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BRIEF REPORTS

A System for In Vivo Imaging of Hepatic Free Fatty Acid Uptake

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Alterations in hepatic free fatty acid (FFA) uptake and metabolism contribute to the development of prevalent liver disorders such as hepatosteatosis. However, detecting dynamic changes in FFA uptake by the liver in live model organisms has proven difficult. To enable noninvasive real-time imaging of FFA flux in the liver, we generated transgenic mice with liver-specific expression of luciferase and performed bioluminescence imaging with an FFA probe. Our approach enabled us to observe the changes in FFA hepatic uptake under different physiological conditions in live animals. By using this method, we detected a decrease in FFA accumulation in the liver after mice were given injections of deoxycholic acid and an increase after they were fed fenofibrate. In addition, we observed diurnal regulation of FFA hepatic uptake in living mice. Our imaging system appears to be a useful and reliable tool for studying the dynamic changes in hepatic FFA flux in models of liver disease.

Keywords: Mouse Model; Visualization; Metabolism; Lipid.

In obesity, plasma level of free fatty acids (FFAs) usually are increased and are associated with an increased risk of hepatosteatosis, the hallmark feature of nonalcoholic fatty liver disease.[1] Some deleterious effects of excessive FFAs on liver function can be prevented by inhibiting fatty acid transport proteins (FATPs) in the liver, thereby reducing FFA hepatic uptake.[2] Thus, quantitative monitoring of long-term hepatic FFA uptake in vivo should be of paramount importance for lipid research and liver-associated metabolic disorders.

We recently developed a bioluminescence imaging (BLI) probe for monitoring FFA uptake (S)-2-(6-[(3-[(15-carboxypentadecyl)disulfanyl]propoxy)carbonyloxy]benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (FFA-Luc).[3] FFA-Luc is a C16 long-chain fatty acid linked to luciferin via a disulfide bond. It is taken up via physiological, compatible, transporter-mediated processes[3] and upon uptake uncages luciferin as a result of reducing the intracellular environment, resulting in cleavage of the disulfide bond. Thus, in luciferase-expressing cells, FFA-Luc uptake results in the proportional generation of photons.[3] By using this probe we could detect FFA uptake from the intestine and distinct sites such as brown adipose tissue in mice that express luciferase under the actin promoter (FVB-Luc$^+$).[3] However, we were not able to determine hepatic fatty acid uptake owing to the high scattering of multiple signals from the abdominal cavity. To circumvent this problem, we generated transgenic mice expressing luciferase under the control of the albumin promoter for liver-specific luciferase expression (L-Luc mice).

After luciferin intraperitoneal injection, although FVB-Luc$^+$ mice showed signal throughout the body, the signal from L-Luc mice was liver specific (Figure 1*A*). To further confirm liver-specific luciferase expression, we harvested several organs and detected light emission only in the liver (Figure 1*B*). The dose-response in L-Luc mice was investigated by injecting 2, 10, and 20 mmol/L of luciferin and 10, 50, and 100 μmol/L of FFA-Luc, followed by monitoring total photon flux for 50 minutes by BLI. The results indicated a dose-dependent increase in total photon flux after both luciferin and FFA-Luc injection (Figure 1*C* and *D*).

Because the probe is taken up by all FFA using tissues,[3] we considered the possibility that luciferin uncaged in extrahepatic tissues could circulate back to the liver and thus contribute to the hepatic BLI signal independent of hepatic FFA uptake. To determine serum levels of FFA-Luc–derived free luciferin, we measured luciferin content in serum samples of FFA-Luc- and luciferin-injected wild-type mice 10 and 20 minutes after injection (Figure 1*E*). Based on the serum luminescence data, we calculated a serum-free luciferin of 0.16 μmol/L and further determined that this serum concentration of luciferin can be achieved using a single 100 μL intraperitoneal injection of 4 μmol/L free luciferin. We injected this dose into the L-Luc mice to determine the signal intensity in the liver generated by free circulating luciferin at a concentration expected to be reached by extrahepatically generated breakdown of the FFA-Luc probe (Figure 1*F*). The results indicated that the maximal total signal generated by 0.16 μmol/L circulating luciferin was less than 4% of the total signal we observed

Abbreviations used in this paper: **BLI, bioluminescence imaging; BODIPY, boron-dipyrromethene; DCA, deoxycholic acid; FATP, fatty acid transport protein; FFA, free fatty acid; FFA-Luc, (S)-2-(6-[(3-[(15-carboxypentadecyl)disulfanyl]propoxy)carbonyloxy]benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid; L-Luc, liver-specific luciferase; ZT, zeitgeber time.**

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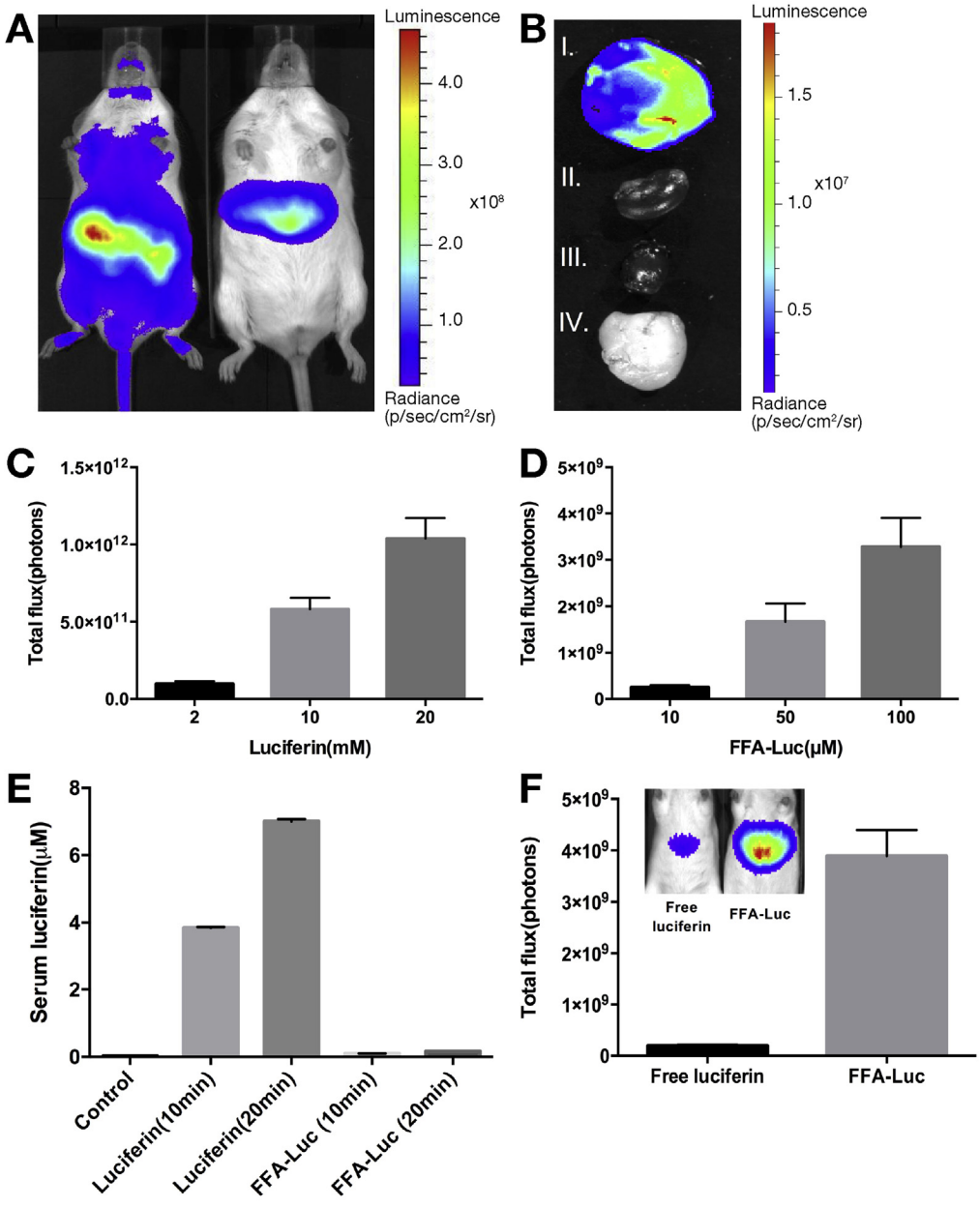

Figure 1. Generation of liver-specific transgenic mice and model validation. (*A*) Ventral luminescent/photographic overlay comparing the BLI of FVB-Luc^{+} (*left*) and L-Luc (*right*) mice 5 minutes after intraperitoneal injection of luciferin. (*B*) Luminescent/photographic overlay of FFA-Luc uptake by the liver (I), kidney (II), heart (III), and white adipose tissue (IV) from L-Luc mice 5 minutes after luciferin administration. Total photon flux (*C*) 0–25 minutes after the injection of luciferin and (*D*) 0–50 minutes after the injection of FFA-Luc at the indicated concentrations. Error bars are ± SEM of 3 independent experiments. (*E*) Detection of free serum luciferin before injection, 10 and 20 minutes after the injection of luciferin and FFA-Luc. Error bars are ± SEM (n = 5). (*F*) Luminescence emitted by free luciferin injected at a dose (100 μL at 4 μmol/L) to match the serum levels of FFA-derived circulating free luciferin (0.16 μmol/L in serum). Error bars are ± SEM (n = 5).

for FFA-Luc, and thus within the range of interanimal variations.

Next, we determined whether our imaging system could be used to detect changes in liver FFA uptake. Our previous study showed that the secondary bile acid, deoxycholic acid (DCA), inhibits FATP5 with a median inhibitory concentration of 0.19 μmol/L in vitro without any apparent toxicity, and showed that DCA is able to reduce the hepatic uptake of various long-chain fatty acids significantly.[4] We measured FFA influx in the liver of DCA-treated L-Luc mice using BLI with FFA-Luc and compared the measurement with a widely used ex vivo FFA uptake assay with fluorescently labeled FA (boron-dipyrromethene [BODIPY]) (Figure 2*A* and *B*). BODIPY incorporates into lipids and has been used as a valuable tool in lipid transport and membrane studies.[5] Although only the bioluminescent method allowed for in vivo detection, both FFA-Luc and BODIPY-FFA–based assays detected a comparable decrease in FFA hepatic uptake of 35% in the DCA-treated group. In addition, we could not detect DCA-induced quenching of luciferase-luciferin bioluminescence (Supplementary Figure 1). We then applied our imaging approach to study the effect of fenofibrate on hepatic FFA uptake. Fenofibrate promotes β-oxidation in the liver,[6] but its effect on FFA uptake has not been explored. We fed L-Luc mice standard chow or a fenofibrate (0.2% wt/wt) diet for 10 days and then analyzed for FFA uptake in the liver. The results showed a significant increase by 40% of hepatic FFA uptake in fenofibrate-treated animals compared with control (Figure 2*C*), suggesting that enhanced FFA

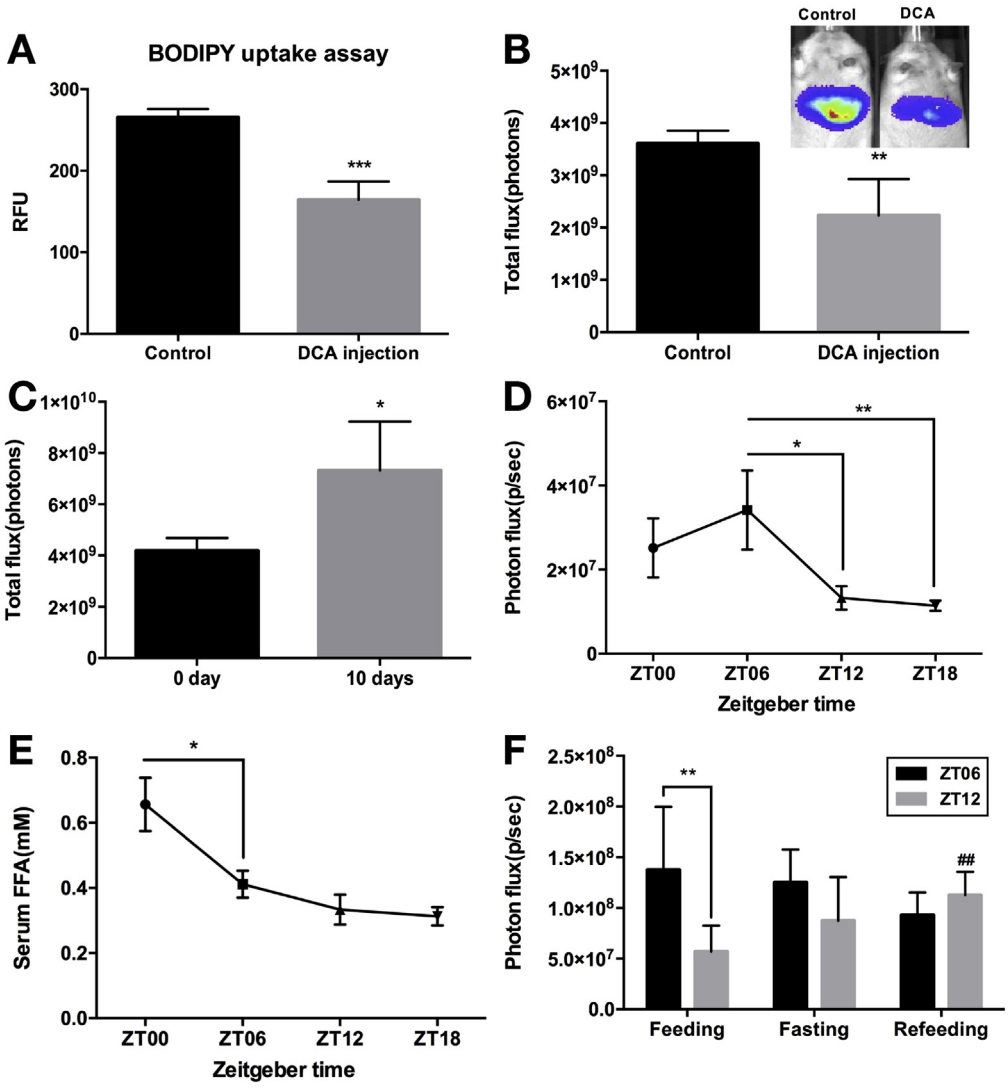

Figure 2. Application of BLI to monitoring the changes in FFA hepatic uptake in different physiological conditions. Acute effects of DCA injection at a concentration of 6.4 mg/kg body weight into L-Luc mice on the reduction of hepatic uptake of (*A*) BODIPY and (*B*) FFA-Luc. Student *t* test (n = 5); $^{**}P < .01$, $^{***}P < .001$. (*C*) Fenofibrate feeding increased FFA-Luc uptake in the liver. Student *t* test (n = 5); $^{*}P < .05$. (*D*) FFA uptake rate after the injection of FFA-Luc into L-Luc mice and (*E*) serum FFA concentration during the light (ZT6, ZT12) and dark (ZT18, ZT0) periods. One-way analysis of variance (n = 5); $^{*}P < .05$, $^{**}P < .01$. (*F*) FFA uptake rate after regular feeding, 24 hours of fasting, and refeeding during the light (ZT6, ZT12) periods. $^{\#\#}P < .01$ between feeding and refeeding at ZT12, $^{**}P < .01$ in a paired Student *t* test. Values are reported with error bars as ± SEM. RFU, relative fluorescence unit.

uptake contributes to a fenofibrate-induced increase in β-oxidation.

We next applied BLI to explore hepatic diurnal changes in liver FFA uptake over a 24-hour period in male L-Luc mice. The highest FFA uptake was observed at zeitgeber time (ZT) 06 (1 PM), midlight phase, whereas the lowest uptake was detected at ZT18 (Figure 2*D*); moreover, significant differences were observed between ZT06 and ZT12 (decreased by 59% of ZT06) and between ZT06 and ZT18 (decreased by 64% of the highest). These results indicate that FFA uptake by the liver is altered across the day and night, suggesting a robust diurnal rhythm. These data agree with the finding that FATP2, one of the major hepatic fatty acid transporters, shows a strong diurnal expression pattern.[7] Importantly, the rhythmic changes in hepatic FFA uptake were not driven by changes in circulation FFA levels (Figure 2*E*).

To confirm our diurnal changes of hepatic FFA uptake reflects actual FFA uptake, we compared hepatic FFA uptake under feeding, fasting, and refeeding states (Figure 2*F*). We observed that ZT06 and ZT12 showed significantly different hepatic uptake in the regular feeding state. However, there was no significant difference in the hepatic FFA uptake after 24-hour fasting or refeeding between ZT06 and ZT12 (Figure 2*F*). Refeeding significantly increased hepatic FFA uptake at ZT12, although it had less effect at ZT06. This means that food manipulation can override the diurnal rhythms of hepatic FFA uptake and have different effects at different time points during the day. Changes in uptake are not driven by serum FFA levels because the FFA level is low when the FFA uptake rate is low (Supplementary Figure 2).

Taken together, we have shown the development of a novel in vivo imaging system and its application for monitoring physiological and pathologic changes to FFA uptake in preclinical models. The data obtained in this study show that FFA uptake could be monitored in real time under various conditions, which, given the importance of FFA hepatic accumulation in physiology, opens up a spectrum of opportunities for studying

fundamental mechanisms underlying lipid metabolism in the liver.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2016.10.002.

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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Materials and Methods

Animal Models

FVB-Luc^{+} (FVB-transgenic[CAG-luc,-green fluorescent protein]L2G85Chco/J) mice were obtained from our in-house breeding colony. Mice were maintained at a 12-hour light/12-hour dark cycle at 22°C and had free access to food and water. To generate a mouse strain with liver-specific luciferase expression (L-Luc mice), mice bearing the Gt(ROSA)26Sor$^{tm1(Luc)Kael}$ allele and Tg(Alb-cre)21Mgn mice were purchased from Jackson Laboratories (Bar Harbor, ME) (stocks 005125 and 003574, respectively) and crossed. Gray-fur mice were bred several times to generate white-fur litters. To examine the effect of fenofibrate, mice were fed a standard laboratory chow diet with or without fenofibrate (0.2% wt/wt) for 10 days, while having free access to drinking water. All animal studies were approved by and performed according to the guidelines of the Animal Care and Use Committee of the University of California, Berkeley.

General Animal Imaging Methods and Data Analysis

A Xenogen IVIS Spectrum instrument (Caliper Life Sciences, Alameda, CA) was used to obtain luminescence images in all animal experiments. Image analysis was performed using the IVIS Living Image software. The total photon flux in each mouse was determined by drawing a region of interest in the animal and integrating photon flux over the total imaging period. Mice were anesthetized before injection and during imaging by isoflurane inhalation.

Intraperitoneal Injection of FFA-Luc and Luciferin

Anesthetized mice were injected intraperitoneally with 100 μL of 200 μmol/L FFA-Luc in 0.1% (wt/vol) bovine serum albumin–containing phosphate-buffered saline or with 100 μL of 2 mmol/L luciferin in phosphate-buffered saline immediately before imaging. Luminescence images were acquired by autoexposure back-to-back for 50 minutes in case of FFA-Luc and 25 minutes in case of luciferin.

Quantification of Free Luciferin in Serum

Sera were collected before and 10 and 20 minutes after intraperitoneal injection of FFA-Luc and luciferin, and 50 μL of serum samples was plated on 96-well plates containing reaction solution; firefly luciferase (L9420; Sigma-Aldrich, St. Louis, MO) and adenosine triphosphate (A2383; Sigma-Aldrich) were added immediately before recording the results using a SpectraMax i3 (Molecular Devices, Sunnyvale, CA). Luminescence signals were measured for 10 seconds, and the amount of luciferin was calculated based on a standard curve constructed using a series of luciferin concentrations.

In Vivo Monitoring of FFA Hepatic Uptake Using Fluorescence-Labeled FA (BODIPY)

The assay was performed as described previously[1] with minor modifications. In brief, mice fasted overnight were anesthetized with isoflurane and injected intraperitoneally with DCA (6.4 mg/kg body weight) and then BODIPY (100 μL of 2 μmol/L solution). After 30 minutes, mice were euthanized, and livers were harvested and homogenized in RIPA buffer. Liver lysates were prepared with 3 volumes of Dole's reagent (heptane:2-propanol:2 N sulfuric acid = 10:40:1 vol/vol/vol), centrifuged at 18,000 $\times$ g for 10 minutes, and clear organic-phase supernatant (top layer) was collected and added to a 96-well plate for fluorescence measurement.

Diurnal Rhythm of FFA Hepatic Uptake

Two cohorts of male mice were maintained on a 12-hour light/12-hour dark cycle; ZT0 referred to lights on and ZT12 referred to lights off. The first injection of FFA-Luc was conducted at ZT06 (1 PM) in 1 group and at ZT18 (1 AM) in the other group, followed by subsequent injections every 30 hours. For the fasting and refeeding study, an additional 2 groups of male mice were measured using a hepatic FFA uptake assay under different feeding states at the ZT6 and ZT12 periods. The signal was measured for 20 minutes during each imaging and the total photon flux was determined. To avoid light application during dark period assessments, we used night vision goggles and dim red lighting. Serum was collected at the same time points and FFA levels were measured using the FFA kit (Sigma-Aldrich).

Measurement of Quenching of Luciferase–Luciferin Bioluminescence

Incubation of different concentration of DCA or fenofibrate with luciferin was performed in 2% dimethyl sulfoxide/phosphate-buffered saline for 10 minutes. At the end of the incubation, an equal volume (100 μL) of a reaction solution containing luciferase and adenosine triphosphate was added and mixed well. Bioluminescence signals were measured for 10 seconds using a SpectraMax i3 (Molecular Devices).

Supplementary References

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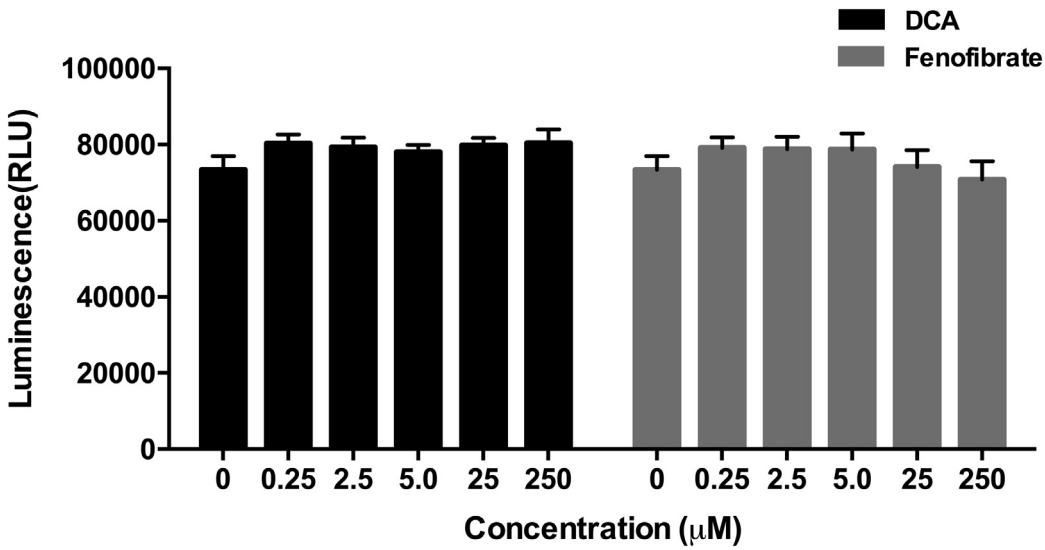
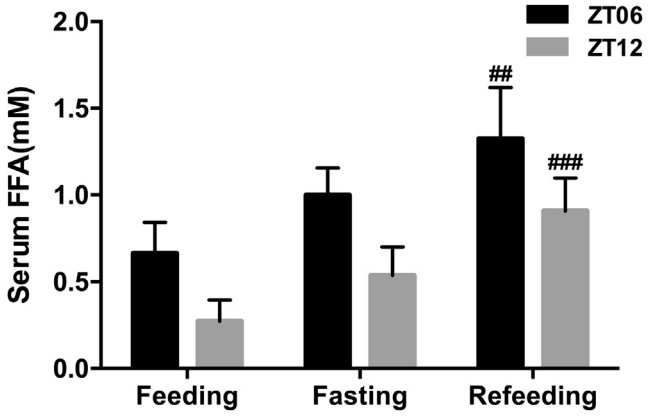


Supplementary Figure 1. Determination of DCA- or fenofibrate-induced quenching of luciferase–luciferin bioluminescence. Luciferin was pre-incubated with varying concentrations of DCA or fenofibrate for 10 minutes, followed by addition of reaction solution containing luciferase and adenosine triphosphate. Relative bioluminescence signals were measured for 10 seconds. Statistical analyses were performed using the Student *t* test. Error bars are ± SEM (n = 4). RLU, relative luminescence units.



Supplementary Figure 2. Serum FFA level after regular feeding, 24 hours of fasting, and refeeding during the light (ZT6, ZT12) periods. Statistical analyses were performed using a paired Student *t* test. $^{\#\#}P < .01$ between feeding and refeeding at ZT06 and $^{\#\#\#}P < .005$ at ZT12. Error bars are ± SEM.