Regulation of Fasting Fuel Metabolism by Toll-Like Receptor 4

Shanshan Pang, Haiqing Tang, Shu Zhuo, Ying Qin Zang, and Yingying Le

OBJECTIVE—Toll-like receptor 4 (TLR4) has been reported to induce insulin resistance through inflammation in high-fat–fed mice. However, the physiological role of TLR4 in metabolism is unknown. Here, we investigated the involvement of TLR4 in fasting metabolism.

RESEARCH DESIGN AND METHODS—Wild-type and TLR4 deficient (*TLR4*$^{-/-}$) mice were either fed or fasted for 24 h. Glucose and lipid levels in circulation and tissues were measured. Glucose and lipid metabolism in tissues, as well as the expression of related enzymes, was examined.

RESULTS—Mice lacking TLR4 displayed aggravated fasting hypoglycemia, along with normal hepatic gluconeogenesis, but reversed activity of pyruvate dehydrogenase complex (PDC) in skeletal muscle, which might account for the fasting hypoglycemia. *TLR4*$^{-/-}$ mice also exhibited higher lipid levels in circulation and skeletal muscle after fasting and reversed expression of lipogenic enzymes in skeletal muscle but not liver and adipose tissue. Adipose tissue lipolysis is normal and muscle fatty acid oxidation is increased in *TLR4*$^{-/-}$ mice after fasting. Inhibition of fatty acid synthesis in *TLR4*$^{-/-}$ mice abolished hyperlipidemia, hypoglycemia, and PDC activity increase, suggesting that TLR4-dependent inhibition of muscle lipogenesis may contribute to glucose and lipid homeostasis during fasting. Further studies showed that TLR4 deficiency had no effect on insulin signaling and muscle proinflammatory cytokine production in response to fasting.

CONCLUSIONS—These data suggest that TLR4 plays a critical role in glucose and lipid metabolism independent of insulin during fasting and identify a novel physiological role for TLR4 in fuel homeostasis. ***Diabetes*** **59:3041–3048, 2010**

Mammals have evolved complex metabolic systems to adapt to food deprivation. Under fasting condition, the triglycerides (TGs) stored in white adipose tissue (WAT) are hydrolyzed to release free fatty acids (FFAs), which become the primary fuel for liver and muscle through fatty acid oxidation (FAO) (1). Meanwhile, de novo fatty acid synthesis is strongly inhibited (2). During prolonged fasting, blood glucose levels are maintained within a narrow range to prevent life-threatening hypoglycemia, mainly through activation of hepatic gluconeogenesis. Glucose can also be spared by skeletal muscle through inhibition of pyruvate dehydrogenase complex (PDC) activity (3,4). PDC catalyzes the formation of acetyl-CoA from pyruvate, leading to irreversible net loss of carbohydrate. These metabolic adaptations to fasting are tightly regulated by several hormones, such as glucagon, glucocorticoids, epinephrine, and, recently reported, fibroblast growth factor 21 (5,6).

TLR4 is one of the mammalian pattern recognition receptors, recognizing pathogen-associated molecules and playing pivotal roles in innate immune response (7). Recently, saturated fatty acids have been reported to enhance the secretion of proinflammatory chemokines and cytokines through TLR4 activation (8–10). The activation of TLR4 by saturated fatty acids is believed to link obesity, inflammation, and insulin resistance (11–15). Mice with either deletion or mutation of TLR4 resist fatty acids- or high-fat diet–induced insulin resistance (11–13,15). Further, hematopoietic cell-specific deletion of TLR4 ameliorates heptic and adipose tissue insulin resistance in high-fat–fed mice (14). Given that these studies clearly demonstrate pathophysiological roles for TLR4 in metabolic disorders, such as obesity and insulin resistance, we propose that TLR4 may also play important roles in metabolic regulation under physiological conditions. In this study, we investigate the involvement of TLR4 in fasting metabolism and provide in vivo evidence that TLR4 plays an essential role in the physiological regulation of fuel homeostasis.

RESEARCH DESIGN AND METHODS

Animal experiments. *TLR4*$^{-/-}$ mice with a C57BL/6 background (16) were kindly provided by Prof. Vincent Deubel and Prof. Baoxue Ge (Institute Pasteur of Shanghai, Chinese Academy of Sciences). Wild-type (C57BL/6) mice were purchased from the Shanghai Laboratory Animal Co. Mice were housed under a 12-h dark/light cycle with free access to standard chow and water. For experiments, 6- to 8-week-old male wild-type and *TLR4*$^{-/-}$ mice were housed individually and fasted at 9:00 A.M. Twenty-four hours later, mice were killed and serum, liver, epididymal fat, and gastrocenimus muscle were collected and snap-frozen in liquid nitrogen for further analysis. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Chinese Academy of Sciences.

For C75 (Sigma) treatment, mice received intraperitoneal injection of 20 mg/kg C75 dissolved in 200 μl RPMI-1640 right before fasting; control mice were injected with 200 μl RPMI accordingly. For the insulin tolerance test, mice were fasted for 24 h and blood glucose was assessed before insulin (novolin) injection (0.25 units/kg i.p.) and at 15, 30, 45, and 60 min after injection. For the pyruvate tolerance test, mice were fasted for 24 h and blood glucose were assessed before pyruvate injection (2g/kg i.p.) and at 20, 40, 60, and 80 min after injection.

Assessment of energy expenditure and respiratory quotient. O_2 and CO_2 consumption were determined in the comprehensive laboratory animal monitoring system (CLAMS; Columbus Instruments) according to the manufacturer's instructions. Animals were acclimated to the system for 18–24 h with free

From the Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China; and the Graduate School of Chinese Academy of Sciences, Shanghai, China.

Corresponding author: Yingying Le, yyle@sibs.ac.cn, or Ying Qin Zang, yqin@sibs.ac.cn.

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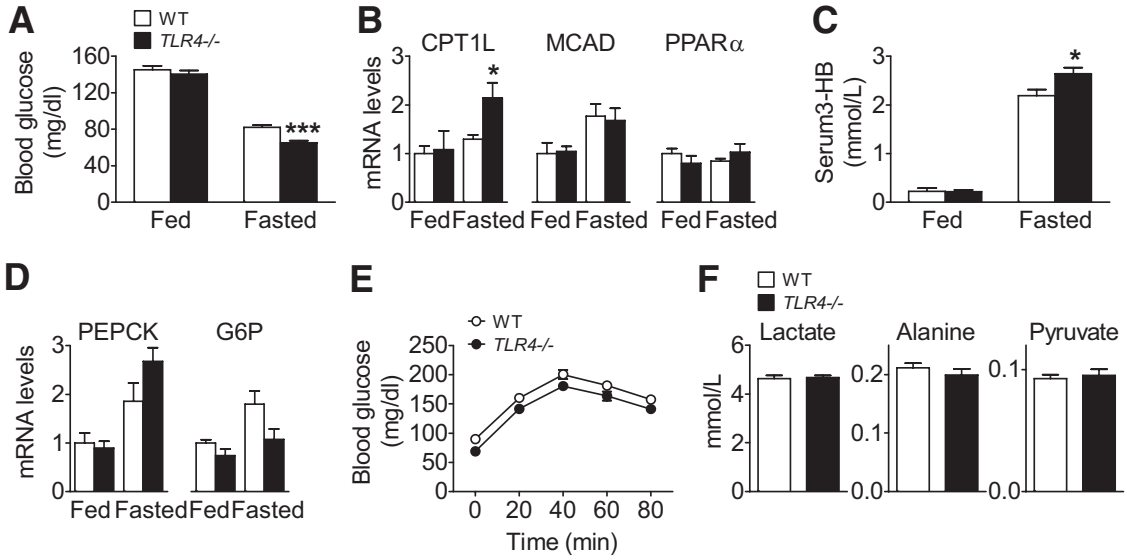

FIG. 1. Severe fasting hypoglycemia and normal hepatic gluconeogenesis in *TLR4*$^{-/-}$ mice. Male wild-type (WT) and *TLR4*$^{-/-}$ mice were either fed or fasted for 24 h. *A*: Blood glucose levels; $n = 9–10$. *B*: mRNA levels of *CPT1*, *MCAD*, and *PPARα* in liver. *C*: Circulating levels of β-hydroxybytyrate. *D*: mRNA levels of *PEPCK* and *G6Pase* (G6P) in liver. *E*: Pyruvate tolerance test after 24 h of fasting; $n = 8–9$. *F*: Serum levels of gluconeogenic substrates in the fasted state. All data shown are means ± SEM; $n = 4–5$ unless otherwise indicated. *$P < 0.05$, ***$P < 0.001$ vs. wild-type mice.

access to food and water, and the measurements were conducted for 24 h from 9:00 A.M. without food supply.

Metabolic parameters analysis. Total fat mass was measured by nuclear magnetic resonance with a Minispec Mq7.5 Analyzer (Bruker, Germany). Blood glucose was determined using a glumeter (Freestyle). Serum levels of TG and FFA were determined by an enzymatic triglyceride assay kit (Applygen, Beijing) and a LabAssay non-esterified fatty acids (NEFA) kit (Wako), respectively. Serum levels of lactate, alanine and pyruvate were determined by enzymatic kits (Biovision). Serum insulin levels were determined by an ELISA kit (Millipore). Tissue TG content was measured as previously described (17). Briefly, frozen liver and gastrocenimus muscle were weighted, homogenized in isopropanol, incubated at 4°C for 1 h, and centrifuged. The supernatants were collected to measure TG concentrations by the enzymatic kit.

Lipolysis assay. Lipolysis studies were performed in explants from freshly isolated edipidymal fat as previously described (18). Briefly, fat explants from mice fasted for 24 h were incubated in Krebs-Ringer buffer (12 mmol/l HEPES, 121 mmol/l NaCl, 4.9 mmol/l KCl, 1.2 mmol/l $MgSO_4$, and 0.33 mmol/l $CaCl_2$) with 3.5% fatty acid–free BSA and 0.1% glucose. Glycerol (Applygen, Beijing) and NEFA (Wako) contents were measured after one-hour incubation.

Muscle fatty acid oxidation. Fatty acid oxidation rate was determined in muscle homogenates as previously described (19) with modifications. Briefly, muscle homogenates were incubated with reaction mixture, in which [^{3}H]oleate was used as substrate. After incubation, reaction medium was added with 2.5 ml methanol:chloroform (1:2) and 1 ml 2 mol/l KCl/2 mol/l HCl to separate the aqueous phase, which contains ^3H_2O and was taken for scintillation counting.

PDC activity assay. Actual PDC activity in gastrocenimus muscle was measured by an assay coupling with arylamine N-acetyltransferase as previously described (20). One unit of PDC activity corresponds to the acetylation of 1 μmol p-(paminophenylazo)benzene sulfonic acid/min at 30°C.

RNA extraction and real-time PCR. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-Free DNase. First-strand cDNA was synthesized with M-MLV reverse transcriptase and oligo (dT) primers. Real-time quantitative PCR was performed on an ABI Prism 7900 sequence detection system (Applied Biosystems), using SYBR Green PCR Master Mix (Applied Biosystems). The results were normalized against 36B4 gene expression. The primer sequences are available upon request.

Western blot analysis. Tissue was homogenized in radioimmunoprecipitation assay lysis buffer and centrifuged to remove the debris. Proteins were separated by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and blocked in 5% nonfat milk at room temperature for 1 h.The membrane was incubated overnight at 4°C with the following primary antibodies: mouse anti-fatty acid synthase (FAS) (BD Transduction Laboratories) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (KANGCHEN, China), rabbit anti–ATP citrate lyase (ACL), IκBα, phosphorylated levels of insulin receptor, phosphorylated levels of Akt, and phosphorylated levels of glycogen synthase kinase (GSK)3β (Cell Signaling). After washing, the blots were incubated with horseradish peroxidase–conjugated anti-mouse or rabbit immunoglobulin G secondary antibody for 1 h at room temperature and then developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Statistical analysis. Data are presented as means ± SEM. Statistical significance was determined using two-tailed unpaired Student's *t* test. A value of $P < 0.05$ was considered significant.

RESULTS

***TLR4*$^{-/-}$ mice exhibit aggravated fasting hypoglycemia.** We first examined the involvement of TLR4 in glucose metabolism in response to fasting. After fasted for 24 h, *TLR4*$^{-/-}$ mice exhibited significantly lower blood glucose levels than wild-type controls (wild type: 82 ± 2.42 mg/dl, *TLR4*$^{-/-}$: 65.11 ± 2.48 mg/dl; $P < 0.001$), whereas no difference was observed in the fed state (Fig. 1*A*), suggesting an essential role for TLR4 in maintaining fasting blood glucose levels.

To explore the mechanisms underlying the severe fasting hypoglycemia, we first focused on hepatic glucose production. We examined the expression levels of key enzymes governing FAO and gluconeogenesis in liver. No differences in the mRNA levels of medium-chain acyl-CoA dehydrogenase (*MCAD*) or *PPARa* were observed between wild-type and *TLR4*$^{-/-}$ mice (Fig. 1*B*). Surprisingly, TLR4 deficiency increased the mRNA levels of carnitine palmitoyltransferase 1 (*CPT1*) in the liver (Fig. 1*B*) and the β-hydroxybytyrate levels in the serum (Fig. 1*C*). mRNA levels of *PEPCK* in *TLR4*$^{-/-}$ mice were comparable with those in wild-type controls, while *G6Pase* only showed a tendency to decrease in *TLR4*$^{-/-}$ mice ($P = 0.071$) (Fig. 1*D*). To further compare the abilities of gluconeogenesis between mice, we performed a pyruvate tolerance test. As expected, wild-type and *TLR4*$^{-/-}$ mice showed similar glycemic responses to pyruvate after fasting (Fig. 1*E*), indicating comparable capacities of gluconeogenesis. Also, no differences in the serum levels of gluconeogenic substrates, including lactate, alanine, and pyruvate, were observed between wild-type and *TLR4*$^{-/-}$ mice in the fasted state (Fig. 1*F*). These data suggest that the fasting

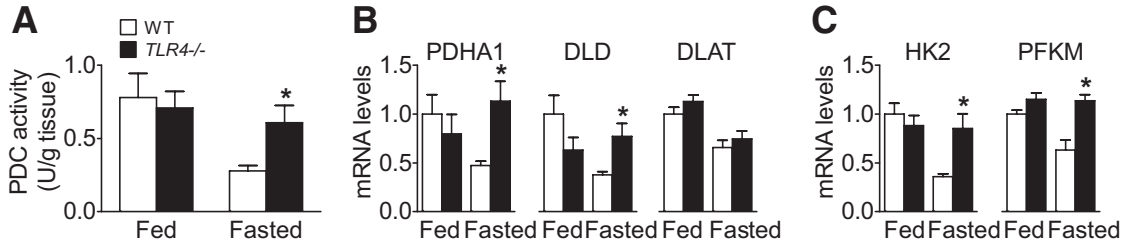

FIG. 2. Reversed PDC activity and glycolytic enzymes expression in skeletal muscle of $^{-/-}$ mice during fasting. Male wild-type (WT) and *TLR4*$^{-/-}$ mice were either fed or fasted for 24 h. *A*: PDC activity in skeletal muscle. *B*: mRNA levels of PDC components in skeletal muscle. *C*: mRNA levels of rate-limiting enzymes of glycolysis in skeletal muscle. All data shown are means ± SEM; $n = 4–7$. *$P < 0.05$ vs. wild-type mice.

hypoglycemia in *TLR4*$^{-/-}$ mice does not result from impaired hepatic gluconeogenesis.

TLR4 deficiency reverses PDC activity and glycolytic enzymes expression in skeletal muscle during fasting. During fasting, glucose could also be spared by blocking the irreversible net loss of glucose in skeletal muscle through PDC inhibition (3,4). PDC consists of three enzymes: pyruvate dehydrogenase (*PDHA1*), dihydrolipoamide acetyltransferase (*DLAT*), and dihydrolipoamide dehydrogenase (*DLD*). We found that fasting significantly inhibited PDC activity in skeletal muscle of wild-type mice. Notably, the inhibition was largely reversed by TLR4 deletion (Fig. 2*A*). In addition, fasting inhibited the mRNA expression of all three enzymes of PDC in wild-type mice, whereas it only inhibited the expression of *DLAT* in *TLR4*$^{-/-}$ mice (Fig. 2*B*). These results suggest that unsuppressed PDC activity in skeletal muscle contributes at least partly to the severe fasting hypoglycemia in *TLR4*$^{-/-}$ mice. We also found that TLR4 deficiency reversed the mRNA levels of hexokinase 2 (HK2) and muscle phosphofructokinase (PFKM), two rate-limiting enzymes of glycolysis, in skeletal muscle during fasting (Fig. 2*C*), suggesting that glycolysis may also be reversed by TLR4 deficiency.

TLR4 deficiency increases lipid levels in circulation and skeletal muscle in response to fasting. We next examined the involvement of TLR4 in lipid metabolism. Compared with wild-type mice, *TLR4*$^{-/-}$ mice exhibited higher levels of TG and FFA in circulation after fasting and similar serum levels of TG and FFA in the fed state (Fig. 3*A* and 3*B*). In liver, TG levels were increased in response to fasting in both wild-type and *TLR4*$^{-/-}$ mice, with a tendency to be higher in *TLR4*$^{-/-}$ mice (Fig. 3*C*). In skeletal muscle, a 2.5-fold increase in TG content was observed in *TLR4*$^{-/-}$ mice in the fasted state only (Fig. 3*D*). These findings indicate that TLR4 deficiency elevates lipid levels in skeletal muscle and circulation in response to starvation.

***TLR4*$^{-/-}$ mice exhibit normal adipose tissue lipolysis and increased muscle fatty acid oxidation during fasting.** The lipid abnormalities in *TLR4*$^{-/-}$ mice might result from increased TG mobilization in WAT, decreased FAO, and/or increased de novo lipogenesis in key metabolic tissues. We found that fasting significantly reduced total fat mass in both wild-type and *TLR4*$^{-/-}$ mice but with less loss in *TLR4*$^{-/-}$ mice (Fig. 4*A*). Consistently, *TLR4*$^{-/-}$ mice exhibited less epididymal fat loss during fasting (Fig. 4*B*). We also performed lipolysis assay in epididymal fat explants from fasted mice. Wild-type and *TLR4*$^{-/-}$ mice exhibited similar glycerol release rates (Fig. 4*C*), indicating comparable lipolysis. Interestingly, *TLR4*$^{-/-}$ mice showed lower NEFA release than wild-type mice, resulting in a significant reduction in NEFA-to-glycerol ratio (Fig. 4*C*), indicating increased fatty acid reesterification. These data suggest that the higher lipid levels in *TLR4*$^{-/-}$ mice do not result from increased fat mobilization.

As to muscle FAO, TLR4 deficiency increased the mRNA levels of *CPT1* and *MCAD* after fasting and had no effect on the expression of *PPARa* (Fig. 4*D*). The mRNA levels of acetyl-CoA carboxylase 2 (*ACC2*), which inhibits CPT1 through its product mitochondrial malonyl-CoA (21), were similar between two sets of mice (Fig. 4*D*), indicating that ACC2-related regulation may not be involved in the augmentation of CPT1 in *TLR4*$^{-/-}$ mice. More importantly, *TLR4*$^{-/-}$ mice exhibited increased muscle FAO rates compared with wild-type mice after fasting (Fig. 4*E*). These observations suggest that FAO does not contribute to the higher lipid levels in *TLR4*$^{-/-}$ mice.

TLR4 is required for inhibition of lipogenic genes in skeletal muscle during fasting. We then examined the expression of enzymes regulating de novo fatty acid synthesis, including *ACC1*, *ACL*, and *FAS*. Fasting decreased the expression of these enzymes in liver, WAT, and muscle (Fig. 5*A–C*). Surprisingly, TLR4 deficiency resulted in a complete reversion of the mRNA levels of *ACC1* and *ACL* and a partial reversion of FAS in muscle but not liver or WAT (Fig. 5*A*-5*C*). TLR4 deficiency also fully reversed the protein levels of ACL and FAS in muscle but not liver (Fig. 5*D*-5*E*). In addition, the mRNA expression of elongation of very long–chain fatty acids, family member 6 (*Elovl6*) and stearoyl-CoA desaturase 1 (*SCD1*), two other important enzymes regulating fatty acid synthesis, showed a pattern simiar to that of FAS in skeletal muscle (Fig. 5*C*). Consistently, the mRNA levels of glycerol-3-

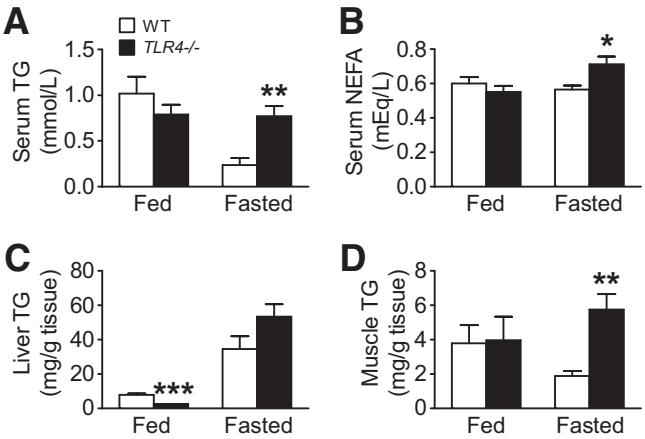

FIG. 3. Increased lipid levels in serum and skeletal muscle of *TLR4*$^{-/-}$ mice in response to fasting. Male wild-type (WT) and *TLR4*$^{-/-}$ mice either fed or fasted for 24 h were examined for serum levels of TG (*A*) and FFA (*B*) and TG levels in liver (*C*) and skeletal muscle (*D*). All data shown are means ± SEM; $n = 4–7$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. wild-type mice.

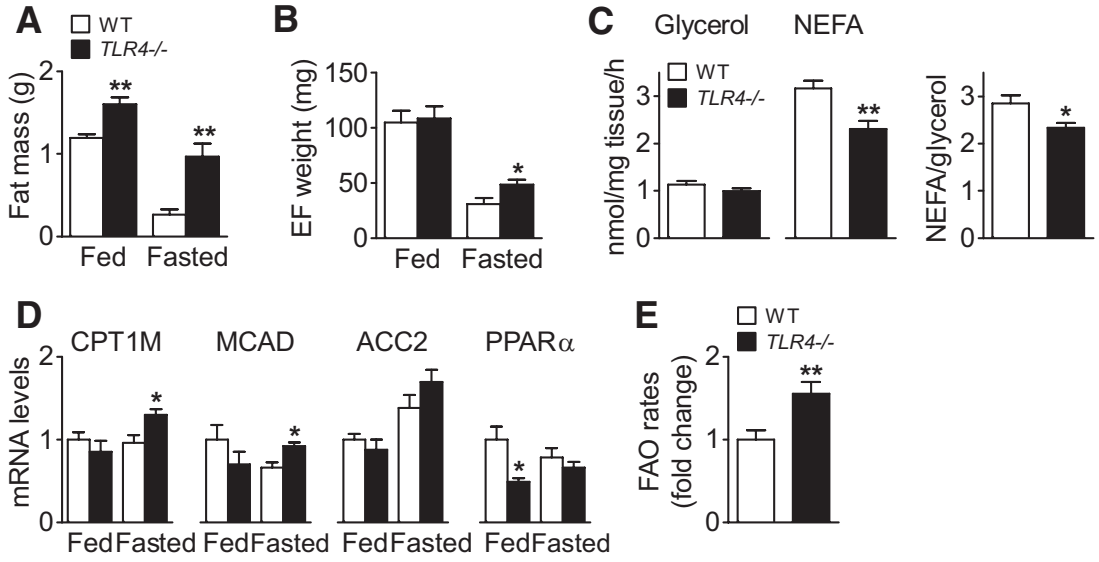

FIG. 4. Effect of TLR4 deficiency on adipose tissue lipolysis and muscle fatty acid oxidation. *A* and *B*: Male mice either fed or fasted for 24 h were examined for total fat mass (*A*) and epididymal fat (EF) weight (*B*). *C*: glycerol and NEFA release from epididymal fat explants from 24 h–fasted mice and the ratio of NEFA to glycerol. *D*: mRNA levels of *CPT1*, *MCAD*, *ACC2*, and *PPARα* in skeletal muscle. *E*: fatty acid oxidation rates measured in muscle homogenates from fasted mice. All data shown are means ± SEM; $n = 4–7$. *$P < 0.05$, **$P < 0.01$ vs. wild-type (WT) mice.

phosphate acyltransferase 1 (*GPAT1*) and diacylglycerol acyltransferase 1 (*DGAT1*), two enzymes controlling TG synthesis, were also higher in skeletal muscle of *TLR4*$^{-/-}$ mice (Fig. 5*C*). The mRNA levels of *SREBP1c* and *PPARγ*, two transcription factors controlling lipogenesis (22,23), were all decreased in the skeletal muscle of wild-type and *TLR4*$^{-/-}$ mice after fasting, but *TLR4*$^{-/-}$ mice had a higher levels of *PPARγ* (Fig. 5*F*), suggesting that PPARγ might be involved in TLR4-dependent inhibition of lipogenic genes in skeletal muscle. Collectively, these data suggest that TLR4 is required for the inhibition of lipogenic genes in skeletal muscle during fasting and provide a possible explanation for the relatively higher TG levels in skeletal muscle but not liver or WAT of *TLR4*$^{-/-}$ mice.

To further test the contribution of lipid synthesis to the serum lipids in *TLR4*$^{-/-}$ mice, we treated mice with C75,

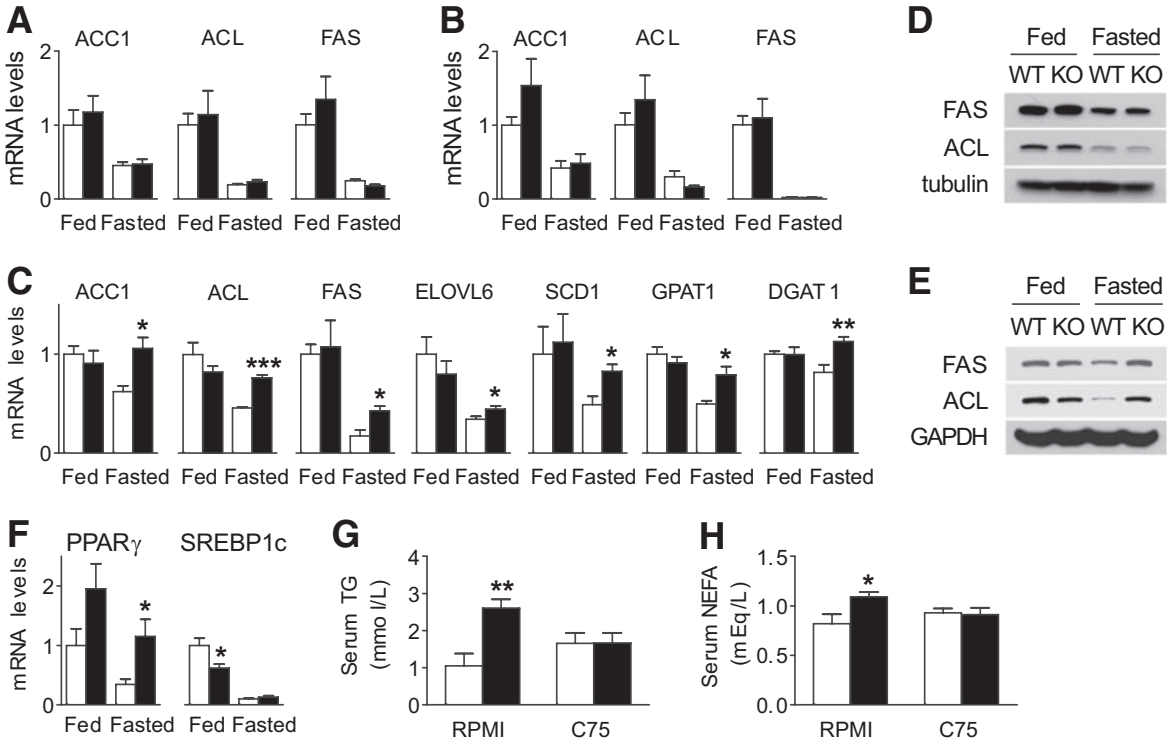

FIG. 5. Effect of TLR4 deficiency on the expression of lipogenic enzymes during fasting. *A–C*: mRNA levels of enzymes regulating de novo fatty acid synthesis in liver (*A*), WAT (*B*), and skeletal muscle (*C*). *D* and *E*: immunoblot analysis of ACL and FAS in liver (*D*) and skeletal muscle (*E*). *F*: mRNA levels of lipogenic transcription factors in skeletal muscle. *G* and *H*: wild-type (WT) and *TLR4*$^{-/-}$ mice either received RPMI-1640 vehicle or C75 were fasted for 24 h and examined for serum levels of TG (*G*) and FFA (*H*). All data shown are means ± SEM; $n = 4–6$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. wild-type mice. □, wild type; ■, *TLR4*$^{-/-}$.

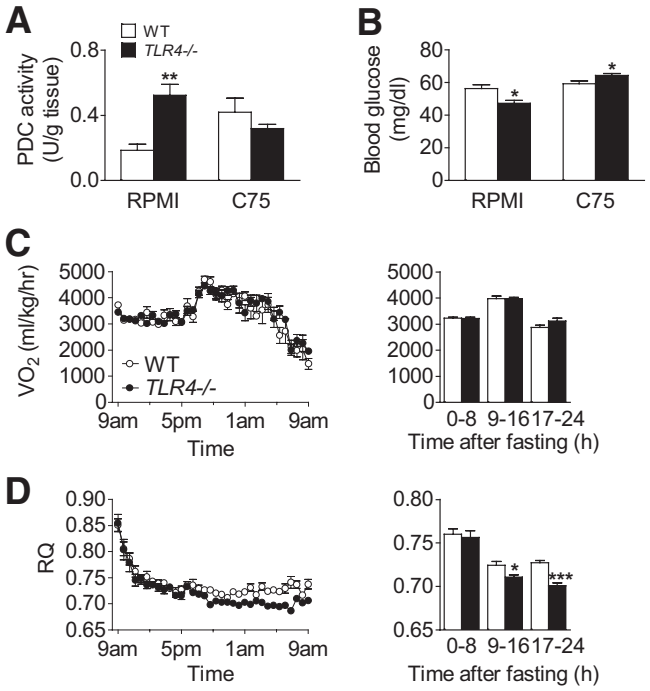

FIG. 6. TLR4 maintains fuel homeostasis during fasting. *A* and *B*: RPMI-1640 vehicle – or C75-treated male mice were fasted for 24 h and examined for PDC activity in skeletal muscle (*A*) and blood glucose levels (*B*). *C* and *D*: Measurements of oxygen consumption (*C*) and RQ (*D*) during fasting were carried out in the metabolic cage during fasting. All data shown are means ± SEM; $n = 4–6$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. wild-type (WT) mice.

a potent FAS inhibitor (24), to suppress de novo fatty acid synthesis. After fasting, vehicle-treated *TLR4*$^{-/-}$ mice showed elevated serum levels of TG and FFA (Fig. 5*G* and 5*H*)—similar to untreated mice. Notably, C75 treatment fully abolished these elevations (Fig. 5*G* and 5*H*). Together with our observation that FAS expression was only reversed in muscle, but not liver and WAT, these data indicate that TLR4 may be involved in fasting serum lipids regulation by governing muscle fatty acid synthesis.

TLR4 maintains homeostasis between glucose and lipid fuel during fasting. Because de novo fatty acid synthesis uses acetyl-CoA oxidized from pyruvate as substrate, we asked whether TLR4 controls PDC activity and blood glucose levels through fatty acid synthesis regulation. As expected, we found that C75 treatment abolished the increase in muscle PDC activity and the severe fasting hypoglycemia resulting from TLR4 deficiency (Fig. 6*A* and 6*B*). These findings indicate that excess acetyl-CoA from increased muscle pyruvate oxidative decarboxylation in *TLR4*$^{-/-}$ mice may enter lipogenesis instead of TCA cycle. Thus, to better understand the effect of TLR4 deficiency on systemic fuel utilization, energy expenditure and respiratory quotient (RQ) were determined. During fasting, total energy expenditure was comparable in *TLR4*$^{-/-}$ mice and wild-type mice (Fig. 6*C*). The RQ of wild-type and *TLR4*$^{-/-}$ mice exhibited a similar pattern, with a rapid decrease after fasting followed by a steady level, indicating the shift of fuel utilization from carbohydrates to fat. However, the RQ of *TLR4*$^{-/-}$ mice decreased to a lower level than that of wild-type mice (Fig. 6*D*), indicating that more fat was used as energy. The lower RQ level in *TLR4*$^{-/-}$ mice is consistent with their increased FAO rates and supports that the products of pyruvate oxidative decarboxylation may be used for lipogenesis. Collectively, these data suggest an important role for TLR4 in maintaining the homeostasis between glucose and lipid fuel during fasting.

Insulin signaling and local inflammation are not involved in TLR4-dependent regulation of fasting metabolism. TLR4 has been reported to modulate insulin action by enhancing proinflammatory cytokine expression (13). However, TLR4 deficiency had no effect on circulating insulin levels and insulin sensitivity during fasting (Fig. 7*A* and *B*). In addition, phosphorylated levels of insulin receptor, Akt, and GSK3β, key molecules of insulin signaling, were comparable in skeletal muscle between wild-type and *TLR4*$^{-/-}$ mice upon insulin stimulation in the

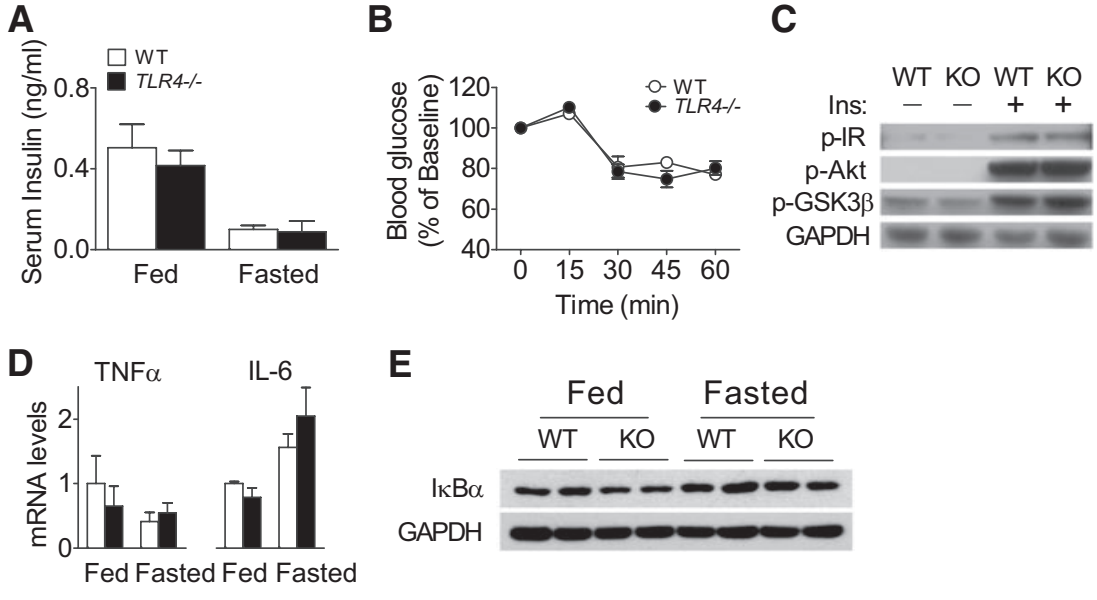

FIG. 7. Insulin signaling and expression of inflammatory genes. Male wild-type and *TLR4*$^{-/-}$ mice were either fed or fasted for 24 h. *A*: Serum insulin levels. *B*: Insulin tolerance tests in the fasted state. *C*: Immunoblot analysis of phosphorylated levels of insulin receptor (p-IR), Akt (p-Akt), and GSK3β (p-GSK3β) in skeletal muscle of mice with or without insulin stimulation after fasting. *D*: mRNA levels of *TNFα* and *IL-6* in skeletal muscle. *E*: Immunoblot analysis of IκBα in skeletal muscle. All data shown are means ± SEM; $n = 4–6$.

fasted state (Fig. 7*C*). We also observed no differences in the mRNA levels of tumor necrosis factor α (*TNFα*) or interleukin-6 (*IL-6*) in skeletal muscle between wild-type and *TLR4*$^{-/-}$ mice (Fig. 7*D*). Nuclear factor-κB is the key molecule mediating TLR4 activation–induced proinflammatory cytokine expression. Consistently, *TLR4*$^{-/-}$ mice had protein levels of IκBα comparable with those of wild-type controls in skeletal muscle after fasting (Fig. 7*E*). These results suggest that TLR4 regulates fasting metabolic adaptations in skeletal muscle independent of insulin and local inflammation.

DISCUSSION

The goal of this study was to investigate the physiological role of TLR4 in metabolism. We found that TLR4 played an important role in glucose and lipid metabolism in response to starvation through regulation of critical metabolic pathways in skeletal muscle, including pyruvate oxidative decarboxylation and de novo lipogenesis.

TLR4$^{-/-}$ mice exhibited exacerbated fasting hypoglycemia along with unimpaired hepatic gluconeogenesis. Strikingly, we observed that starvation inhibited the PDC activity in skeletal muscle of wild-type mice and that this inhibition was markedly reversed in TLR4-deficient mice. Inhibition of PDC activity is essential for glucose preservation because acetyl-CoA could not be reconverted to pyruvate. The decline in PDC activity permits cycling of carbon between glycolysis and gluconeogenesis and thus maintains blood glucose even if glycolysis is active (3). Besides, Jeoung et al. (25) have reported that partially reversed fasting PDC activity by pyruvate hydrogenase kinase 4 deletion, a critical PDC kinase, results in exacerbated fasting hypoglycemia in mice. Thus, although other mechanism(s) may also be involved, our results clearly suggest an essential role for TLR4 in sparing glucose during starvation, at least in part, through PDC inhibition in skeletal muscle. Interestingly, the reversed PDC activity is accompanied with the reversed mRNA expression of two of its components, *PDHA1* and *DLD*. Thus, although the PDC activity is generally controlled by reversible phosphorylation (26), regulation at the transcriptional level may also be involved. Intriguingly, the protein levels of PDHA1 in mitochondrial extracts do not change during fasting (25); we therefore measured the mRNA levels of superoxide dismutase 2 (*SOD2*), a reference mitochondrial marker. We found that fasting did not affect the expression of *SOD2* in wild-type mice (data not shown), indicating that the suppressive effect is specific to components of PDC.

During fasting, PDC inhibition is closely paralleled by a reduced rate of glycolysis. Intriguingly, the mRNA expression of glycolytic enzymes, *HK2* and *PFKM*, was also reversed in *TLR4*$^{-/-}$ mice, which is indicative of reversed glycolysis. These results indicate that TLR4 may be involved in inhibiting muscle glucose disposal through coordinate suppression of glycolysis and pyruvate oxidative decarboxylation during fasting. In addition, we found that, in murine primary muscle cells, activation of TLR4 by lipopolysaccharide had no effect on either PDC activity or expression of *HK2* and *PFKM* (data not shown), indicating that the regulation of these enzymes by TLR4 during fasting may not be direct.

TLR4$^{-/-}$ mice also exhibited higher lipid levels in muscle and circulation in the fasted state. These mice showed less fat loss but similar adipose lipolysis capacity compared with wild-type mice, ruling out the contribution of fat mobilization to serum lipid elevation. In fat lipolysis assay, we unexpectedly observed a decreased ratio of NEFA to glycerol in fasted *TLR4*$^{-/-}$ mice, indicating that the higher serum lipid may contribute to less fat loss, possibly through increased fatty acid reesterification. *TLR4*$^{-/-}$ mice also showed increased expression of FAO genes and FAO rates in muscle, as well as reduced RQ levels during fasting, arguing against decreased FAO as a contributor to lipid abnormalities.

In terms of lipogenesis, FAS inhibitor C75 treatment abolished the elevations of serum lipids in *TLR4* mice. Although the effect of C75 is not tissue specific, TLR4 deficiency reversed FAS expression in skeletal muscle only, not liver or WAT, during fasting, indicating that C75 effect on muscle FAS may account for its abolishment on serum lipid increases. It is therefore likely that reversed muscle lipogenesis in *TLR4*$^{-/-}$ mice may contribute to blood lipid increases during fasting, although other tissue (like the skeleton) may also be involved. We also found that TLR4 deletion increased the mRNA levels of *CD36* but had no effect on *LPL* expression in the skeletal muscle during fasting (data not shown), arguing against the possibility that TLR4 deficiency increases circulating lipid levels by inhibiting muscle FFA or TG uptake. Thus, how dysregulated muscle lipogenesis under a fasting condition contributes to the lipid disturbance in circulation requires further investigation.

Liver and WAT are widely considered major lipogenic tissues. However, muscle tissue may also actively participate in de novo lipogenesis. Several studies around the 1970s showed that liver and dissectible adipose tissue usually contribute to no more than 40% of total fatty acid synthesis rate in rodents. Instead, the rest of carcass, mainly muscle and skeleton, is the major site of fatty acid synthesis (27–32). Although there was one study that suggested that the intermuscular fat in carcass might contribute to lipogenesis (33), several following studies using dissected muscle without fat showed that muscle tissue may be an important lipogenic tissue because of its large mass (27,34,35). Interestingly, in the fasted state, liver's contribution to lipogenesis becomes almost negligible (31,36), whereas muscle may be one of the predominant tissues for lipogenesis (36). Recently, several myotube cell-based studies have also supported an active capacity of muscle in de novo lipogenesis. The expression of SREBP1c, a key lipogenic transcription factor, is clearly detectable in muscle at a level close to the liver (37). Upon insulin or glucose stimulation, muscle SREBP1c regulates the transcription of lipogenic enzymes, such as FAS and ACL (37,38). Glucose also stimulates muscle de novo lipogenesis, which in turn results in lipid accumulation in myotubes (38,39). Taken together, these studies support an active role for muscle in lipogenesis, although its role(s) under physiological or pathophysiological conditions requires further investigation.

Glucose and lipid are tightly related in metabolism. In the glucose–fatty acid cycle, FAO suppresses PDC activity by increasing the ratio of acetyl-CoA to CoA (40). Intriguingly, TLR4 may control glucose metabolism through regulation of de novo fatty acid synthesis, because FAS inhibition abolished the fasting hypoglycemia and muscle PDC activity increase in *TLR4*$^{-/-}$ mice. Since de novo fatty acid synthesis uses acetyl-CoA as substrate, it is conceivable that de novo fatty acid synthesis regulates

muscle PDC activity by altering the ratio of acetyl-CoA to CoA. These findings indicate that not only lipid oxidation but also lipid synthesis may affect muscle glucose metabolism.

Currently, it is unclear how TLR4 is activated and regulates these metabolic pathways during fasting. Unlike its reported role in insulin resistance (13), TLR4-dependent regulation of fasting metabolism is neither insulin nor inflammation dependent. During starvation, the increases in fatty acids have been reported to modulate muscle PDC activity (41,42). However, although direct FAO is generally thought to mediate the acute effect of FFA on PDC activity (41), it is not responsible for this long-term (starvation) regulation of muscle PDC (43), indicating the existence of alternative mechanism(s). Because saturated fatty acids have been reported as the agonists for TLR4 (8–10), it is possible that TLR4 activation by FFA regulates muscle PDC activity during starvation. Hormone signaling, such as triiodothyronine, is critical for PDC activity modulation during the fed-to-starved transition (41,44), raising the possibility that TLR4 regulates fasting metabolism through cross-talk with other hormone signaling. These interesting hypotheses require further investigation.

In summary, our findings identify TLR4 as a physiological regulator of fuel metabolism independent of insulin and identify a novel physiological role for TLR4 in metabolism. Muscle lipogenesis may play an important role in maintaining fasting fuel homeostasis.

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