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ORIGINAL ARTICLE

Cocoa Butter and Safflower Oil Elicit Different Effects on Hepatic Gene Expression and Lipid Metabolism in Rats

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Abstract The aim of this study was to compare the effects of cocoa butter and safflower oil on hepatic transcript profiles, lipid metabolism and insulin sensitivity in healthy rats. Cocoa butter-based high-fat feeding for 3 days did not affect plasma total triglyceride (TG) levels or TG-rich VLDL particles or hepatic insulin sensitivity, but changes in hepatic gene expression were induced that might lead to increased lipid synthesis, lipotoxicity, inflammation and insulin resistance if maintained. Safflower oil increased hepatic β-oxidation, was beneficial in terms of circulating TG-rich VLDL particles, but led to reduced hepatic insulin sensitivity. The effects of safflower oil on hepatic gene expression were partly overlapping with those exerted by cocoa butter, but fewer transcripts from anabolic pathways were altered. Increased hepatic cholesterol levels and increased expression of hepatic CYP7A1 and ABCG5 mRNA, important gene products in bile acid production and cholesterol excretion, were specific effects elicited by safflower oil only. Common effects on gene expression included increased levels of p8, DIG-1 IGFBP-1 and FGF21, and reduced levels of SCD-1 and SCD-2. This indicates that a lipid-induced program for hepatic lipid disposal and cell survival was induced by 3 days of high-fat feeding, independent on the lipid source. Based on the results, we speculate that hepatic TG infiltration leads to reduced expression of SCD-1, which might mediate either neutral, beneficial or unfavorable effects on hepatic metabolism upon high-fat feeding, depending on which fatty acids were provided by the diet.

Keywords Dietary fat · Hepatic lipid metabolism · Gene expression

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Abbreviations

StD	Standard diet
CBD	Cocoa butter-enriched diet
SOD	Safflower oil-enriched diet
FA	Fatty acid(s)
SFA	Saturated fatty acid(s)
MUFA	Monounsaturated fatty acid(s)
PUFA	Polyunsaturated fatty acid(s)
TG	Triglyceride(s)
CE	Cholesteryl ester(s)
HDL	High density lipoprotein
LDL	Low density lipoprotein
VLDL	Very low density lipoprotein
GH	Growth hormone
IL	Interleukin
TNFα	Tumor necrosis factor α
FGF21	Fibroblast growth factor 21
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
LXR	Liver X receptor

FXR	Farnesoid X receptor
PPARα	Peroxisome proliferator-activated receptor α
SREBP	Sterol regulatory element binding proteins
IRS	Insulin receptor substrate
GSK3β	Glycogen synthase kinase 3β
STAT	Signal transducer and activator of transcription
SOCS2	Suppressor of cytokine signaling 2
EGR1	Early growth response 1
AMPK	AMP-activated protein kinase
CPT	Carnitine palmitoyl transferase
ACC	Acetyl-CoA carboxylase
SCD	Stearoyl-CoA desaturase
IGFBP-1	Insulin-like growth factor-binding protein 1
CYP7A1	Cytochrome P450, family 7, subfamily A
ABCG5	ATP-binding cassette, sub-family G, member 5
3-PGDH	3-Phosphoglycerate dehydrogenase
PSAT1	Phosphoserine aminotransferase 1
RPLP	Ribosomal protein long-chain protein Pθ

Introduction

Numerous studies have been performed on the effects of dark chocolate or cocoa in relation to public health [1, 2]. Improved immune function [3, 4], cardiovascular status [5] and insulin sensitivity [6, 7] are among the reported positive health effects of cocoa. Cocoa contains flavonoids, which are believed to mediate the beneficial effects of dark chocolate. Flavan-3-ol has recently received much attention due to its cardioprotective properties [1]. However, the lipid content of chocolate and cocoa is relatively high, and with a high percentage of saturated fatty acids (SFA).

Epidemiological studies have shown that intake of excess SFA is the principal lifestyle-related cause of insulin resistance and obesity-related diseases in humans [8]. Carbohydrate tolerance and whole-body insulin sensitivity have been shown to be impaired with consumption of diets enriched with SFA in healthy human subjects [9, 10] and experimental animals [11–13]. The concept that dietary SFA produces hypercholesterolemia is also widely accepted. However, cocoa butter has been shown to have little or no effect on plasma cholesterol levels and the development of atherosclerosis when fed to animals [14–16] and humans [17, 18]. It has been suggested that this neutral effect of cocoa butter might be related to its relatively low digestibility [19] and lymphatic absorption [20]. Furthermore, stearic acid, which is the main saturated fatty acid in cocoa butter, has been shown to have a neutral effect on the plasma lipid profile [21]. Ingested cocoa has also been shown to reduce de novo lipid synthesis in rat liver [22], which might explain some of the positive health effects of cocoa. Whether cocoa butter would exert similar effects on the liver is not known.

It is well accepted that hepatic fat accumulation is linked to insulin resistance [23], and that fatty liver is an independent predictor of type 2 diabetes, the metabolic syndrome and cardiovascular disease [24, 25]. Since fatty liver leads to failure of insulin to suppress hepatic glucose production, subjects with a fatty liver have an increased risk of developing hyperglycemia, glucose intolerance and peripheral insulin resistance. Furthermore, hepatic insulin resistance includes the decreased ability of insulin to suppress triglyceride-rich VLDL particle production in the liver [26], leading to hypertriglyceridemia and low HDL cholesterol concentration [27]. Hepatic insulin resistance is thus likely to be an important factor of the metabolic syndrome.

Short term (3 days) high-fat feeding in rats results specifically in hepatic fat accumulation and has been used as a model to study the mechanisms underlying hepatic insulin resistance [28, 29]. Three days of high-fat feeding, using safflower oil as lipid source, is sufficient to cause a threefold increase in hepatic lipid content and to suppress insulin sensitivity [29]. Safflower oil is rich in linoleic acid (18:2n-6) and has been shown to increase oxidative and inflammatory stresses [30–32], which are thought to be involved in the development of e.g. hepatic insulin resistance. Since hepatic insulin signaling results in increased de novo lipogenesis, suppressed hepatic insulin sensitivity might be protective for the liver during situations of increased dietary fat. Short-term oxidative stress, in the absence of lipotoxicity and inflammation, might thus be part of a cellular defense system where reduced insulin signaling protects the liver from further lipid loading. It has been proposed that insulin resistance is good when viewed in its original evolutionary context, but becomes pathological upon persistent stimulation by oxidative stress [33].

In contrast to the neutral effect of cocoa butter on plasma cholesterol levels, linoleic acid-enriched diets have beneficial effects on circulating cholesterol levels [34–36]. The aim of this study was to get clues as to whether this difference can be attributed to differences at the level of hepatic metabolism. The lipid content of chocolate and cocoa is relatively high, but the effects of ingested cocoa butter on markers for hepatic lipid metabolism and insulin signaling have to our knowledge not been addressed before. In the present study we sought (1) to answer whether short-term cocoa butter feeding is neutral, beneficial or detrimental regarding hepatic lipid metabolism, and (2) to identify similarities as well as differences between cocoa butter and safflower oil regarding effects on plasma lipoproteins, hepatic gene expression, lipid metabolism and insulin sensitivity using short-term high-fat feeding in rats.

Experimental Procedure

Animal Experiments

Seven week old male Sprague–Dawley rats (Scanbur BK AB) were maintained on standard chow (R36, Lactamin, Sweden) for a week before start of the experiment. Rats were thereafter fed for 3 days with either standard, cocoa butter- or safflower oil-enriched R36 diets. The compositions of these diets are shown in Table 1. The fatty acid compositions of the high-fat diets were the following; the safflower-enriched diet contained 77% linoleic, 15% oleic, 6% palmitic and 2% stearic acid; whereas the cocoa butter-enriched diet contained 36% stearic, 33% oleic, 25% palmitic and 3% linoleic acid. Food was removed late in the evening (11 p.m.), so that the animals were without food for 12 h before they were sacrificed.

Four rats from each group were injected intraperitoneally with insulin (Actrapid, Novo Nordisk), at a dose of 5 mU/g body weight, and four with saline only. Immediately before and 40 min after the injection, a drop of blood was collected from the tip of the tail and analyzed for blood glucose, using a Precision Xtra glucometer and test strips (Abbot Scandinavia AB). The rats were sacrificed and tissues removed and frozen in liquid nitrogen. The animal experiments were approved by the regional Ethics Committee on Animal Experiments.

Plasma Analysis

Blood was collected from the vena cava in 10-ml tubes containing 17.5 mg EDTA (B&D). Plasma was obtained by centrifugation at 3,000 g for 10 min. The resulting supernatants were removed and analyzed for insulin (RIA kits from Millipore) and lipids. Lipoprotein separation, by size-exclusion chromatography on individual samples from each animal, and lipid content calculations were performed as described previously [37]. Plasma levels of cytokines (IL-1β, IL-6, IL-17 and TNFα) were determined using Rat Cytokine/Chemokine Milliplex Kit (Millipore) and a Luminex instrument (LABScan 100, Luminex), according to the manufacturer's instructions.

Hepatic Lipid Content

Cellular lipids were extracted from the rat liver using chloroform and methanol (2:1 v/v), using the Folch method [38]. The extracts were dried, dissolved and analyzed for triacylglycerides and total (free and esterified) cholesterol, using kits from Roche and Calbiochem, respectively. Samples were analyzed in triplicate and the results expressed as microgram lipid per milligram liver weight.

Fatty Acid Oxidation

Hepatic β-oxidation was assayed by monitoring the palmitoyl-CoA-dependent reduction of NAD to NADH in sub-fractionated liver homogenates, as described previously [39]. Liver tissues (300 mg) were homogenized in 3 ml ice-cold sucrose-solution (0.25 M sucrose, 10 mM HEPES, 1 mM EDTA) using a Potter–Elvehjem glass-Teflon homogenizer. The homogenates (1 ml) were centrifuged for 10 min at 500 g, the supernatants withdrawn and centrifuged for 10 min at 10,000 g, and the resulting pellet re-suspended in sucrose-solution. The samples were added to the reaction buffer to a final concentration of 30 mM phosphate buffer pH 7.5, 0.2 mM NAD, 0.1 mM coenzyme A, 12 mM DTT, 0.15 mg/ml BSA (fatty acid free) and 0.01% triton X-100 (Sigma–Aldrich, Inc). The initial rate of NADH production was determined upon addition of palmitoyl-CoA (Sigma–Aldrich, Inc) (10 µg/ml) at 37 °C. The absorbance at 340 nm was measured spectrophotometrically.

Table 1 Diet compositions

	R36 standard		Safflower oil-enriched		Cocoa butter-enriched	
	Component (g/100 g diet)	Energy (%)	Component (g/100 g diet)	Energy (%)	Component (g/100 g diet)	Energy (%)
Moisture	10	–	10	–	10	–
Ash	6	–	6	–	6	–
Fiber	3.5	0.8	2.7	0.5	2.7	0.5
Carbohydrate	58	67.8	40.1	36.3	40.1	36.3
Protein	18.5	22.2	12.2	11.4	12.2	11.4
Fat	4	9.2	29.5	51.8	29	51.8
Total calories (kJ/100 g)	128.8		166.1		166.1	

Analysis of Gene Expression by Quantitative Real-Time PCR

Total RNA was isolated by homogenization of frozen rat livers using a polytrone PT-2000 (Kinematica AG) and TRIzol® Reagent (Invitrogen Life Technologies) according to the protocol supplied by the manufacturer. The RNA concentration was determined spectrophotometrically at 260 and 280 nm. The quality of the RNA samples was examined using a RNA 6000 Nano Bioanalyzer according to the manufacturer's instructions (Agilent Technologies).

5 μg total RNA was treated with DNase (RQ1) and reverse transcribed using iScript™ reverse transcriptase and 5× buffer for a First Strand cDNA synthesis (Bio-Rad Laboratories AB). The purity of the synthesized cDNA was checked by agarose gel electrophoresis. Subsequently, 2 μl of each first strand cDNA served as a template in a 20 μl PCR reaction mix containing the primers for the gene of interest (see Table 2) and iQ SYBR Green Supermix (Bio-Rad Laboratories AB). Quantification of gene expression was performed according to the manufacturer's protocol using DNA Engine Opticon™2 real-time PCR detection system (MJ Research). A relative standard curve was constructed with serial dilutions (1:1, 1:5 and 1:25) using a pool of the cDNA generated from all animals used in the study. The amplification program consisted of 1 cycle of 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, annealing temperature for 10 s, 72 °C for 30 s; fluorescent intensity was measured at a specific acquisition temperature for each gene. The protocol was validated for each gene of interest by checking melting curves for the absence of primer–dimers or other unwanted amplicons. The level of individual mRNAs was normalized with the level of the housekeeping gene ribosomal protein long-chain protein Pθ (RPLP). Results are expressed in arbitrary units.

Immunoblotting

Whole liver cell lysates were obtained by homogenizing 100 mg of liver in 1 ml RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, 1 μg/ml of aprotinin, leupeptin, and pepstatin), using a polytrone PT-2000 (Kinematica AG), followed by 20 min of centrifugation (12,000*g*). The resulting supernatants were collected and proteins resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked for 1 h in Tris-buffered saline (TBS; 10 mM Tris pH 8.0, 150 mM NaCl) containing 0.1% (v/v) Tween-20 and 5% (w/v) milk powder or 5% (w/v) bovine serum albumin, incubated overnight at 4 °C with the antibody of interest diluted in TBS-T with 1% milk or bovine serum albumin. Antibodies for detecting IGFBP1 (1:500) were from Abcam; p-STAT3-Tyr705, STAT3, p-STAT5-Tyr694 (1:500), SOCS2, STAT5, p-AMPK-Thr172 and AMPK (1:1,000) were from Cell signaling; SCD-1 (1:500), SREBP-1, IRS (1:2,000), p-IRS1-Ser307 (1:1,000) and β-actin (1:50,000) were from SantaCruz. The membranes were washed and incubated with secondary antibody for 1 h in room temperature according to the data sheet provided by the company. After additional washing steps antibody binding was visualized using an ECL detection system (Pierce Biotechnology, Inc). Densitometry analysis was performed using the software Quantity One 4.6.5 Basic (Bio-Rad) to compare the amount of the antibody of interest to β-actin.

Akt Phosphorylation

Whole liver cell lysates were obtained by homogenizing 1 g of liver in 3 ml RIPA buffer, as described above). The resulting supernatants were collected and the degree of insulin signaling was analyzed by measuring the degree of insulin-dependent phosphorylation of Akt and GSK3β. Akt activation was determined by analyzing the amount of phosphorylated Akt (p-Akt-Ser473) in relation to total Akt, using commercially available ELISA kits (Biosource). Samples were analyzed in triplicate and the results determined as unit p-Akt per ng total Akt.

Expression Profiling Using Microarrays

Microarrays containing 70-mer oligonucleotide probes for 27,649 rat protein-coding genes were fabricated and used

Table 2 Primers

Accession no.	Gene		Forward primer (5′–3′)	Reverse primer (5′–3′)
NM_013144	IGFBP-1	Insulin-like growth factor-binding protein 1	GTGGAATGCCATTAGCACCT	CAGCAAACAGTGCGAGACAT
NM_130752	FGF21	Fibroblast growth factor 21	ACACCGCAGTCCAGAAAGTC	TCACTTTGATCCTGAGGCCT
NM_139192	SCD-1	Stearoyl-coenzyme A desaturase1	GATATCCACGACCCCAGCTC	TACCTTATCAGTGCCCTGGG
NM_058208	SOCS2	Suppressor of cytokine signaling 2	GACGGGAAATTCAGATTGG	ACTTCTGCCGACTCAGCATT
NM_022402	RPLP	Ribosomal protein long-chain protein Pθ	CAGCAGGTGTTTGACAATGG	AAAGGGTCCTGGCTTTGCTC

to obtain transcript profiles, essentially as described previously [40]. A 40-µg amount of the total RNA from each sample were DNase-treated using RNeasy MiniElute Cleanup kit (QIAGEN) according to the supplied protocol and RNA concentrations measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). The microarray experiments were performed using the Pronto!™ Plus Direct Systems (Corning Incorporated and Promega Corporation, NY), according to the manufacturer's instructions using 5 µg total RNA from each sample. In the first set of experiments, each hybridization compared Cy3-labeled cDNA reverse transcribed from RNA isolated from rats fed the standard diet with Cy5-labeled cDNA isolated from rats fed the high-fat diets. Each experiment was analyzed using individual samples and dye-swopping. The Cy3-labelled cDNA was mixed with Cy5-labelled cDNA and purified using the Chip-Shot™ Membrane Clean-Up System. The probes were next mixed with 45 µl hybridization solution, added to the arrays and placed in sealed hybridization chambers at 42 °C. After an overnight incubation (18–19 h), arrays were washed, dried and scanned to create images, using a Genepix 4200A laser scanner (Axon Instruments, Union City, CA, USA). For each array, the photomultiplier tubes and power of the laser were adjusted so that the overall count ratio of Cy5 to Cy3 signal was approximately 1. Image analysis was performed using the GenePix 6.0 software (Axon Instruments). Automatic flagging was used to localize absent or very weak spots, which were excluded from further analysis.

The analyzed image files were filtered for spots showing signals at least two times above background. The fluorescence ratios of the spots were further normalized with the Lowess (Locally Weighted Scatter Plot Smoother) method using the statistical language R (R 1.3.1 software). Identification of differentially expressed genes (due to high-fat feeding) was performed using the SAM 1.21 (Significance Analysis for Microarray) software incorporated in Microsoft office Excel program. A 5% false discovery rate was used as a first cut-off. Genes with a greater than 1.5-fold increase or decrease were considered as being regulated, even though smaller changes in gene expression may also have important biological consequences. The results are represented as the mean of at least three independent determinations. All data are available from the NCBI Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo/) using the series entry GSE13936.

Statistical Analysis

All data were subjected to analysis of variance (one-way ANOVA) followed by Fisher's post-hoc analysis and expressed as means ± SE. Differences between groups were considered statistically significant when the probability that they occurred by chance was <0.05.

Results

High-Fat Diet-Mediated Effects on Lipid Profiles and Hepatic Gene Expression

Rats fed a cocoa butter-enriched diet (CBD; 52% of the energy from cocoa butter) for 3 days were compared to rats fed safflower oil-enriched diet (SOD; 52% of the energy from safflower oil) or standard diet (StD; 9% of the energy from cocoa butter). Hepatic triglyceride (TG) content was increased in animals fed high-fat diets, as compared to those fed StD (Fig. 1a). No difference in TG content was observed between the two different high-fat diets. In contrast, only SOD-fed rats had increased levels of hepatic cholesterol. Safflower oil exerted the biggest effects on plasma lipids. As illustrated in Fig. 1b, the SOD-fed rats had the lowest plasma levels of TG and cholesterol. CBD-fed rats had similar plasma TG content as the rats on StD, but lower levels of plasma cholesterol.

To determine the effects of the different high-fat diets on lipoproteins, plasma lipid profiles were generated from the three diet groups. Again, SOD had the greatest impact on plasma lipids. As shown in Fig. 1c, ingested safflower oil decreased TG-rich VLDL particles and increased cholesterol-rich VLDL particles. At the same time, HDL cholesterol was reduced. No difference was observed between the rats fed CBD and StD. Thus, in spite of similar hepatic lipid content, the two different high-fat diets elicited different effects on plasma lipoprotein particles. The results are in line with previous publications [34], and indicate lipid-specific effects (i.e. dependent on lipid source) on hepatic lipid and lipoprotein metabolism.

To get an overview of high-fat diet-mediated alterations within the liver, whole-genome rat oligo microarrays were used to generate hepatic transcript profiles (using livers from StD-fed animals as control). Surprisingly, the CBD had a much greater impact on gene expression than the SOD. With a 5% false discovery rate and a cut-off at 1.5-fold difference, 822 transcripts were significantly different between CBD- and StD-fed animals, whereas only 151 transcripts were altered in response to SOD-feeding. Most effects were lipid-specific (i.e. dependent on lipid source), but 73 transcripts were similarly altered in the two high-fat groups (Fig. 2a). The high-fat-regulated transcripts were grouped according to cellular functions and some interesting results are listed in Table 3. All data are available from the NCBI Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo/) using the series entry GSE13936.

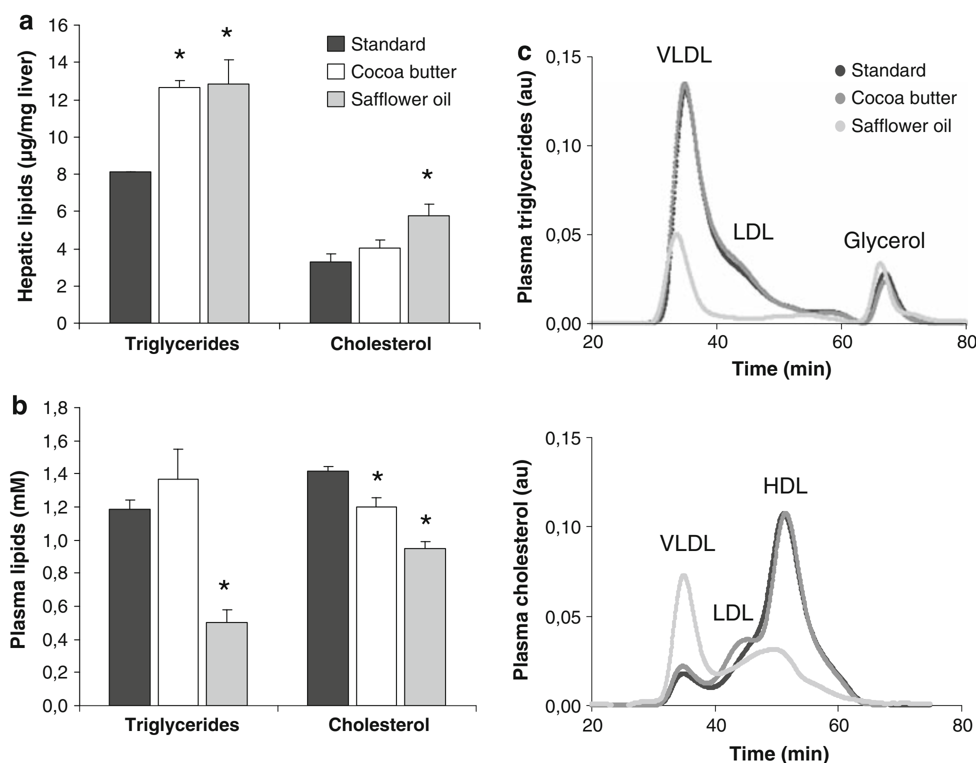

Fig. 1 Effects of short-term high-fat feeding on lipid levels in liver and plasma. Triglyceride and cholesterol levels were measured in **a** hepatic lipid extracts or **b** plasma and **c** lipoprotein profiles were generated from rats fed a standard diet or either cocoa butter- or safflower oil-enriched diets. Data are represented as means ± SE, and * indicates significant differences compared to standard diet ($n = 4$). The chromatograms represent the average from determinations performed on individual samples

High-Fat Diet-Mediated Changes in Gene Expression Independent on Lipid Source

As mentioned above, hepatic TG content was increased to the same extent in the two high-fat groups, which might elicit a similar response on certain genes. Among the gene products similarly altered in the two high-fat groups, the biggest changes (more than 2.5-fold) were observed for stearoyl-CoA desaturase 1 (SCD-1) and 2 (SCD-2), fibroblast growth factor 21 (FGF21), nuclear protein 1 (p8) and serine dehydratase (Table 3). Reduced levels of serine dehydratase might indicate reduced amino acid catabolism. Similarly, increased levels of glucokinase (Table 3) indicate enhanced capacity for hepatic glucose uptake and enhanced activity through anabolic pathways.

The p8 protein is a transcription factor that regulates the expression of genes involved in cell defense against adverse effects of stress [41]. Such stress-mediated survival mechanisms within the liver involve protein kinase C delta binding protein (DIG-1) and insulin-like growth factor-binding protein 1 (IGFBP-1) [42]. Both DIG-1 and IGFBP-1 mRNA were increased in our high-fat fed animals (Table 3). Based on microarray data IGFBP-1 mRNA levels were only increased in CBD-fed rats, but quantitative RT-PCR showed increased IGFBP-1 mRNA levels in both fat-fed groups (Fig. 2b). However, IGFBP-1 protein was increased in CBD-fed rats only (Fig. 3a).

As shown in Fig. 2b, the high-fat-mediated induction of FGF21 mRNA was confirmed using RT-PCR. Since FGF21-treatment in animals has been shown to increase fat utilization and to reduce hepatosteatosis [43], the rate of β-oxidation was compared between the groups. As shown in Fig. 4a, the SOD-fed rats had a 2.7-fold increase in fatty acid (FA) turnover rate. A trend ($p = 0.07$) towards increased β-oxidation was also observed in the CBD-fed rats.

Among the lipid-induced effects on gene products from metabolic pathways, the biggest changes were observed for SCD-1 and 2. The high-fat-mediated reduction of SCD-1 mRNAs was confirmed using RT-PCR (Fig. 2b) and reduced SCD-1 protein levels were demonstrated using immunoblotting (Fig. 3b). This is an interesting finding since SCD-1 deficiency leads to activation of lipid oxidation, reduced TG synthesis and storage within the liver [44]. The lowest levels of both mRNA and protein were observed in the SOD-fed rats. Increased AMP-activated protein kinase (AMPK) activity has been shown to mediate SCD-1 dependent effects on hepatic β-oxidation [45]. We analyzed the degree of AMPK activation, i.e. the degree of phosphorylation, but the higher rate of β-oxidation in the SOD group was not paralleled by increased AMPK phosphorylation (Fig. 4b).

Both PUFA and monounsaturated fatty acids (MUFA) activate farnesoid X receptor (FXR), and thereby decrease the mature form of sterol regulatory element binding

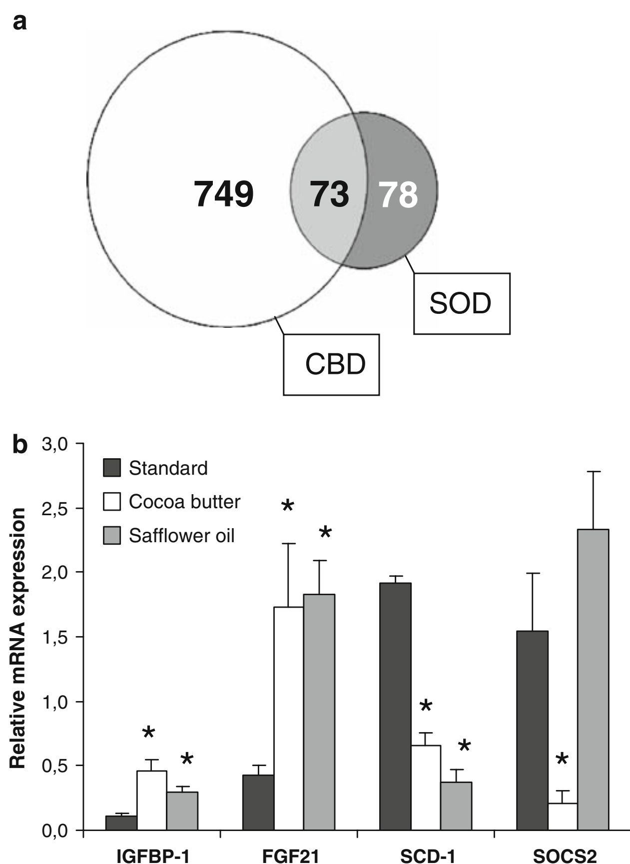

Fig. 2 Effects of short-term high-fat feeding on hepatic gene expression. **a** Overview of results obtained from microarray analysis comparing the effects of cocoa butter and safflower oil on hepatic gene expression. Whole-genome rat oligo microarrays were used to generate transcript profiles from rats fed a standard diet (StD) or either cocoa butter- (CBD) or safflower oil- (SOD) enriched diets ($n = 4$). StD-fed animals were used as control. A 5% false discovery rate was used and transcripts with a greater value than 1.5-fold increase or decrease (compared to StD) were considered differentially expressed. The total number of hepatic gene products affected by CBD (*no fill*), SOD (*black fill*), or both (*gray fill*) are indicated. **b** Effects of short-term high-fat feeding on selected hepatic transcript levels. Hepatic mRNA was isolated from rats fed standard or high-fat diets. Expression levels were quantified by real-time PCR and normalized to the housekeeping gene RPLP. Data are represented as means ± SE, and * indicates significant differences compared to standard diet ($n = 4$)

protein-1c (SREBP-1c), as well as the expression of its target lipogenic genes such as SCD-1 in liver [46]. We therefore compared the content of hepatic SREBP-1 between the three diet groups. Surprisingly, the mature form of SREBP-1 (68 kDa) was elevated at the expense of unprocessed SREBP-1 (125 kDa) in both CBD- and SOD-fed rats (Fig. 4c). This observation suggests that SCD-1 expression can be repressed upon high-fat feeding through a mechanism that does not depend on reduced SREBP-1 maturation.

Taken together, these results indicate that a lipid-induced program for hepatic lipid disposal and cell survival might be operating upon 3 days of high-fat feeding. The former might involve increased levels of FGF21 and reduced levels of SCD-1 and SCD-2, whereas the latter includes increased levels of p8, DIG-1 and IGFBP-1.

Cocoa Butter Diet-Mediated Changes in Gene Expression

As summarized in Table 3, cocoa butter had a greater impact on hepatic genes from all functional groups listed, as compared to safflower oil. Rats fed CBD had increased levels of gene products from anabolic pathways, including biosynthesis of amino acids and lipids with cholesterogenesis being represented by the largest number of transcripts (Table 3). Several genes from the glycolytic pathway and de novo lipid synthesis were increased about twofold, including transketolase, pyruvate kinase, malic enzyme 1, ATP citrate lyase and spot 14. ATP citrate lyase catalyzes the formation of acetyl-CoA and oxaloacetate, and acetyl-CoA serves several important biosynthetic pathways such as fatty acid, cholesterol and bile acid synthesis. However, the biggest changes in expression levels (more than fivefold) were observed for 3-phosphoglycerate dehydrogenase (3-PGDH), phosphoserine aminotransferase 1 (PSAT1), asparagine synthase and early growth response 1 (EGR1). Higher levels of 3-PGDH and PSAT1 indicate a greater capacity for serine synthesis and serine-dependent pathways, such as the biosynthesis of phosphatidylserine and glutathione.

The effect on EGR1 might be related to a CBD-specific effect on inflammatory mediators, including the observed increase in expression of macrophage migration inhibitory factor and interleukin 17 receptor B (Table 3). This is in line with previous reports on EGR1 as a master switch to trigger the expression of inflammatory genes involved in e.g. atherosclerosis [47]. Since EGR1-negative mice have been shown to exhibit decreased expression of hepatic IL-6 mRNA [48], this and other cytokines (IL-1β, IL-17, and TNFα) were measured in plasma from the different rats. However, only one animal (from the SOD group) had detectable levels of inflammatory cytokines (IL-6, 945 pg/ml; IL-1β, 168 pg/ml). We further measured the degree of activation (phosphorylation) of the transcription factor STAT3, which is known to be activated in response to IL-6 and other cytokines [49]. There was no indication of enhanced STAT3 signaling in these rats, as determined by immunoblotting (data not shown).

Within the group of transcripts for signaling molecules, suppressor of cytokine signaling 2 (SOCS2) showed the greatest response to CBD-feeding. Reduced expression of SOCS2 mRNA was confirmed using RT-PCR (Fig. 2b),

Table 3 High-fat diet-induced changes in hepatic gene expression

Accession no.	Gene name	Effect of diet (high-fat vs. standard)	
		Cocoa butter	Safflower oil
Signaling molecules			
Endocrine, paracrine and autocrine factors			
NM_013144	Insulin-like growth factor binding protein 1	4.61	
NM_130752	Fibroblast growth factor 21	3.30	3.91
NM_031051	Macrophage migration inhibitory factor	1.79	
XM_343169	Adipsin	1.67	
NM_199115	Angiopoietin-like protein 4	0.64	
NM_001004274	Insulin-like growth factor binding protein 4	0.63	
NM_053329	Insulin-like growth factor binding protein, acid labile subunit	0.58	
NM_012549	Endothelin 2	0.42	
NM_031351	Attractin	0.60	0.60
Membrane-bound receptors			
NM_053019	Arginine vasopressin receptor 1A	2.21	
XM_224604	Interleukin 17 receptor B	1.88	
NM_199114	Fibroblast growth factor receptor-like 1	1.67	
NM_013123	Interleukin 1 receptor, type I	0.63	
NM_013036	Somatostatin receptor 4	0.62	
NM_012704	Prostaglandin E receptor 3	0.61	
NM_017183	Interleukin 8 receptor, beta	0.60	
NM_012630	Prolactin receptor	0.50	
NM_017018	Histamine receptor H 1	0.47	
NM_012550	Endothelin receptor type A	0.44	
Intracellular signaling mediators			
NM_199405	Leucine carboxyl methyltransferase 1	2.52	1.74
NM_053857	Eukaryotic translation initiation factor 4E binding protein 1	2.43	2.31
NM_134449	PKC delta binding protein (DIG-1)	2.12	2.11
NM_013055	Mitogen activated protein kinase kinase kinase 12	0.51	
XM_343472	Cytokine inducible SH2-containing protein	0.46	
NM_058208	Suppressor of cytokine signaling 2	0.27	
Transcription factors			
NM_012551	Early growth response 1	6.76	
NM_053611	Nuclear protein 1 (p8)	4.26	3.40
NM_013149	Aryl hydrocarbon receptor	1.74	
XM_217192	RAR-related orphan receptor alpha	1.68	
NM_001100966	SREBP cleavage activating protein	1.54	
NM_031668	MYB binding protein (P160) 1a	1.53	
NM_175582	Inhibitor of DNA binding 4	1.52	
NM_013086	cAMP responsive element modulator	0.65	
NM_012805	Retinoid X receptor alpha	0.60	0.58
NM_012524	CCAAT/enhancer binding protein (C/EBP) alpha	0.57	
NM_012543	D site albumin promoter binding protein	0.54	
NM_138875	Jun D proto-oncogene	0.54	
NM_022671	One cut domain, family member 1 (HNF6 alpha)	0.35	
Cellular metabolism			
Amino acid turnover			
NM_031620	3-phosphoglycerate dehydrogenase	8.42	
NM_198738	Phosphoserine aminotransferase 1	6.21	
NM_013079	Asparagine synthetase	5.50	

Table 3 continued

Accession no.	Gene name	Effect of diet (high-fat vs. standard)	
		Cocoa butter	Safflower oil
NM_012571	Glutamate oxaloacetate transaminase 1	0.55	0.54
NM_017159	Histidine ammonia lyase	0.53	
NM_022619	Solute carrier family 7, member 2	0.46	0.52
NM_053962	Serine dehydratase	0.09	0.38
Carbohydrate turnover			
NM_012565	Glucokinase	4.00	2.44
NM_016987	ATP citrate lyase	2.22	
NM_022592	Transketolase	2.22	
NM_012600	Malic enzyme 1	1.81	
NM_022268	Liver glycogen phosphorylase	1.80	
NM_012624	Pyruvate kinase	1.59	
NM_031151	Malate dehydrogenase, mitochondrial	1.54	
NM_012621	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	1.53	
NM_022215	Glycerol-3-phosphate dehydrogenase 1	0.63	
Cholesterol and bile acid turnover			
NM_019238	Farnesyl diphosphate farnesyl transferase 1	2.69	
NM_031062	Mevalonate (diphospho) decarboxylase	2.12	
NM_053607	Cholate-CoA ligase	2.01	
NM_017268	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	1.89	
NM_017136	Squalene epoxidase	1.87	
NM_022389	7-dehydrocholesterol reductase	1.72	
NM_031049	Lanosterol synthase	1.72	
NM_031840	Farensyl diphosphate synthase	1.66	
NM_024143	Bile acid-CoA ligase	1.63	
NM_173307	ATP-binding cassette, subfamily A, member 5	0.46	
NM_053754	ATP-binding cassette, subfamily G, member 5		2.34
NM_012942	CYP7A1		3.64
Fatty acid turnover			
NM_001006995	Acetyl-CoA acetyltransferase 2	2.00	
NM_017075	Acetyl-CoA acetyltransferase 1	1.63	
NM_012703	Thyroid hormone responsive protein, SPOT14	1.59	
NM_053445	Fatty acid desaturase 1	1.71	
NM_031344	Fatty acid desaturase 2	1.57	
NM_173137	Fatty acid desaturase 3	1.69	
NM_053365	Fatty acid binding protein 4	0.64	
NM_138907	Mitochondrial acyl-CoA thioesterase 1	0.63	0.42
XM_234398	Peroxisomal acyl-CoA thioesterase 2B	0.63	
NM_012597	Lipase, hepatic	0.62	
NM_144750	Lysophospholipase	0.50	0.44
NM_173151	CTP:phosphocholine cytidylyltransferase	0.48	
NM_031841	Stearoyl-CoA desaturase 2	0.27	0.08
NM_139192	Stearoyl-CoA desaturase 1	0.22	0.10
Retinol metabolism			
NM_022407	Aldehyde dehydrogenase family 1, member A1	2.40	
NM_017158	CYP2C7	0.50	

Total hepatic RNA was extracted from rats fed different diets ($n = 4$) and the effect of cocoa butter or safflower oil on gene expression was studied using rat whole-genome oligo arrays. The data were obtained using SAM at a false discovery rate of 5% and are represented as mean fold changes. All data are available from the NCBI Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo/) using the series entry GSE13936

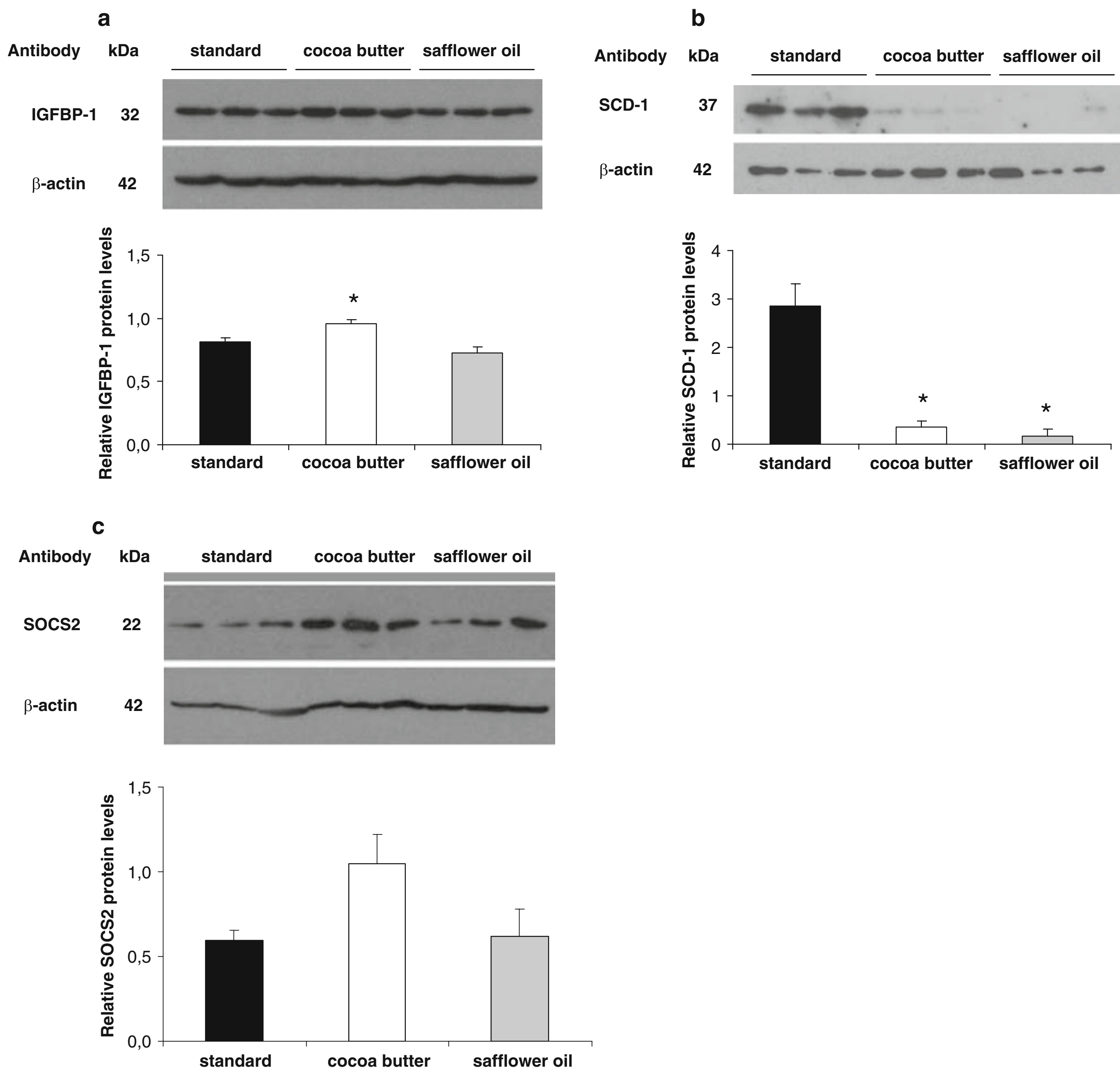

Fig. 3 Effects of short-term high-fat feeding on selected hepatic protein levels. Livers from rats fed standard or high-fat diets were used to extract whole-cell lysates. Protein levels of **a** IGFBP-1, **b** SCD-1 and **c** SOCS2 were analyzed by immunoblotting (*upper section*) and quantified by densitometry analysis in relation to β-actin (*lower section*). Data are represented as means ± SE, and * indicates significant differences compared to standard diet ($n = 3$)

but a trend ($p = 0{,}112$) towards increased SOCS2 protein levels were demonstrated using immunoblotting (Fig. 3c). SOCS2 is a negative regulator of growth hormone (GH)-mediated signaling and gene regulation, which includes down-regulation of its own GH-dependent expression [50, 51]. Reduced GH signaling, supposedly due to increased SOCS2 protein in the CBD group, might thus explain the reduced expression of SOCS2 mRNA as well as the observed effects on other GH-regulated transcripts in these animals (Table 3). Those include IGFBP1 [52], insulin-like growth factor binding protein acid labile subunit [53], prolactin receptor [54], CYP2C7 [55], SCD-1 [40, 56] and SCD-2 [51].

Taken together, the results described above suggest that rats fed a cocoa butter-enriched diet for 3 days might have a higher capacity to synthesize fatty acids, phospholipids, cholesterol and bile acids, as compared to rats fed safflower oil. Altered GH-signaling and expression of GH-regulated genes in the CBD-fed rats might lead to further perturbations in hepatic lipid metabolism.

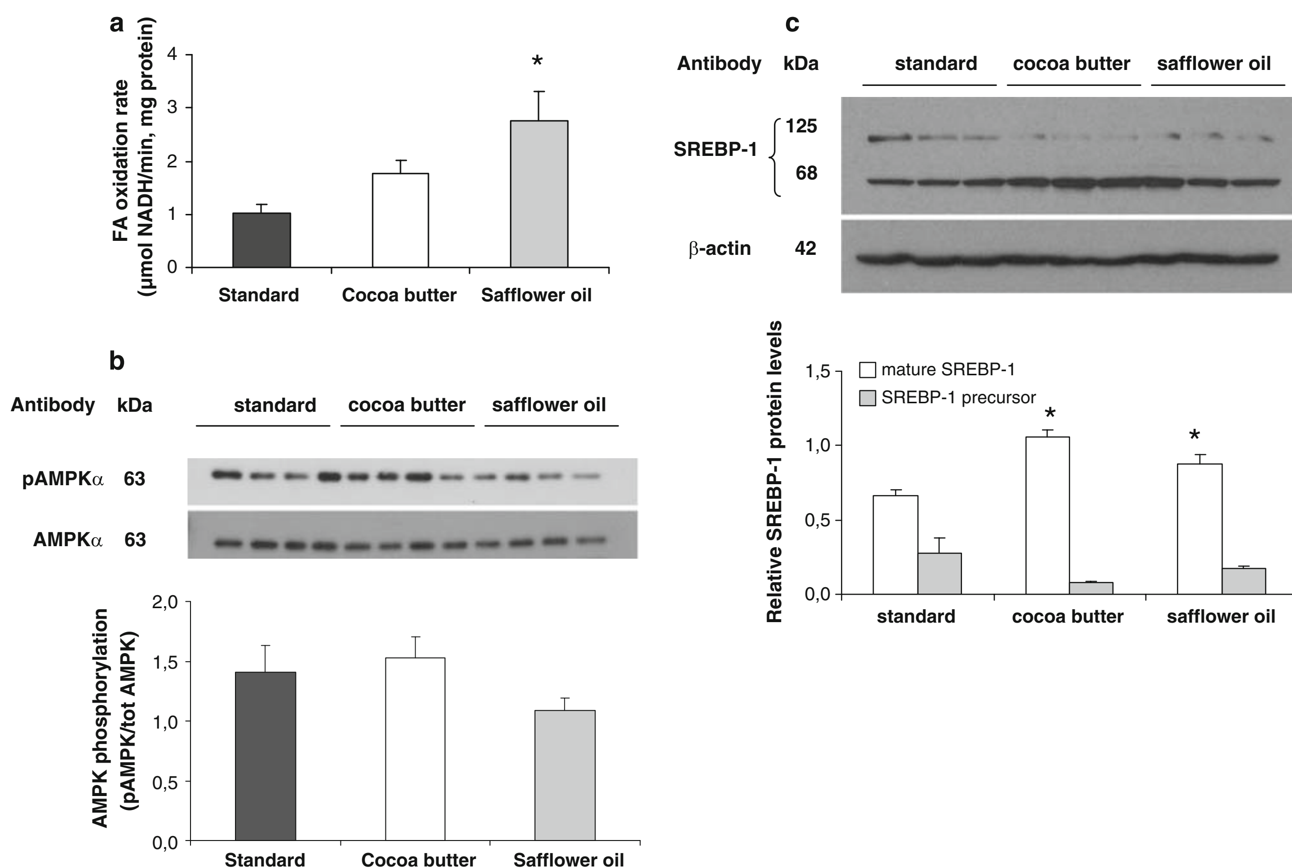

Fig. 4 Effects of short-term high-fat feeding on hepatic fatty acid oxidation rates and SREBP-1 protein levels. **a** Fatty acid oxidation rates were determined in liver homogenates ($n = 4$), whereas **b** AMPK phosphorylation ($n = 4$) and **c** SREBP-1 maturation ($n = 3$) were determined in whole-cell lysates by immunoblotting (*upper section*) and quantified by densitometry analysis in relation to total AMPK or β-actin (*lower section*). Data are represented as means ± SE, and * indicates significant differences compared to standard diet

High-Fat Diet-Mediated Effects on Hepatic Insulin Sensitivity

Finally, hepatic insulin sensitivity was compared between the three diet groups. The effect of insulin treatment (i.p. injection) was investigated using the degree of Akt-activation (phosphorylation at Ser473) as a measure of insulin receptor signaling [57]. All rats responded to insulin through increased Akt-phosphorylation, but the safflower oil-fed rats were only half as sensitive as the other animals (Fig. 5a). This indicates that, in spite of a similar degree of hepatic lipid content, hepatic insulin signaling was more efficient in the CBD-fed rats as compared to those fed with safflower oil. Cellular stress and free fatty acids (among other things) are known to induce insulin resistance through the activation of several serine/threonine kinases that phosphorylate and inactivate IRS1. In the liver, phosphorylation of IRS1 at Ser307 has been suggested to play a role in the development of high-fat feeding-induced insulin resistance [58]. To find an explanation for the lipid-dependent differences in Akt-phosphorylation we next compared the degree of IRS1-phosphorylation. There was no indication of enhanced phosphorylation of IRS1 at Ser307 in the SOD-fed rats, as determined by immunoblotting (data not shown).

One important function of hepatic insulin signaling is to suppress TG-rich VLDL particle production [24]. Comparing rats on StD with those on CBD, a similar reduction in both plasma TG levels (Fig. 5c) and TG-rich VLDL particles (Fig. 5d) were observed. The SOD-fed rats did not respond, which might reflect reduced hepatic insulin sensitivity or alternatively be due to the already low levels of TG-rich VLDL in these rats. All rats responded equally well to insulin at the level of blood glucose lowering, and their basal glucose levels were the same (Fig. 5b). Basal insulin levels were also similar between the groups (StD 1.57 ± 0.15 ng/ml; CBD 1.10 ± 0.07 ng/ml; SOD 1.62 ± 0.23 ng/ml).

Discussion

In the present study, short-term cocoa butter feeding in healthy rats was neutral regarding serum lipids and hepatic

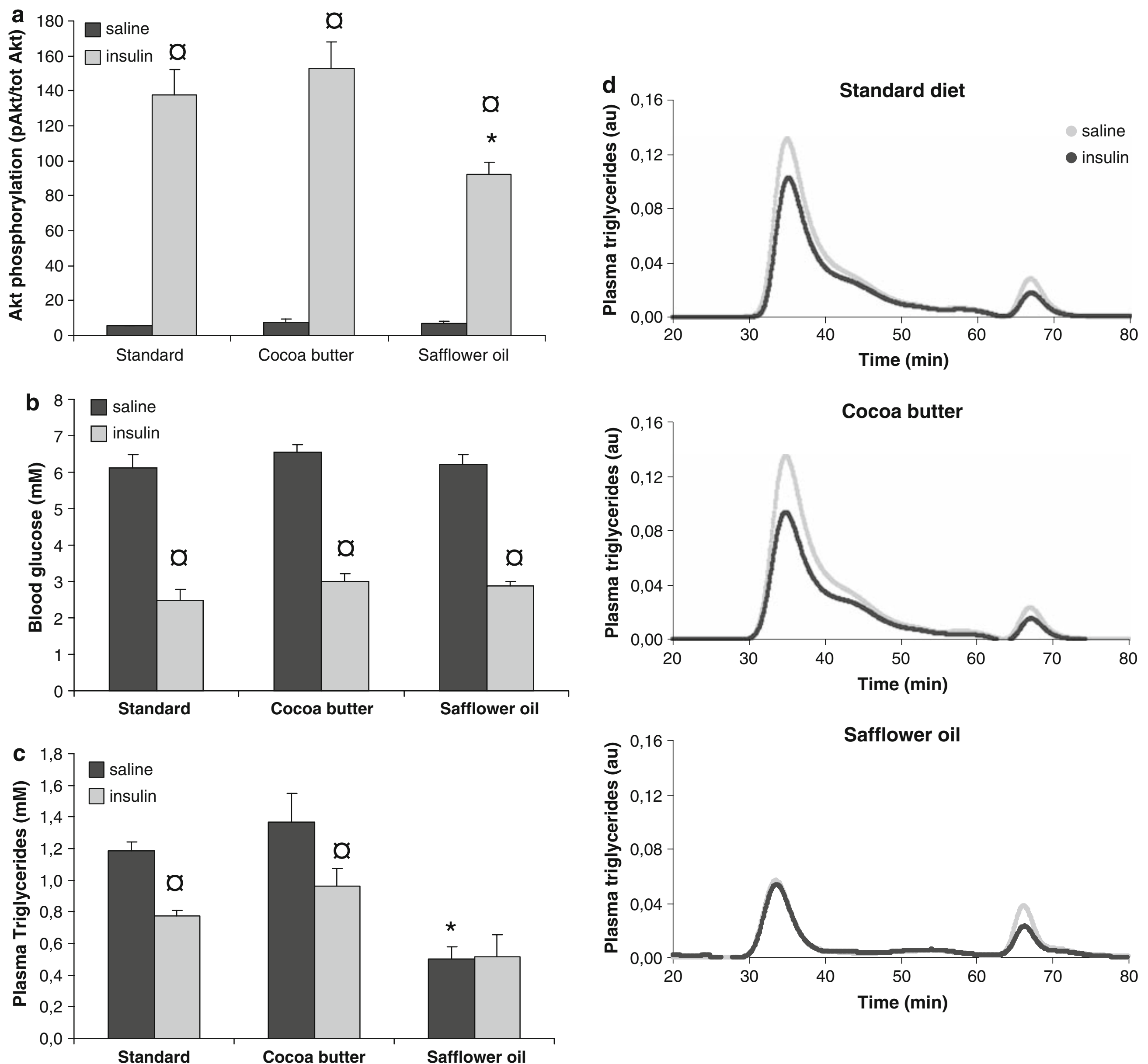

Fig. 5 Effects of short-term high-fat feeding on effects of insulin treatment. Rats fed standard or high-fat diets were subjected to 40 min of saline or insulin treatment. **a** The ratio between phosphorylated and unphosphorylated Akt was determined in whole liver cell lysates using ELISA. Levels of **b** glucose in blood and **c** TG in plasma were determined, and **d** plasma lipoprotein profiles were generated. Data are represented as means ± SE, * indicates significant differences compared to standard diet, and ¤ indicates significant differences compared to saline treatment ($n = 4$). The chromatograms represent the average from determinations performed in individual samples

insulin sensitivity, but changes in hepatic gene expression were induced that might lead to increased lipid synthesis, lipotoxicity, inflammation and insulin resistance if maintained. Safflower oil increased hepatic β-oxidation, was beneficial in terms of circulating TG-rich VLDL particles, but led to reduced hepatic insulin receptor signaling. The effects of safflower oil on hepatic gene expression were partly overlapping with those exerted by cocoa butter, but fewer transcripts from anabolic pathways were altered. Increased hepatic cholesterol levels and increased expression of hepatic CYP7A1 and ABCG5 mRNA, important gene products in bile acid production and cholesterol excretion, were specific effects elicited by safflower oil only. Common effects on gene expression included increased levels of p8, DIG-1, IGFBP-1 and FGF21, as well as reduced levels of SCD-1 and SCD-2. This indicates that a lipid-dependent program for hepatic lipid disposal and cell survival was induced by 3 days of high-fat

feeding, independently of the lipid source. The observation that cocoa butter exerted a similar degree of SCD-1 reduction as did safflower oil has to our knowledge not been described before. The mechanism behind this effect is as yet unknown, but data presented herein suggest that it is independent of reduced SREBP-1 maturation.

The diet-specific effects on plasma lipids are in agreement with previous studies [34], and might partly be explained by differences in hepatic FA oxidation rates. The level of TG in plasma is most often related to VLDL production in the liver, and this in turn is dependent on whether FA are being used for FA oxidation or re-esterification [27]. The enhanced FA oxidation rate in the SOD-fed rats indicates that both de novo FA synthesis [45] and lipoprotein formation was reduced [59], leading to lowered levels of plasma TG. Another explanation might be provided by diet-specific FA compositions in combination with reduced hepatic expression of SCD-1.

SCD-1 is a microsomal enzyme required for the biosynthesis of oleate and palmitoleate, which are the major MUFA of membrane phospholipids, TG, and cholesteryl esters (CE). SCD-1 gene expression is highly regulated by dietary factors; induced by carbohydrates or cholesterol but repressed by PUFA to maintain lipid homeostasis [60, 61]. Cholesterol has been shown to override PUFA-mediated repression of the SCD-1 gene [62], which might redistribute FA from FA oxidation to esterification, CE formation and VLDL production. SCD-1 deficiency has been linked to reduced plasma VLDL secretion [63], lending further support to the notion that oleoyl-CoA and palmitoleyl-CoA produced by SCD-1 are necessary to synthesize CE targeted to hepatic VLDL secretion. In the present study, low expression of SCD-1 might thus contribute to the reduced plasma levels of TG-rich VLDL particles observed in the SOD-fed rats. Cocoa butter has a higher content of MUFA compared to safflower oil (33 vs. 15%). Since MUFA (mainly oleate) are supplied to a greater extent by the CBD, reduced synthesis of oleoyl-CoA and palmitoleyl-CoA might not affect VLDL secretion to the same extent as in the SOD-fed rats. Supposedly, the higher rate of β-oxidation upon SOD-feeding might in part be a consequence of reduced VLDL secretion and re-esterification of FA into CE.

Hepatic cholesterol was increased, VLDL cholesterol increased and HDL cholesterol reduced after 3 days SOD-feeding, whereas there were no such changes due to CBD-feeding. Since reduced reverse cholesterol transport is well-known to promote atherosclerosis, this indicates a potential proatherogenic effect of the former dietary regimen. The SOD-mediated increase in hepatic cholesterol might lead to liver X receptor (LXR) stimulation and thus explain the increased expression of CYP7A1 [64] and the sterol transporter ABCG5 [65]. This effect of safflower oil on gene products involved in hepatic cholesterol turnover has to our knowledge not been described before but might be related to the reduced expression of SCD-1 and capacity to export CE in VLDL particles in the SOD-fed rats.

Although it is well accepted that inhibition of SCD-1 prevents many aspects of the metabolic syndrome (diet-induced obesity, hepatic steatosis, insulin resistance, hypertriglyceridemia), it was recently shown that low levels of SCD-1 might promote aortic atherosclerosis in a mouse model of hyperlipidemia and atherosclerosis [66]. It was suggested that inhibition of SCD-1 in the liver results in secretion of VLDL particles that are highly enriched in SFA-rich CE, giving rise to SFA-CE-rich LDL particles. Increased delivery of SFA to macrophages leads to accumulation of SFA, enhanced inflammatory cytokine secretion and a proinflammatory phenotype. In the present study, reduced expression of SCD-1, in the absence of sufficient levels of dietary MUFA (i.e. in the SOD group only), was paralleled by increased hepatic cholesterol, increased VLDL cholesterol and reduced HDL-cholesterol. Whether this effect would persist upon longer periods of high dietary intake of safflower oil is not known and should be addressed.

The finding that SOD exerted a greater effect on FA oxidation as compared to CBD is in accordance with previous studies and might be related to its higher content of PUFA, which are known to be potent activators of PPARα and modulators of lipid metabolism [67]. Upon PPARα activation, CPT-I and II, peroxisomal and mitochondrial β-oxidation enzymes, enzymes of ketogenesis, and omega-oxidation enzymes are induced and create an increased capacity for FA oxidation [59]. However, none of these gene products were induced in the SOD-fed rats in this study (see GEO; http://www.ncbi.nlm.nih.gov/geo/). Increased β-oxidation has also been shown to be an effect of SCD-1 deficiency [44], but the CBD-fed rats had almost equally reduced SCD-1 expression without the same increase in FA oxidation. Increased AMPK activity has been shown to mediate the SCD-1 dependent effects on hepatic β-oxidation [45]. This pathway involves AMPK-dependent repression of acetyl-CoA carboxylase (ACC), reduced ACC-mediated formation of malonoyl-CoA, and thereby de-repression of CPT-Ia enzymatic activity, leading to enhanced transfer of FA into the mitochondria [45, 68]. However, a higher rate of β-oxidation was not paralleled by an increased degree of AMPK phosphorylation in the SOD-fed rats.

The high-fat diet-mediated increase in FGF21 mRNA is interesting in this regard, since FGF21 has been shown to increase β-oxidation. FGF21, a hormone induced by fasting, is predominantly expressed in the liver and has beneficial effects on glucose homeostasis and insulin sensitivity both in rodents [69], monkeys [70] and man [71]. Furthermore, FGF21-treatment in animals was recently

shown to mediate increased energy expenditure, fat utilization and lipid excretion, to reduce hepatosteatosis and improve glycemia [43]. The mechanisms behind these effects were recently shown to include peroxisome proliferator-activated receptor gamma coactivator protein-1α [72]. Whether there is any link between increased FGF21 levels and reduced SCD-1 expression has to await further studies.

It has also been shown that FGF21 induces SOCS2, blocks GH-dependent STAT5b signaling and alters the expression of GH-regulated genes during starvation [73]. Among other GH-suppressed gene products [74, 75], hepatic IGFBP-1 expression was found to be induced by FGF21 [73]. Our finding that FGF21 was induced by high-fat feeding in parallel with increased IGFBP-1 and increased FA oxidation is in line with the reports cited above, and adds to the picture of FGF21 as a sensor of FA availability, during starvation as well as during hepatic fatty infiltration. In the present study, 3 days of cocoa butter feeding seemed to induce the level of hepatic SOCS2 protein and altered the expression of genes previously shown to be under the regulation of GH, such as IGFBP1, insulin-like growth factor binding protein acid labile subunit, prolactin receptor, CYP2C7, SCD-1, SCD-2 as well as SOCS2. A down-regulation of SOCS2 mRNA in the CBD-fed rats indicates that GH sensitivity might become normalized with time and, since GH has lipogenic effects in the liver [76–78], lead to enhanced fatty infiltration if the high-fat feeding is maintained.

Circulating fasting levels of IGFBP-1 were recently shown to reflect hepatic lipid content and insulin sensitivity in non-diabetic humans, with IGFBP-1 levels being positively correlated with hepatic insulin sensitivity and inversely related to liver fat content [79]. Furthermore, low circulating IGFBP-1 concentrations have been linked with increased risk of macrovascular disease in humans [80]. Although the cause and effect relationship has not been proven, increased hepatic lipid content might lead to elevated levels of hepatic and circulating IGFBP-1, beneficial for metabolic health. Furthermore, stress-mediated survival mechanisms within the liver of rodents have been shown to involve IGFBP-1 [42]. In this study, we have shown that the p8 protein, DIG-1 and IGFBP-1 were induced by short-term high-fat feeding. The p8 protein is a transcription factor that regulates the expression of genes involved in cell defense, whereas DIG-1 is a protein kinase C delta binding protein that increases the protein stability of p53. When the expression of p53 target genes such as IGFBP-1 is increased, a survival pathway is initiated, which is thought to give the cell an opportunity to repair low-level damage. Since high levels of lipids within the hepatocyte are toxic to the cell, this high-fat response might include ways to reduce the hepatic lipid content. Another interesting gene product of importance during situations of nutrient overload, and induced by high-fat feeding in the present study, is the eukaryotic translation initiation factor 4E-binding (eIF4E-binding) protein 1 (4E-BP1). The combined disruption of 4E-BP1 and 4E-BP2 in mice has been shown to increase their sensitivity to diet-induced obesity and insulin resistance [81]. It was suggested that 4E-BP1 and 2 play important roles as metabolic brakes in the development of obesity and type 2 diabetes. 4E-BP1 was induced to the same extent (more than twofold) in both CBD- and SOD-fed rats (Table 3).

The notion that SOD-feeding reduces hepatic insulin sensitivity has been described before [28, 29]. Our finding that 3 days on SOD reduces insulin-mediated phosphorylation of Akt in the absence of any detectable change in p-IRS1-Ser307 verifies the results presented by Samuel et al. [29]. In the same study, reduced activation of Akt was paralleled by blunted insulin-mediated suppression of hepatic glucose output (HGO). This important measure of hepatic insulin sensitivity was not investigated in the present study, and it can not be ruled out that cocoa butter feeding would lead to the same detrimental effect. The possibility that short-term HGO regulation is independent of Akt-phosphorylation should be investigated, but it would also be interesting to compare the impact of different high-fat feeding protocols on other hepatic effects of insulin, such as lipid and glycogen synthesis. Furthermore, there are Akt-independent signaling pathways that might have been differently affected by the high-fat diets. Further studies are required to address this and to find the SOD-mediated mechanism that blocks insulin receptor signaling up-stream of Akt. Specifically, the hypothesis that a greater FA oxidation rate, as a consequence of high dietary intake of safflower oil or similar fat sources, might lead to enhanced oxidative stress and insulin resistance should be tested.

As indicated above, the difference in insulin sensitivity at the level of Akt and GSK3β between CBD- and SOD-fed rats does not exclude that hepatic insulin resistance might develop upon excess intake of cocoa butter. On the contrary, our transcript profiles could be interpreted as an increased capacity for lipid synthesis within the liver of CBD-fed rats, which might increase the risk of developing steatosis and insulin resistance. This is in contrast to the effects of cocoa ingestion, which was reported to suppress the expression of genes for enzymes involved in fatty acid synthesis in rat liver [22]. This beneficial effect of cocoa might therefore depend on lipophobic molecules absent in the butter fraction of cocoa.

Taken together, we have described short-term effects of high-fat feeding on hepatic gene expression in relation to lipid profiles and insulin signaling. Three days of a high dietary intake of cocoa butter led to changes within the

liver that might be part of a defense program triggered by the increase in hepatic TG. This included reduced expression of SCD-1, which could result in reduced synthesis of oleoyl-CoA and palmitoleyl-CoA of importance for VLDL secretion. However, the high content of oleate in cocoa butter (i.e. provided by the diet) might explain why there were no changes in plasma lipoproteins or hepatic cholesterol levels, in spite of reduced SCD-1. Thus, 3 days on this diet might not be a metabolic problem, but since there were other changes in transcript levels indicative of increased activity through anabolic pathways, it might be speculated that longer treatment duration would lead to steatosis and insulin resistance. In comparison, 3 days on a safflower oil-enriched diet resulted in similar defense-related changes, but this was paralleled by reduced TG-rich VLDL and HDL cholesterol in plasma, increased hepatic cholesterol, increased FA oxidation and reduced insulin signaling. The low expression of SCD-1 in the absence of sufficient MUFA supplied by the diet might result in a reduced ability to produce CE and VLDL, leading to reduced TG-rich VLDL in the circulation, increased cholesterol and fatty acids in the liver, and as a consequence of the latter, increased FA oxidation, ROS formation and insulin resistance. In summary, we speculate that increased hepatic TG leads to reduced expression of SCD-1, which might mediate either neutral, beneficial or unfavorable effects on hepatic metabolism upon high-fat feeding, depending on which fatty acids were provided by the diet.

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