Metabolic adaptation to intermittent fasting is independent of peroxisome proliferator-activated receptor alpha

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Guolin Li [1,2,3,**,5], Chad N. Brocker [1,5], Tingting Yan [1,5], Cen Xie [1], Kristopher W. Krausz [1], Rong Xiang [4], Frank J. Gonzalez [1,*]

ABSTRACT

Background: Peroxisome proliferator-activated receptor alpha (PPARA) is a major regulator of fatty acid oxidation and severe hepatic steatosis occurs during acute fasting in *Ppara*-null mice. Thus, PPARA is considered an important mediator of the fasting response; however, its role in other fasting regiments such as every-other-day fasting (EODF) has not been investigated.
Methods: Mice were pre-conditioned using either a diet containing the potent PPARA agonist Wy-14643 or an EODF regimen prior to acute fasting. *Ppara*-null mice were used to assess the contribution of PPARA activation during the metabolic response to EODF. Livers were collected for histological, biochemical, qRT-PCR, and Western blot analysis.
Results: Acute fasting activated PPARA and led to steatosis, whereas EODF protected against fasting-induced hepatic steatosis without affecting PPARA signaling. In contrast, pretreatment with Wy-14,643 did activate PPARA signaling but did not ameliorate acute fasting-induced steatosis and unexpectedly promoted liver injury. *Ppara* ablation exacerbated acute fasting-induced hypoglycemia, hepatic steatosis, and liver injury in mice, whereas these detrimental effects were absent in response to EODF, which promoted PPARA-independent fatty acid metabolism and normalized serum lipids.
Conclusions: These findings indicate that PPARA activation prior to acute fasting cannot ameliorate fasting-induced hepatic steatosis, whereas EODF induced metabolic adaptations to protect against fasting-induced steatosis without altering PPARA signaling. Therefore, PPARA activation does not mediate the metabolic adaptation to fasting, at least in preventing acute fasting-induced steatosis.

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Keywords PPARA; PPARalpha; Intermittent fasting; Every-other-day fasting; Steatosis; Adaptive fasting response

1. INTRODUCTION

Numerous studies have shown that intermittent fasting can prevent or delay the onset of metabolic diseases [1—3] including hepatic steatosis [4—6], while a single bout of acute fasting promotes hepatic lipid accumulation [7]. These studies indicate that the physiological response and underlying metabolic alterations associated with repeated fasting-refeeding cycles and a single acute fast are very different. In fact, acute fasting can impose systemic metabolic stress in most species [8], while fasting followed by refeeding triggers metabolic reprogramming, restores metabolic homeostasis, and is accompanied by additional alterations that protect against subsequent periods of food deprivation [8]. In this context, and according to the

physiological description of stress response and adaptive response [9,10], the physiological response to the initial damage caused by a single bout of acute fasting should be considered a 'stress response', while the protective effects that result from the habitual response to repeated stress followed by a recovery period that is imposed by fasting-refeeding cycles would be an 'adaptive response'. The adaptive response that results from fasting, and in particular refeeding, regimens may consequently lead to the many beneficial metabolic effects associated with intermittent fasting.
Intermittent fasting as an intervention in human populations is somewhat impractical; however, the development of therapeutics that target fasting associated pathways are of great interest as they avoid having to impose a strict dietary regime. Given that fasting is

[1]Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA [2]Laboratory of Aging Biochemistry, College of Life Sciences, Hunan Normal University, Changsha 410081, China [3]The Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Sciences, Hunan Normal University, Changsha, Hunan 410081, China [4]The State Key Laboratory of Medical Genetics & School of Life Sciences, Central South University, Changsha 41001, China

[5] These authors contributed equally to this study.

*Corresponding author. Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA. E-mail: gonzalef@mail.nih.gov (F.J. Gonzalez).

**Corresponding author. Laboratory of Aging Biochemistry, College of Life Sciences, Hunan Normal University, Changsha 410081, China. E-mail: hnsdlgl@hunnu.edu.cn (G. Li).

characterized by the depletion of hepatic glycogen, increased lipid utilization, and elevated serum ketone bodies [8] and that peroxisome proliferator-activated receptor alpha (PPARA) is a major regulator of fatty acid oxidation (FAO) and ketogenesis, this ligand-activated nuclear receptor is considered a key mediator of the fasting response [11–13]. Indeed, *Ppara*-null or liver-specific *Ppara*-null mice show a dramatic reduction of FAO, and display severe hepatic steatosis and hypoglycemia after acute fasting [11,14–16]. These studies provide evidence of a role for PPARA during fasting but provide no evidence for its role during refeeding. Therefore, it's difficult to differentiate whether PPARA mediates a stress response to fasting, an adaptive response to fasting, or both. Moreover, current evidence on the role of pharmacological PPARA activation using a variety of agonists in steatosis is inconsistent. Some studies support the view that activation of PPARA can ameliorate steatosis in several models [17–19], some do not [20–22], and one report even suggested that PPARA can promote steatosis [23]. Collectively, current evidence indicates that although PPARA governs FAO, and loss of PPARA exacerbates acute fasting-induced steatosis and hypoglycemia in mice, the concept that PPARA is a mediator of the adaptive response to fasting remains to be elucidated. If PPARA is indeed responsible for the metabolic adaptation to fasting, it would be reasonable to expect that 1) pharmacological activation of PPARA prior to fasting would simulate the adaptive response and substantially ameliorate acute fasting-induced steatosis, and 2) mice pre-conditioned by repeated fasting-refeeding cycles would activate PPARA and suppress acute fasting-induced steatosis.

Therefore, in the current study, wild-type and *Ppara*-null mice were placed on an every-other-day fasting (EODF) regimen and/or treated with the potent PPARA agonist Wy-14643 to explore the effect of PPARA on the adaptive response to fasting. The results suggests that although PPARA deficiency aggravates acute fasting-induced steatosis, EODF elicits a pronounced metabolic adaptation to prevent fasting-induced hepatic steatosis independent of PPARA.

2. MATERIALS & METHODS

2.1. Mice
All procedures involving mice were performed under protocols approval by the National Cancer Institute Animal Care and Use Committee. Six-week-old male C57BL/6N mice were purchased from Charles River Laboratories. Six-to eight-week-old wild-type (WT) or *Ppara*-null (*Ppara*$^{-/-}$) mice on the C57BL/6N background were described previously [24,25]. Mice were housed in a temperature- and light-controlled vivarium with free access to water and standard rodent chow (NIH-07, Envigo, Huntingdon, United Kingdom), a standard control grain diet (F3028, Bio-Serve, Flemington, NJ), or a standard control grain diet containing 0.1% Wy-14643 (Bio-Serv, Flemington, NJ). Male 2- to 4-month-old mice were used in all experiments unless otherwise indicated.

2.2. EODF and Wy-14643 treatment
Mice were randomly divided into control (CON) and intermittent fasting groups. All mice were fed with standard chow diet and co-housed (2 mice per cage) for two weeks prior to study initiation to allow for acclimation to the animal facility. The CON group was allowed unrestricted, *ad libitum* access to a chow diet, while the intermittent fasting group was fed on alternating 24 h periods of free access to food followed by fasting for 20 cycles, referred to as every-other-day fasting (EODF). After the pre-conditioned intermittent fasting period, the EODF mice were divided in half; one set was killed at the end of a 24 h feeding period, and the other set was killed at the end of a 24 h fasting period. Control mice were also split into two groups with half of the animals killed at the end of a 24 h feeding period and the remaining half killed after a 24 h fasting period to match the fasted EODF group. In the *Ppara*-null versus WT mouse study, mice were fasted 16 h due to the pronounced steatosis and liver damage observed in fasted *Ppara*-null mice in response to prolonged fasting. For the Wy-14643 studies, the mice were allowed unrestricted access to a standard control grain diet for two weeks to allow for acclimation to the grain diet, and then randomly separated into two groups and placed on either a diet containing 0.1% Wy-14643 (100 mg/kg/day) or a matching grain control diet for two days. Half of the mice were then killed in the fed state, and the other half were killed after fasting.

2.3. Respiratory exchange ratio assay
Respiratory exchange ratios (RER) were measured by indirect calorimetry in mice after 14 cycles of EODF treatment using a 12-chamber Environment Controlled CLAMS (Columbus Instruments, Columbus, OH) with one mouse/chamber as previously described [26]. After a 48 h chamber acclimatization, the mice were monitored for 24 h during the fed state followed by 20 h during the fasting state. During testing, water was provided *ad libitum*.

2.4. Serum glucose and fasting blood glucose assay
For assaying serum glucose, blood samples were collected into tubes containing no anticoagulant from the retro-orbital sinus per a standard procedure (Parasuraman et al., 2010) and then were sequentially clotted for 30 min on ice, centrifuged at 2,000 g for 15 min at 4 °C, the top yellow serum layer, and glucose measured using a Glucometer (Bayer, Pittsburgh, PA). Fasting blood glucose was detected using a Glucometer (Bayer, Pittsburgh, PA) by tail bleeds after fasting for 16 h.

2.5. Serum biochemical assays
Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assessed in a 96-well microplate using a commercial ALT or AST assay kit (Catachem, Bridgeport, CT), and monitored at 340 nm for 5 min with a microplate reader (BioAssay Systems, Harvard, CA). Serum ketone bodies and non-esterified fatty acids (NEFA) were assayed using Wako Clinical Diagnostics kits (Wako USA, Richmond, VA). Serum insulin levels were tested with an ultra-sensitive mouse insulin ELISA kit (90080, Crystal Chem, Downers Grove, IL).

2.6. Liver lipid and glucose analysis
For analysis of liver lipid and glucose content, 20 mg of frozen liver was homogenized in 400 μl of 50 mM Tris + 5% Triton-X 100, then samples were heated to 80–100 °C and cooled to room temperature (repeated twice). After centrifugation, the supernatants were diluted 1–5 fold, lipids were quantified using Wako Clinical Diagnostics kits (Wako USA, Richmond, VA), and glucose concentrations assayed using a Glucometer (Bayer, Pittsburgh, PA).

2.7. Serum acylcarnitines profiling
Serum acylcarnitines were quantified as previously described [27]. In brief, 5 μl serum was diluted with 45 μl acetonitrile containing 0.1 μM D3-palmitoylcarnitine and centrifuged at 16,000 g for 15 min at 4 °C. The supernatant was removed, dried under vacuum, re-dissolved in HPLC-grade water containing 2% acetonitrile, and assayed by LC-MS. All MS data were analyzed by Waters MassLynx software.

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2.8. Total RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was extracted from frozen tissues using TRIzol reagent per the manufacturer's instructions. The purities and concentrations of total RNA samples were determined by a NanoDrop spectrophotometer (ND-1000, Thermo-Fisher, Waltham, MA). Fifty ng of total RNA was reverse transcribed using cDNA Synthesis SuperMix (Biotool.com). Real-time PCR was carried out in an ABI 7900HT Fast Real-Time PCR System (AB Applied Biosystems, Warrington, UK) with SYBR Green PCR master mix (AB Applied Biosystems) and gene-specific primers. The sequences for the forward and reverse primers used to quantify mRNA are listed in Supplementary Table 1. The following conditions were used for real-time PCR: 95 °C for 5 min, then 95 °C for 15 s, and 60 °C for 1 min in 40 cycles. The $2^{-\Delta\Delta CT}$ method [28] was used to analyze the relative changes in gene expression normalized against 18S rRNA expression. qRT-PCR experiments were designed and performed according per Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [29].

2.9. Western blot analysis

Liver was lysed in RIPA buffer supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo-Fisher, Waltham, MA) and 1 mM PMSF. Protein concentrations were determined using Pierce BCA Protein Assay Kit (Pierce, Rockford, IL). Twenty-five micrograms of protein per lane were loaded onto a 4−12% Criterion TGX Precast Gel (Bio-Rad, Hercules, CA) then transferred to PVDF membranes using a Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA). Membranes were blocked in 5% non-fat milk followed by overnight incubation with primary antibody at 4 °C. Primary antibodies used are as follows: anti-ACADM (55210, Proteintech, Rosemont, IL) and anti-CPT2 (ab181114, Abcam, Cambridge, MA). Following primary antibody incubation, the blots were washed and incubated with an anti-rabbit IgG HRP-conjugated secondary antibody (#7074S, Cell Signaling Technology, Danvers, MA). Blots were stripped with Restore Western Blotting Stripping Buffer (Thermo-Fisher, Waltham, MA) and re-probed with anti-ACTB for normalization (ab8227, Abcam, Cambridge, MA). Blot imaging was performed on a ChemiDoc™ MP System (Bio-Rad, Hercules, CA) after exposing blot to Clarity Western ECL Blotting Substrate (Bio-Rad, Hercules, CA).

2.10. Hematoxylin and eosin (H&E) staining

All tissues were fixed in 4% paraformaldehyde for 24 h at room temperature, dehydrated, and embedded into paraffin. The tissues were sectioned into 4 μm slices and stained with hematoxylin and eosin. Digital images were collected at 40× magnification with a Keyence BZ-X710 fluorescence microscope (Keyence Inc., Japan). Images shown are representative results of at least three biological replicates.

2.11. Statistical analysis

All results are expressed as means ± SEM. Significance was determined by t-test or two-way ANOVA with Tukey posttest using Prism 7.0 software (GraphPad Software). A P value of less than 0.05 was considered significant.

3. RESULTS

3.1. Acute fasting induced hepatic steatosis and increased *Ppara* expression

In mammals, one of the most pronounced physiological changes that occurs during fasting is the metabolic transition from use of primarily carbohydrates as an energy source in the fed state to use of lipids in the fasted state. Whole-body RERs can reveal whether lipid or glucose is primarily being used as a fuel source. Indeed, this transition did occur in mice as their whole-body RER in the fasting state were close to 0.7 (Figure S1), an index of fat utilization [30,31]. After 24 h of fasting, there was an obvious accumulation of lipids in the liver (Figure 1A,B), most likely due to the result of increased adipose lipolysis and subsequent transport of fatty acids to the liver [32]. Hepatic PPARA modulates genes involved in peroxisomal and mitochondrial FAO and is considered an important transcription factor that mediates the adaptive response to starvation [11,33]. The expression of PPARA and its target gene mRNAs *Fgf21* and *Cpt1a* were increased in acute-fasted mice (Figure 1K), although the expression of another PPARA target gene, *Acadm*, that is directly involved in FAO, was unchanged at both the mRNA (Figure 1K) and protein levels (Figure 1F). PPARA activation under these conditions promotes the transport of circulating lipids into the liver where they may act as endogenous PPARA ligands during fasting [34,35]. These results are consistent with the current view that PPARA is responsible for the metabolic adaptation to fasting, which stems from the fact that *Ppara*-null mice exhibit exacerbated fasting-induced hepatic steatosis [11,13,14]. However, given that prolonged metabolic adaptation to fasting mainly occurs during refeeding cycles [8], with only evidence from a fasting state, it is difficult to discriminate whether the function of PPARA is responsible for the stress response to fasting or the metabolic adaptation to fasting. To clarify this concept, mice were subjected to Wy-14,643 administration or intermittent fasting to explore the relationship between adaptive response to fasting and PPARA activation.

### 3.2. Wy-14,643 activated PPARA but did not ameliorate acute fasting-induced steatosis

First, the effect of PPARA activation on acute fasting was evaluated by pretreating mice with the potent PPARA agonist Wy-14643. A 2-day Wy-14643 treatment activated PPARA target gene mRNA expression in both fed and fasted mice (Figure 1K), and resulted in a corresponding increase in ACADM and CPT proteins in the fed state (Figure 1F). *Acadm* mRNA and ACADM protein were further increased after acute fasting, whereas, CPT2 protein levels decreased significantly (Figure 1F). The drop in CPT2 expression is surprising as this protein is required for the transport of long-chain fatty acids into the mitochondria for β-oxidation. As expected, Wy-14643 also decreased serum triglycerides (TG) in the fed state (Figure 1C), but it did not ameliorate acute fasting-induced hepatic steatosis (Figure 1A,B). Wy-14643 treatment also did not change the levels of serum NEFA (Figure 1D), ketone bodies (Figure 1E), and insulin (Figure 1G) in the fed state. Although Wy-14643 treatment did not affect blood glucose levels (Figure 1H), it promoted hepatic glucose depletion (Figure 1I) and liver injury as revealed by elevated serum ALT activities (Figure 1J) in both the fed and fasted states. In the fed state, Wy-14643 suppressed expression of the rate-limiting genes involved in gluconeogenesis (*Fbp1*), glycogenesis (*Gys2*), and glycogenolysis (*Pygl*) in the liver (Figure 1K). RER results clearly showed that Wy-14,643 treatment decreased glucose utilization during the fed state and accelerated fuel switching to fat during the fasting period (Figure S1). While it cannot be concluded that the hypolipidemic effect of Wy-14,643 is owing to its inhibition of glucose metabolism, these results provide a potential explanation for why Wy-14,643 treatment did not ameliorate hepatic steatosis. Collectively, these data indicate that pharmacological PPARA activation prior to acute fasting does not improve hepatic steatosis but appears to exacerbate liver damage.
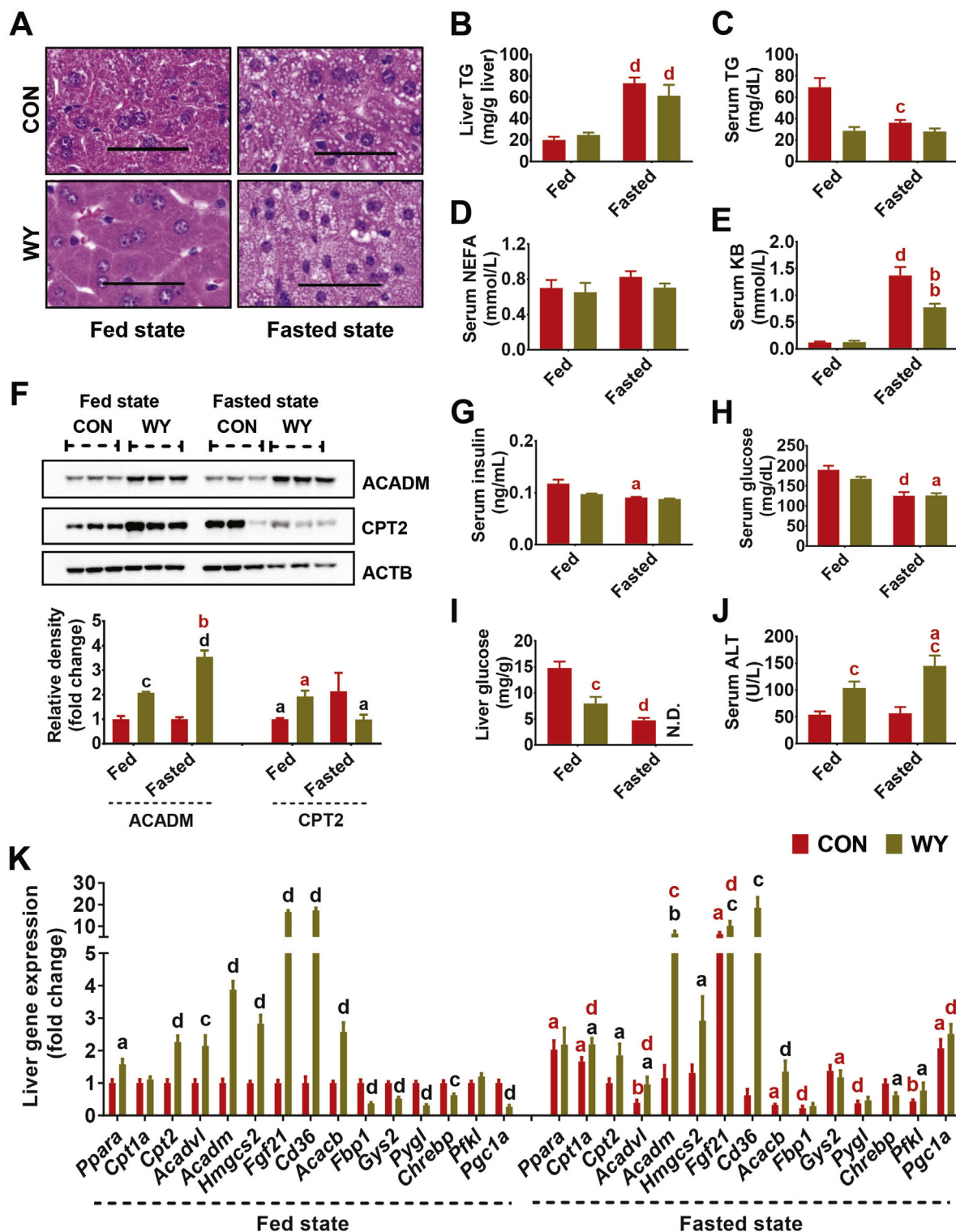
Figure 1: The effect of Wy-14643 on acute fasting-induced liver steatosis. (A) Representative H & E staining of liver in CON (upper) and Wy-14643-treated (lower) mice in the fed (left) and fasted (right) states. Scale bar = 100 μm. (B−C) Average liver (B) and serum (C) triglycerides in CON and Wy-14,643-treated mice in both the fed and fasted states (n = 7−8 mice). (D−E) Serum NEFA (D) and ketone bodies (E) levels of CON and Wy-14643-treated mice in both the fed and fasted states (n = 7−8 mice). (F) Western blot analysis of whole cell extracts from livers of WT mice. Blots were probed with antibodies targeting ACADM, ACTB, and CPT2. Densitometry values were normalized to ACTB expression for each lane and presented as fold change of CON mice in the fed state. (G−J) Serum insulin (G), serum glucose (H), liver glucose (I), and serum ALT (J) levels of CON and Wy-14643-treated mice in both the fed and fasted states (n = 7−8 mice). (K) Liver mRNA expression (fold change to CON fed mice) in both the fed and fasted states (n = 7−8 mice). Data are presented as mean ± SEM. Different lowercase letters indicate statistical significance (a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.005$; and d, $p < 0.001$). Black letters show the effects of WY [Wy-14643 versus control (CON) within the same feeding state], and red letters show the effects of fasting (fasted versus fed state within the same treatment).
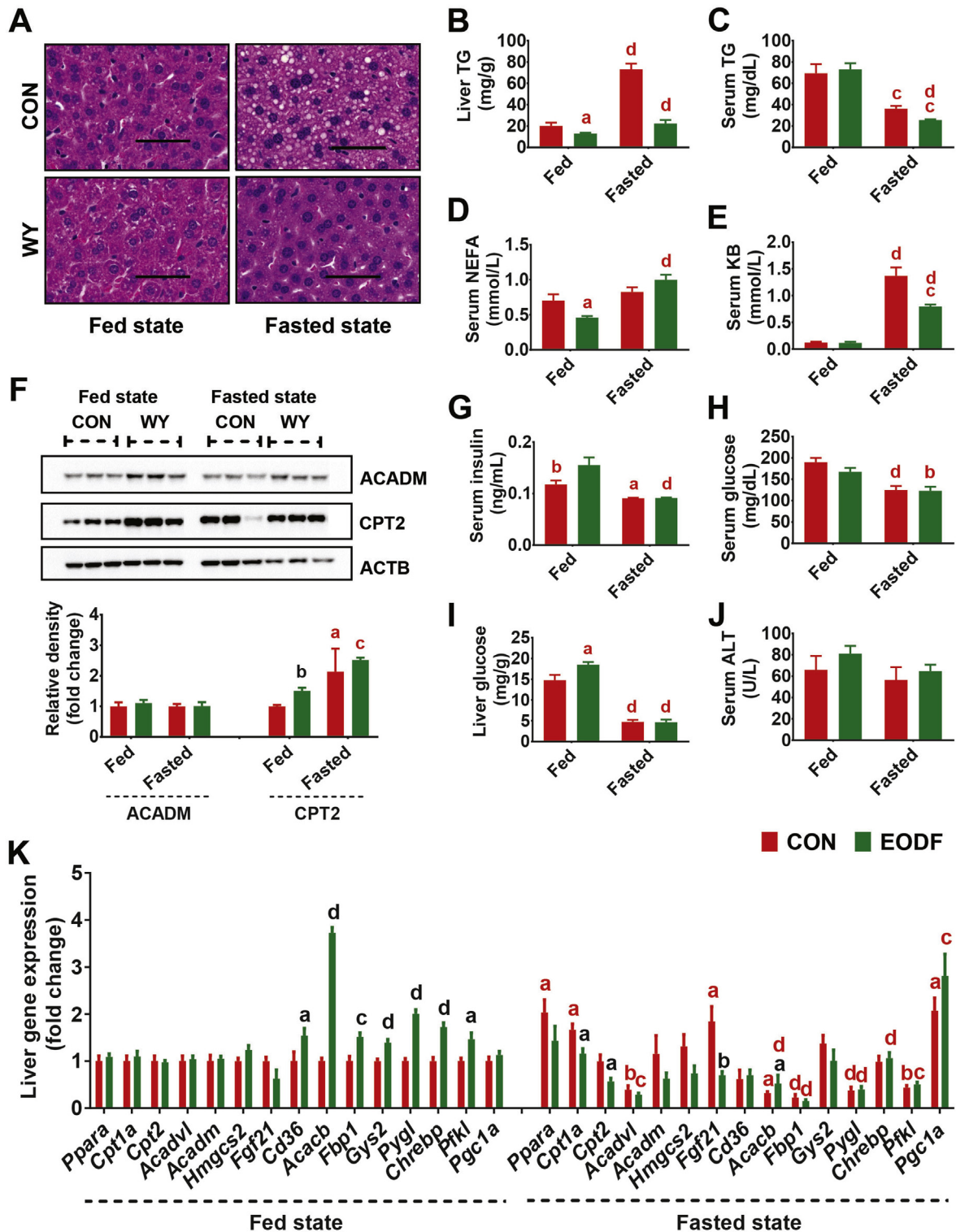
Figure 2: The effect of EODF on acute fasting-induced liver steatosis. (A) Representative H & E staining of liver in CON (upper) and EODF (lower) mice in the fed (left) and fasted (right) states. Scale bar = 100 μm. (B—C) Average liver (B) and serum (C) triglycerides in CON and EODF mice in both the fed and fasted states (n = 7—8 mice). (D—E) Serum NEFA (D) and ketone bodies (E) levels of in CON and EODF mice in both the fed and fasted states (n = 7—8 mice). (F) Western blot analysis of whole cell extracts from livers of WT mice. Blots were probed with antibodies targeting ACADM, ACTB, and CPT2. Densitometry values were normalized to ACTB expression for each lane and presented as fold change of CON mice in the fed state. (G—J) Serum insulin (G), serum glucose (H), liver glucose (I), and serum ALT (J) levels of CON and EODF mice in both the fed and fasted states (n = 7—8 mice). (K) Liver mRNA expression (fold change to CON mice) in both the fed (K) and fasted (L) states (n = 7—8 mice). Data are presented as mean ± SEM. Different lowercase letters indicate statistical significance (a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.005$; and d, $p < 0.001$). Black letters show the effects of EODF [EODF versus control (CON) within the same feeding state], and red letters show the effects of fasting (fasted versus fed state within the same treatment).
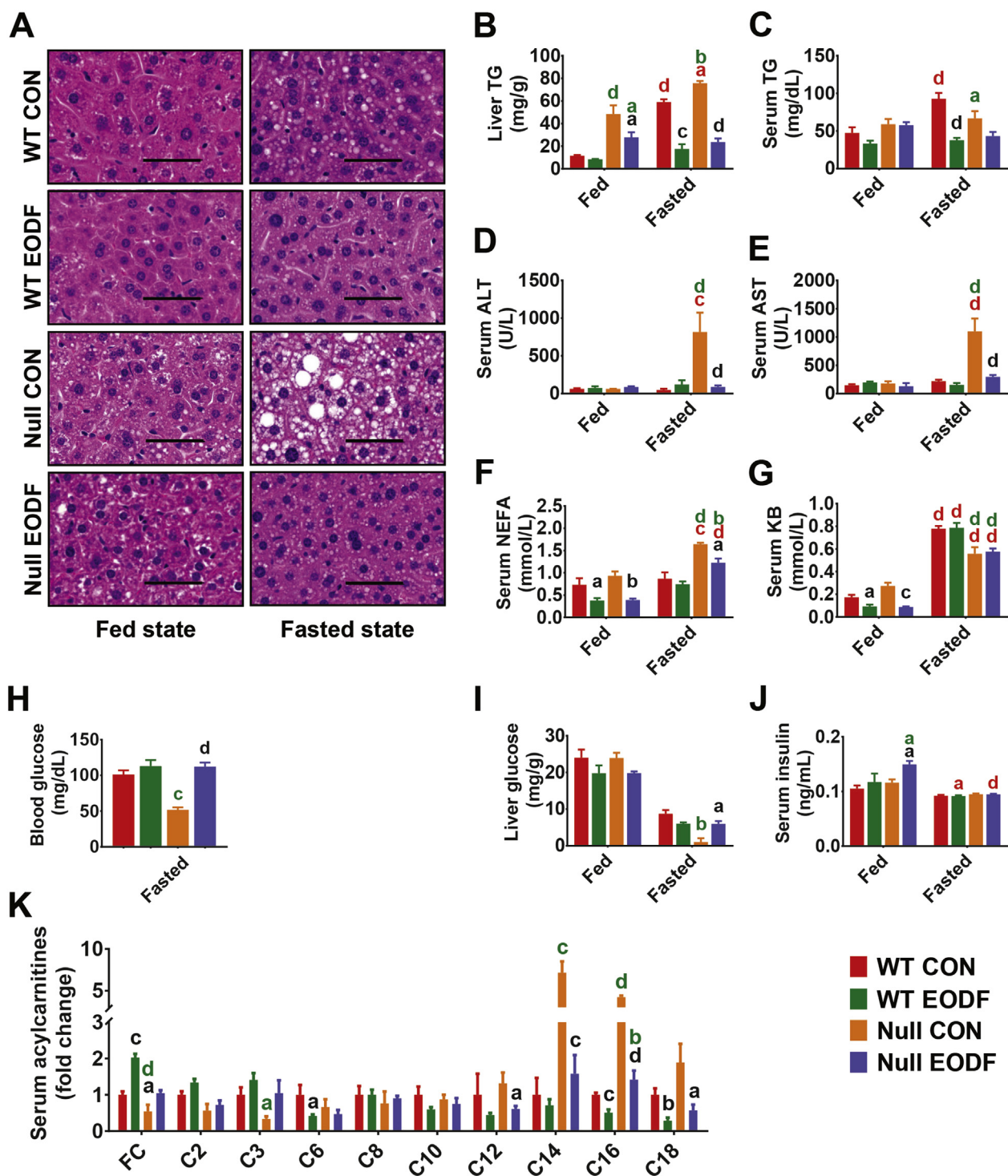
Figure 3: EODF reversed acute fasting-induced hepatic steatosis in *Ppara*-null mice. (A) Representative H & E staining of liver in CON (row 1) and EODF (row 2) wild-type (WT) mice or CON (row 3) and EODF (row 4) *Ppara*-null mice in the fed (left) and fasted (right) states. Scale bar = 100 μm. (B−C) Average liver (B) and serum (C) triglycerides of wild-type (WT) and *Ppara*-null mice fed CON and EODF in both the fed and fasted states (n = 4−5 mice). (D−G) Serum ALT (D), AST (E), NEFA (F), and ketone bodies (G) levels of wild-type (WT) and *Ppara*-null mice fed CON and EODF in both the fed and fasted states (n = 4−5 mice). (I−J) Average liver (I) and serum (J) glucose levels of wild-type (WT) and *Ppara*-null mice fed CON and EODF in both the fed and fasted states (n = 4−5 mice). (K) Serum acylcarnitines profiling in wild-type (WT) mice and *Ppara*-null mice in the fasted state after treated with or without EODF. (n = 4−5 mice). Data are presented as mean ± SEM. Different lowercase letters indicate statistical significance (a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.005$; and d, $p < 0.001$). Black letters show the effects of EODF (EODF versus CON within the same strain and the same feeding state), red letters show the effects of fasting (fasted versus fed state within the same treatment and the same strain), and green letters show the effects of *Ppara*-null (*Ppara*-null versus wild-type mice within the same treatment and the feeding state). FC, free carnitine.
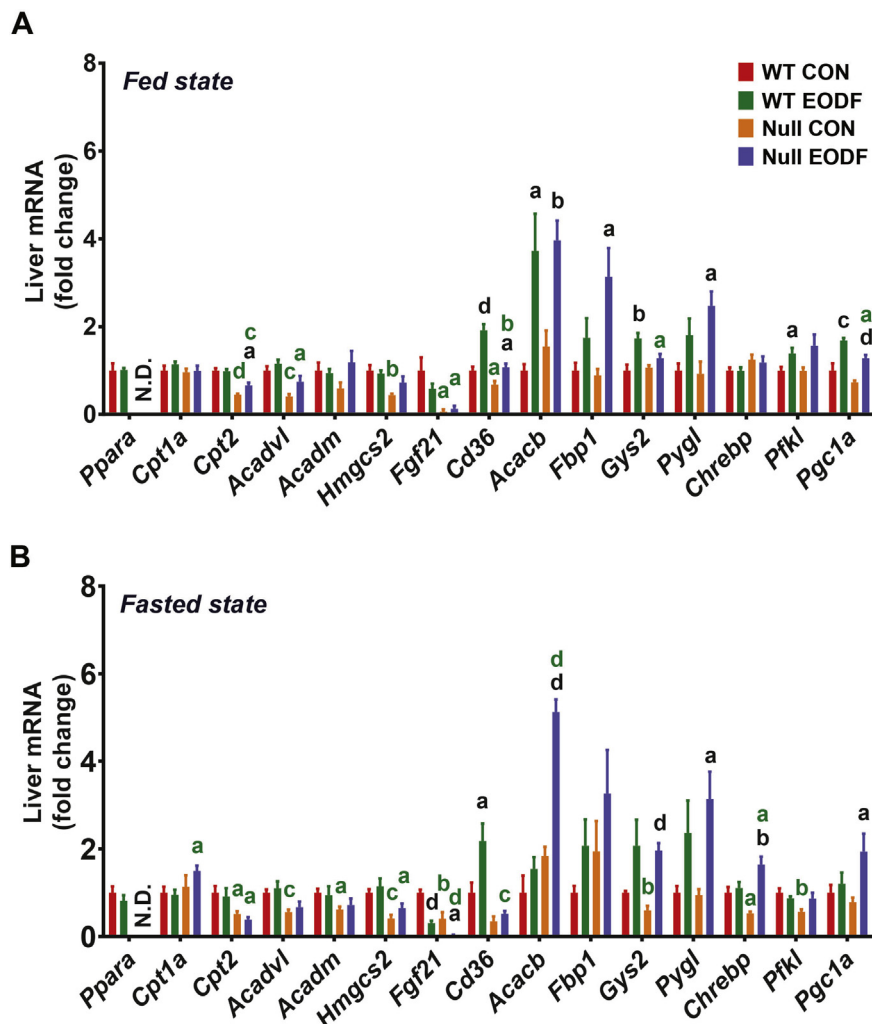
Figure 4: Effects of EODF on liver gene expression in wild-type and *Ppara*-null mice. (A—B) Fold change of *Ppara* mRNA and PPARA target gene mRNAs, FAO key gene mRNAs, and critical genes involved in glucose metabolism in mouse liver after normalized against wild-type (WT) CON mice in the fed state (A) and fasted state (B), n = 4—5 mice/group. Data are presented as mean ± SEM. Different lowercase letters indicate statistical significance (a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.005$; and d, $p < 0.001$). Black letters show the effects of EODF (EODF versus CON within the same strain and the same feeding state), and green letters show the effects of *Ppara*-null (*Ppara*-null versus *Ppara* WT mice within the same treatment and the feeding state).

3.3. EODF prevented acute fasting-induced hepatic steatosis but did not activate PPARA

Intermittent fasting was recently reported to have beneficial health effects [3]. However, it is not clear whether intermittent fasting can impart the metabolic adaptation necessary to ameliorate acute fasting-induced steatosis. To elucidate the impact of intermittent fasting on steatosis, mice were pre-conditioned by EODF, then split into two cohorts with one group killed during the fed state and the other killed at the end of the final fasting cycle. The EODF mice illustrated an obvious resistance to acute fasting-induced steatosis (Figure 2A,B). EODF treatment significantly decreased liver TG in both the fed and fasted states (Figure 2B), serum TG in the fasted state (Figure 2C), serum non-esterified free fatty acids (NEFA) in the fed state (Figure 2D), and serum ketone body levels in the fasted state (Figure 2E). EODF did not upregulate the expression of hepatic *Ppara* mRNA or most PPARA target gene mRNAs (*Cpt1a, Cpt2, Acadvl,* and *Acadm*) in the fed state (Figure 2K), and did not upregulate ACADM protein levels in either fed and fasted states or CPT2 protein in the fasted state (Figure 2F). Intriguingly, EODF increased serum insulin levels (Figure 2G), although

EODF did not affect serum glucose obviously in either fed or fasted states (Figure 2H), intermittent fasting did enhance liver glucose levels (Figure 2I). Moreover, no mice exhibited liver injury (Figure 2J). In line with the above data, EODF upregulated the expression of *Fbp1, Gys2, Pygl, Chrebp,* and *Pgc1a* mRNAs involved in glucose metabolism in the fed state (Figure 2K). RERs of EODF mice in the fed state supported elevated glucose utilization (Figure S1). Collectively, compared to PPARA activation by Wy-14,643, these data indicate that EODF had an opposite effect on most fasting responses. EODF prevents acute fasting-induced steatosis but does not appear to strongly activate PPARA, while Wy-14,643 activates PPARA but does not alleviate steatosis. Taken together, the above data indicate that the beneficial effects of intermittent fasting do not require PPARA activation.

3.4. EODF reversed acute fasting-induced hepatic steatosis in *Ppara*-null mice

To further explore the role of PPARA in EDOF, *Ppara*-null mice and matched wild-type mice were exposed to the EODF regimen at similar endpoints to characterize its contribution. Consistent with current

evidence [11,13], PPARA ablation promoted fatty acid accumulation in the liver (Figure 3A,B) and suppressed the expression of many target genes directly involved in FAO, including *Cpt2, Acadvl,* and *Acadm* (Figure 4A,B). Acute fasting resulted in significantly more severe hepatic steatosis in *Ppara*-null mice than in wild-type mice (Figure 3A,B). PPARA deficiency reduced the fasted serum free carnitines and short-chain acylcarnitines, but promoted the accumulation of long-chain acylcarnitine species (Figure 3K). These data are consistent with earlier reports that PPARA plays a role in regulating hepatic lipid metabolism and that PPARA deficiency aggravates fasting-induced steatosis [11,13]. To explore the role of PPARA during the metabolic response to fasting and refeeding cycles, both wild-type mice and *Ppara*-null mice were placed on an EODF regimen. The results demonstrated that EODF-induced adaptation appeared to occur independent of PPARA (Figures 3 and 4). EODF prevented acute fasting-induced hepatic steatosis in both wild-type mice and *Ppara*-null mice (Figure 3A,B), suppressed the fasted serum TG levels (Figure 3C), and prevented fasting-induced hepatic injury in *Ppara*-null mice as indicated by serum ALT and AST activities (Figure 3D,E). EODF lowered serum NEFA and ketone bodies concentrations in the fed state (Figure 3F,G). Although EODF did not rescue expression of *Cpt2, Acadvl,* and *Acadm* mRNAs encoding critical FAO enzymes in the fasted state in *Ppara*-null mice (Figure 4B), intermittent fasting did promote an increase in fasting serum free carnitine and short-chain acylcarnitines levels while preventing the accumulation of long-chain acylcarnitine species in both wild-type and *Ppara*-null mice (Figure 3K). These findings suggest that the EODF-induced metabolic adaptation to fasting is not mediated by enhancing FAO but rather by other mechanisms that might reduce fatty acid transport to the liver. One possibility is increased metabolism of fatty acids *in situ* in adipose tissue, through activating beige fat in white adipose tissue [36]. Another possible explanation is that EODF enhances glucose utilization and storage in the fed state. This is supported by data indicating that EODF in *Ppara*-null mice increased fasting blood and liver glucose concentrations to levels that were similar to those in wild-type mice (Figure 3H,I, and Figure S2). Moreover, EODF enhanced serum insulin levels in the EODF *Ppara*-null mice in the fed state (Figure 3J). EODF upregulated the expression of many genes involved in glucose metabolism in both the fed and fasted states (Figure 4). EODF-induced alterations in gene expression occurred independent of PPARA status and did not include modulation of many typical PPARA targets (Figure 4). Collectively, all the above evidence indicates that the intermittent fasting-induced metabolic adaptation takes place primarily independent of PPARA activation.

4. DISCUSSION

PPARA is a ligand-activated nuclear transcription factor that plays an essential role in the regulation of energy metabolism by modulating the expression of genes involved in fatty acid transport and oxidation. As PPARA deficiency in mice suppresses FAO gene expression and exacerbates fasting-induced steatosis, PPARA is considered an important mediator of metabolic adaptations to acute fasting [11,13,14]. The current study aimed to elucidate the role of PPARA in response to fasting and, at the same time, assess whether pre-conditioning mice using an EODF regiment or a PPARA agonist could prevent the hepatic steatosis that accompanies prolonged fasting. Evidence from the current study revealed that pharmacological activation of PPARA prior to acute fasting does not prevent or reduce fasting-induced hepatic steatosis. In fact, pre-conditioning using the PPARA agonist Wy-14643 increased liver damage as indicated by a pronounced elevation of

serum ALT levels. This observation is in agreement with recent clinical data suggesting that PPARA agonists had no effect on preventing or treating hepatic steatosis [20—22] as well as another report showing an increase in hepatic TG accumulation after treatment with the PPARA agonist fenofibrate [23]. Evidence from the current study indicates that EODF prevented acute fasting-induced steatosis. However, the EODF-associated improvements in hepatic steatosis and fatty acid metabolism occurred independent of PPARA activity, as EODF in *Ppara*-null mice also protected against lipid accumulation. These results are somewhat counterintuitive as PPARA activation would be expected to increase hepatic FAO and prevent lipid buildup, and PPARA is commonly described as a mediator of fasting response [11,13,14]. However, these results are in agreement with recent findings on the adaptive mechanism for the EODF regimen [36]. EODF can selectively induce white fat beiging and increase beige thermogenesis, which subsequently ameliorates hepatic steatosis and other pathological outcomes associated with metabolic disease. Intriguingly, the EODF-induced adipose beiging also occurred independent of PPARA [36].

Fasting promotes a pronounced shift from utilizing carbohydrates to fatty acids as a primary energy source. A primary finding from the current study demonstrates that the metabolic adaptation to EODF includes enhanced glucose storage and utilization in the fed state. Intriguingly, EODF nearly reversed acute fasting-induced hepatic steatosis, even in *Ppara*-null mice. Although the mechanism for fasting-induced fatty liver might vary from other models of steatosis, aberrant hepatic lipid accumulation commonly results from an imbalance between fatty acid mobilization, transport, and catabolism [37]. The present findings suggest that intermittent fasting both promotes carbohydrate utilization and decreases hepatic steatosis. Intermittent fasting, therefore, may provide a potentially novel strategy for regulating the balance between TG uptake and removal and subsequently represent a therapeutic option for the treatment of fatty liver. An important finding in this study is that EODF can effectively restore deficiencies in fat catabolism caused by loss of PPARA. In the fasted state, PPARA deficiency causes an obvious disturbance in fatty acid metabolism as there is a significant decrease and increase in serum short-chain and long-chain acylcarnitines, respectively, which is consistent with a previous report [38]. Intriguingly, EODF restores serum acylcarnitines in *Ppara*-null mice to levels that correspond to those of wild-type animals, and the efficiency seems to be comparable to carnitine supplementation [38]. Thus, EODF-associated pathways are promising therapeutic targets for treating diseases that stem from defects in fatty acid metabolism.

In conclusion, pharmacological PPARA activation cannot ameliorate acute fasting-induced hepatic steatosis, while EODF induces a clear metabolic adaptation to fasting and substantially prevents fasting-induced hepatic steatosis independent of PPARA. Therefore, PPARA activation may not mediate the main metabolic adaptation to fasting, at least in preventing acute fasting-induced steatosis

Original Article

CONFLICT OF INTEREST

The authors declare no competing financial interests or conflicts of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2017.10.011.

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