Hepatic oleate regulates liver stress response partially through PGC-1α during high-carbohydrate feeding

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Supplementary materials and methods

Animal and diets

Mice were weaned at 21 days of age with free access to water and standard chow diet (Purina #5008) and were housed and bred in a pathogen-free barrier facility that operated with a 12h light/12h dark cycle. All mice were euthanized by isoflurane anesthesia overdose. Blood was collected by cardiac puncture, tissues rapidly removed, snap-frozen in liquid nitrogen, and stored at –80°C. All *in vivo* experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

To induce lipogenic diet-induced hepatic stress, mice were fed the HSVLF diet (2.6% fat, 76.7% carbohydrate, 20.7% protein, 3.53 kcal/g; TD.03045; Harlan Teklad, Inc.) or high carbohydrate (HC) diet (11.8% fat, 70.4% carbohydrate, 17.8% protein, 4.0 kcal/g; TD.98090; Harlan Teklad, Inc.) at 10-12 weeks of age. For experiments of dietary 18:1n-9 and 18:0, HSVLF was supplemented with 20% (w/w) oleate (as triolein) or stearate (as tristearin) (Sigma Aldrich). For the male and female SCD1 LKO, DLKO studies, as well as the male wildtype (WT), GKO, GLS5 and GLS3 studies, mice were 4hr fasted prior to euthanization. Female WT, GKO, GLS5 and GLS3 mice were nonfasted at euthanization.

Fatty acid analyses

Tissue and plasma lipids were extracted following a modified Folch method (1). Lipid fractions including triglyceride (TG), cholesterol ester (CE), phospholipid (PL) and free fatty acid (FFA) were separated by thin layer chromatography (TLC) with heptane/isopropyl ether/acetic acid (60/40/3) (2). The fatty acid composition of each lipid

fraction was analyzed using gas liquid chromatography (GLC) as described (2). For mitochondrial phosphatidylcholine and phosphatidylethanolamine fractions, lipids were separated by TLC, the PL band re-extracted and subjected to a second TLC to separate the individual PL species (3). For liver microsomal phospholipids, microsomes were prepared as previously described (2). Microsomal lipids were extracted, separated by TLC, and phospholipids were then separated using a $CHCl_3$/MeOH/acetic acid (60/30/5) solvent system. Phosphatidylcholine and phosphatidylethanolamine fractions were extracted and transmethylated using boron trifluoride in 14% methanol. Heptadecanoic acid internal standards were added to samples to quantify fatty acids from each lipid fraction.

Insulin tolerance tests, blood glucose and plasma ALT activity

Nonfasted mice were dosed with 0.75 U/kg body weight of human insulin (Novo Nordisk) via intraperitoneal injection. Blood was collected at 0, 15, 30, 45 and 60 minutes post-injection. Blood glucose was measured using a glucose oxidase and peroxidase enzymatic method. Plasma ALT activity was measured using the 5,1 FS Vitros Chemistry System (Ortho Clinical Diagnostics).

RNA and Real-time PCR

To perform real-time PCR, total RNA was isolated from snap-frozen tissue using Tri-Reagent (Molecular Research Center, Inc), followed by DNase I (Ambion) digestion. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). The relative mRNA levels were analyzed by Power SYBR green master mix (Applied Biosystems) and real-time PCR using ABI Applied Biosystems 7500 Fast Real-

time PCR System and normalized to 18s ribosomal RNA. Primer sequences are available upon request.

Histology

Liver and epididymal white adipose tissue were collected and fixed in 10% buffered formalin. Tissues were then paraffin-embedded, sectioned and stained with hematoxylin and eosin to visualize cell morphology. Sections were imaged with a Nikon Eclipse E800 microscope (Nikon) with 200X magnification.

Western Blotting

Whole tissue lysates were prepared by 1X passive lysis buffer containing 2 mM Na_3VO_4, 2 mM PMSF, 2% protease inhibitor cocktail (Sigma Aldrich). Equal amounts of protein from either individual mice or pooled from 3 mice were used for western blot analysis. Proteins were separated by SDS-PAGE, transferred to PVDF membrane (Millipore), blocked, incubated with primary antibody (Santa Cruz Biotechnology) at 4°C overnight. Target proteins were detected by horseradish peroxidase-conjugated secondary antibodies and visualized by Pierce ECL™ Western Blotting Detection Reagents.

Isolation of liver mitochondria and endoplasmic reticulum

Liver mitochondria were isolated as previously described with minor modifications (4). Fresh liver tissue was immediately placed into ice-cold isolation buffer (320 mM sucrose, 1 mM EGTA, 10 mM Tris-Base, pH 7.4) and homogenized on ice using a Kontes Teflon homogenizer (Kontes Glass, Vineland, NJ). The homogenate was then centrifuged at 1,380 x*g* for 3 min at 4°C, supernatant collected and centrifuged at 7,800 x*g* for 5 min at 4°C. The pellet was resuspended in 2 ml of 20% (v/v) Percoll (Sigma Aldrich), and

centrifuged at 11,200 x*g* for 10 min at 4°C. The pellet was washed with ice-cold isolation buffer and centrifuged at 7,800 x*g* for 5 min at 4°C. Pellets were washed twice and resuspended in ice-cold isolation buffer.

Hepatic ER was fractionated as described with minor modifications (5). Fresh liver was minced in homogenization buffer (37.5 mM Tris, 0.5 M sucrose, 1% dextran, 5 mM $MgCl_2$, pH 6.5) containing β-mercaptoethanol (70µl/100ml buffer) at a 4:1 ratio (v/w), homogenized and centrifuged at 5,080 x*g* for 15 min. The top 2/3 portion of the pellet was resuspended with 1.5mL of homogenization buffer, then layered onto 1.2 M sucrose containing β-mercaptoethanol (7µl/20ml buffer) at a 1.2:1 ratio (v/v) and ultracentrifuged at 129,000 x*g* for 30 min. The pellet was resuspended in the supernatant collected from the first centrifugation and diluted 3 fold with ER buffer (55 mM Tris, 5 mM $MgCl_2$, pH 7.0) containing β-mercaptoethanol (35µl/100ml buffer) and centrifuged at 8,300 x*g* for 5 min. The supernatant was collected and layered onto a discontinuous sucrose gradient: 2.0 M, 1.5 M, 1.3 M sucrose with a volume ratio of 3:4:4, and ultracentrifuged at 112,400 x*g* for 90 min. The band between 1.3 M and 1.5 M sucrose and the band between 1.5 M and 2.0 M sucrose were collected as ER fraction. The ER collection was diluted with ER buffer and centrifuged at 112,400 x*g* for 20 min. The ER pellet was resuspended in buffer R (10 mM Tris, 0.25 M sucrose, 0.1 M PMSF, pH 7.4).

Mitochondria membrane potential, MTT assay, membrane fluidity, reactive oxygen species

The isolated liver mitochondria were used for measuring the membrane potential as described with modifications using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' -tetraethylbenzimidazolylcarbocyanine iodide) (AnaSpec) (6). Isolated mitochondria were

incubated with 1.5 mg JC-1/mg protein for 1 min at room temperature in dark. The fluorescence of monomer was measured at excitation 485 nm/emission 535 nm, and that of J-aggregates was measured at excitation 535 nm/emission 590 nm using a Tecan fluorescent plate reader. The ratio between J-aggregates and monomer was calculated to represent the membrane potential of isolated mitochondria.

Mitochondria MTT assay was performed as described with minor modifications (7). Mitochondria were incubated in KCl buffer (125 mM KCl, 2 mM K2HPO4, 1 mM MgCl2, 20 mM HEPES, pH 7.4) with 2.5 mg/ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) in PBS with 6 mM succinic acid for 1 hour at 37°C. The reaction was terminated by solubilization solution containing 50% N,N'-dimethylformamide and 20% sodium dodecyl sulfate (pH 4.8). Absorbance was read at 570 nm.

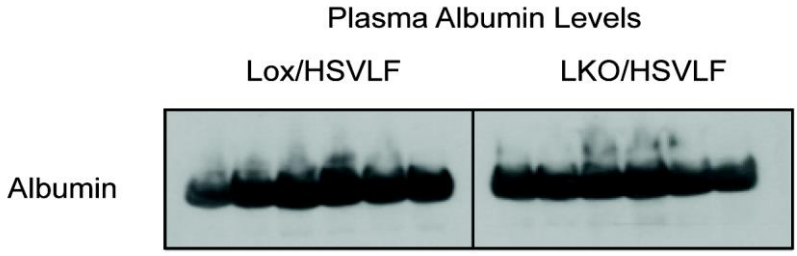
Mitochondrial membrane fluidity was evaluated by fluorescence anisotropy using dye TMA-DPH (Invitrogen) as described (8). Isolated liver mitochondria (0.5mg/ml by protein content) were incubated with 3mM TMA-DPH for 30 min at 37°C with agitation. Fluorescence polarization was measured by a QuantaMaster Model C-60/2000 Spectrofluorimeter (Photon Technologies International) with excitation at 350nm and emission at 430nm. Anisotropy was normalized to mitochondrial protein concentration. Light scattering and intrinsic fluorescence was corrected by subtraction of the fluorescence signal obtained from unlabeled mitochondria samples and the fluorescence from buffer with the label only.

Mitochondrial reactive oxygen species (ROS) was measured in isolated liver mitochondria using fluorescent probe H_2DCFDA (Invitrogen) as described (9). Briefly the isolated liver mitochondria (200µg by protein content) were incubated with 10µM

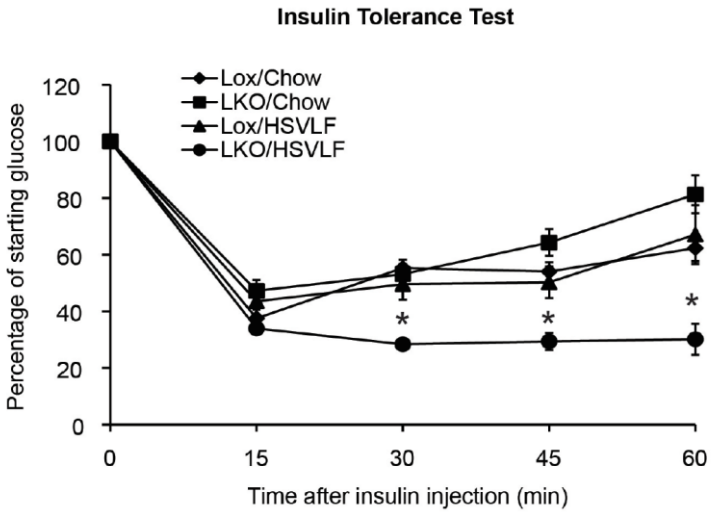
H_2DCFDA dye and 5mM Succinate for 5 min, then the plate was set to a Biolumin 920 plate reader (Molecular Dynamics). The plate was automatically shaken for each cycle during an incubation period of 70 min at 30°C, starting at 0 min, fluorescence was recorded every 10 min during the 70 min incubation (excitation at 485 nm and emission at 520 nm). The fluorescence readings were corrected by subtracting a blank control.

Electron microscopy analysis of liver sections

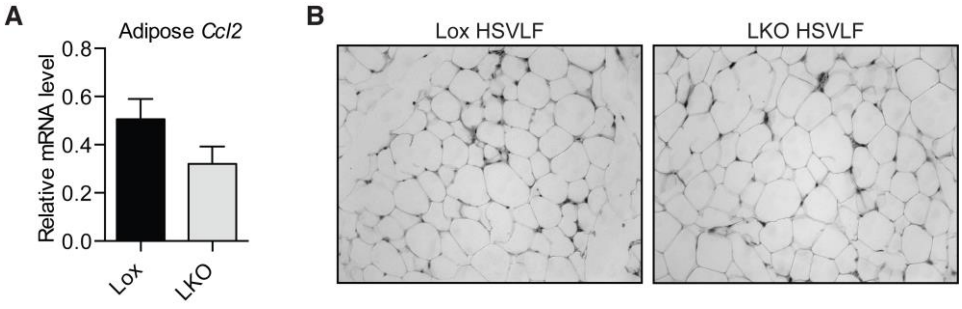
Liver tissues were immersion-fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1M sodium phosphate buffer, pH 7.4 overnight at 4°C. Tissue was post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, further dehydrated in propylene oxide and embedded in Epon epoxy resin. Semi-thin 1μm sections were cut with a Leica EM UC6 Ultramicrotome and observed under a light microscope to establish proper orientation. At the appropriate level, ultra-thin sections were cut with the same microtome and collected on 200 mesh copper grids. Sections were contrasted with Reynolds lead citrate and 8% uranyl acetate in 50% EtOH. Sections were imaged with a Philips CM120 electron microscope and a MegaView III side mounted digital camera.



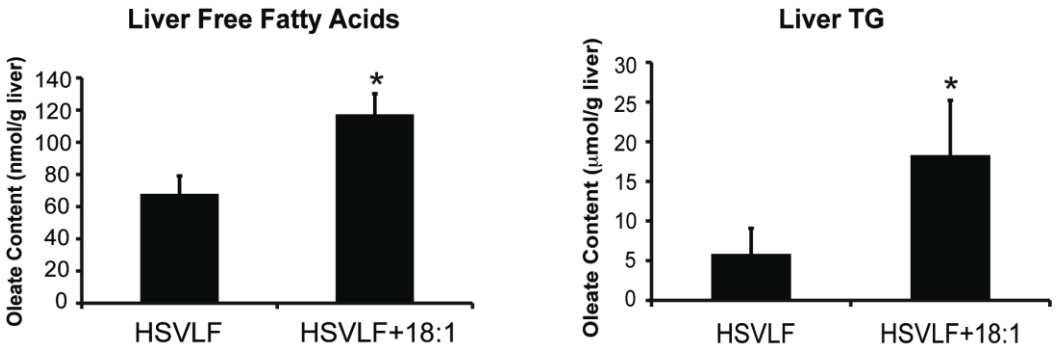
Supplementary Fig. 1. Plasma albumin protein levels are not altered by high-sucrose very low-fat (HSVLF) feeding. Lox and LKO mice were fed the HSVLF diet for 10 days. Immunoblot analysis was used to determine protein levels of plasma albumin. Each lane represents an individual animal.



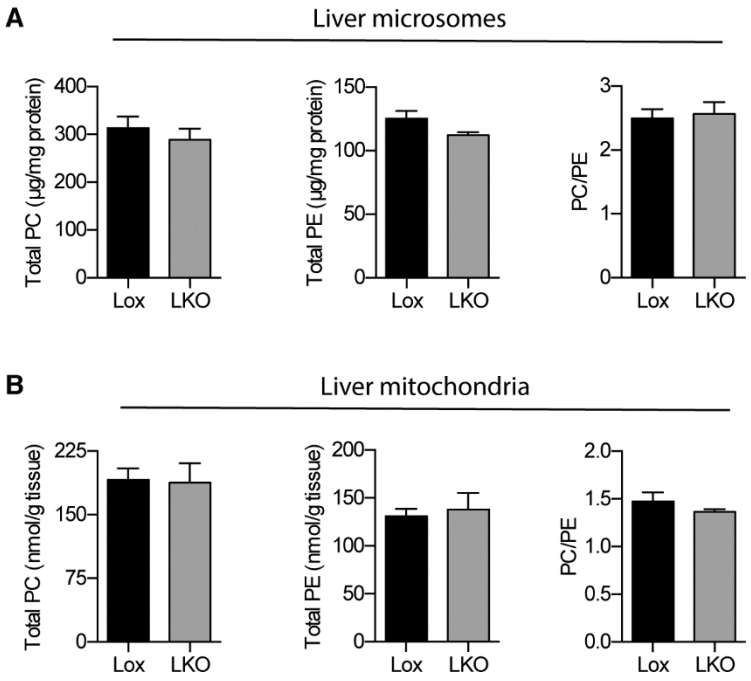
Supplementary Fig. 2. High-sucrose very low-fat (HSVLF) diet-induced liver inflammation does not reduce insulin sensitivity in LKO mice. Lox and LKO mice were fed a chow or HSVLF diet for ten days. Nonfasted mice were intraperitoneally injected with 0.75 U insulin/kg body weight and blood glucose was measured at 0, 15, 30, 45 and 60 minutes post-injection (n=5/group). Data are expressed as percentage of the starting glucose level. Values are mean ± SEM, **P* < 0.05 vs. HSVLF-fed Lox by student's two-tailed *t* test.



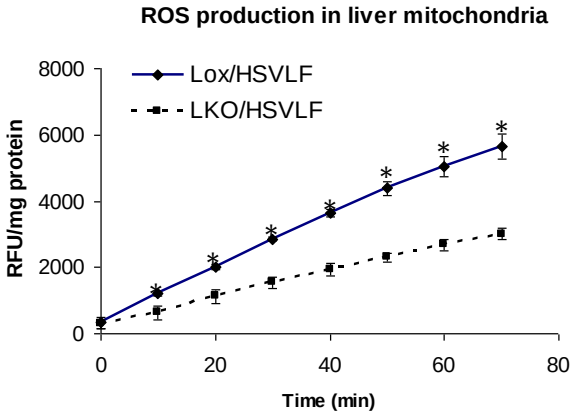
Supplementary Fig. 3. Liver-specific deletion of SCD1 does not induce adipose tissue inflammation. Lox and LKO mice were fed the HSVLF diet for 10 days mice (n=5–8/group) (A) Relative gene expression of *Ccl2* in epididymal white adipose tissue. (B) epididymal WAT morphology using H and E stain were assessed. Values are mean ± SEM.



Supplementary Fig. 4. Triolein supplementation increases oleate concentration in liver lipids. LKO mice were fed the high-sucrose very low-fat (HSVLF) diet with or without 20% w/w oleate (18:1) for 10 days (n=4–5/group). Gas liquid chromatography was used to analyze the fatty acid composition of free fatty acids (left) and hepatic TG (right). Values are mean ± SEM, *$P < 0.05$ by student's two-tailed *t* test.



Supplementary Fig. 5. HSVLF feeding does not influence liver microsomal or mitochondrial phospholipid composition in SCD1 liver knockout mice. Lox and LKO mice were fed high-sucrose very low-fat (HSVLF) for ten days. Phospholipids were extracted, PC and PE species were separated and total levels were measured by gas chromatography and the PC/PE ratio was determined in (A) liver microsomes (n=5–6/group) and (B) liver mitochondrial (n=6/group). Values are mean ± SEM.



Supplementary Fig. 6. Mitochondrial reactive oxygen species are reduced in SCD1 LKO mice. Lox and LKO mice were fed a high-sucrose very low-fat (HSVLF) for ten days. Mitochondria were isolated from fresh liver tissue as described in Methods. Mitochondrial reactive oxygen species (ROS) were measured by a fluorescent probe H_2DCFDA as described in Methods. Values are mean ± SEM (n=4). *$P < 0.05$ by student's two-tailed *t* test.

Supplementary Table 1. Body and tissue weights of female mice fed HSVLF diet for 10 days

	WT	GKO	GLS5	GLS3
Body wt (g)	21.7 ± 0.5	18.1 ± 0.5 *	20.6 ± 0.4 #	18.9 ± 0.7 *
Total food intake (g)	30.5 ± 1.3	30.7 ± 1.3	42.1 ± 1.0 *#	30.6 ± 0.8 ^
Subcutaneous WAT (g)	0.28 ± 0.03	0.13 ± 0.02 *	0.28 ± 0.01 #	0.14 ± 0.02 *
Gonadal WAT (g)	0.33 ± 0.02	0.12 ± 0.01 *	0.31 ± 0.03 #	0.17 ± 0.02 *
Liver TG (μg/mg tissue)	32.7 ± 6.4	3.9 ± 0.4 *	8.1 ± 1.3 *#	6.2 ± 1.0 *

Data are mean ± SEM

*P<0.05 vs. WT; #P<0.05 vs. GKO; ^P<0.05 vs. GLS5

Supplementary Table 2. Body and tissue weights of male mice fed HSVLF diet for 14 days

	Lox	PGC-1‧ LKO	SCD1 LKO	DLKO
Body wt (g)	26.2 ± 0.3	25.3 ± 0.7 #	22.1 ± 0.9 *	23.4 ± 0.8 *
Total food intake (g)	46.7 ± 2.9	44.8 ± 2.7	38.0 ± 1.6	41.9 ± 2.1
Liver wt (g)	1.23 ± 0.04	1.32 ± 0.07	1.27 ± 0.08	1.23 ± 0.04
Subcutaneous WAT (g)	0.26 ± 0.04	0.26 ± 0.02	0.24 ± 0.02	0.22 ± 0.02
Gonadal WAT (g)	0.43 ± 0.05	0.47 ± 0.03	0.31 ± 0.02	0.28 ± 0.04

Data are mean ± SEM; * P<0.05 vs. Lox; #P<0.05 vs. SCD1 LKO

Supplementary Table 3. Body and tissue weights of female mice fed HSVLF diet for 14 days

	Lox	PGC-1▪ LKO	SCD1 LKO	DLKO
Body wt (g)	22.1 ± 0.8	21.2 ± 0.6	19.0 ± 0.9 *	20.1 ± 0.7
Total food intake (g)	41.0 ± 1.4	43.2 ± 1.8	42.5 ± 1.9	39.3 ± 1.3
Liver wt (g)	1.05 ± 0.06	1.03 ± 0.05	1.04 ± 0.03	1.16 ± 0.06
Subcutaneous WAT (g)	0.21 ± 0.02	0.21 ± 0.02	0.20 ± 0.04	0.24 ± 0.02
Gonadal WAT (g)	0.25 ± 0.04	0.27 ± 0.03	0.21 ± 0.05	0.23 ± 0.01

Data are mean ± SEM; * $P<0.05$ vs. Lox

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