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Cafeteria Diet-induced Obesity Causes Oxidative Damage in White Adipose

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Abstract

Obesity continues to be one of the most prominent public health dilemmas in the world. The complex interaction among the varied causes of obesity makes it a particularly challenging problem to address. While typical high-fat purified diets successfully induce weight gain in rodents, we have described a more robust model of diet-induced obesity based on feeding rats a diet consisting of highly palatable, energy-dense human junk foods – the “cafeteria” diet (CAF, 45-53% kcal from fat). We previously reported that CAF-fed rats became hyperphagic, gained more weight, and developed more severe hyperinsulinemia, hyperglycemia, and glucose intolerance compared to the lard-based 45% kcal from fat high fat diet–fed group. In addition, the CAF diet-fed group displayed a higher degree of inflammation in adipose and liver, mitochondrial dysfunction, and an increased concentration of lipid-derived, pro-inflammatory mediators. Building upon our previous findings, we aimed to determine mechanisms that underlie physiologic findings in the CAF diet. We investigated the effect of CAF diet-induced obesity on adipose tissue specifically using expression arrays and immunohistochemistry. Genomic evidence indicated the

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CAF diet induced alterations in the white adipose gene transcriptome, with notable suppression of glutathione-related genes and pathways involved in mitigating oxidative stress. Immunohistochemical analysis indicated a doubling in adipose lipid peroxidation marker 4-HNE levels compared to rats that remained lean on control standard chow diet. Our data indicates that the CAF diet drives an increase in oxidative damage in white adipose tissue that may affect tissue homeostasis. Oxidative stress drives activation of inflammatory kinases that can perturb insulin signaling leading to glucose intolerance and diabetes.

Keywords

obesity; inflammation; oxidative stress; genomics; microarray; 4-HNE

INTRODUCTION

Obesity poses an alarming global public health concern [1,2]. Despite efforts aimed at ameliorating obesity, recent projections have estimated that 51% of the United States population will be obese by 2030 [3]. While typical high-fat purified pellet diets are successful in promoting obesity in mice and rats, we have described a diet-induced obesity model that incorporates a variety of highly palatable, energy-dense foods regularly consumed by humans – the “cafeteria” style (CAF) diet [4,5] that recapitulates obesity-like findings in humans. The CAF diet included a smorgasbord style offering of standard chow pellets, plus 3 human food choices offered daily. We previously reported that rats who ate the CAF diet developed hyperphagia and displayed significantly increased weight gain compared to other diet groups, including a commonly used lard-based high fat diet, consisting of 45% kilocalories-derived from fat and added sucrose. CAF-fed rats developed severe hyperinsulinemia, hyperglycemia, and glucose intolerance. Using a combination of metabolomic strategies and histological tissue analysis, we previously observed a higher degree of inflammation in white and brown adipose and liver compared to rats fed a traditional, purified high-fat diet, low-fat diet, and SC-fed rats [5]. In addition, concentrations of mitochondrial-derived lipid mediators that promote obesity-associated inflammation were found to be significantly elevated in CAF fed rats [4,5]. Metabolomic and immunohistologic measures suggested dramatic adipose tissue dysfunction however, underlying mechanisms remained unclear. Thus, we sought to investigate mechanistic underpinnings of CAF diet-induced inflammation and adipose dysfunction using global gene expression profiling to identify relevant genes and pathways altered with ingestion of the CAF diet. We now report that CAF diet-induced obesity resulted in significant alterations in white adipose tissue gene expression profiles that were associated with blunted protection from oxidative stress and an increase in oxidative damage. Thus, oxidative damage is one CAF diet-mediated pathway that could be responsible for greater adipose inflammation and systemic metabolic dysfunction in this model of diet induced obesity.

MATERIALS AND METHODS

Animals and diet treatments

All procedures were performed with the approval of the Duke University Institutional Animal Care and Use Committee. Male Wistar rats (~200g, 7-8 weeks old), purchased from Harlan Laboratories (Dublin, VA), were housed 2 rats/cage in the Duke University animal housing facility. Rats were maintained on a 12 hour light/dark cycle and given *ad libitum* access to standard chow (SC, Harlan Teklan 7001, Dublin, VA) for 2 weeks before being randomized onto experimental diets. At 9-10 weeks of age (~300g body weight, N = 5 for CAF and N = 4 for SC), rats were either maintained on the SC diet, or were switched to a CAF diet consisting of human snack food provided along with the SC pellet diet (Table 1). For a detailed description of the CAF diet components, refer to Sampey et al [5]. Briefly, the CAF diet included human food purchased at a supermarket and was provided in excess, including cookies, cereals, cheese, processed meats, crackers, etc. [5]. To estimate total gram and overall caloric intake of the CAF diet, items were weighed prior to and after consumption, and corrected for drying. The snack food items varied daily according to the fat, protein, and carbohydrate content as listed in Supplementary Table S2 of Sampey et al. [5]. Fat intake was the most drastically altered macronutrient with an estimated intake of 55% kcal from fat per day in CAF-fed rats. In addition, kilocalories from simple carbohydrate consumption was increased 500% (from 36 kcal to 180 kcal) in the CAF-fed group compared to the SC-fed groups. There were no added sugars to the SC diet; any sugars present were derived from whole-grains (corn, oats, etc.). Simple sugars were <5% of total carbohydrates in SC7001 (see Supplementary Table S3 from [5]). After 15 weeks on diet, rats were fasted for 6 hours and epididymal white adipose tissue (WAT) was collected. A portion of the WAT was isolated for mRNA isolation and another portion was fixed and paraffin-embedded for IHC.

RNA isolation

QIAzol Lysis Reagent was used to isolate mRNA from 100mg WAT samples (Qiagen, Valencia, CA, USA and [5,6]). mRNA quantity and quality were analyzed by Nanodrop (Thermoscientific, Wilmington, DE) and Bioanalyzer 2100 (Agilent, Wilmington, DE), respectively. For microarray analyses, cDNA was synthesized at the Functional Genomics Core at UNC-Chapel Hill. N = 5 for CAF diet group and 4 for SC diet group.

Gene expression microarrays

Gene expression quantification was conducted at the Functional Genomics Core Facility at UNC-Chapel Hill using genome-wide transcript microarrays (Affymetrix Rat Gene 1.0 ST, Santa Clara, CA). Microarrays where subjected to quality assessment and processed by robust multi-array average to produce transcript-level expression estimates using the aroma.affymetrix R package [7]. Transcripts were annotated with rat gene symbols using Affymetrix's annotation (RaGene-1_0-st-v1.na28.rn4.transcript.csv). Expression values were then log2 transformed. See Supplemental table ("S") Tables 1 and 2 for the complete gene expression data sets. Full gene names are in S Table 3.

4-hydroxynonenal immunohistochemistry

Sections of WAT from rats fed SC or CAF diets were stained for 4-hydroxynonenal (4-HNE), a marker of cellular lipid peroxidation [8]. 5μm sections were stained using an anti-4-HNE primary antibody (1:800 dilution in Dako antibody diluent, Abcam, Cambridge, MA) and biotinylated goat anti-mouse secondary antibody (1:500 dilution in Dako antibody diluent, Jackson ImmunonResearch, West Grove, PA). Positive 4-HNE staining was visualized using a Vectastain© Elite ABC peroxidase system (1:50 dilution, Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine (DAB, Thermo Fisher Scientific, Pittsburgh, PA) development. N=3/group. Digital images of stained sections were generated by scanning slides using an Aperio Image Scope Digital Slide Scanner (Vista, CA). Total positive DAB staining was quantified in 5 random microscopic fields using Aperio ImageScope Software (Vista, CA and [6,9]).

Statistical methods

Statistical analysis of microarrays (SAM) analysis was used to identify genes that were significantly different between CAF and SC diet groups with a false discovery rate (FDR) of < 0.05 [10]. Pathway enrichment and functional clusters of gene ontology were identified from differentially expressed genes between CAF and SC using DAVID [11]. DAVID functional pathway analysis was used to identify the genes whose expression changes were responsible for defining whether a pathway was modulated in the CAF diet group. For 4-HNE staining, unpaired Student's *t*-test was used using GraphPad Prism 6 (La Jolla, CA). A p-value < 0.05 was considered significant.

RESULTS & DISCUSSION

Genomic profiling of WAT revealed that CAF diet-fed rats displayed blunted anti-oxidant capacity and reduced anti-inflammatory mediators compared to control SC-fed rats

Obesity is recognized as a state of low-grade, chronic inflammation which may be perpetuated, in part, by increased oxidative stress [12,13]. The most striking finding through expression analysis was significantly blunted anti-oxidant pathways in CAF-fed WAT compared to SC-fed controls. Gene expression analysis of WAT mRNA from rats fed either SC or CAF revealed that expression of 377 genes were significantly elevated and 392 genes were significantly decreased in the CAF diet group compared to the SC group (S Table 1 and S Table 2, respectively; full gene names can be found in S Table 3). A subset of genes are up- and down- regulated genes are highlighted in Table 2 and Table 3, respectively. The 34 most regulated genes are highlighted in Figure 1A. DAVID gene ontology (GO) analysis of differentially expressed genes between CAF diet and SC diet-fed groups revealed several enriched functional clusters of GO pathways of importance to the Metabolic Syndrome and inflammatory state of the WAT tissue. A total of 12 GO pathways were up-regulated in the CAF diet rats compared to the SC control group (FDR ≤ 0.25) (Figure 1B). Seven functional pathways were down-regulated in the CAF-fed WAT tissue compared to SC controls (FDR ≤ 0.25) including Drug metabolism, Glutathione metabolism, and Metabolism of xenobiotics by cytochrome P450, mainly driven by decreased expression of glutathione S-transferases (Figure 1C).

The profound loss of multiple glutathione-S transferase isoforms in WAT from rats fed a CAF style diet suggested that this rapid model of obesity promoted severe oxidative stress. Glutathione (GSH), the major anti-oxidant produced by cells [14], is a free radical scavenger that plays a vital role in protecting cells from oxidative damage. Glutathione S-transferases catalyze the addition of GSH to molecules – for example, peroxidized lipids and xenobiotics -- for the purpose of making the molecule water-soluble and, thus, able to be excreted [15]. Suppressed GST family gene expression, like that observed in the CAF-fed group, leads to down-regulation of multiple functional pathways, namely drug metabolism and metabolism of xenobiotics by cytochrome P450, as determined by DAVID bioinformatics analysis.

Furthermore, expression profiling and DAVID pathway analysis demonstrated increased expression of fatty acid binding proteins and a reduction in key anti-inflammatory genes. CAF-diet increased expression of several genes related to lipid metabolism, including *Elvol6*, fatty acid binding protein 3 and fatty acid binding protein 5. *Elvol6* is a microsomal enzyme that catalyzes the elongation of C12, 14, and 16 saturated and monounsaturated fatty acids, and in this way, can modulate intracellular fatty acid composition [16]. Elevated *Elvol6* may lead to accumulation of stearate (18:0), a pro-inflammatory saturated fatty acid [17] and blocked insulin-stimulated activation of AKT, thus impairing insulin signaling [18]. *Elvol6* can also induce endoplasmic reticulum stress and apoptosis [19]. Fatty acid binding proteins are intracellular lipid chaperones that play central roles in fatty acid transport, trafficking, and export [20,21,22]. *Fabp3* (H-FABP) and *Fabp5* (E-FABP) regulate glucose uptake or non-alcoholic fatty liver disease, respectively [23,24]. In addition, *Fabp5* expression increases with reactive oxygen species production and oxidative stress [25,26]. Thus, CAF-diet upregulated important mediators of lipid biology that lead to altered metabolism and insulin resistance. In addition, CAF-induced obesity also significantly decreased expression of *anti-inflammatory* cytokines *Il-33* and *cd22* in WAT. IL-33 induces helper T cells (Th2), mast cells, eosinophils, and basophils to produce type 2 cytokines and is negatively associated with BMI and diabetes [27,28]. Type 2 cytokines promote alternative activation of macrophages which is central to maintenance of insulin sensitivity [29,30]. CD22 is a lectin that blunts the immune response in B cells [31]. Together, downregulation of these cytokines may lead to a lack of control of inflammation, leading to chronic inflammation associated with obesity.

Lipid peroxidation was significantly increased in WAT from CAF-fed rats compared to SC-fed controls potentially due to reduced 4-HNE excretion secondary to CAF down-regulation of glutathione S-transferase alpha 4 (*Gsta4*)

To test for evidence of oxidative stress based on genomic findings, 4-hydroxynonenal (4-HNE) was used as a marker to detect oxidative damage in the WAT tissue. 4-HNE is an α,β-unsaturated hydroxyalkenal which is produced during the process of lipid peroxidation; therefore, 4-HNE is a specific marker of polyunsaturated omega-6 lipid (e.g. linoleic and arachidonic acids) damage with oxidative stress [32]. IHC staining for 4-HNE, a marker of cellular oxidative stress, demonstrated that WAT from CAF-fed rats had more than double 4-HNE staining than the SC controls (Figure 2 A-C, $p = 0.03$). Curtis et al. reported that increased 4-HNE resulted in impaired adipocyte mitochondrial function and increased superoxide production [33]. Certain glutathione S-transferases are responsible for mitigating

and maintaining 4-HNE levels within cells. Herein, we provide evidence that CAF-diet exposure induced a striking decrease in glutathione metabolism-related genes (Table 3 and S Table 2). Notably, glutathione S-transferase alpha 4 (*Gsta4*) was decreased by CAF-diet compared to SC-fed WAT. *Gsta4* catalyzes the addition of a glutathione molecule onto 4-HNE to facilitate its excretion. Importantly, *Gsta4* is the glutathione S-transferase with the highest affinity for 4-HNE, and therefore, the glutathione S-transferase primarily responsible for metabolizing 4-HNE to reduce oxidative stress burden [34,35,36]. Down-regulation of *Gsta4* resulted in increased mitochondrial reactive oxygen species production, as well as increased carbonylation of metabolism-associated proteins including fatty acid binding proteins [37,38]. Taken together, CAF-diet down-regulated *Gsta4* expression may have elevated levels of 4-HNE, as detected in the WAT. Similar to our findings, other gene expression analyses of white adipose from diet-induced obesity models, including one utilizing a slightly different CAF style diet, have reported decreased expression of glutathione-associated genes with obesity [39,40], lending support to the relevance of this antioxidant pathway to obesity-induced defects.

A limitation to using the CAF diet is that nutritional information may only be obtained from what is provided on packaging of the items that were purchased. Since the CAF diet is not a defined, purified diet, there are many unknowns that may contribute to the oxidative stress detected in adipose tissue. Despite these limitations, studies in humans parallel our results. Indeed, in obese adults and children, elevated markers of oxidative stress and suppressed glutathione metabolites correlated with dysregulated adipokine levels, which contribute to the development of Metabolic Syndrome [41,42,43]. Furthermore, elevated 4-HNE has been reported in obese humans [41,44,45]. Importantly obesity-induced oxidative damage may be reversible: reducing inflammation through anti-inflammatory supplementation or weight loss reduced the expression of oxidative damage-promoting genes and oxidative damage [46,47]

In conclusion, feeding rodents a cafeteria-style diet consisting of highly palatable, energy dense human junk foods resulted in significant adipose oxidative damage potentially due to a down-regulation of glutathione metabolic pathways. Our results corroborate observations of enhanced oxidative burden in adipose tissue of obese humans therefore demonstrating that the cafeteria-style model of diet-induced obesity is a relevant model of human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CAF cafeteria diet

FDR	false discovery rate
SAM	significance analysis of microarrays
SC	standard control diet
WAT	white adipose tissue

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Highlights

- Cafeteria diet is a robust model of human obesity.
- Cafeteria diet down-regulated genes involved in glutathione metabolism.
- Elevated oxidative damage in white adipose tissue was detected.

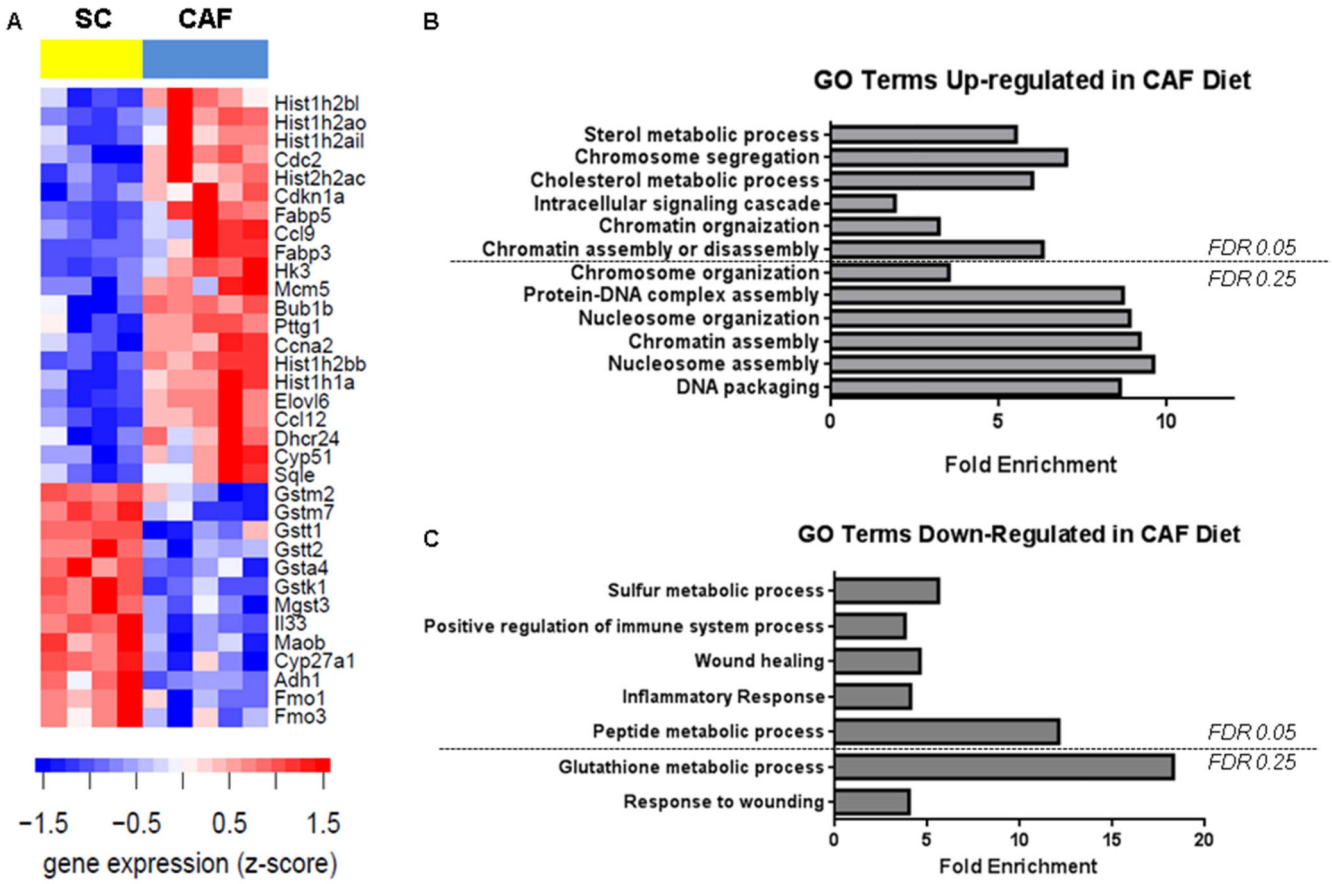

Figure 1. A. Gene expression and ontology analysis revealed CAF-diet downregulation of genes limiting oxidative stress and inflammation in WAT

A. Gene expression is displayed as a heat map in which columns are samples, rows are genes and shading indicates expression level according to the legend. Gene expression values were transformed to z-scores. Representative genes were selected from the significantly differentially gene lists (Table S1 and S2). DAVID Gene Ontology analysis of genes differentially expressed between CAF versus SC was used to identify functional clusters of gene ontology (GO) categories significantly over-represented and upregulated (**B**) or downregulated (**C**) in CAF-fed adipose versus SC-fed; an False discovery rate (FDR) cutoff of either 0.05 or 0.25 (as indicated) was used. Data are presented as the fold enrichment for each ontology group. Data presented are the comparison of changes in gene expression in CAF group with respect to the SC group.

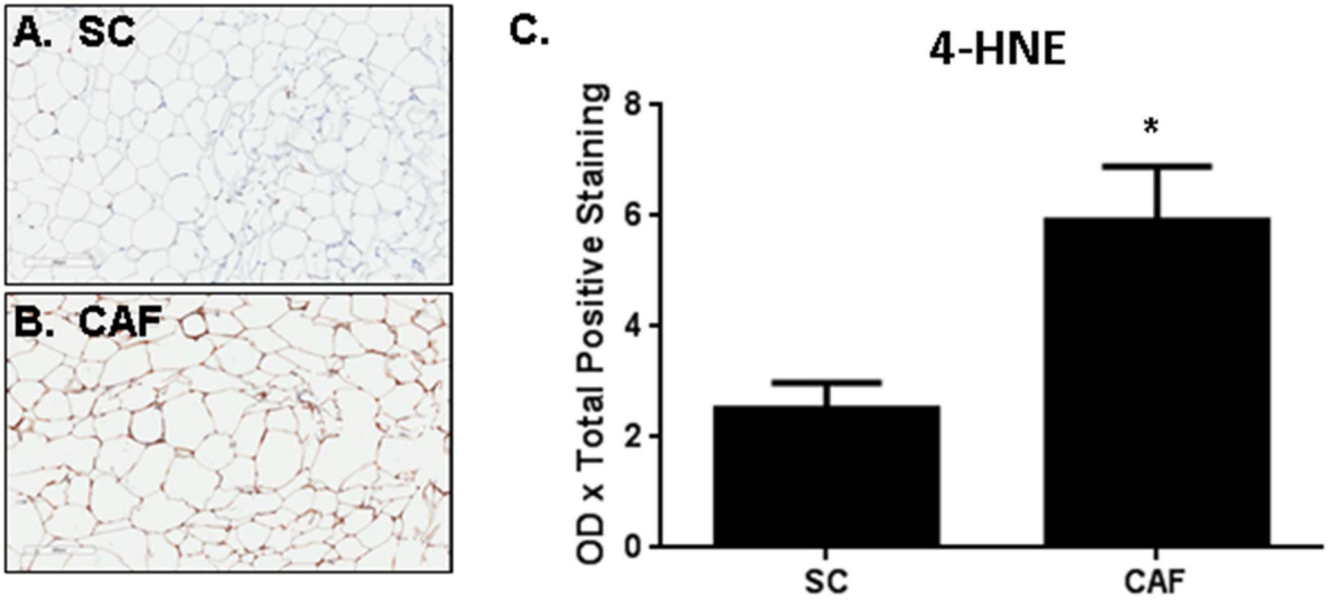

Figure 2. Oxidative stress is increased in WAT from CAF diet-exposed rats

Anti-4-HNE IHC was performed in WAT collected from rats fed (**A**) SC or (**B**) CAF diet for 15 weeks. Representative 20× images are shown. **C**. Quantification of mean optical density x total positive staining ± SEM. N = 3. *p-value = 0.03.

Table 1
Composition of standard chow (SC) and cafeteria (CAF) diets

	SC7001	Cafeteria
Name	Standard chow (SC)	Cafeteria (CAF)
Manufacturer	Harlan Teklad	Misc
Catalog number	SC7001	3 items + SC7001
FAT		
kcal/gm	3.83	varies daily
% fat/weight	4%	
% fat/kcal	12%	45-53%
Fat sources:	Porcine fat Linoleic acid (1%/wt)	See Table 2 of ref. 3
PROTEIN		
% protein /wt	25%	~20%
% prot/kcal	34%	
Protein sources:	Dehulled soybean meal, porcine meat, dehydrated alfalfa meal, dried whey, casein, purified amino acids	See Table 2 of ref. 3
CARBOHYDRATE		
%carb/wt	66%	~35%
% carb/kcal	54%	
Carbohydrate sources	corn, wheat, barley, oats	See Table 2 of ref. 3

Table 2
Selected adipose genes significantly up-regulated with CAF diet exposure

Selected genes of interest with increased expression in WAT from rats fed CAF diet are listed. An FDR < 0.05 (q value ≤ 5), determined by SAM analysis, was considered significant. See Supplemental table S Table 1 for a complete list of gene expression data.

Gene Name	Gene Symbol	q-value
ELOVL family member 6, elongation of long chain fatty acids	*Elovl6*	0.0
Fatty acid binding protein 3	*Fabp3*	1.5
Fatty acid binding protein 5	*Fabp5*	1.5
Hexokinase 3 (white cell)	*Hk3*	1.5
Chemokine (C-C-motif) Ligand 9	*Ccl9*	2.3
Lipin 2	*Lpin2*	2.5
Chemokine (C-C-motif) Ligand 12	*Ccl12*	3.7
24-dehydrocholesterol reductase	*Dhcr24*	3.8
Acyl-coenzyme A dehydrogenase, long chain	*Acadl*	3.8
Cytochrome P450-51	*Cyp51*	4.1
MAPK-KK 11	*Mapk11*	4.1
Adipose differentiation-related protein	*Adfp*	4.7
Acyl-coenzyme A dehydrogenase, C-2 to C-3 short chain	*Acads*	4.7

Table 3
Selected adipose gene significantly down-regulated with CAF diet exposure

Selected genes of interest with decreased expression in WAT from rats fed CAF diet are listed. An FDR < 0.05 (q value ≤ 5), determined by SAM analysis, was considered significant. See Supplemental table S Table 2 for a complete list of gene expression data.

Gene Name	Gene Symbol	q-value
Glutathione S-transferase, theta 3	*Gstt3*	0.0
Interleukin 33	*Il33*	0.0
Glutathione S-transferase, mu 7	*Gstm7*	0.9
Glutathione S-transferase, kappa 1	*Gstk1*	0.9
Glutathione S-transferase, a4	*Gsta4*	0.9
Glutathione S-transferase, theta 1	*Gstt1*	1.7
Glutathione S-transferase, mu 2	*Gstm2*	2.5
Glutathione S-transferase, theta 2	*Gstt2*	3.4
Cytochrome P450, family 27, subfamily a, polypeptide 1	*Cyp27a1*	3.7
Glutathione S-transferase, mu 6-like	*Gstm6l*	4.1
CD22 molecule	*Cd22*	4.7