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Maintenance of liver glycogen during long-term fasting preserves energy state in mice

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Abstract

Glycogen shortage during fasting coincides with dramatic changes in hepatic adenine nucleotide levels. The aim of this work was to study the relevance of liver glycogen in the regulation of the hepatic energy state during food deprivation. To this end, we examined the response of mice with sustained increased liver glycogen content to prolonged fasting. In order to increase hepatic glycogen content, we generated mice that overexpress protein targeting to glycogen (PTG) in the liver (PTG^{OE} mice). Control and PTG^{OE} mice were fed *ad libitum* or fasted for 36 h. Upon fasting, PTG^{OE} mice retained significant hepatic glycogen stores and maintained hepatic energy status. Furthermore, we show that liver glycogen controls insulin sensitivity, gluconeogenesis, lipid metabolism, and ketogenesis upon nutrient deprivation.

Keywords

glycogen; fasting; energy state; gluconeogenesis; AMPK; liver

Food deprivation in mammals results in significant changes to metabolism in order to sustain energy and the availability of metabolic substrates [1]. Hormones such as glucagon, insulin, catecholamines, fibroblast growth factor 21 (FGF21), and glucocorticoids play important roles in this process. To maintain blood glucose concentration during the early stage of fasting, liver glycogen is mobilized by glycogenolysis. Furthermore, during prolonged fasting and thus glycogen depletion, hepatocytes use lactate, pyruvate, glycerol, and amino acids to synthesize glucose through gluconeogenesis [2]. Moreover, fatty acids are mobilized

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Author contributions

IL-S and JGG conceived the study, designed all experiments, interpreted the data, wrote the manuscript, and directed the study. IL-S, AB, and AA performed the experiments. IL-S analyzed the data. JD generated the animal model and contributed to interpret the data. All authors approved the final version of the manuscript to be published. IL-S is responsible for the integrity of the work as a whole.

Data accessibility

The data are available on request from the authors.

from white adipose tissue to liver, where they are oxidized to acetyl-CoA, which is then used to synthesize ketone bodies [3].

Fasting provokes a reciprocal rise and fall in hepatic AMP and ATP concentrations [4,5], and it has been described that reductions in hepatic energy state due to metabolic stress require cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) [5]. Also, liver AMP-activated kinase (AMPK) is critical for stabilizing liver energy state during nutrient deprivation [6].

Liver glycogen is synthesized by the hepatic isoenzyme of glycogen synthase (LGS or GYS2). LGS activity is regulated by phosphorylation at multiple sites, which causes its inactivation. Conversely, LGS is activated by dephosphorylation. Protein phosphatase 1 (PP1) is regulated by a group of glycogen-targeting subunits (G subunits) that serve to localize PP1 to glycogen particles, leading to dephosphorylation and activation of LGS. In this regard, G_L (PPP1R3B) and protein targeting to glycogen (PTG, also known as PPP1R5 or PPP1R3C) are the two main G subunits expressed in the liver, and overexpression of either one increases glycogen content [7,8].

In this study, we have analyzed the impact of liver glycogen in the regulation of hepatic energy state during food deprivation. To this end, we have generated mice that overexpress PTG in the liver and that maintain relatively high levels of hepatic glycogen even when fasted. Our results demonstrate that liver glycogen regulates the hepatic energy state upon nutrient deprivation.

Materials and Methods

Animals

All procedures were approved by the Barcelona Science Park Animal Experimentation Committee and carried out in accordance with the European Community Council Directive and the National Institute of Health guidelines for the care and use of laboratory animals. Mice that overexpress PTG in the liver (PTG^{OE}) were generated as previously described [8]. Mice were housed with *ad libitum* access to water and a standard chow diet (Envigo, Indianapolis, IN, USA). When the animals were 6 months old, a cohort from each genotype had their food removed at the beginning of the dark cycle (19:00) and were sacrificed after a 36-h fast. A separate cohort of each genotype was allowed *ad libitum* access to food and was sacrificed at 08:00. Prior to sacrifice, whole blood was collected from the tail of the animals in EDTA-coated tubes. It was then centrifuged, and plasma was collected for analysis. After sacrifice, tissues were collected and stored at -80°C until analysis.

Liver and skeletal muscle biochemical analysis

Glycogen was measured as previously described [9]. Briefly, frozen tissue was homogenized in 4 volumes of 30% KOH and heated at 100°C for 15 min. Aliquots were then spotted on 31ET paper (Whatman, Sigma-Aldrich, St Louis, MO, USA). The papers were washed three times with 66% ethanol, which precipitates glycogen. The dried papers were incubated with amyloglucosidase (25 U L^{-1} Sigma) in 100 mM sodium acetate buffer at pH 4.8 to [C0] digest glycogen, and glucose release was determined by the reaction with hexokinase and

glucose 6-phosphate dehydrogenase, following the original method described by Chan [10]. The intracellular concentration of ATP and AMP was measured in perchloric acid extracts by HPLC, as previously described [11]. Lactate was measured in perchloric acid extracts using a commercial spectrophotometric kit (Horiba, ABX, Montpellier, France). Triacylglycerols (TGs) in liver were quantified in 3 mol·L⁻¹ KOH and 65% ethanol extracts based on the method described by Salmon and Flatt [12] using a kit (Sigma-Aldrich, St Louis, MO, USA).

Glycogen isolation

Liver samples were homogenized in 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM NaF, 1% NP-40, 1 mM PMSF, and a protease inhibitor cocktail tablet (Roche, Basel, Switzerland) (buffer A). Homogenates were centrifuged at 16 000 *g* for 15 min at 4 °C. The resulting supernatant was ultracentrifuged at 100 000 *g* for 30 min (Optimax TX-rotor TLA 55). Glycogen was recovered in the pellet. The pellet was resuspended in the same volume as the supernatant.

Blood biochemical analysis

Blood glucose levels were measured using a glucometer (Ascensia Breeze 2; Bayer Healthcare, Leverkusen, Germany). Plasma insulin and FGF21 were determined by ELISA (Crystal Chem, Downers Grove, IL, USA; BioVendor—Laboratori medicina a.s, Brno, Czech Republic). Plasma β-hydroxybutyrate was determined using a commercial kit (Sigma-Aldrich). Plasma free fatty acids (FFAs) were determined using a commercial kit (Abcam, Cambridge, UK).

Pyruvate tolerance test

For the pyruvate tolerance test, mice received an i.p. injection 2 g·kg⁻¹ of sodium pyruvate (Sigma-Aldrich) dissolved in PBS. Blood glucose concentration was measured via tail bleed before and at times indicated after injection. All glucose measurements were made using an Ascensia Breeze 2 glucometer.

Western blot analysis

Western blots (WBs) were performed in liver samples homogenized in buffer A. Homogenates were centrifuged at 16 000 *g* for 15 min at 4 °C. Proteins were resolved in 10% or 15% acrylamide gels for SDS/PAGE and transferred to Immobilon membranes (Millipore, Sigma-Aldrich). The following antibodies were used: PEPCK (a kind gift from Dr. E. Beale); p-FoxO1 Thr24 (9464, Cell Signaling, Danvers, MA, USA); FoxO1 (9454, Cell Signaling); GK (peptide 414–428 raised by Sigma Genosys (Cambridge, UK)); p-AMPKβ Ser108 (4181, Cell Signaling); AMPKβ (4150, Cell Signaling); p-AMPKα Thr172 (2531, Cell Signaling); AMPKα (2532, Cell Signaling); p-AKT Thr308 (4056, Cell Signaling); p-AKT Ser473 (9271, Cell Signaling); AKT (9272, Cell Signaling); p-GSK3β Ser9 (9336, Cell Signaling); and GSK3β (sc7291, Santa Cruz, Dallas, TX, USA). Proteins were detected by the ECL method (Immobilon Western Chemiluminescent HRP Substrate, Millipore, Sigma-Aldrich). Loading control of the WB membrane was performed using the REVERT total protein stain.

RNA preparations and quantitative RT–PCR

Tissue preparation, RNA extraction, RT–PCR, and quantitative real-time PCR analyses were performed as described [13]. The following TaqMan primer/probe sets (Applied Biosystems, Madrid, Spain) were used for quantitative RT–PCR: BDH1 (Mm00558330_m1); BDH2 (Mm00459075_m1); HMGCS2 (Mm00550050_m1); CD36 (Mm01135202_g1); PPAR α (Mm00440939_m1); CPT1 α (Mm01231183_m1); PEPCK (Mm00440636_m1); PCX (Mm00500992_m1); FBP1 (Mm00490181_m1); FGF21 (Mm00840165_g1); and 18S ribosomal (18S) (Mm03928990_g1). 18S was used as a housekeeping gene.

Lean weight and fat weight

Lean weight and fat weight were measured using magnetic resonance imaging (EchoMRI System, EchoMRI LLC; Houston, TX, USA).

Statistics

Data are expressed as mean \pm SEM. *P* values were calculated using two-way ANOVA with *post hoc* Tukey's tests as appropriate. For pyruvate tolerance test (PTT) and area under the curve (AUC), Student's *t*-test was performed.

Results

Effects on body weight, lean, and fat mass

In order to increase hepatic glycogen content, we generated mice that overexpress PTG in the liver (PTG^{OE} mice) [8]. To study the role of liver glycogen during the adaptive response to fasting, we compared control and PTG^{OE} mice that had been fed, or fasted for 36 h. Control and PTG^{OE} mice lost about 6 g of body weight during fasting, accounting for approximately 20% of body weight loss. Of this loss, 2 g corresponded to fat and 4 g to lean weight (Fig. 1). This percentage of weight loss in control animals is in agreement with previous studies [14]. Fasting caused a significant decrease in blood glucose levels in control and PTG^{OE} mice, although the decrease was attenuated in the latter (Fig. 1E). The plasma insulin concentration, which was already decreased in fed PTG^{OE} mice, was greatly reduced in fasted control and PTG^{OE} animals (Fig. 1F).

Effects on energy state

As we previously described [8], PTG^{OE} mice overaccumulated hepatic glycogen under fed conditions. Following a 36-h fast, liver glycogen was completely depleted in control mice, while in PTG^{OE} mice it was partially mobilized down to levels in the same range as those in fed control animals (Fig. 2A). Hepatic glucose 6-phosphate (G6P), the primary intracellular metabolite of glucose, was significantly reduced after fasting in control mice but not in PTG^{OE} mice (Fig. 2B). Upon fasting, hepatic ATP and AMP levels in control mice decreased (Fig. 2C) and increased (Fig. 2D), respectively, resulting in an increase in the AMP/ATP ratio (Fig. 2E) compared with fed control counter-parts. Nonetheless, the changes in hepatic nucleotide levels mediated by fasting were abolished in PTG^{OE} mice (Fig. 2C–E), thus preserving the energy state of the organ. Furthermore, low energy states under fasting conditions are associated with the activation of AMPK [2,15]. The phosphorylated AMPK β /

total AMPK β ratio was decreased in fasted PTG^{OE} mice compared with fasted control animals, indicating a lower activation state of the enzyme (Fig. 3A,G). However, the phosphorylated AMPK α /total AMPK α ratio was similar in all the experimental groups (Fig. 3B,H). Another interesting observation was that both total AMPK β and total AMPK α were increased in the livers of PTG^{OE} mice compared with control mice (Fig. 3A,E; B,F).

To determine whether these increases were due to AMPK binding to glycogen, liver glycogen was precipitated by ultracentrifugation. AMPK α and AMPK β were detected in the pellet, thereby indicating that a fraction of the enzyme was bound to glycogen (Fig. 3I). However, most of the enzyme was found in the supernatant.

Effects on gluconeogenesis

To study the impact of liver glycogen on the regulation of gluconeogenesis during fasting, the protein expression of hepatic PEPCK and the expression of several gluconeogenic genes were measured. After 36 h of fasting, hepatic PEPCK protein (Fig. 4A,C) and gene expression (Fig. 4D) were increased in control mice. However, this effect was attenuated in PTG^{OE} mice (Fig. 4A,C,D). Fasting also increased the expression of the gluconeogenic genes pyruvate carboxylase (PCX) and fructose biphosphatase 1 (FBP1) in control mice, but not in PTG^{OE} counterparts (Fig. 4D). Fasted PTG^{OE} mice showed an increase in liver lactate (Fig. 4E), indicative of less demand for lactate use for gluconeogenesis. Glucokinase expression was significantly higher in fasted PTG^{OE} mice (Fig. 4A,B). To more directly assess the role of liver glycogen in gluconeogenesis, mice were challenged with the gluconeogenic precursor, pyruvate. An improved pyruvate tolerance was shown in PTG^{OE} mice (Fig. 4F) as represented by a significant reduction in AUC (Fig. 4G), suggesting that liver glycogen attenuates gluconeogenesis in the liver. The PTT was performed after a 6-h fast and not after 36-h fast, like the rest of the experiments. At the later time point, the initial concentrations of blood glucose were different (Fig. 1E) and this may hamper the interpretation of the results.

Effects on hepatic insulin signaling

Hepatic insulin signaling was analyzed in fed and fasted mice of both genotypes. The phosphorylation of protein kinase B (AKT) (Fig. 5A–C) and glycogen synthase kinase 3 β (GSK3 β) (Fig. 5A,D) was increased in the liver of fasted PTG^{OE} mice compared with fasted control animals, thereby indicating a higher activation state of the insulin signaling cascade. AKT signals through multiple downstream pathways, including the Forkhead box protein O1 (FoxO) family of transcription factors. Fasting decreased the phosphorylation of FoxO1 in the liver of control mice, but this effect was attenuated in PTG^{OE} mice (Fig. 5A,E).

Effects on lipid metabolism and ketogenesis

Fasting has a significant impact on lipid metabolism [16]. Prolonged fasting elicited increased hepatic TG accumulation in control mice, but this increase was attenuated in PTG^{OE} mice (Fig. 6A). Fasted control mice and PTG^{OE} mice showed an increased concentration of FFAs, but no significant differences between the genotypes were found (Fig. 6B). The liver is the primary source of ketone bodies. Fasting increased plasma β -hydroxybutyrate concentration in control mice; however, as previously described for a

shorter period of fasting [8], the increase was attenuated in PTG^{OE} animals (Fig. 6C). The expression of genes associated with ketogenesis was examined next. The expression of BDH1 increased in the liver of fasted control mice but not in that of fasted PTG^{OE} animals (Fig. 6E). BDH2 expression decreased after 36 h of food deprivation (Fig. 6E) and HMGCS2 expression increased upon fasting in both genotypes, although the increase was less marked in PTG^{OE} mice (Fig. 6E). Given that hepatic FFA β -oxidation/ketogenesis increases during fasting [17], we examined the impact of liver glycogen on the expression of genes involved in fatty acid oxidation, namely CD36, PPAR α , CPT1 α , and FGF21. The expression of all these genes was attenuated in PTG^{OE} animals (Fig. 6F). FGF21, a key regulator of the metabolic response to fasting, is secreted by the liver in nutrient-deficient states [18–20]. Control mice, but not PTG^{OE} animals, showed a significant increase in plasma levels of FGF21 after a 36-h fast (Fig. 6D). This parameter correlated with the hepatic expression of FGF21 (Fig. 6F). This result is consistent with recent findings that demonstrate that the liver is the primary source of circulating FGF21 [21].

Effects on skeletal muscle metabolism upon fasting

To address the potential role of liver glycogen on skeletal muscle metabolism during fasting, we measured various metabolites in the gastrocnemius muscle. Thirty-six hours of food deprivation did not alter the concentration of glycogen, lactate, or ATP in the gastrocnemius (Table 1) in either genotype. However, the concentration of AMP and the AMP/ATP ratio were increased to the same extent in the muscle in both genotypes after fasting (Table 1).

Discussion

Here, we report that liver glycogen is a regulator of the hepatic energy state of the liver in response to long-term fasting. We show that glycogen controls the hepatic energy state, gluconeogenesis, lipid metabolism, ketogenesis, and insulin sensitivity upon nutrient deprivation.

In order to increase liver glycogen stores, we generated mice overexpressing PTG specifically in this organ. These animals show a doubling of glycogen concentration in the fed state. Our results show that, in spite of the long period of fasting, PTG^{OE} mice experienced only a 50% decrease in hepatic glycogen stores. An explanation for this observation is that, although glycogenolysis is active, glycogen stores are constantly replenished by glycogen synthase. Remarkably, PTG^{OE} mice showed an increased capacity to maintain plasma glucose under a 36-h fast, while the percentage of body weight loss upon long-term fasting was similar to that of control mice.

The hepatic energy state is defined by adenine nucleotide levels and particularly by the AMP/ATP ratio [5]. Adenine nucleotide concentrations in the mouse liver are highly sensitive to acute physiological stressors, as well as to metabolic disease, and the hepatic energy state is substantially reduced following fasting [5]. Previous studies from our laboratory have demonstrated that liver glycogen levels regulate hepatic ATP content in mice fed a high-fat diet (HFD) [8,11]. In the present study, we show that liver glycogen contributes to maintaining the hepatic energy charge after long-term fasting (36 h). As expected, fasted control mice presented dramatic changes in hepatic adenine nucleotide

levels. However, fasted PTG^{OE} mice maintained ATP and AMP concentrations in the same range as those in fed control mice, thus abolishing the decrease in the hepatic energy state associated with fasting. Remarkably, PTG^{OE} mice also showed reduced PEPCK expression. It has been proposed that higher hepatic AMP/ATP ratios correlate with increased total PEPCK protein content. Mice with liver-specific deletion of PEPCK preserved hepatic energy state in response to an overnight fast, thereby supporting the notion that PEPCK-mediated gluconeogenesis is needed to induce changes in the hepatic energy state [5].

Given that the gluconeogenesis pathway consumes ATP, a decrease in PEPCK, as occurred in PTG^{OE} mice, could partly explain the higher levels of ATP found in the livers of these animals upon fasting. Hepatic lactate, a precursor of gluconeogenesis, was higher in fasted PTG^{OE} mice, indicative of less demand for lactate use for gluconeogenesis. Accordingly, PTG^{OE} mice showed improved pyruvate tolerance in the PTT.

Remarkably, AMPK was less active in fasted PTG^{OE} mice. However, the total levels of the enzyme were increased in these animals. Research over the past two decades has revealed that the regulation of AMPK is influenced by glycogen availability [22]. A significant body of evidence demonstrates that AMPK binds glycogen [23,24]. Therefore, the increase in this enzyme in the fasted PTG^{OE} mice could be due to the higher levels of glycogen. To verify this hypothesis, we precipitated glycogen by ultracentrifugation and found that indeed a fraction of AMPK was bound to glycogen particles, especially in PTG^{OE} mice. Nevertheless, we found that most of the AMPK was in the non-glycogen-bound fraction. Therefore, the lesser activation but higher levels of AMPK cannot be attributed solely to a physical interaction between AMPK and glycogen. Our results support the idea that AMPK senses cellular energy in the form of AMP/ATP but may also be able to sense the availability of energy reserves in the form of glycogen, as previously described [24].

Hepatic insulin signaling is mediated by the liver insulin receptor, which signals through the downstream kinase AKT, to coordinate hepatic metabolism [25,26]. AKT signals through multiple downstream pathways, including the FoxO family of transcriptional factors and GSK3. Fasting PTG^{OE} mice showed an increase in the hepatic insulin signaling cascade (increased phosphorylation of AKT, GSK3 β , and FoxO1), in spite of maintaining low insulin levels. Therefore, the liver of these animals became more sensitive to insulin. FoxO1 is a substrate of AKT and it contains three AKT phosphorylation sites (Thr24, Ser256, and Ser319) [27]. During the fasting state, FoxO1 is activated by dephosphorylation and translocated into the nucleus, which leads to the transcriptional induction of PEPCK [28]. The livers of fasted PTG^{OE} mice showed an increase in the activation of AKT, which in turn phosphorylates FoxO1. The phosphorylation of FoxO1 induces its nuclear exclusion and cytoplasmic retention, thereby suppressing PEPCK gene expression and attenuating gluconeogenesis. In this regard, it has been described that LGS knockout mice show an increase in the liver gluconeogenic enzyme PEPCK [29] and decreased phosphorylation of components of the hepatic insulin signaling pathway, as well as phosphorylation of FoxO1 [30]. Conversely, the liver of streptozotocin diabetic rats, in which hepatic glycogen had been increased by the expression of an active form of LGS, also show a reduction in PEPCK and other gluconeogenic enzymes [31].

Another interesting finding was that GK protein expression did not decrease in the liver of PTG^{OE} mice upon fasting, despite low insulin levels. The expression of GK in this organ is highly dependent on the presence of insulin, and several lines of evidence support the notion that AKT activation is involved in the induction of hepatic GK [32–34]. The increase in AKT phosphorylation in the liver of PTG^{OE} mice upon fasting could explain the higher levels of GK found in these animals. Furthermore, the increased GK, combined with the higher blood glucose concentration, may account for the observed increase in G6P.

Fasting increases hepatic TGs in rodents [35]. This fasting-induced hepatic steatosis results from an excessive uptake of circulating fatty acids derived from adipose tissue lipolysis. The amount of hepatic TG content was lower in fasted PTG^{OE} mice than control mice. We propose that the impaired hepatic fatty acid uptake caused by downregulation of CD36 expression in the liver of fasted PTG^{OE} mice is responsible for the reduced TG content in these animals. CD36 is an important regulator of hepatic fatty acid uptake under conditions of elevated FFAs [36]. Hepatic CD36 disruption in various models of hepatic steatosis significantly improves steatosis by lowering TG, diacylglycerol, and cholesterol ester content [36,37]. It could be argued that that lower CD36 expression in the liver of fasted PTG^{OE} mice would cause an increase in FFAs. In this regard, Wilson CG et al. [36] addressed the role of CD36 in regulating liver lipid content by specifically deleting CD36 in two steatosis models, one genetic (JAK2L) and another nutritional (HFD feeding) in which the elevated CD36 expression was associated with profound lipid accumulation in liver. Those authors conclusively showed that hepatocyte-specific CD36 disruption significantly reduced liver TGs without affecting FFA concentration. Only mice with global CD36 deletion showed a significant increase in FFAs, caused by severely impaired fatty acid uptake into peripheral tissues (muscle, heart, and adipose tissue) [38]. However, we are unable to explain what happens to the FFAs not taken up in fasted PTG^{OE} mice.

In liver, the intracellular accumulation of diacylglycerol triggers the activation of novel protein kinases C, with subsequent impairments in insulin signaling [39]. There is an inverse relationship between hepatic TG stores and hepatic insulin sensitivity [30,40,41]. The higher sensitivity to insulin found in fasted PTG^{OE} mice could be due to the lower hepatosteatosis found in these animals.

PPAR α , a nuclear hormone receptor activated by FFAs, is another transcription factor that is central to the metabolic shift—from glucose to fat utilization—initiated by fasting [42]. Upon activation of PPAR α , fatty acid oxidation is stimulated and increased amounts of acetyl-CoA are produced. A large proportion of acetyl-CoA derived from fatty acid β -oxidation is utilized in ketogenesis [43,44]. Fasted PTG^{OE} mice presented decreased expression of genes related to ketogenesis and β -oxidation, resulting in lower levels of β -hydroxybutyrate. Our results are in agreement with those of Mehta et al., who reported that enhanced glycogen storage buffers the onset of the fasting-induced metabolic shift to β -oxidation [45]. AMPK regulates cellular lipid metabolism mostly through an increase in CPT1 activity and the subsequent activation of fatty acid oxidation [46,47]. Fasted PTG^{OE} mice showed lower fatty acid oxidation compared with control mice. This observation could be related to the lower activation state of AMPK in these animals. Of particular relevance in the regulation of ketogenesis and fatty acid oxidation is the ‘hormone-like’ FGF21 [20]. The

hepatic expression of this molecule is PPAR α -dependent, and it is induced by fasting [19,20]. An additional important finding of this work is that, upon fasting, PTG^{OE} mice did not show an increase in FGF21 in plasma or liver. These observations thus suggest that liver glycogen contributes to regulating the secretion of FGF21.

In summary, our data indicate that, during fasting, hepatic glycogen contributes to the control of adenine nucleotide in the liver through the decrease in gluconeogenesis. In the presence of glycogen, the liver is less predisposed to the fasting state. On the basis of our findings, we conclude that glycogen is more than just a simple storage form of glucose and that it plays an active role as a gauge of liver energy metabolism.

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Abbreviations

| | |
|--------------------------------|---|
| AKT | protein kinase B |
| AMP | adenosine monophosphate |
| AMPK | AMP kinase |
| ATP | adenosine triphosphate |
| AUC | area under the curve |
| CPT1α | carnitine palmitoyltransferase 1 α |
| FFA | free fatty acid |
| FBP1 | fructose biphosphatase 1 |
| FGF21 | fibroblast growth factor 21 |
| FoxO1 | forkhead box protein O1 |
| G subunits | glycogen-targeting regulatory subunits |
| G6P | glucose 6-phosphate |
| GK | glucokinase |
| GSK3 | glycogen synthase kinase 3 |
| LGS | liver glycogen synthase |
| PCX | pyruvate carboxylase |
| PEPCK | phosphoenolpyruvate carboxykinase |

| | |
|---------------------------------|---|
| PKA | protein kinase A |
| PP1 | protein phosphatase 1 |
| PPAR α | peroxisome proliferator-activated receptor α |
| PTG | protein targeting to glycogen |
| PTT | pyruvate tolerance test |
| TG | triacylglycerol |

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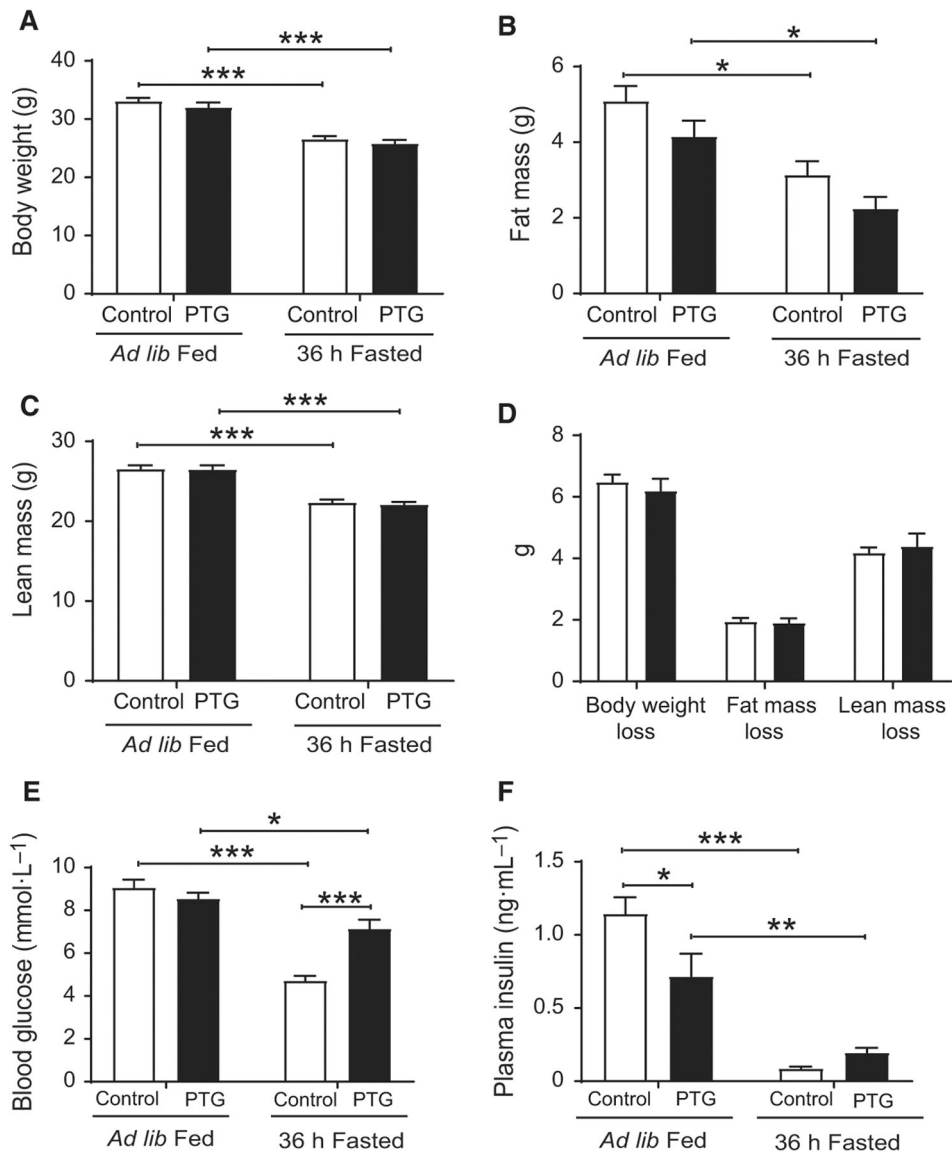


Fig. 1. Body weight, lean, and fat mass of control (white bars) and PTG^{OE} (black bars) mice under fed or fasted conditions. Animals were either allowed *ad libitum* access to food or were subjected to a 36-h fast. (A) Body weight. (B) Fat weight. (C) Lean weight. (D) Body weight loss, fat weight loss, and lean weight loss. (E) Blood glucose concentration. (F) Plasma insulin levels. Data are presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. $n = 15$ for body, fat, and lean weight, and $n = 8$ for glucose and insulin.

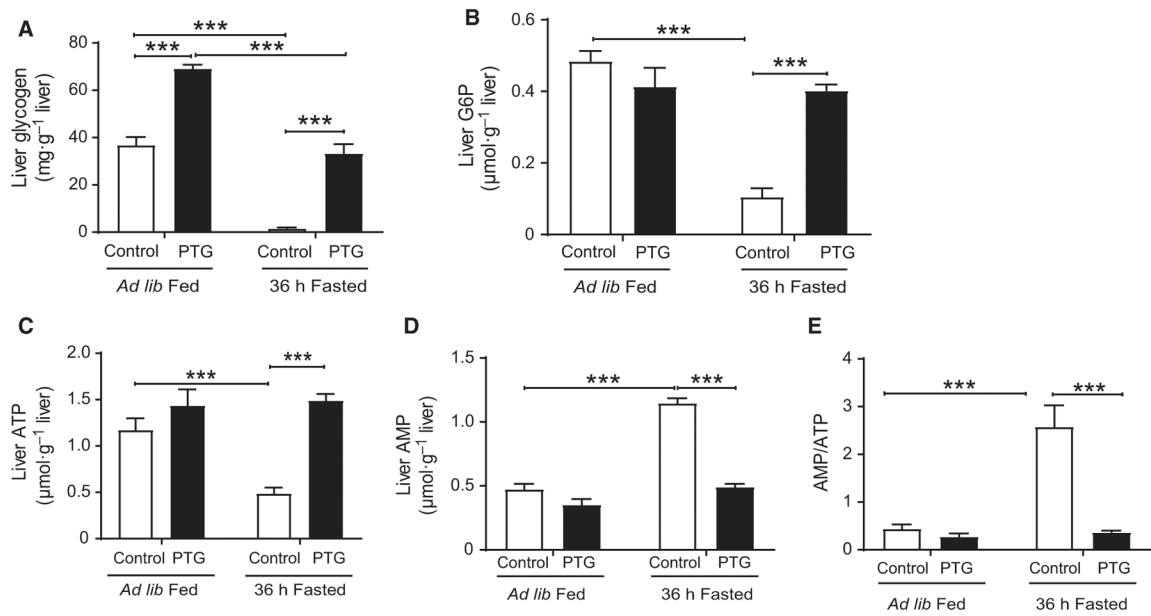


Fig. 2. Hepatic energy state of control (white bars) and PTG^{OE} (black bars) mice under fed or fasted conditions. (A) Glycogen content. (B) G6P content. (C) ATP concentration. (D) AMP concentration. (E) AMP/ATP ratio. Data are presented as mean \pm SEM. *** $P < 0.001$. $n = 6-8$.

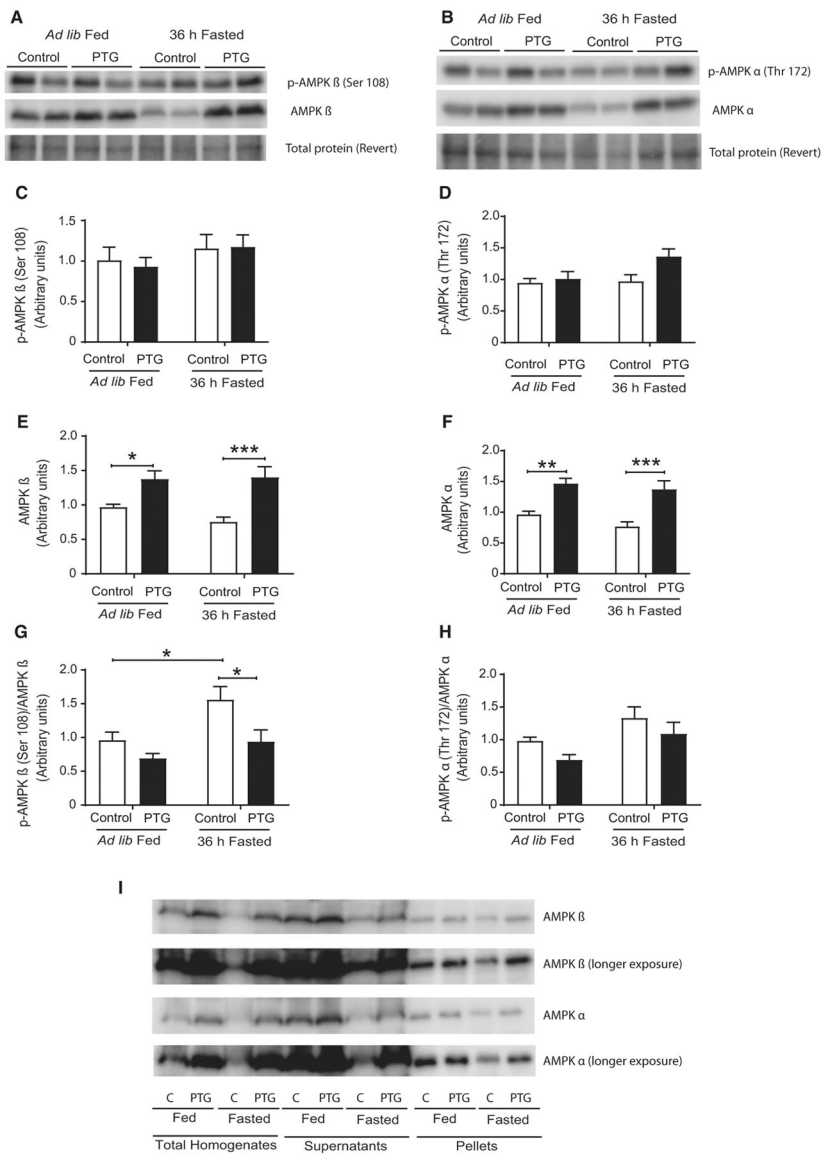


Fig. 3. AMPK of control (white bars) and PTG^{OE} (black bars) mice under fed or fasted conditions. (A, E) Total and (A, C) phosphorylated AMPK β and (B, F) total and (B, D) phosphorylated AMPK α measured by western blot. (G) Phosphorylated to total AMPK α is provided as a ratio (A.U). (H) Phosphorylated to total AMPK α is provided as a ratio (A.U). (I) Amount of AMPK β and AMPK α in homogenates, supernatant, and 100 000 g pellet fraction. Data are presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. $n = 8$.

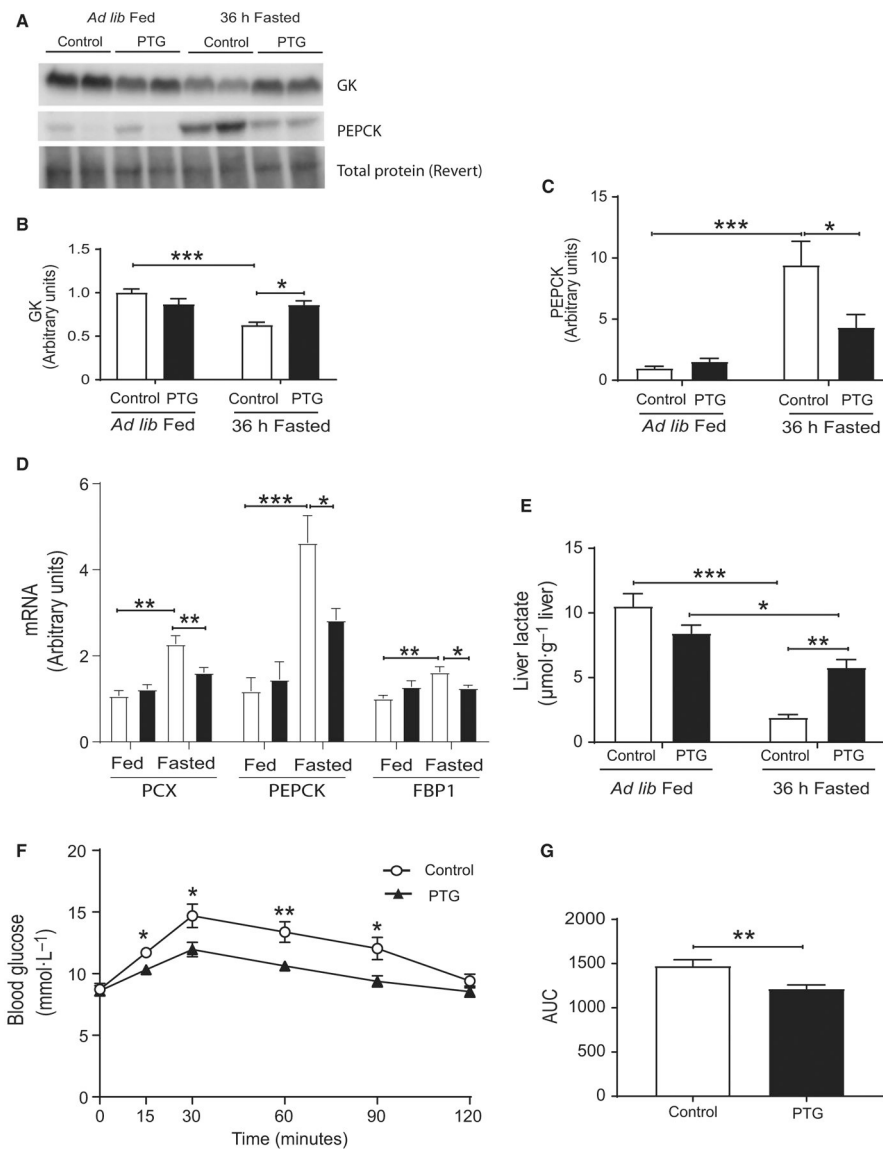


Fig. 4. Hepatic gluconeogenesis of control (white bars) and PTG^{OE} (black bars) mice under fed or fasted conditions. (A) Western blot of liver lysates. (B) Amount of GK measured by western blot. (C) Amount of PEPCK measured by western blot. (D) Expression of genes associated with gluconeogenesis (PCX, PEPCK, and FBP1). (E) Liver lactate content. (F) Pyruvate tolerance test after 6-h fasting. (G) AUC. AUC was calculated from blood glucose profiles during pyruvate tolerance test. Data are presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. $n = 8$ for western blot, $n = 8$ – 28 for gene expression, and $n = 6$ – 8 for pyruvate tolerance test.

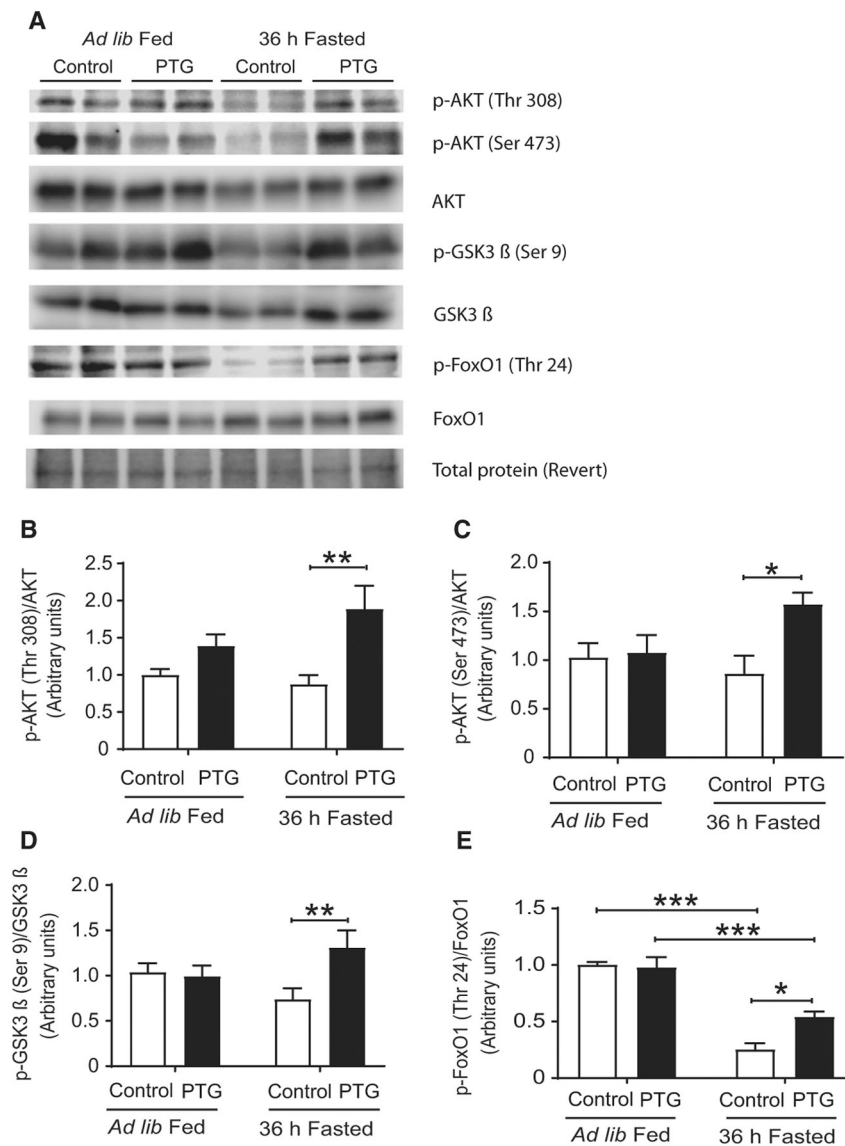


Fig. 5. Hepatic insulin signaling of control (white bars) and PTG^{OE} (black bars) mice under fed or fasted conditions. (A) Western blot of liver lysates. (B) Amount of total and phosphorylated AKT in threonine 308 measured by western blot. (C) Amount of total and phosphorylated AKT in serine 473 assessed by western blot. (D) Amount of total and phosphorylated GSK3 β assessed by western blot. (E) Amount of total and phosphorylated FoxO1 assessed by western blot. Data are presented as mean \pm SEM. * P < 0.05, ** P < 0.01, and *** P < 0.001. n = 8.

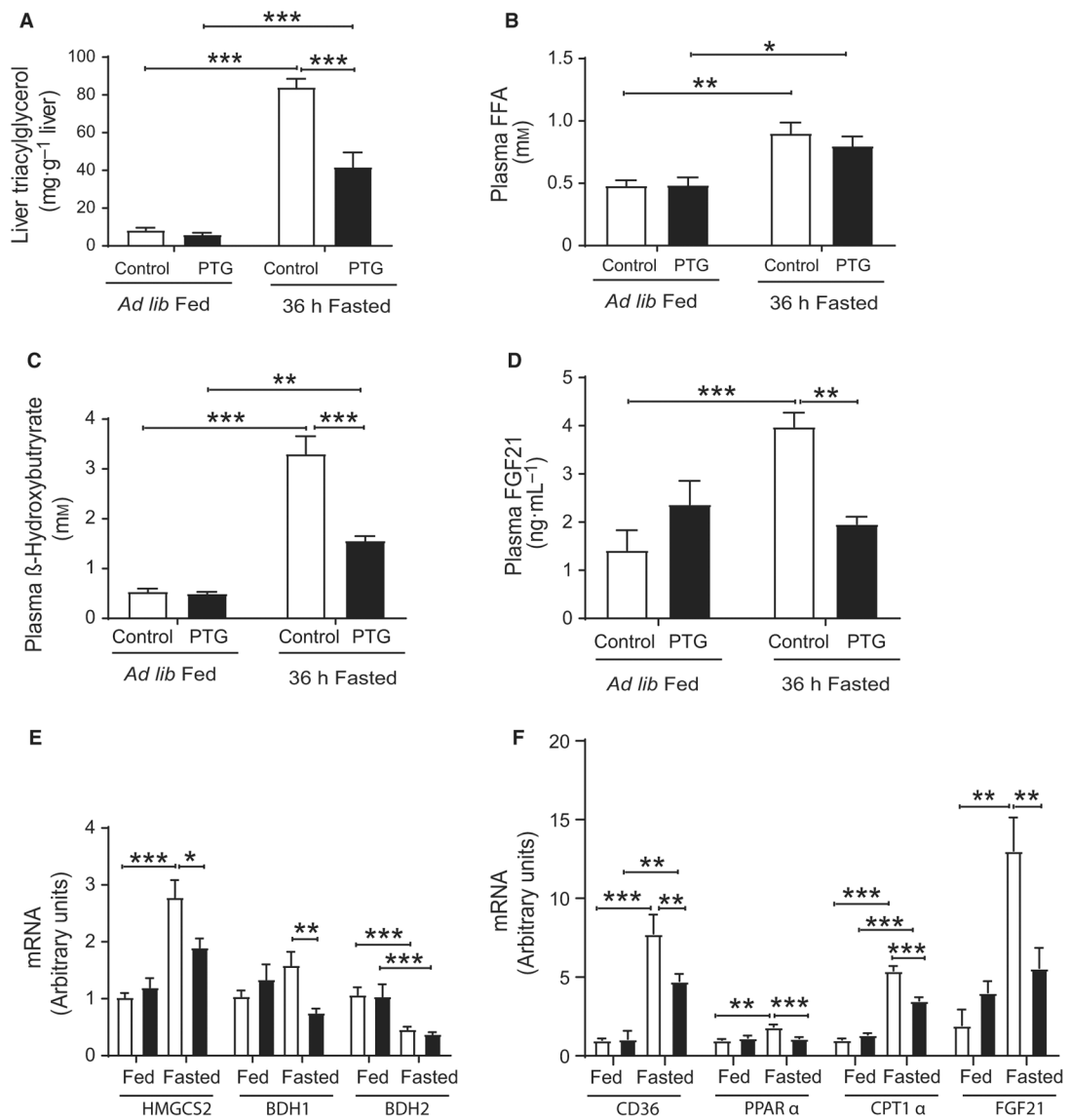


Fig. 6. Hepatic TG, liver β -oxidation, and ketogenesis of control (white bars) and PTG^{OE} (black bars) mice under fed or fasted conditions. (A) Hepatic TG concentration. (B) Plasma FFA levels. (C) Plasma β -hydroxybutyrate levels. (D) Plasma FGF21 concentration. (E) Expression of genes associated with ketogenesis (HMGCS2, BDH1, and BDH2). (F) Expression of genes associated with β -oxidation (CD36, PPAR α , CPT1 α , and FGF21). Data are presented as mean \pm SEM. * P < 0.05, ** P < 0.01, and *** P < 0.001. n = 6 for hepatic TG, n = 7–8 for plasma metabolites and n = 8–28 for gene expression.

Skeletal muscle metabolism of control and PTG^{OE} mice. Glycogen, lactate, ATP, AMP, and AMP/ATP ratio were measured in the gastrocnemius muscle. Data are presented as mean \pm SEM.

Table 1.

| | Fed | | Fasted | |
|-----------------------------------|-------------------|-------------------|--------------------------------|--------------------------------|
| | Control | PTG ^{OE} | Control | PTG ^{OE} |
| Glycogen (mg per g tissue) | 1.4 \pm 0.1 | 1.4 \pm 0.1 | 1.3 \pm 0.1 | 1.3 \pm 0.1 |
| Lactate (μ mol per g tissue) | 7.7 \pm 0.6 | 8.4 \pm 0.3 | 7.8 \pm 0.5 | 7.4 \pm 0.5 |
| ATP (μ mol per g tissue) | 5.2 \pm 0.2 | 4.9 \pm 0.3 | 5.0 \pm 0.1 | 4.8 \pm 0.2 |
| AMP (μ mol per g tissue) | 0.033 \pm 0.003 | 0.026 \pm 0.003 | 0.073 \pm 0.018 [*] | 0.077 \pm 0.014 [*] |
| AMP/ATP | 0.007 \pm 0.001 | 0.005 \pm 0.001 | 0.015 \pm 0.004 [*] | 0.016 \pm 0.003 [*] |

^{*} $P < 0.05$ between fed and fasted mice from the same genotype. $n = 4-8$.