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Ablation of Lgr4 enhances energy adaptation in skeletal muscle via activation of Ampk/Sirt1/Pgc1α pathway

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ABSTRACT

Leucine-rich repeat-containing G protein-coupled receptor 4 (*Lgr4*) is a newfound obese-associated gene. Previous study reveals that heterozygous mutation of *Lgr4* correlates with decreased body weight in human. In our recent study, we demonstrate that Lgr4 ablation promotes browning of white adipose tissue and improves whole-body metabolic status. However little is known about its role in other metabolic tissues. Herein, we show that *Lgr4* homozygous mutant ($Lgr4^{m/m}$) mice show increased respiratory exchange ratio (RER, closer to 1.0) than wild-type mice at 12:00 AM (food-intake time for mice) while decreased RER (closer to 0.75) at 12:00 PM (fasting for mice), indicating a glucose-prone versus fatty acid-prone metabolic pattern, respectively. Furthermore, Lgr4 ablation increases lipid oxidation-related gene expression while suppresses glucose transporter type 4 (Glut4) levels in skeletal muscle under fasting condition. These data suggest that Lgr4 ablation enhances the flexibility of skeletal muscle to switch energy provider from glucose to fatty acid in response to glucose depletion. We further reveal the activation of Ampk/Sirt1/Pgc1α pathway during this adaptive fuel shift due to Lgr4 ablation. This study suggests that Lgr4 might serve as an adaptive regulator between glucose and lipid metabolism in skeletal muscle and reveals a potentially new regulator for a well-established adaptive network.

1. Introduction

Skeletal muscle, as the massive organ in our body, contributes to 80% of insulin-induced glucose uptake and is the key metabolic site for both glucose and fatty acid. Energy deprivation often gives an acute challenge to skeletal muscle, forcing muscle to replace glucose oxidation with fatty acid to maintain metabolic and nutrient homeostasis [1]. A number of evidences demonstrate that under the conditions of glucose depletion, such as fasting or exercise, AMP-activated protein kinase (Ampk) activated by increased AMP/ATP ratio initiates an adaptive signaling process, recruiting a number of factors including peroxisome proliferator-activated receptor g (Pparγ), peroxisome proliferator-activated receptor g coactivator-1a (Pgc1α), and their target genes, to promote fuel shift, which involves formation of oxidative muscle fiber [2], elevated fatty acid oxidation, and mitochondrial biogenesis [1,3].

In skeletal muscle cell, one key metabolic enzyme that regulates nutrient switch is pyruvate dehydrogenase kinase-4 (Pdk4), which prevents pyruvate from entering tricarboxylic acid (TAC) cycle by phosphorylating pyruvate dehydrogenase (PDH), thereby favoring fatty acid over glucose oxidation [4]. Under the condition of short glucose supply, increased Pdk4 levels mediate fuel shift from glucose to fatty acid oxidation in muscle cell. Interestingly, one booster of Pdk4 is Pgc1α, a well-known regulator in energy metabolism and coactivator of other activators above [5]. *Pgc1α* induces *Pdk4* expression via *Pgc1α*/estrogen-related receptor a (Errα)

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pathway, consistent to its role in lipid metabolism and glucose oxidation—positive in the former, but negative in the later [4,6,7]. In skeletal muscle, Pgc1α is able to switch muscle fiber type from fast-twitch fiber to slow-twitch fiber, which is characterized as more mitochondrial mass and oxidative capacity [2]. Moreover, Pgc1α has been reported to coactivate Pparγ to regulate nonshivering thermogenesis and mitochondrial oxidation in adipose tissue, which is also seen in other metabolic organs, such as heart and skeletal muscle [7]. Recently, an Ampk-Sirt1-Pgc1α pathway has been identified to be critical to the muscle's adaptive selection on energy source. Ampk is a pivot sensor of cellular AMP/ATP ratio. It interprets AMP/ATP information into intracellular NAD^+/NADH, and thus influencing the activity of NAD-dependent deacetylase sirtuin-1 (*Sirt1*), which deacetylate Pgc1α, as well as other Pdk4 activators including FoxO1 and Forkhead box O3 (FoxO3), resulting in the alteration of Pdk4 and fuel-preference shift of skeletal muscle [8–10]. However, the upstream regulators of Ampk signaling in fuel adaptation are largely unknown.

Leucine-rich repeat-containing G-protein coupled receptor 4 (Lgr4) has been reported to play important roles in the function of many organs [11–15]. In our previous study, we demonstrate Lgr4 is a new regulator for energy metabolism by regulating white-to-brown fat switch [16]. Ablating Lgr4 remarkably upregulates browning biomarkers uncoupling protein 1 (Ucp 1) as well as Pgc1α in white adipocytes. Metabolic improvements, including enhanced energy expenditure and glucose tolerance, have been observed in Lgr4 ablation mice as well, indicating profound metabolic effects of Lgr4 on energy metabolism. Yet, how Lgr4 regulate energy metabolism in other metabolic organs than adipose tissue remains to be explored. In the present study, we mainly focused on the metabolic effects of Lgr4 on skeletal muscle, revealing a potent role of Lgr4 in regulating energy adaptation of skeletal muscle.

2. Materials and methods

2.1. Animals

Lgr4 homozygous mutant (*Lgr4*$^{m/m}$) mice were generated as previously described [16] and were housed in metabolic cage at 22 ± 2 °C with 12 h-to-12 h light–darkness cycles. Male *Lgr4*$^{m/m}$ and littermates aged ~4 months were used in the experiment. Respiratory exchange ratio (RER) at 12:00 PM (light condition) and 12:00 AM (dark condition) was analyzed with metabolic chambers (Columbus Instruments). Mice were fasted overnight before introperitoneal glucose tolerance test (IPGTT) and muscle tissues harvesting. The glucose and insulin dosage for IPGTT and insulin-stimulation test were consistent to the previous report [16]. Dissected tissues were frozen and stored at −80 °C instantly after obtained. The Animal Care Committee of Shanghai Jiao Tong University school of Medicine approved all animal experiments.

2.2. NEFA and glycerol measurement

We measured non-esterified fatty acid (NEFA) and glycerol level with whole blood from retro-orbital vein using commercial kits (WaKo for NEFA, Sigma for glycerol).

2.3. RNA isolation and quantitative real-time PCR

Total RNA was extracted from gastrocnemius muscle tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA), following manufacturer's instruction and converted to cDNA from 1 μg RNA by using the Reverse Transcription system (Promega, Madison, WI, USA). Real-time PCR was performed with LC480 system (Roche) using SYBER Green Supermix (Takara). Primers used in this study are listed in supplementary Table 1.

2.4. Protein preparation and western blot

Proteins extracted from dissected tissue were prepared using radioimmunoprecipitation assay buffer. All of these protein samples were subjected to concentration determination and western blotting. Primary antibodies for Ampk, p-Akt, Akt, Glut4 (Cell signaling Technology), Sirt1 (Upstate), Pgc1α (Millipore), Ucp2 (R & D Systems), Ucp3 (Pierce) were used to detect target proteins, while α-tubulin (Cell signaling Technology) and β-actin (Santa Cruz) were used as internal controls. The blotting bands were visualized using Odyssey infrared imaging system (LI-COR) following the manufacturer's guide. The representative blotting bands were repeated three times or more.

2.5. Statistical analysis

The data presented in this study is all analyzed by two-tailed Student *t*-test, and showed as mean ± s.e.m.

3. Results

3.1. Ablating Lgr4 inhibits fatty acid release with abundant glucose supply and increases fatty acid oxidation with deficiency of glucose supply

To explore the role of Lgr4 in glucose and fatty acid balance, we first examined the systematic change of glucose and lipid metabolism due to Lgr4 ablation. In our previous study, *Lgr4*$^{m/m}$ mice presented higher glucose dissipation under the challenge of introperitoneal glucose tolerance test (IPGTT) compared with wild-type mice [16]. When challenged with glucose after overnight fasting, wild-type mice showed a rapid decrease of plasma non-esterified fatty acid (NEFA) normalized with glycerol (Fig. 1a), indicating decreased fatty acids release. Interestingly, a much lower NEFA level was detected in *Lgr4*$^{m/m}$ mice under the same glucose challenge (Fig. 1a), suggesting a potentially reduced lipid oxidation in Lgr4 ablation mice in response to abundant glucose input. This hypothesis was further supported by the changes in respiratory exchange ratio (RER), which was higher in *Lgr4* mutant mice than in wild-type mice at 12:00 AM (food-intake time for mice) [17], with a ratio of closer to 1.0 (Fig. 1b), suggesting a glucose-prone metabolic pattern. Interestingly, at 12:00 PM (fasting-time for mice) [17], RER in *Lgr4*$^{m/m}$ mice was significantly reduced with a ratio of closer to 0.75, a number that indicates fatty acid as the primary fuel for energy metabolism (Fig. 1b), suggesting Lgr4 ablation enhances lipid utilization in depletion of glucose. These data suggest that *Lgr4* mutant mice is more sensitive to the change of nutrient supply, and present a higher intensity of adaptation in terms of switching fuel. Thus, Lgr4 might act as a negative factor in fuel adaptive shift.

3.2. Under fasting condition, Lgr4 ablation increases lipid oxidation-related gene expression while suppresses glucose transporter type 4 (Glut4) expression in skeletal muscle

As skeletal muscle is the massive metabolic organ that possesses the ability to change energy source based on flux of nutrient input, we next examined the levels of lipid oxidation-related genes in skeletal muscle of both genotypes in response to glucose depletion. As shown in Fig. 2, most oxidative biomarkers at transcriptional level showed no statistical differences except Peroxisome proliferative activated receptor (*Pparγ*), which presents a significantly higher level in *Lgr4* mutant mice (Fig. 2a). Interestingly, pyruvate

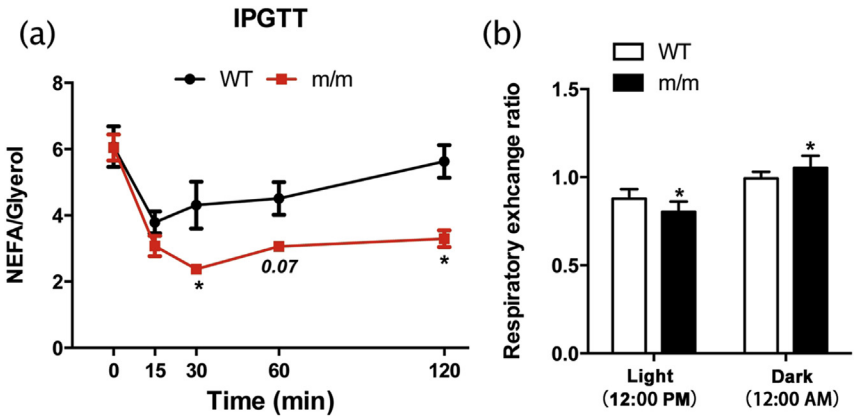

Fig. 1. Ablating Lgr4 reduces the release of fatty acid into circulation with abundant glucose supply while increases fatty acid utilization with glucose shortage. (a) Serum NEFA/Glycerol ratio of *Lgr4* mutant mice decreases under the challenge of acute glucose administration. (n = 7–9 for each group) (b) Respiratory exchange ratio decreases at 12:00 AM, while increases at 12:00 PM in *Lgr4* mutant mice (n = 8 for each group). NEFA, non-esterified fatty acid. WT, wild-type mice. m/m, *Lgr4* mutant mice. *P < 0.05.

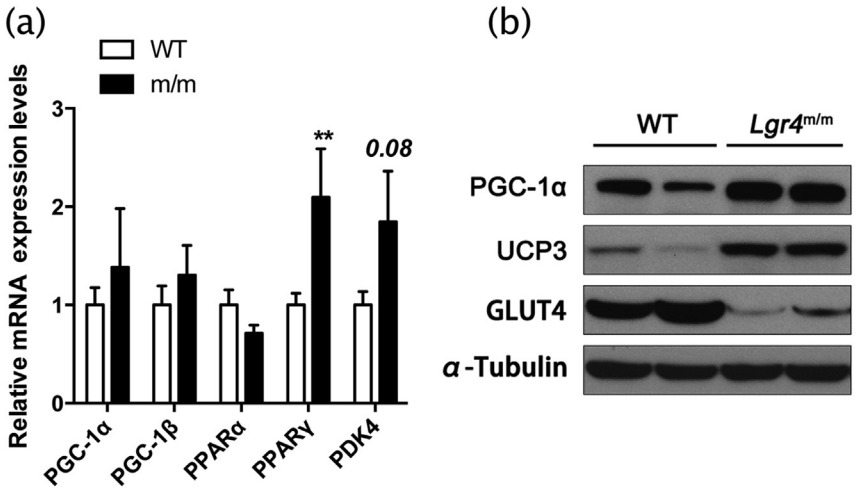

Fig. 2. Lgr4 ablation increases lipid oxidation-related gene expression and reduces Glut4 protein level in skeletal muscle under fasting condition. (a) Relative mRNA levels of *Pgc1α*, *Pgc-1β*, *Pparα*, *Pparγ* and *Pdk4* in skeletal muscle of wild-type and *Lgr4*$^{m/m}$ mice (n = 9 for each group). (b) The protein levels of Pgc1α, Ucp3, and Glut4 in skeletal muscle of wild-type and *Lgr4*$^{m/m}$ mice. All of these mice were fasted overnight before tissue harvesting. WT, wild-type mice. m/m, *Lgr4* mutant mice. *P < 0.05; **P < 0.01.

dehydrogenase kinase 4 (*Pdk4*), a Pgc1α-induced regulator that favors fatty acid oxidation over glucose, showed an increased trend in *Lgr4* mutant mice with p value 0.08 (Fig. 2a). Significantly, at protein levels, a strong elevation of Pgc1α and uncoupling protein 3 (Ucp3) was observed in *Lgr4* mutant mice compared with wild-type mice. Meanwhile, Glut4, the primary glucose transporter in muscle, was robustly reduced in *Lgr4* mutant mice (Fig. 2b), indicating increased lipid oxidation and mitochondrial thermogenesis while decreased glucose transportation in *Lgr4* mutant mice. These data suggest that a stronger lipid-prone metabolic pattern occurs in skeletal muscle due to Lgr4 ablation in the context of fasting, which might partially explain the enhancement of adaptive fuel switching induced by Lgr4 ablation.

3.3. Ablation of Lgr4 activates Ampk/Sirt1/Pgc1α pathway in skeletal muscle of fasting mice

Based on the recent findings that AMPK, SIRT1 and Pgc1α act together as an energy-sensor network that favors lipid-metabolism over glucose in skeletal muscle when energy is insufficient [8–10], we detected weather this pathway is involved in the Lgr4 ablation-induced improvement of metabolic adaptation in response to glucose deficiency. As expected, the protein levels of Ampk, Sirt1 and Pgc1α were significantly increased in gastrocnemius muscle of *Lgr4* mutant mice. Ucp3, as an indicator of mitochondrial oxidation [18], was also increased (Fig. 3). These data suggest that Lgr4

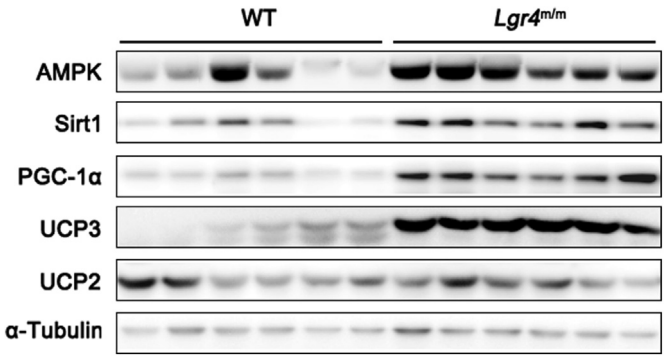

Fig. 3. AMPK/SIRT1/Pgc1α pathway is activated in skeletal muscle of *Lgr4*$^{m/m}$ mice after overnight fasting. The proteins levels of AMPK, SIRT1, Pgc1α, as well as UCP3 and UCP2 were measured in the skeletal muscle of wild-type and *Lgr4*$^{m/m}$ mice under fasting condition. WT, wild-type mice. m/m, *Lgr4* mutant mice.

ablation might improve fuel adaptation of skeletal muscle through intensifying Ampk/Sirt1/Pgc1α pathway.

3.4. Ablation of Lgr4 enhances insulin sensitivity in muscle

To further examine the metabolic outcome of Lgr4 ablation on skeletal muscle, we performed insulin challenge test to evaluate the insulin sensitivity of skeletal muscle. *Lgr4* mutant mice showed a significantly higher phosphorylated serine/threonine-protein kinase (p-Akt) level 10 min after intraperitoneal insulin injection (Fig. 4), indicating a higher insulin sensitivity of skeletal muscle in *Lgr4* mutant mice. This is consistent with the systematical improvement of glucose metabolism we observed in our previous study [16].

4. Discussion

Fuel switch between glucose and fatty acid in response to energy supplement is crucial for energy homeostasis, and skeletal muscle is important for the energy adaptation. Energy sensors such as Ampk and Sirt1 have been reported in controlling fuel adaptive selection upon nutrient status. Interestingly, our study here presented an enhanced energy shifting, as well as an intensified Ampk/Sirt1/Pgc1α signaling pathway in skeletal muscle in ablation of Lgr4, which might indicate a possible involvement of LGR4 in regulating energy adaptation in skeletal muscle.

According to previous studies [1,8–10], an energy adaptive network consisting of Ampk, Sirt1, Pgc1α and their targeted genes could be activated under the condition of energy depletion (fasting, exercise, etc.), and Ampk acts as the initiative sensor, which senses intracellular AMP/ATP ratio, triggering fuel adaptive process. However, the molecular interaction between environmental energy depletion and intracellular Ampk expression is still unclear. Our data showed a significantly intensified AMPK and its sequential signaling in ablation of Lgr4, a G protein-coupled receptor located on the cellular membrane, supporting a negative regulation of Lgr4 on *Ampk*-induced energy adaptive network. Although further studies are needed to confirm the subtle connection of Lgr4 and Ampk, our results provide a possible interactive mode that transfer extracellular energy depletion into intracellular AMP/ATP ratio through a certain group of membrane receptors.

Pgc1α acts as another key player in the energy adaptive network, because of its direct regulatory effects on fuel oxidative genes, including *Pdk4* [5], and *Ucp3*, [3]. Pgc1α act as a strong activator in mitochondrial biogenesis and fatty acid oxidation not only via co-activating multiple transcriptional factors to upregulate expression of oxidative genes, including *Ucp1* in adipose tissue and *Ucp3* in skeletal muscle [3], but also resulting from a muscle fiber switch from fast-twitch to slow-twitch fiber [2]. On the other hand, most reports supported a negative role of Pgc1α in glucose transportation [19] and oxidation [5]. Thus, up-regulation of Pgc1α is a pivot step in fasting-induced fuel adaptation. Our data showed that Lgr4 ablation resulted in a significant increment of Pgc1α in response to fasting, and a corresponding change of its downstream signaling including UCP3 [3] and GLUT4 [19], supporting that LGR4 might act as a suppressive factor for the glucose-to-lipid fuel transition in response to fasting. .Moreover, the significant increment of Pgc1α is also observed in adipose tissue of *Lgr4* mutant mice [16], further suggesting regulatory effects of Lgr4 on Pgc1α expression. Yet, based on the significant effect of Pgc1α on skeletal muscle fiber transition, further study is still needed to confirm more detailed mechanism of LGR4 in regulating fuel transition in skeletal muscle.

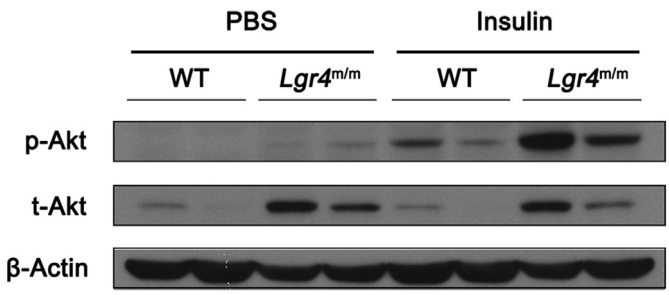

Fig. 4. Ablation of Lgr4 enhances insulin sensitivity of muscle tissue. The protein levels of phosphorylated AKT (p-AKT) and total AKT (t-AKT) in skeletal muscle of wild-type and *Lgr4*$^{m/m}$ mice were measured after insulin and PBS treatment. WT, wild-type mice. m/m, *Lgr4* mutant mice.

We also observed that insulin sensitivity in skeletal muscle is increased with LGR4 ablation, consistent to the whole-body improvement of insulin tolerance in LGR4 mutant mice [16]. Since the low basic insulin level during fasting, we consider that the enhanced fuel adaptation in response to fasting in LGR4 mutant mice is probably independent to such alteration of insulin sensitivity.

In summary, our study demonstrates that LGR4 might participate in regulating fuel transition from glucose to fatty acid in skeletal muscle in response to fasting by downregulating Ampk/Sirt1/Pgc1α pathway. Though further study is needed to confirm how LGR4 regulates the interaction between extracellular nutrient status and intracellular Ampk level, our work provides a potentially new regulator for energy adaptation in skeletal muscle.

Author contributions

R.L. and J.H. conceived the project and designed the experiments. Y.S., J.W. and Y.K. carried out most of the experiments. J.W., R.L. and Y.S. wrote the paper. S.Z, and W.L. carried NEFA and glycerol examination. M.C., Y.Z. and T.N. contributed with the mice breeding, genotyping and animal experiments. J.S. assisted with statistical analysis. Q.M., Z.Z. and G.N. contributed comments and advice on the manuscript. All authors were involved in editing the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.066.

Transparency document

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