Regulation of fasting fuel metabolism by Toll-like receptor 4

Running title: TLR4 regulates fasting metabolism

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**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 investigate the involvement of TLR4 in fasting metabolism.

**Research design and methods** - Wild-type (WT) and TLR4 deficient (TLR4/-) mice were either fed or fasted for 24 hours. Glucose and lipid levels in circulation and tissues were measured. Glucose and lipid metabolism in tissues, as well as the expression of related enzymes, were 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.

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 (FGF21) (5,6). TLR4 is one of the mammalian pattern-recognized 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 disruption or mutation of TLR4 resist to fatty acids or high-fat-diet induced insulin resistance (11-13,15). Further, hematopoietic cell-specific deletion of TLR4 ameliorates hepatic and adipose tissue insulin resistance in high-fat-fed mice (14). As 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 C57BL/6 background (16) were kindly provided by Prof. Vincent Deubel and Prof. Baoxue Ge (Institute Pasteur of the Shanghai, CAS). WT (C57BL/6) mice were purchased from the Shanghai Laboratory Animal Co. Ltd. Mice were housed under a 12-hr dark/light cycle, with free access to standard chow and water. For experiments, 6-8 weeks old, male WT and TLR4/-/- mice were housed individually and fasted at 9:00 am. Twenty four hours later, mice were sacrificed, 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, CAS.

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

Assessment of energy expenditure and respiratory quotient. O₂ and CO₂ 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 18-24h with free access to food and water, and the measurements were conducted for 24h from 9:00 am without food supply.

Metabolic parameters analysis. Total fat mass was measured by nuclear magnetic resonance (NMR) 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 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 1h, 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 epididymal fat as previously described (18). Briefly, fat explants from mice fasted for 24h were incubated in Krebs-Ringer buffer (12mM HEPES, 121mM NaCl, 4.9mM KCl, 1.2mM MgSO4 and 0.33mM CaCl2) 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 were determined in muscle homogenates as previously described (19), with modifications. Briefly, muscle homogenates were incubated with reaction mixture, in which [³H]-Oleate was used as substrate. After incubation, reaction medium were added with 2.5ml of methanol:chloroform (1:2) and 1ml of 2M KCl/2M HCl to separate the organic from aqueous phase, which contains ³H₂O 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 of
p-(paminophenylazo)benzene sulfonic acid per 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 ABI Prism 7900 sequence detection system (Applied Biosystems), using SYBR Green PCR Mater 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 RIPA lysis buffer and centrifuged to remove the debris. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and blocked in 5% nonfat milk at room temperature for 1h. The membrane was incubated overnight at 4°C with the following primary antibodies: mouse anti-FAS (BD Transduction Laboratories) and GAPDH (KANGCHEN, China), rabbit anti-ACL, IκBα, phospho-IR, phospho-Akt, and phospho-GSK3β (Cell Signaling). After washing, the blots were incubated with HRP-conjugated anti-mouse or rabbit IgG secondary antibody for 1h at room temperature, and then developed with the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific).

**Statistical analysis.** Data are presented as the mean ± 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 24hrs, **TLR4-/-** mice exhibited significantly lower blood glucose levels than WT controls (WT = 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. 1A), 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) and PPARα were observed between WT and **TLR4-/-** mice (Fig. 1B). Surprisingly, TLR4 deficiency increased the mRNA levels of carnitine palmitoyltransferase 1 (CPT1) in the liver (Fig. 1B) and the β-hydroxybutyrate levels in the serum (Fig. 1C). mRNA levels of PEPCK in **TLR4-/-** mice were comparable to those in WT controls, while G6Pase only showed a tendency to decrease in **TLR4-/-** mice (*P* = 0.071) (Fig. 1D). To further compare the abilities of gluconeogenesis between mice, we performed pyruvate tolerance test (PTT). As expected, WT and **TLR4-/-** mice showed similar glycemic responses to pyruvate after fasting (Fig. 1E), indicating comparable capacities of gluconeogenesis. Also, no differences in the serum levels of gluconeogenic substrates, including lactate, alanine and pyruvate, were observed between WT and **TLR4-/-** mice in the fasted state (Fig. 1F). These data suggest that the fasting 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 WT mice. Notably, the inhibition was largely reversed by TLR4 deletion (Fig. 2A). In addition, fasting inhibited the mRNA expression of all three enzymes of PDC in WT mice, while
only inhibited the expression of DLAT in TLR4-/− mice (Fig. 2B). 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. 2C), 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 WT mice, TLR4-/− mice exhibited higher levels of TG and FFA in circulation after fasting, while had similar serum levels of TG and FFA in the fed state (Fig. 3A and 3B). In liver, TG levels were increased in response to fasting in both WT and TLR4-/− mice, with a tendency to be higher in TLR4-/− mice (Fig. 3C). In skeletal muscle, a 2.5 fold increase in TG content was observed in TLR4-/− mice in the fasted state only (Fig. 3D). 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 WT and TLR4-/− mice, but with a less loss in TLR4-/− mice (Fig. 4A). Consistently, TLR4-/− mice exhibited less epididymal fat (EF) loss during fasting (Fig. 4B). We also performed lipolysis assay in EF explants from fasted mice. WT and TLR4-/− mice exhibited similar glycerol release rates (Fig. 4C), indicating comparable lipolysis. Interestingly, TLR4-/− mice showed lower NEFA release than WT mice, resulting in a significant reduction in NEFA/glycerol ratio (Fig. 4C), 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, while had no effect on the expression of PPARα (Fig. 4D). 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. 4D), indicating 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 WT mice after fasting (Fig. 4E). 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, ATP citrate lyase (ACL) and fatty acid synthase (FAS). Fasting decreased the expression of these enzymes in liver, WAT and muscle (Fig. 5A-5C). 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. 5A-5C). TLR4 deficiency also fully reversed the protein levels of ACL and FAS in muscle, but not liver (Fig. 5D-5E). In addition, the mRNA expression of elongation of very long chain fatty acids, family member 6 (Elov6) and stearoyl-CoA desaturase 1 (SCD1), another two important enzymes regulating fatty acid synthesis, showed a similar pattern to FAS in skeletal muscle (Fig. 5C). Consistently, the mRNA levels of glycerol-3-phosphate acyltransferase 1 (GPAT1) and diacylglycerol acyltransferase 1 (DGAT1), two enzymes controlling TG synthesis, were also higher in skeletal muscle of TLR4-/− mice (Fig. 5C). The mRNA levels of SREBP1c and PPARγ, two transcription factors controlling lipogenesis (22,23), were all decreased in the skeletal muscle of WT
and TLR4-/− mice after fasting, but TLR4-/− mice had a higher levels of PPARγ (Fig. 5F), 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, 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. 5G and 5H), similar to untreated mice. Notably, C75 treatment fully abolished these elevations (Fig. 5G and 5H). 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 resulted from TLR4 deficiency (Fig. 6A and 6B). 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 were comparable in TLR4-/− mice and WT mice (Fig. 6C). The RQ of WT and TLR4 -/- mice exhibited 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 WT mice (Fig. 6D), indicating 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. 7A and 7B). In addition, phosphorylated levels of insulin receptor, Akt and GSK3β, key molecules of insulin signaling, were comparable in skeletal muscle between WT and TLR4-/− mice upon insulin stimulation in the fasted state (Fig. 7C). We also observed no differences in the mRNA levels of TNFα or IL-6 in skeletal muscle between WT and TLR4-/− mice (Fig. 7D). NF-κB is the key molecule mediating TLR4 activation-induced proinflammatory cytokine expression. Consistently, TLR4-/− mice had comparable protein levels of IκBα to WT controls in skeletal muscle after fasting (Fig. 7E). These results suggest that TLR4 regulates fasting metabolic adaptations in skeletal muscle independent of insulin and local inflammation.

**DISCUSSION**

The goal of this study is 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 WT mice, and this inhibition was markedly reversed in TLR4 deficient mice. Inhibition of PDC activity is essential for glucose preservation, because acetyl-CoA could not be re-converted 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 have reported that partially reversed fasting PDC activity by PDK4 deletion, a critical PDC kinase, results in exacerbated fasting hypoglycemia in mice (25). 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 does 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 WT mice (data not shown), indicating 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, 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 (LPS) had no effect on both PDC activity and expression of HK2 and PFKM (data not shown), indicating 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 WT mice, ruling out the contribution of fat mobilization to serum lipid elevation. In fat lipolysis assay, we unexpectedly observed 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, but 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 fasting condition contributes to the lipid disturbance in circulation requires further investigation.

Liver and WAT are widely considered as major lipogenic tissues. However, muscle tissue may also actively participate in de novo lipogenesis. Several studies around 1970s’ have shown 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 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 due to 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 also support 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 needs further investigations.

Glucose and lipid are tightly related in metabolism. In glucose-fatty acid cycle, fatty acid oxidation (FAO) suppresses PDC activity by increasing the ratio of [acetyl-CoA]/[CoA] (40). Intriguingly, TLR4 may controls glucose metabolism through regulation of de novo fatty acid synthesis, as 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]/[CoA]. These findings indicate that not only lipid oxidation but also lipid synthesis may affect muscle glucose metabolism.

Currently, we are 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). As 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 remain 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.

Author contributions. S.P. researched data, contributed to discussion, wrote manuscript, reviewed/edited manuscript; H.T. researched data, contributed to discussion, reviewed/edited manuscript; S.Z. researched data, contributed to discussion; Y.Q.Z contributed to discussion, reviewed/edited manuscript; Y.L. researched data, contributed to discussion, reviewed/edited manuscript.

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FIGURE LEGENDS

**Fig. 1.** Severe fasting hypoglycemia and normal hepatic gluconeogenesis in TLR4/-/- mice. Male WT and TLR4/-/- mice were either fed or fasted for 24hrs. (A) Blood glucose levels; n = 9-10. (B) mRNA levels of CPT1, MCAD and PPARα in liver. (C) Circulating levels of β-hydroxybytytrate. (D) mRNA levels of PEPCK and G6Pase in liver. (E) Pyruvate tolerance test after 24hrs of fasting; n = 8-9. (F) Serum levels of gluconeogenic substrates in the fasted state. All data shown are mean ± SEM; n = 4-5 unless indicated. *P<0.05, ***P<0.001 versus WT mice.

**Fig. 2.** Reversed PDC activity and glycolytic enzymes expression in skeletal muscle of TLR4/-/- mice during fasting. Male WT and TLR4/-/- mice were either fed or fasted for 24hrs. (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 mean ± SEM; n = 4-7. *P<0.05 versus WT mice.

**Fig. 3.** Increased lipid levels in serum and skeletal muscle of TLR4/-/- mice in response to fasting. Male WT and TLR4/-/- mice either fed or fasted for 24hrs were examined for serum levels of TG (A) and FFA (B), TG levels in liver (C) and skeletal muscle (D). All data shown are mean ± SEM; n = 4-7; *P<0.05, **P<0.01, ***P<0.001 versus WT mice.

**Fig. 4.** Effect of TLR4 deficiency on adipose tissue lipolysis and muscle fatty acid oxidation. (A-B) Male mice either fed or fasted for 24hrs were examined for total fat mass (A) and EF weight (B). (C) Glycerol and NEFA release from EF explants from 24hrs 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 mean ± SEM; n=4-7; *P<0.05, **P<0.01 versus WT mice.

**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-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-H*) WT and TLR4−/− mice either received RPMI vehicle or C75 were fasted for 24hrs, and examined for serum levels of TG (*G*) and FFA (*H*). All data shown are mean ± SEM; n = 4-6; *P<0.05, **P<0.01, ***P<0.001 versus WT mice.

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

**Fig. 7.** Insulin signaling and expression of inflammatory genes. Male WT and TLR4−/− mice were either fed or fasted for 24hrs. (*A*) Serum insulin levels. (*B*) Insulin tolerance tests in the fasted state. (*C*) Immunoblot analysis of phosphorylated levels of insulin receptor (IR), Akt and 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 IκBa in skeletal muscle. All data shown are mean ± SEM; n = 4-6.

**Figure 1**
Figure 5

A

B

D

C

E

F

G

H

Liver

WAT

ACC1

ACL

FAS

mRNA levels


Figure 6

A

![Graph A](image)

PDC activity (U/g tissue)

WT

TLR4−/−

RPMI

C75

B

![Graph B](image)

Blood glucose (mg/dl)

WT

TLR4−/−

RPMI

C75

C

![Graph C](image)

VO₂ (ml/kg/hr)

WT

TLR4−/−

9am 5pm 1am 9am

Time

0-8 9-1617-24

Time after fasting (h)

D

![Graph D](image)

RQ

WT

TLR4−/−

9am 5pm 1am 9am

Time

0-8 9-1617-24

Time after fasting (h)

Figure 7

A

![Graph A](image)

Serum insulin (ng/ml)

WT

TLR4−/−

Fed

Fast

B

![Graph B](image)

Blood glucose (% of Baseline)

WT

TLR4−/−

0 15 30 45 60

Time (min)

C

![Graph C](image)

WT KO WT KO

Ins:

p-IR

p-Akt

p-GSK3β

GAPDH

D

![Graph D](image)

mRNA levels

TNFα

IL-6

Fed

Fast

Fed

Fast

GAPDH

E

![Graph E](image)

IkBα

GAPDH

Fed

Fast

WT

KO

WT

KO