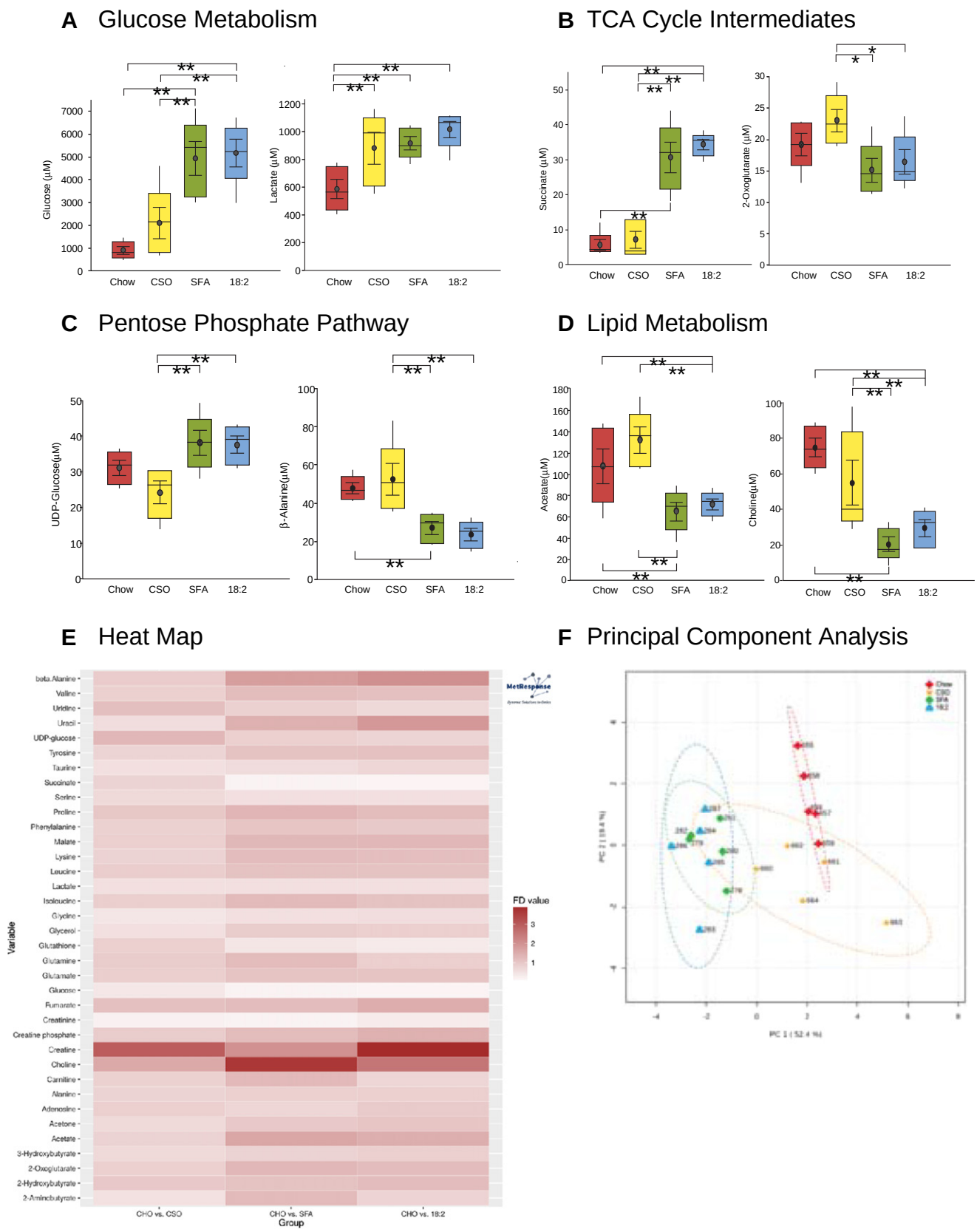


**Fig. 6 – $^{1}$H 2D NMR metabolomics of livers. Liver metabolites were analyzed by 2D NMR to examine patterns of various metabolites. CSO-fed mice displayed metabolic profiles identical to chow-fed mice with respect to glucose metabolism (A), TCA cycle (B), pentose phosphate pathway (C), and lipid metabolism (D) intermediates. E, A heat map of differences in individual metabolites. F, Principal component analysis suggests that metabolic phenotype of CSO-fed mice is similar to chow-fed mice, whereas SFA- and 18:2-enriched mice are more lipogenic. n = 6 animals per group; *P < .05 18:2 vs chow-fed group.**

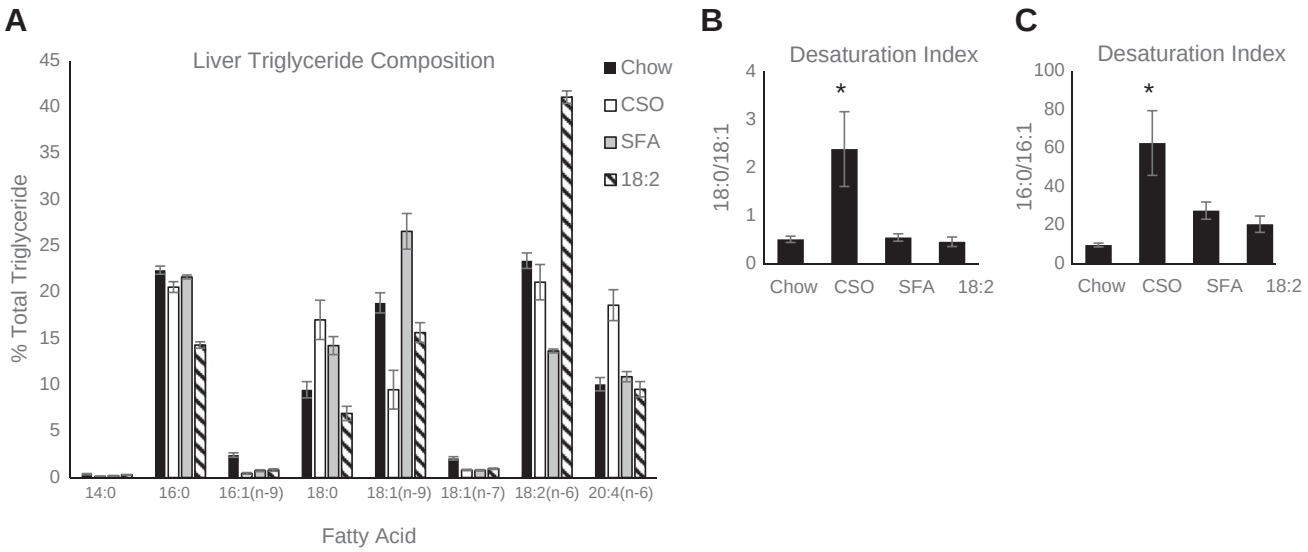


**Fig. 7 – Fatty acid composition of liver TGs. A, Total TGs were separated from livers of mice fed the 4 diets for 8 weeks. TG fatty acid composition was determined by GC-MS and expressed as g fatty acid/100 g lipid. Desaturation indices of 18-carbon (B) and 16-carbon (C) species suggest an impairment in Δ9 desaturase activity. Values are presented as means ± SEM.**

### 3.4. *NMR metabolomic profile*

To better understand the metabolic implications of the different diets, we screened more than 40 individual small metabolites from livers of mice of the 4 groups. Major intermediary metabolites that represent glycolysis, Tricarboxylic acid cycle (TCA) cycle, pentose phosphate pathway, and lipid metabolism were selected for display (Fig. 6A-D). Consistent with RER data, glycolysis intermediates such as glucose, Uridine diphosphate glucose (UDP-glucose), and lactate levels were lower in CSO-fed mice vs SFA- and 18:2-fed groups; similar patterns were seen with TCA cycle intermediates such as succinate and 2-oxoglutarate, indicating lowering of glucose oxidation in CSO diet. Meanwhile, $\beta$-alanine, acetate, and choline levels were higher in CSO-fed mice compared with SFA- and 18:2-fed groups, indicating more lipid oxidation profile in CSO diet. No differences were observed between chow-fed and CSO-fed mice, whereas CSO-fed vs the SFA- and 18:2-fed groups all significantly differed between the selected metabolites. These data imply that the metabolic profiles of CSO-fed animals are more similar to profiles of the chow-fed mice than they are to the profiles of the animals fed the other fat-enriched diets. Additionally, individual metabolite differences shown by heat map indicate that the majority of changes in the livers of high-fat–fed mice occurred with the SFA- and 18:2-rich diets but not with the CSO-rich diet (Fig. 6E). Despite the fact that CSO lipid composition is composed primarily of 18:2n6 and saturated fatty acids, the livers from animals fed CSO did not differ from the livers of chow-fed mice (Fig. 6F). CSO-fed animals do not display the lipogenic phenotype of the SFA- or 18:2-fed mice; instead, they appear more like chow-fed animals despite the fact that the lipid contents of their diet were similar to these latter groups.

### 3.5. *TG fatty acid composition of livers*

Because there were notable differences among the metabolic profiles of livers of mice fed CSO- vs SFA- and 18:2-rich diets, we questioned whether there were differences in liver TG composition that could explain the metabolic differences. As we noted with the TG composition among each of the diets, there were expected differences in liver 16:0, stearic acid (18:0), and 18:2n-6; however, the most notable differences were among Δ9 desaturation products. In the CSO diet, 16:1n9 was higher than the other 3 diets (Fig. 1B); yet in the liver, its level was the lowest (Fig. 7A), with a similar profile for oleic acid (18:1n9). The desaturation indices (and especially the 16:0/16:1n9 ratio) reveal that Δ9 desaturase activity, which is catalyzed by stearoyl-CoA desaturase-1 (SCD1), was suppressed (Fig. 7B). It has been well established that HFDs lead to increased SCD1 activity [8-10]. However, both 16- and 18-carbon fatty acid desaturation levels were lower in CSO-fed mice (Fig. 7C).

### 3.6. *DHSA mediates the effect of CSO on lipid metabolism*

The metabolic phenotype of CSO-fed mice strongly resembled that of liver-specific SCD1 knockout mice [11]. Those animals display reduced hepatic lipid content and de novo lipogenesis with increased desaturation indices (SFA/MUFA). Cottonseed products can contain various impurities, including the bioactive secondary metabolite gossypol, which is known to inhibit various dehydrogenase enzymes through an uncharacterized mechanism [12,13]. Based upon the elevated levels of LDHa and LDHb in liver of the CSO-fed mice, we suspected that gossypol contamination of the CSO diets might be responsible for the metabolic phenotype. We

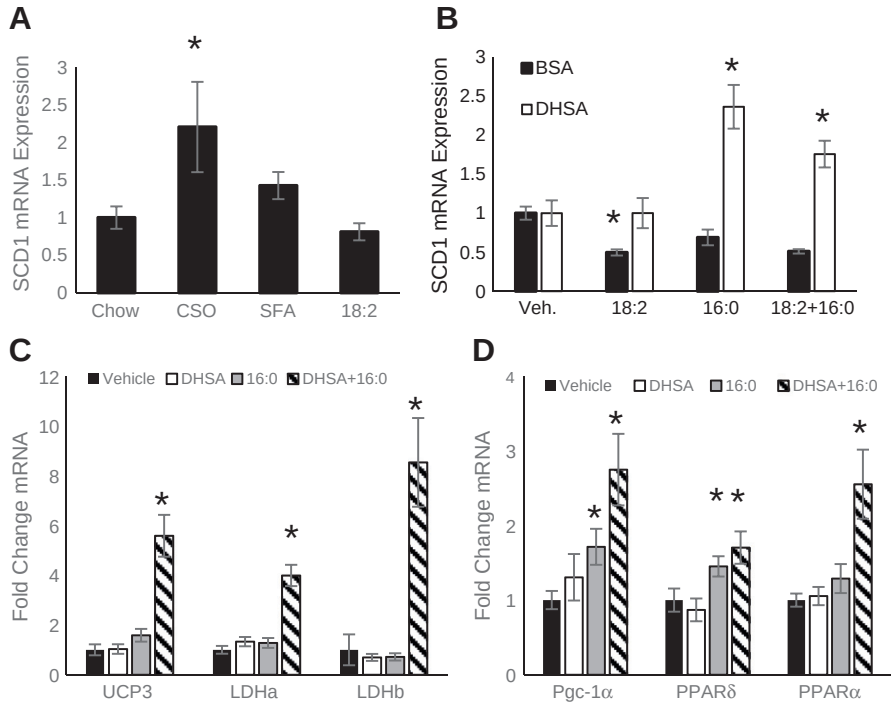


**Fig. 8 – DHSA replicates the effects of CSO on SCD1 activity in vitro. A, Mice fed CSO-rich diets have higher SCD1 expression despite lower MUFAs, indicating impaired desaturase activity. FL83-B cells were treated with 10 μmol/L palmitic acid (16:0), 1 μmol/L DHSA, or both (DHSA + 16:0), and changes in UCP3, LDHa, and LDHb, along with their transcriptional activators Pgc-1α, PPARδ, and PPARα, were assessed. The changes in response to DHSA replicated the changes observed in animals fed CSO. n = 6/group; *P < .05. Values are presented as means ± SEM.**

performed liquid chromatography/mass spectrometry (MS) assays on the CSO diets, but we were unable to detect gossypol within a lower limit of detection of 5 ppb (data not shown).

Crude CSO also contains approximately 1% esterified cyclopropyl fatty acids (CPFAs), but the oil-refining process generally removes most of these from the oil. However, we found that the CSO diet contained approximately 0.3% DHSA (data not shown), which is a cyclopropanyl intermediate in the synthesis of sterculic acid [14,15]. Eighteen-carbon CPFAs are known inhibitors of SCD1, as they contain a cyclopropyl ring (C3H4) between carbons 9 and 10 where SCD1 typically catalyzes oxidation of saturated fatty acid C-C bonds [16]. As a result of the cyclopropyl ring, it is believed that CPFAs are irreversibly bound to the active site of SCD1, thereby rendering the enzyme inactive.

We and others have shown that in the presence of SCD1 inhibitors, SCD1 mRNA and protein expressions increase [16,17], likely as a response to increased SFA content. SCD1 expression also increases with SFA-rich diets and typically is associated with reduced saturated fatty acid and increased MUFA levels. However, in the CSO-fed animals, liver SCD1 expression increased without a concomitant reduction in saturated fatty acids, indicating that desaturase activity is inhibited (Fig. 8A). SFA-fed mice increased SCD1 expression, and 18:2-fed animals showed no change. These results are in agreement with previous studies where saturated fatty acids increase SCD1 expression and n-6 PUFAs suppressed lipogenic gene expression via binding to promoter sterol response elements [18,19]. To confirm these effects in a cell-based model, we treated mouse FL83-B hepatocyte-like cells with 10 μmol/L 18:2n6, 16:0, or 18:2n6 + 16:0 in the presence or absence of 1 μmol/L DHSA. It is well known that inhibition of SCD1 activity can be reflected in increased mRNA expression, a phenomenon likely due to positive feedback from increased levels of saturated fatty acids. As expected, 18:2n6 suppressed SCD1 gene expression (0.5 ± 0.04-fold vs Veh; $P = .0001$), and in the presence of DHSA, 18:2n6 did not change relative to control (Fig. 8B). Similarly, 16:0 treatment reduced SCD1 expression alone (0.7 ± 0.1-fold vs Veh; $P = .02$) but increased significantly in the presence of DHSA (2.4 ± 0.3-fold vs Veh; $P = .001$). The combined response of 18:2n6 + 16:0 decreased SCD1 but, in the presence of DHSA, increased its expression. We further confirmed these results by examining the expression of previously known targets of liver-specific SCD1 inhibition. The patterns of UCP3, LDHa, LDHb (Fig. 8C), Pgc-1α, PPARδ, and peroxisome proliferator activated receptor alpha (PPARα) (Fig. 8D) are all in agreement with previously published work [11] and our animal models in the current study. These data support the concept that the combination of 16:0, which increases the requirement for SCD1 activity, along with DHSA and 18:2n-6, which block SCD1 activity and expression, can prevent lipogenic adaptations in the liver.

## 4. Discussion

Our hypothesis was rejected for n-6 PUFAs. However, although DHSA was not the hypothesized mediated of

PPARδ, it was determined to be effective. Herein, we have effectively demonstrated that CSO can prevent the metabolic adaptations associated with an HFD. CSO is composed primarily of linoleic (53%), palmitic (24%), and oleic acids (18%), and based on its composition, it is generally regarded as an "unhealthy" oil. We compared mice fed a CSO-enriched diet to mice fed chow, cocoa butter (high SFA)–enriched, or safflower oil (high 18:2n6)–enriched diets and found that the CSO-fed mice largely protected the animals from HFD-induced metabolic adaptations. The livers of CSO-fed mice were nearly identical to chow-fed mice, whereas cocoa butter– and safflower oil–fed mice displayed highly lipogenic livers. We have found the mechanism to be due to the presence of a bioactive lipid that is specific to CSO, identified as DHSA. DHSA in conjunction with linoleic acid suppressed SCD1 activity to produce a phenotype similar to the SCD1 liver knockout model. Our results clearly indicate that CSO should be viewed as a "healthy oil" with potent bioactive properties toward SCD1.

SCD1 is the rate-limiting enzyme in de novo lipid synthesis. It converts long-chain saturated fatty acids into MUFAs, and its expression is critical for maintaining metabolic function in nearly every tissue. SCD1 activity in liver is required to produce MUFAs during neutral lipid synthesis for efficient esterification, storage, and fluidity [17]. Interestingly, when SCD1 activity is absent in the liver, mice that are on a high-CHO, very low-fat (HCVLF) diet are completely protected from steatosis and fatty liver disease [11]. Under normal conditions, an HCVLF diet will cause extensive lipid accumulation, steatosis, inflammation, and liver insulin resistance due to the high rate of lipogenesis [20]. However, when SCD1 is genetically deleted from the liver, mice on an HCVLF diet have almost no accumulation of TG.

As a result of its ability to block lipid accumulation, SCD1 has been sought as a target for antiobesity and anti-inflammatory treatments. This is due in part to enhanced lipid oxidation in liver, muscle, and heart through as-yet-unidentified mechanisms [4,21,22]. It is thought that reductions in 18:1 synthesis block overall TG esterification, which in turn channels FFAs into β-oxidation. If this is occurring as a result of DHSA, it is possible that channeling FFAs to oxidation may promote PPARδ activation. It is noteworthy that both PPARδ and PPARα increased in response to SFA + DHSA, and although it was not assessed in the current project, it would be important to assess the mechanism by which DHSA is promoting PPAR activity.

Extensive efforts have been made to recapitulate the SCD1 knockout phenotype using pharmacological methods; however, side effects, such as dry skin (alopecia) and dry eye, have prevented their widespread use in clinical trials. Based on the results of this study, it would be interesting to determine the impact of DHSA supplementation on the development or reversal of nonalcoholic steatohepatitis. Currently, there are no consistently effective therapies for nonalcoholic steatohepatitis, with the exception of weight loss, and the ability of DHSA in the form of CSO, either crude or refined, to safely block de novo lipogenesis may provide a beneficial therapeutic approach.

Limitations of the current work include a confined mouse model consuming 50% of energy from a single food source, lack of sufficient energy expenditure above caged activity levels, and not directly measuring SCD1 activity. Despite these limitations, we were able to define the effect of CSO and DHSA on metabolism in mice. A CSO-enriched diet appears to have components that allow it to stimulate tissue metabolism at the molecular level beyond what is seen in matched PUFA- or SFA-enriched diets. These observations demonstrate that CSO may offer distinct metabolic benefits not seen with standard fatty acid supplementation. Moreover, these findings further demonstrate the importance of experiments that include commonly consumed dietary foods rather than purified macronutrients to study bioactive lipids. Taken together, our observations support the hypothesis that CSO may stimulate energy expenditure without weight gain and may promote several favorable molecular adaptations in both liver and skeletal muscle. As a result of these findings, current efforts are under way to analyze the content of each diet to identify potential feed metabolites which may be contributing to each of the adaptations revealed to us thus far.

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