Acyl-CoA Thioesterase 1 (ACOT1) Regulates PPARα to Couple Fatty Acid Flux with Oxidative Capacity During Fasting

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ABSTRACT

Hepatic acyl-CoA thioesterase 1 (ACOT1) catalyzes the conversion of acyl-CoAs to fatty acids (FAs) and Coenzyme A. We sought to determine the role of ACOT1 in hepatic lipid metabolism in C57Bl/6J male mice one week following adenovirus-mediated Acot1 knockdown. Acot1 knockdown reduced liver triglyceride (TG) due to enhanced TG hydrolysis and subsequent FA oxidation. In vitro experiments demonstrated that Acot1 knockdown led to greater TG turnover and FA oxidation, suggesting that ACOT1 is important for controlling the rate of FA oxidation. Despite increased FA oxidation, Acot1 knockdown reduced the expression of peroxisome proliferator-activated receptor α (PPARα) target genes. While overexpression increased PPARα reporter activity, suggesting ACOT1 regulates PPARα by producing FA ligands. Moreover, ACOT1 exhibited partial nuclear localization during fasting and cAMP/PKA signaling, suggesting a local regulation of PPARα. As a consequence of increased FA oxidation and a reduction in PPARα activity, Acot1 knockdown enhanced hepatic oxidative stress and inflammation. The effects of Acot1 knockdown on PPARα activity, oxidative stress, and inflammation were rescued by supplementation with Wy-14643, a synthetic PPARα ligand. Taken together, we demonstrate that ACOT1 regulates fasting hepatic FA metabolism by balancing oxidative flux and capacity.
During times of fasting, FAs are released from adipose tissue and readily taken up by the
liver where they can be oxidized in the mitochondria via β-oxidation (1,2). In order to be transported into the mitochondria, FAs must be converted to acyl-CoAs by long chain acyl-CoA synthetases (3). A family of acyl-CoA thioesterases catalyze the reverse reaction – the hydrolysis of the fatty acyl-CoA thioester bond resulting in the production of Coenzyme A and a free FA (4). This reaction seemingly removes acyl-CoAs from mitochondrial β-oxidation. In the liver, ACOT1 is the primary cytosolic thioesterase isoform (5). PPARα induces Acot1 expression through a distal response element in the promoter region of the gene (6,7). However, fasting induces Acot1 expression in whole body Ppara-null mice as well as liver-specific Ppara knockout mice (6,7), suggesting that PPARα is sufficient, but not necessary for Acot1 expression. Therefore, ACOT1 is speculated to be involved in FA trafficking during periods of increased hepatic FA influx and oxidation (8,9).

PPARα is a transcription factor that governs the expression of genes involved in FA oxidation and is necessary to increase FA oxidative capacity during times of fasting (7). A wide variety of lipid species are speculated to serve as ligands for PPARα activation including free FAs (10–12). In addition to its role in FA oxidation, PPARα promotes the expression of anti-inflammatory genes (13) as well as suppresses expression of inflammatory genes (14). As such, PPARα ligands are potential therapeutic agents for the prevention or treatment of inflammation (14).

Mitochondrial FA oxidation produces a mild amount of ROS at complex I and III of the electron transport chain (15). This ROS production is balanced by antioxidant activity that protects the mitochondria from oxidative stress (16). However, during times of increased FA oxidation, increased membrane potential can cause a large amount of ROS production that can exceed antioxidant capacity leading to oxidative stress (15,17,18). Regulating the rate of FA
oxidation in the cell is critical for minimizing ROS and oxidative stress that can occur during times of increased oxidation. Overexpression of ACOT1 in cardiomyocytes reduces FA oxidation and ROS production in mice with diabetic cardiomyopathy (19). These data suggest a potential involvement of ACOT1 in promoting oxidative capacity through substrate regulation and signaling. Despite high expression in the liver during fasting, the involvement of ACOT1 in lipid metabolism has yet to be characterized. Herein, we identify ACOT1 as a key enzyme that links FA flux and trafficking to PPARα signaling, ROS, and inflammation.

RESEARCH DESIGN AND METHODS

Mouse handling. The Institutional Animal Care and Use Committee of the University of Minnesota approved all protocols used in this study. Seven to nine week old male C57BL/6J mice were purchased from Harlan Laboratories (Madison, WI) and housed in a controlled temperature (20°-22°C) and light (12-hour light/12-hour dark) vivarium. Mice had free access to water and were fed a purified diet (TD.94045) from Harlan Teklad (Madison, WI). One week before sacrifice, mice received a tail vein injection of an adenovirus harboring either scramble short-hairpin RNA (shRNA) described previously (20) or shRNA targeted to Acot1 mRNA. Acot1 shRNA adenovirus was generated from Open Biosystems (Huntsville, AL) based on accession number NM_012006.2 (antisense sequence AAACACTCACTACCCAACTGT). For the studies involving Wy-14643, mice were fed the purified diet supplemented with 0.1% WY-14643 (Selleckchem, Houston, TX), a synthetic PPARα ligand, for 7 days (21) An additional group of mice were fed a 45% high fat diet (HFD) (TD.06415) for 12 weeks prior to a tail vein injection of the adenovirus treatment. One week post transfection all mice were fasted overnight (16 hours) and harvested unless noted otherwise.
**Hepatocyte experiments.** Pulse-chase studies with isotopes were conducted in primary hepatocytes isolated from 16 h fasted mice as previously described (20, 22). A Seahorse XF analyzer was used to determine total oxygen consumption rate (OCR) in primary hepatocytes isolated from control and Acot1 knockdown mice after an overnight fast. Cells were seeded in a collagen coated XF cell culture plates at 3 x 10⁴ cells/well and incubated at 37° for 4 hours. Media was changed to XF Assay media with 5.5 mM glucose, 2 mM glutamax, and 1 mM pyruvate and placed in a CO₂ chamber for an hour. Basal oxygen consumption was measured for 30 minutes, followed by an injection of either BSA control or 500 µM of oleate and OCR was determined for 30 minutes. Finally, antimycin A was injected at 1 µM to determine non-mitochondrial oxygen consumption. Average basal OCR and OCR in response to BSA control or 500µM of oleate was calculated.

**TG hydrolase activity.** Tissues were lysed in a buffer consisting of 20 mM Tris-HCl, 150 mM NaCl, and 0.05% Triton X-100, pH 8.0. Lysates were spun at 15,000xg for 15 minutes. Infracnatant was collected and mixed with 1,2-Di-O-lauryl-rac glycerol-3-(glutaric acid 6-methylresorufin ester) and incubated at 30°C for 1 hour. Kinetic readings were taken every 2 minutes at 530 nm excitation and 590 nm emission. Readings were compared to a standard curve generated with free resorufin.

**Metabolite analyses.** Serum β-hydroxybutyrate, tissue TGs and tissue and serum FAs were analyzed as described previously (20).
RNA isolation and analysis. mRNA was isolated from tissue or cells using Trizol Reagent (ThermoFisher, Waltham, MA) according to manufacturer’s protocol. cDNA was generated using SuperScript® VILO (Invitrogen, Carlsbad, CA). Quantitative real-time polymerase chain reaction was performed using SYBR Select (ThermoFisher, Waltham, MA).

Protein preparation and western blotting. Protein was isolated from hepatocytes and liver tissue in lysis buffer and quantified by BCA. ACOT1 protein was determined by western blotting using 80 μg of protein. Following SDS-PAGE, proteins were transferred to a PDVF membrane and probed for ACOT1 with a custom antibody. The *Mus musculus* ACOT1/ACOT2 antibody was developed in rabbit by injecting the antigen CSVAAVGNTISYKDET-amide (21st Century Biochemicals, Marlboro, MA).

ROS determination. Cellular ROS and reactive nitrogen species (RNS) was determined using the OxiSelect In Vitro ROS/RNS Assay Kit (Cell Biolabs, Inc., San Diego, CA).

Plasmids and cloning. DsRed-Express N1 plasmid was purchased from Clonetech (Mountain View, CA). Acot1 cDNA was amplified from a pCMV6 Acot1 plasmid purchased from Origene (Rockville, MD). Acot1 cDNA was cloned into the multiple cloning site of DsRed-Express N1 by restriction enzyme digestion and T4 DNA ligase ligation. S232A point mutation was achieved using the QuikChange site-directed mutagenesis kit from Agilent and primers previously reported (9).

Cell and Tissue Imaging. Tissue sections were either froze in Tissue-Tek® O.C.T. (VWR,
Eagan, MN) or fixed in 10% formalin and subsequently paraffin embedded. Slides were
deparaffinized and blocked in 3% BSA, incubated with ACOT1 antibody overnight, stained with
DAPI, and mounted. Immunohistochemistry for Cd45 was determined as well as Oil Red O and
H&E stains from the University of Minnesota’s BioNet Histology and IHC Lab. AML12 cells
were cultured in DMEM media containing 10% FBS, 1% pen/strep, and 0.1% insulin-transferrin-
selenium solution. Cells were lipid loaded overnight in serum free DMEM with 500 µM oleate,
then pre-treated with 30 µM H89, a PKA inhibitor, for one hour before a 10 minute treatment
with 10 µM of a cell permeable cAMP analogue, 8-Bromoadenosine 3’,5’-cyclic monophosphate
(8-Br-cAMP). Cells were fixed, stained for ACOT1, and DAPI and then mounted. COS7 cells
were transfected using Effectene (Qiagen, Germantown, MD) transfection reagent with ACOT1-
DsRed-Express. Cells were then treated and fixed as described for the AML12 studies.

**PPARα Reporter assays.** COS7 or L cells were transfected with PPARα reporter construct
(pSG5-GAL4-Ppara), firefly luciferase reporter plasmid (DK-MH-UASluc), control Renilla
luciferase plasmid (pRLSV40), and empty DsRed-Express N1 plasmid, Acot1-DsRed-Express
N1, or Acot1-S232A-DsRed-Express N1. Cells were treated in serum free media with 500 µM
oleate for 16 hours then treated with 8-Br-cAMP or vehicle for 6 hours before harvesting for
assessment of reporter activity using the Dual-Luciferase Reporter Assay System (Promega,
Madison, WI). Firefly luciferase activity was normalized to Renilla luciferase activity.

**Thioesterase Activity Assay.** Thioesterase activity was determined as previously described (24)
with minor modifications. Frozen liver samples were homogenized with a dounce homogenizer
for 15 seconds. Homogenates were centrifuged at 100,000g at 4°C for 1 hour and supernatants
were collected. Samples were diluted in assay buffer (50 mM KCl, 10 mM HEPES) and 1mg of protein was loaded per well. DTNB was added at a working concentration of 50µM and read at 405 nm at 37°C. Palmitoyl Co-A was added at 20 µM and read at 405 nm at 37°C for 3-5 minutes. CoA concentration as determined against a standard curve. Values were reported as nmoles CoA/min/mg protein.

**Statistical Analysis.** Values are expressed as the mean ± SEM. Statistical significance was determined by Student’s t-test.

**RESULTS**

**ACOT1 is increased during fasting.**

To assess the expression pattern and cellular distribution of ACOT1, we harvested mice that were fed or fasted for 16 hours. As expected, fasting resulted in a large induction of *Acot1* mRNA (Fig. 1A), which correlated to an increase in ACOT1 protein expression (Fig. 1B). Importantly, ACOT1 and ACOT2 are 93.7% homologues (25) and therefore the antibody generated for these studies recognizes both ACOT1 and ACOT2. However, ACOT2 is exclusively a mitochondrial protein (24) and previous groups have shown that ACOT2 runs below ACOT1 on Western blots (26). Therefore, to exhibit the specificity of our antibody, we compared our samples to those of mitochondrial preps from mouse liver overexpressing *Acot2* (24). ACOT1 in liver lysates runs above ACOT2 as shown in Supplemental Fig. 1. ACOT2 is barely detectable in whole liver lysates compared to ACOT1. Consistent with the Western blotting, ACOT1 exhibited low expression during the fed state but was robustly increased after 16 hours of fasting. As such, our studies primarily focused on mice in the fasted state when
ACOT1 is expressed. ACOT1 has been described as a cytosolic protein, however to assess its cellular localization we stained liver sections for ACOT1. As expected, ACOT1 was not present in the fed state but was abundant during fasting. Although ACOT1 was largely cytosolic, we observed ACOT1 in the nucleus in response to fasting (Fig. 1C), which was subsequently confirmed with Western blotting of nuclear fractions (Fig. 1D), suggesting a potentially undocumented nuclear role of ACOT1.

_Acot1_ knockdown reduces fasting liver TG by enhancing TG turnover and increases oxidation of endogenous and exogenous FAs.

To determine the contribution of ACOT1 to hepatic energy metabolism, we used adenoviral delivered shRNA to knockdown _Acot1_ in the liver. Seven days post transduction, control mice (cont. shRNA) or liver _Acot1_ knockdown mice (_Acot1_ shRNA) (n=7-9) were fasted for 16 hours prior to sacrifice. We observed a significant reduction (∼60%) in both _Acot1_ mRNA (Supplemental Fig. 2A), and protein (∼60%) (Supplemental Fig.2B). Immunohistochemistry exhibited reduced cytosolic and nuclear ACOT1 with _Acot1_ knockdown (Supplemental Fig. 2C). This reduction in ACOT1 expression correlated to reduced thioesterase activity (Supplemental Fig. 2D).

_Acot1_ knockdown significantly reduced hepatic triglyceride content as determined by enzymatic assays and imaging of lipid droplets in H&E and Oil Red O stained liver sections (Fig. 2A-B). There were no effects of _Acot1_ knockdown on liver lipid droplet accumulation in fed mice consistent with its low expression in the fed state (Supplemental Fig. 3A). The reduced liver TG observed in response to _Acot1_ knockdown could arise from a reduction in _de novo_ lipogenesis and/or TG synthesis, enhanced TG secretion in the form of VLDL, or enhanced TG
breakdown and FA oxidation. Thus, we assessed each of these possibilities to determine which pathway(s) were altered leading to reduced hepatic TG content in response to Acot1 knockdown. To assess de novo lipogenesis and TG synthesis, we performed radiolabel experiments in primary hepatocytes isolated from mice transduced with scramble control or Acot1 shRNA adenoviruses. We incubated cells for 2 hours with $^{14}$Cacetate or $^3$Hglycerol and measured incorporation of the label into the TG fraction. Rates of incorporation of either acetate or glycerol into the TG fraction were similar between treatment groups (Supplemental Fig. 4A-B) suggesting that de novo lipogenesis and TG synthesis are unaffected by Acot1 knockdown. Acot1 knockdown had little effect on serum TG (Supplemental Fig. 4C) or hepatic TG secretion based on serum TG from animals treated with tyloxapol (Supplemental Fig. 4D), suggesting no change in VLDL secretion. These results were confirmed with radiolabeling studies in primary hepatocytes, which showed that Acot1 knockdown had no effect on secreted TG (Supplemental Fig. 4E). Together, these data suggest that changes in de novo lipogenesis, TG synthesis, or VLDL assembly and secretion are unaffected by Acot1 knockdown. We next tested the effects of Acot1 knockdown on TG hydrolysis. There was a significant increase in TG hydrolase activity in tissue homogenates from livers treated with Acot1 shRNA compared to control livers (Fig. 2C). Enhanced lipolysis paralleled an increase in serum β-hydroxybutyrate (Fig. 2D) indicative of enhanced FA oxidation. We again confirmed these effects were specific to the fasted since Acot1 knockdown did not alter β-hydroxybutyrate in fed mice (Supplemental Fig. 3B). These data support a role for ACOT1 in mitigating the flux of acyl-CoAs to mitochondrial β-oxidation in the fasted state. To further assess alterations in FA flux, in vitro pulse-chase experiments were performed. Similar to the unchanged TG synthesis from acetate or glycerol (Supplemental Fig. 4A-B), incorporation of $^{14}$Coleate into the cellular TG pool was similar between treatment
groups (Fig. 2E). Consistent with increased TG hydrolysis in livers from Acot1 knockdown mice, turnover of [14C]TG was significantly greater with Acot1 knockdown in primary hepatocytes (Fig. 2F). Additionally, FA oxidation was significantly increased in both the pulse (Fig. 2G) and chase periods (Fig. 2H) in the Acot1 knockdown hepatocytes.

We next assessed oxygen consumption rate (OCR) in primary hepatocytes from control and Acot1 knockdown mice using the Seahorse XF analyzer. As expected Acot1 knockdown hepatocytes exhibited a significantly increased in OCR with the addition of FAs compared to BSA control (Supplemental Fig. 5); although basal rates were numerically higher they did not reach significance. To confirm the enzymatic importance of ACOT1 in regulating FA oxidation and TG turnover, we next transfected L cells with an empty DsRed-Express N1 vector (EV), Acot1 DsRed-Express N1 (Acot1), or a catalytically dead mutant Acot1-S232A-DsRed-Express N1 (Acot1 S232A) and performed a pulse-chase with [14C]oleate. Expression and thioesterase activity of these mutants were confirmed in (Supplemental Fig. 6). Cells transfected with Acot1 exhibited less FA oxidation and more TG retention (Supplemental Fig. 7) compared the EV and Acot1 S232A mutant, indicating the catalytic activity of ACOT1 is necessary for its regulation of FA oxidation and TG turnover. Taken together, these data suggest that Acot1 knockdown reduces hepatic TG levels through increased TG turnover and enhanced oxidation of both exogenous and endogenous FAs.

ACOT1 regulates PPARα activity.

Since there was an observed increase in FA oxidation, we next measured the expression of target genes of PPARα, the principal transcription factor governing hepatic FA oxidation (7). Surprisingly, Acot1 knockdown reduced expression of PPARα and PGC1α target genes (Fig. 3A-
B). In addition, catalase protein expression, a marker of peroxisome content, and mitochondrial DNA abundance was reduced with Acot1 knockdown (Fig. 3C-D). When combined with the aforementioned effects on FA oxidation, these results suggest that ACOT1 uncouples FA oxidation from the upregulation of genes and machinery involved in oxidative metabolism. We next speculated that the overexpression of ACOT1 could drive PPARα activity. To test this, COS7 cells and L cells were transfected with an EV, Acot1 or Acot1-S232A plasmids and treated with vehicle or 8-Br-cAMP. The combination of 8-Br-cAMP and ACOT1 enhanced PPARα reporter activity more than 8-Br-cAMP alone in COS7 cells (Fig. 4A) and L cells (Fig. 4B). Moreover, induction of PPARα reporter activity by ACOT1 was dependent on its catalytic activity, suggesting that ACOT1 regulates PPARα through production of FA ligands. Lastly, Acot1 knockdown in AML12 cells blocked the induction of PPARα target gene expression by 8-Br-cAMP (Supplemental Figure 8). Together these data suggest that ACOT1 synergizes with cAMP to promote PPARα activity.

**8-Br-cAMP promotes ACOT1 nuclear translocalization.**

Although characterized as a cytosolic protein, the data in Fig. 1C-D and Supplemental Fig. 2C, suggest that ACOT1 is also present in the nucleus. Since ACOT1 has no predicted nuclear localization sequence (27), yet has numerous putative PKA phosphorylation sites (28), we suspected that nuclear location of ACOT1 could be driven by elevated cAMP/PKA signaling during fasting. To test the translocation of ACOT1 under β-adrenergic stimulation, AML12 cells were treated with H89, a PKA inhibitor, for 1 hour followed by 8-Br-cAMP for an additional 10 minutes before cells were fixed and stained for ACOT1. Nuclear ACOT1 increased with the addition of 8-Br-cAMP, however this effect was blocked by the PKA inhibitor H89 (Fig. 4C-D).
Nuclear localization of ACOT1 was also confirmed in COS7 cells transfected with Acot1 DsRed-Express N1 treated with vehicle or 8-Br-cAMP (Fig. 4E). ACOT1 signal was detectable in the nucleus in response to 8-Br-cAMP, but not when treated with a vehicle. Together, these data suggest that ACOT1 regulates PPARα activity in response to cAMP/PKA signaling, potentially due to its nuclear localization and production of local FA ligands.

**ACOT1 is important for mitigating oxidative stress due to enhanced FA oxidation during fasting.**

Enhanced FA oxidation can lead to the production of ROS in the electron transport chain leading to oxidative stress (29). In response to Acot1 knockdown, we observed enhanced FA oxidation (Fig. 3D) yet a reduction in oxidative machinery as indicated by reduced expression of PPARα targets (Fig. 4A-E). Such effects could lead to enhanced ROS generation and oxidative stress. Supportive of this logic, Acot1 knockdown livers exhibited significantly more ROS than the control livers (Fig. 5A). In addition to increased ROS, Acot1 knockdown increased the expression of oxidative stress markers heme oxygenase 1 (Ho-1) and uncoupling protein 2 (Ucp2) (Fig. 5B). Increases in oxidative stress promotes inflammation (15) and PPARα is well-documented to be important for anti-inflammatory pathways (13,14). Thus, the reduction in PPARα targets along with increased oxidative stress suggests Acot1 knockdown could promote inflammation. Indeed, there was a significantly increase in expression of inflammatory markers Tnfa, Cd11c, Il-1β, and F4/80 (Fig. 5C). Consistent with enhanced inflammatory gene expression, Cd45 immunohistochemistry revealed enhanced immune cell infiltration in livers with Acot1 knockdown (Fig. 5D). Similarly, immune cell infiltration can also be observed in the H&E micrographs (Fig. 3B). These results suggest that ACOT1 mitigates oxidative stress and
inflammation during fasting, potentially through enhancing PPARα activity.

**The effects of Acot1 knockdown can be rescued by PPARα agonism.**

To further explore the potential role of ACOT1 in supplying FA ligands for PPARα activation, we attempted to rescue the effects of ACOT1 knockdown by feeding mice a diet supplemented with a synthetic PPARα ligand, Wy-14643. Mice were treated with adenovirus as described above and fed a purified diet or a diet supplemented with 0.1% Wy-14643 (30) for one week. As expected Wy-14643 significantly increased expression of ACOT1, however, Acot1 shRNA adenovirus significantly blunted this induction (Fig. 6A). Wy-14643 ablated the effects of Acot1 knockdown on reducing liver TG (Fig. 6B-C). However, Acot1 knockdown and Wy-14643 independently and together increased TG hydrolase activity (Fig. 6D). As seen above, Acot1 knockdown increased serum β-hydroxybutyrate in mice fed the control diet, while Wy-14643 significantly increased serum β-hydroxybutyrate to a similar level between treatment groups (Fig. 6E). Moreover, Wy-14643 rescued the expression of PPARα target genes in mice treated with Acot1 shRNA (Fig. 6F) as well as catalase protein expression (Fig. 6G). Since Wy-14643 is able to rescue the Acot1 knockdown phenotype, it is likely that ACOT1 is responsible for providing FA ligands for activation of PPARα.

We next explored the possibility that Wy-14643 could also rescue the oxidative stress and inflammation observed in response to Acot1 knockdown. Wy-14643 was able to normalize the expression of oxidative stress marker Ho-1, but was unable to normalize Ucp2 expression in Acot1 knockdown livers (Fig. 7A). Wy-14643 also normalized the expression of inflammatory genes and prevented immune cell infiltration in livers of Acot1 knockdown mice (Fig. 7B-C). In summary, these data suggest that ACOT1 regulates PPARα as a means to influence hepatic
energy metabolism, oxidative stress, and inflammation.

**The effects of Acot1 knockdown in diet induced steatosis.**

Since Acot1 knockdown resulted in oxidative stress and inflammation, hallmarks of liver disease, we investigated the effects of Acot1 knockdown in mice fed a high fat diet. Mice were fed a 45% fat diet for 12 weeks prior to an adenovirus delivery of cont. shRNA or Acot1 shRNA (Supplemental Fig. 9A-B). Abundant lipid droplet accumulation as observed liver of mice fed the high fat diet, but Acot1 knockdown had similar hepatic TG content compared to controls (Supplemental Fig. 9C-D). Acot1 knockdown increased serum β-hydroxybutyrate (Supplemental Fig. 9E) despite unchanged expression of PPARα (Supplemental Fig. 9F) or PGC1α (Supplemental Fig. 9G) target genes. Despite the lack of reductions in gene expression, mitochondria content was reduced with Acot1 knockdown (Supplemental Fig. 9H). As expected, Acot1 knockdown solicited a robust induction of inflammatory and oxidative stress markers (Supplemental Fig. 9I). Acot1 knockdown also increased ROS (Supplemental Fig. 9J) and fibrosis (Supplemental Fig. 9K). Together these data suggest that under high fat diet induced steatosis, ACOT1 is protective against inflammation and oxidative stress that leads to fibrosis.

**DISSCUSSION**

The liver imports FAs proportional to their concentration in the blood. Thus, increased rates of FA metabolism must match this uptake during times of fasting. Upon entering the liver, hepatic FAs are first converted to acyl-CoA molecules prior to their entry into most metabolic pathways (3). ACOT1 catalyzes the reverse reaction, producing free FAs (5), which should slow the flux of FAs to downstream metabolic pathways. Consistent with this logic, hepatic
knockdown of ACOT1 increased the oxidation of FAs in vivo and in vitro (Fig. 2), while having no observed effect on TG synthesis or VLDL secretion (Supplemental Fig. 4). Taken together, these results suggest that ACOT1 specifically regulates FAs destined to oxidative pathways, potentially by removing acyl-CoAs as substrates. By slowing FA oxidation, ACOT1 may serve to protect the liver from detrimental effects of excessive FA oxidation.

PPARα is a dominant transcription factor in the liver, responsible for the up-regulation of genes involved in FA oxidation during fasting (7,31). Acot1 knockdown led to enhanced FA oxidation, which would be expected to correlate to greater expression of several PPARα target genes. However, PPARα targets were reduced (Fig. 3A) following Acot1 knockdown despite the greater oxidative rate. Since FAs serve as endogenous ligands to activate PPARα (10), we speculated that ACOT1 provides FA ligands to activate PPARα. Therefore, administration of a synthetic PPARα ligand (e.g. Wy-14643) should rescue the effects of hepatic Acot1 knockdown on PPARα signaling. Wy-14643 treatment largely rescued the phenotype resulting from ACOT1 knockdown including normalization of FA oxidation and PPARα target genes (Fig. 6). We also tested if ACOT1 thioesterase activity was necessary for its regulation of PPARα. Expressing ACOT1 in COS7 or L cells increased PPARα reporter activity during 8-Br-cAMP stimulation. However, a catalytically dead ACOT1 mutant (Acot1 S232A) had no effect on PPARα activity compared to the empty vector control (EV) (Fig. 4A-B), supporting the theory that ACOT1 thioesterase activity provides FA ligands to regulate PPARα. Surprisingly, we found no difference in cytosolic or nuclear free FAs with Acot1 knockdown (Supplemental Fig. 10). Free FAs are lipotoxic substances that are tightly regulated inside cells (1). It is possible that the ACOT1-derived FA pool may be too small or transient to see distinct differences in total quantity, or that FAs are selectively channeled to influence PPARα activity. ACOT1
overexpression alone was not sufficient to increase PPARα reporter activity, only during cAMP/PKA signaling did ACOT1 solicit PPARα activation (Fig. 4A-B). We observed nuclear localization of ACOT1 in response to 8-Br-cAMP (Fig. 4C-E), suggesting nuclear localization may be necessary for ACOT1 to activate PPARα. Similarly, Acot1 knockdown reduced nuclear ACOT1 (Supplemental Fig. 2C) and subsequently reduced PPARα transcripts (Fig. 3A). Thus, these data show that ACOT1 produces FA ligands to activate PPARα, increasing FA oxidative capacity. This regulation may be dependent on the nuclear localization of ACOT1 in response to fasting and cAMP/PKA signaling.

Normal mitochondrial oxidation produces a mild stress on cells resulting in the production of ROS (15). However, ROS production is balanced by mitochondrial antioxidant activity that protects the mitochondria from oxidative stress. During times of increased FA oxidation, ROS can exceed antioxidant capacity and lead to oxidative stress (32). In addition, PPARα signaling limits oxidative stress (33,34) and promotes the expression of anti-inflammatory genes (13). There was greater hepatic ROS and expression of oxidative stress markers in hepatic Acot1 knockdown mice (Fig. 5A-B) that paralleled elevated rates of FA oxidation (Fig. 2) and reduced PPARα target gene expression (Fig. 3A). Additionally, there was greater expression of inflammatory genes and immune cell infiltration in response to Acot1 knockdown (Fig. 5C-D). Thus, the observed inflammation in response to Acot1 knockdown could be due to reduced PPARα dependent anti-inflammatory gene expression. Wy-14643 treatment rescued the oxidative stress and inflammation seen in Acot1 knockdown livers (Fig. 7). As such, ACOT1 appears to protect the liver from oxidative stress and inflammation via promotion of PPARα signaling.

Since oxidative stress and inflammation are key events in the progression of NAFLD (35),
perhaps it is not surprising that Acot1 knockdown increased oxidative stress and inflammation ultimately leading to fibrosis in response to high fat feeding. Current evidence suggests that high fat diets increases FA oxidation (36), which correlates with the increase in serum β-hydroxybutyrate we observed in mice on a HFD (Supplemental Fig. 8E) compared to basal mice (Fig. 2D). This increase in FA oxidation is associated with the oxidative stress and inflammation that occurs as the disease progresses (37). Our findings support the importance of FA oxidation and its complications in the progression of NAFLD from steatosis to fibrosis and implicate ACOT1 as playing an important role in balancing FA oxidation, ROS and inflammation.

To our knowledge these are the first studies to characterize the physiological importance of ACOT1 in hepatic fasting lipid metabolism. This study highlights a significant role for ACOT1 in regulating FA oxidation, PPARα activity, oxidative stress, and inflammation during fasting. In support of a protective role of ACOT1, humans with fewer copy numbers of the 14q24.3 locus where ACOT1 resides are more likely to develop non-alcoholic steatohepatitis, a disease in which oxidative stress and inflammation are the defining characteristics (38). Similarly, overexpression of ACOT1 improves diabetic cardiomyopathy by reducing FA oxidation and ROS production (19). Thus, when combined, the few studies evaluating ACOT1 all highlight a potential beneficial role of ACOT1 in mitigating the negative effects aberrant lipid metabolism.

Based on our current findings, ACOT1 plays a pivotal role in protecting livers from excess FA oxidation and the ensuing oxidative stress and inflammation while simultaneously promoting PPARα activity (Fig. 8). In addition, ACOT1 locates to the nucleus during fasting, suggesting a potential local regulation of PPARα via the production of FA ligands. Thus, this work highlights the importance of ACOT1 as a branch point in regulating FA flux to oxidative
pathways and controlling the transcriptional regulation of FA oxidation.

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FIGURE LEGENDS

Figure 1. Hepatic ACOT1 is increased in fasting. C57Bl/6J mice were either fed or fasted overnight (16 hours). (A) Acot1 expression was increased with fasting as analyzed by RT-PCR (n=5). (B) Western blot and densitometry of ACOT1 in fed and fasted mice. (C) Fixed liver sections from fed or fasted (16 hours) mice were probed with the ACOT1 primary antibody and Alexa 488 secondary. (D) Western blots of nuclear preps from fed and fasted mice. *P<0.05.

Figure 2. Acot1 knockdown increases hepatic FA oxidation. (A) Acot1 knockdown reduced hepatic TG. (B) H&E and Oil Red O stains of liver tissue show reduce lipid droplets in the Acot1 shRNA treatment group (n=7-9). (C) TG hydrolase activity was increased in liver tissue homogenates of mice treated with Acot1 shRNA (n=4). (D) Serum β-hydroxybutyrate was
increased following hepatic \textit{Acot}1 knockdown in 16 hour fasted mice. (E) \textit{Acot}1 knockdown did not influence $[^{14}\text{C}]$oleate incorporation into cellular triglyceride (TG). (F) TG turnover as measured by $[^{14}\text{C}]$TG loss during the chase period was increased in response to \textit{Acot}1 knockdown. \textit{Acot}1 knockdown increased the oxidation of exogenous (G) and endogenous (H) FAs as measured by $[^{14}\text{C}]$acid-soluble metabolites (ASM) in the media (n=3).

\textbf{Figure 3. ACOT1 regulates expression of PPARα target genes, PGC1α target genes, and peroxisome and mitochondria abundance}. Expression of (A) PPARα target genes and (B) PGC1α target genes were reduced with \textit{Acot}1 knockdown (n=7). (C) Catalase protein expression, a marker of peroxisome abundance, was reduced with \textit{Acot}1 knockdown; densitometry represents n=5. (D) Mitochondrial DNA abundance was reduced with \textit{Acot}1 knockdown.

\textbf{Figure 4. ACOT1 regulates PPARα activity and partially localizes to the nucleus}. (A) COS7 and (B) L cells were transfected with an empty DsRed Express N1 (EV), \textit{Acot}1-DsRed Express N1 (\textit{Acot}1), or \textit{Acot}1-S232A-DsRed Express N1, and reporter assay components (n=6-12). Cells were harvested and luciferase activity was assessed. ACOT1 translocates to the nucleus in AML12 cells in response to treatment with 8-Br-cAMP. Pretreatment with H89 blocked the translocation of ACOT1 in response to 8-Br-cAMP as measured by (C) immunofluorescent staining and (D) nuclear fractionation and Western blotting. (E) An ACOT1 fusion construct (DsRed) shows partial nuclear localization in response to 8-Br-cAMP in COS7 cells. *P<0.05.

\textbf{Figure 5. Hepatic \textit{Acot}1 knockdown increases inflammation and oxidative stress in mice}. (A) \textit{Acot}1 knockdown increased intracellular ROS measured by OxiSelect \textit{in vitro} ROS/RNS Kit (n=4). (B) \textit{Acot}1 knockdown increased expression of the oxidative stress genes heme oxygenase 1 (\textit{Ho}-1) and uncoupling protein 2 (\textit{Ucp}2). (C) Expression of inflammatory markers are increased in mice treated with \textit{Acot}1 shRNA (n=7). (D) CD45 immunohistochemistry stains
from liver tissues revealed increased immune cell infiltration following *Acot1* knockdown (n=2). *P<0.05.

**Figure 6. Wy-14643 rescues effects of Acot1 knockdown.** (A) *Acot1* mRNA following adenovirus treatments and 0.1% Wy-14643 supplementation (n=7-9). (B) H&E staining shows that Wy-14643 reduced and normalized lipid droplet accumulation in the *Acot1* knockdown treatment group. (C) Wy-14643 normalized liver TG levels between control and *Acot1* shRNA treatment groups. (D) TG hydrolase activity and (E) serum β-hydroxybutyrate concentrations are increased and normalized following Wy-14643 (n=4). (F) Wy-14643 increases PPARα target gene expression and rescued the effects of *Acot1* knockdown. (G) Catalase protein expression is increased by Wy-14643 and normalized in livers of *Acot1* shRNA treated mice (n=2). *P<0.05 vs. cont. shRNA group. #P<0.05 vs. control diet.

**Figure 7. Wy-14643 rescues inflammation and oxidative stress markers.** (A-B) Wy-14643 normalized the expression of most oxidative stress markers (A) and reduced the inflammatory markers observed with *Acot1* knockdown (n=7-9) (B). (C) Wy-14643 normalized macrophage infiltration assessed by Cd45 immunohistochemistry staining (n=2). *P<0.05 vs. cont. shRNA group. #P<0.05 vs. Purified control diet.

**Figure 8. Proposed mechanism of ACOT1 in fasting liver lipid metabolism.** ACOT1 expression during fasting mitigates FA flux toward oxidation in the cytosol. Under fasting and elevated cAMP/PKA stimulation, nuclear ACOT1 is important in the activation of PPARα. Fasting with reduced ACOT1 enhances FA oxidation with reduced PPARα activity that leads to oxidative stress and inflammation. Therefore, ACOT1 is an important enzyme for balancing oxidative capacity, oxidative stress, and inflammation with PPARα activity during fasting.
Figure 1

203x271mm (300 x 300 DPI)
Figure 2

267x254mm (300 x 300 DPI)
Figure 3

190x254mm (300 x 300 DPI)
Figure 4

203x270mm (300 x 300 DPI)
Figure 5

212x254mm (300 x 300 DPI)
Figure 6

304x406mm (300 x 300 DPI)
Figure 7

190x254mm (300 x 300 DPI)
Figure 8

229x254mm (300 x 300 DPI)
Supplemental Figure 1. ACOT1 antibody distinguishes between ACOT1 and ACOT2. (A)

The ACOT1 antibody recognizes mouse ACOT1 and ACOT2. Liver homogenates from fed and 16 hour fasted mice were compared to mitochondrial preps from mice overexpressing Acot2.
Supplemental Figure 2. Hepatic Acot1 knockdown reduces ACOT1 protein and thioesterase activity. Acot1 expression is reduced in response to Acot1 knockdown as analyzed by RT-PCR (A) and Western blotting of liver lysates (B) 7 days post transduction (n=7-9). (C) Immunohistochemistry of ACOT1 from fix liver sections indicates reduced ACOT1 protein with Acot1 knockdown. (D) Acot1 knockdown reduced thioesterase activity (n=3-4). *P<0.05.
Supplemental Figure 3. *Acot1* knockdown effects on hepatic lipid metabolism in the fed state. Control and *Acot1* knockdown mice were harvested in the fed state. (A) Liver tissue were frozen in Tissue-Tek® O.C.T. for H&E and Oil Red O staining. (B) Serum was assessed for β-hydroxybutyrate.
Supplemental Figure 4. Liver specific knockdown of Acot1 does not affect de novo lipogenesis, TG synthesis, or VLDL secretion. Pulse-chase experiments were conducted with [$^{14}$C]glycerol, [$^{14}$C]acetate, and [$^{14}$C]oleate. Incorporation of [$^{14}$C]acetate (A) and [$^{14}$C]glycerol (B) into the triglyceride (TG) fraction after the 2 hour pulse was similar between treatments. (C-D) Acot1 knockdown does not alter fasting serum TG (C) or changes in serum TG in response to tyloxapal to measure rates of VLDL secretion (D). Media [$^{14}$C]TG after the 6 hour chase was similar between groups (E).
Supplemental Figure 5. Hepatic Acot1 knockdown increases oxygen consumption. Primary hepatocytes were isolated from control and Acot1 knockdown mice. OCR was determined in seahorse analyzer XF in the presence of (A) BSA control or (B) 500 uM oleate.
Supplemental Figure 6. *Acot1* point mutation S232A. *Acot1*-DsRed Express N1 (*Acot1*) and *Acot1*-S232A-DsRed Express N1 (*Acot1* S232A) express similarly in COS7 cells (A) compared to the DsRed Express N1 (EV) yet *Acot1*-S232A has no thioesterase activity.
Supplemental Figure 7. ACOT1 overexpression on lipid trafficking. L cells were transfected with Acot1-DsRed Express N1 (Acot1) and Acot1-S232A-DsRed Express N1 (Acot1 S232A) and treated with trace $^{14}$C-oleate along with 500 µM unlabeled oleate bound to BSA (3:1 molar ratio) overnight, then chased for 6 hours with isotope free media. Compared to the DsRed Express N1 (EV), and catalytically dead mutant, Acot1-S232A-DsRed Express N1 (Acot1 S232A), Acot1-DsRed Express N1 (Acot1) had less (A) FA oxidation and greater (B) TG retention.
**Supplemental Figure 8.** *Acot1* knockdown prevent cAMP induced PPARα target gene expression. AML12 cells were transduced with control shRNA or *Acot1* shRNA. Twenty-four hours later cells were treated with 500 µM oleate overnight followed by vehicle (veh) or 8-Br-cAMP for 6 hours prior to harvest. (A) *Acot1* expression in control and *Acot1* knockdown AML12 cells. (B) PPARα target gene expression. *P*<0.05 vs. veh. #P<0.05 vs. control shRNA.
Supplemental Figure 9. Hepatic Acot1 knockdown exacerbates complications on a high fat diet. Acot1 expression is reduced as measured by (A) Western blotting and (B) RT-PCR (n=8-10). Acot1 knockdown had no effect on hepatic TG (C) H&E and (D) liver TG quantification. (E) Acot1 knockdown increased serum β-hydroxybutyrate. Acot1 knockdown had no effect on
(F) PPARα and (G) PGC1α target gene expression, yet significantly reduced total (H) mitochondrial abundance. (I) Acot1 knockdown increased expression of inflammatory markers and oxidative stress genes. (J) Acot1 knockdown increased intracellular ROS measured by OxiSelect in vitro ROS/RNS Kit (n=4). (K) Sirius Red staining indicated more fibrosis with Acot1 knockdown.
Supplemental Figure 10. Hepatic Acot1 knockdown had no effect on hepatic FA content.

(A) Total liver FAs were not changed with Acot1 knockdown as well as no significant change in abundance of (B) specific FA species. (C) Nuclear preps were isolated from liver tissue, FAs were extracted via DOLE extraction method, and quantified.