Activation of Lactate Receptor Positively Regulates Skeletal Muscle Mass in Mice

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Summary

G protein-coupled receptor 81 (GPR81), a selective receptor for lactate, expresses in skeletal muscle cells, but the physiological role of GPR81 in skeletal muscle has not been fully elucidated. As it has been reported that the lactate administration induces muscle hypertrophy, the stimulation of GPR81 has been suggested to mediate muscle hypertrophy. To clarify the contribution of GPR81 activation in skeletal muscle hypertrophy, in the present study, we investigated the effect of GPR81 agonist administration on skeletal muscle mass in mice. Male C57BL/6J mice were randomly divided into control group and GPR81 agonist-administered group that received oral administration of the specific GPR81 agonist 3-Chloro-5-hydroxybenzoic acid (CHBA). In both fast-twitch plantaris and slow-twitch soleus muscles of mice, the protein expression of GPR81 was observed. Oral administration of CHBA to mice significantly increased absolute muscle weight and muscle weight relative to body weight in the two muscles. Moreover, both absolute and relative muscle protein content in the two muscles were significantly increased by CHBA administration. CHBA administration also significantly upregulated the phosphorylation level of p42/44 extracellular signal-regulated kinase-1/2 (ERK1/2) and p90 ribosomal S6 kinase (p90RSK). These observations suggest that activation of GRP81 stimulates increased the mass of two types of skeletal muscle in mice *in vivo*. Lactate receptor GPR81 may positively affect skeletal muscle mass through activation of ERK pathway.

Keywords

Skeletal muscle • G protein-coupled receptor 81 • Muscle mass • p42/44 extracellular signal-regulated kinase-1/2 • p90 ribosomal S6 kinase

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Introduction

Skeletal muscle exhibits high plasticity and changes its mass in response to external stimuli. It is well known that mechanical loading induced by resistance training is a typical stimulus for skeletal muscle hypertrophy [1]. It is generally accepted that skeletal muscle mass is highly regulated by the balance between protein synthesis and degradation [2]. The protein synthesis in skeletal muscle has been suggested to be mediated by anabolic signals, such as p42/44 extracellular signal-regulated kinase-1/2 (ERK1/2) and its downstream target p90 ribosomal S6 kinase (p90RSK) [3]. In skeletal muscle cells, the ERK/p90RSK signaling pathway is activated by mechanical loading [4] and has been suggested to be involved in mechanical loading-induced skeletal muscle hypertrophy [5]. Additionally, the protein kinase B (Akt)/ mammalian target of rapamycin (mTOR) pathway and its downstream targets p70 S6 kinase (p70S6K) are also included in anabolic signals and involved in muscle hypertrophy [6-8]. However, the detailed mechanism of muscle hypertrophic response to resistance training has not been elucidated. For instance, blood-flow-restriction training is a training under vascular occlusion. This training increases the size of human skeletal muscle [9,10] and the level of blood circulating metabolites, such as interleukin-6 (IL-6), growth hormone (GH), and lactate [10,11]. In particular, IL-6 is thought to stimulate muscle satellite cells and play important roles in muscle hypertrophy [12]. IL-6 may also directly mediate protein synthesis through activation

of anabolic signaling pathways [13]. Furthermore, GH has been shown to play an anabolic role in regulating skeletal muscle mass [14]. Therefore, there is a possibility that the metabolites contribute to muscle hypertrophy following resistance training.

Lactate is one of the secretions released from skeletal muscle cells. It is generally accepted that lactate synthesis in skeletal muscle cells is stimulated by high-intensity exercise, and cytosolic lactate is oxidized to pyruvate in mitochondria. Especially, lactate synthesized in fast-twitch muscle, which has few mitochondria, is transported by circulation to slow-twitch muscle for use as an energy source [15-17]. To our knowledge, the effect of intracellular lactate on not only protein synthesis but also muscle mass is unknown, but it has been suggested that extracellular lactate contributes to increased muscle mass [18-20]. Administration of the lactate-containing compound to mice has been reported to enhance the effects of low-intensity exercise, increasing skeletal muscle mass and muscle satellite cell regulatory factors [18]. Our previous study also demonstrated that extracellular lactate causes C2C12 myotube hypertrophy by activating the anabolic intracellular ERK pathway [19], which stimulates proliferation and differentiation of muscle cells [21,22]. In addition, we have recently shown that oral administration of lactate stimulates not only skeletal muscle hypertrophy but also regeneration of injured skeletal muscle via the activation of muscle satellite cells [20]. However, the molecular mechanisms of lactate-induced hypertrophic responses remain unclear.

It has been reported that G protein-coupled receptor 81(GPR81), which is a selective receptor for lactate, is expressed in rodent skeletal muscle cells [19,23]. Moreover, the expression of GPR81 in human skeletal muscle has been confirmed at both mRNA [23] and protein [24] levels. GPR81-mediated activation of the anabolic ERK-intracellular signals triggered by both lactate and GPR81 agonist has been reported in cultured rat L6 skeletal muscle cells [25]. However, the physiological role of GPR81 in skeletal muscle cells has not been fully elucidated. We hypothesize that the hypertrophic effects of lactate are mediated by GPR81. In fact, in our previous study using cultured skeletal muscle cells, the administration of a GPR81 agonist led to hypertrophy of C2C12 myotubes through activation of the ERK/p90RSK pathway [19]. In contrast, GPR81 agonist administration did not significantly change the Akt/mTOR/p70S6K pathway [19]. However, there is no evidence showing that GPR81 has a hypertrophic effect on skeletal muscle *in vivo*. Therefore, in the present study, we focused on ERK pathway and investigated the effects of GPR81-agonist administration on the ERK pathway and skeletal muscle mass in mice.

Mammalian skeletal muscles are classified into fast- and slow-twitch muscle based on their contractile properties. The responses to extracellular stimuli depend on these muscle types [26-28]. It is well known that monocarboxylate transporters (MCTs) are present in the plasma membrane of skeletal muscle, and lactate is transported across the plasma membrane by MCTs [29]. MCT1 and MCT4 are predominantly expressed in slow- and fast-twitch fibers, respectively. MCTs play a key role of not only export of lactate from muscle cells but also import of lactate into muscle cells. The uptake of extracellular lactate into muscle cells is stimulated by MCT1, while the export of intracellular lactate from muscle cells is stimulated by MCT4 [30]. On the other hand, a selective receptor for lactate GPR81 is expressed in skeletal muscle cells [19,23], and activation of ERK pathway downstream of GPR81 is triggered by lactate as well as GPR81 agonist [19,25]. However, it is unclear whether there is a difference in GPR81 expression between slow- and fast-twitch skeletal muscles.

Thus, MCTs and GPR81 may be involved in skeletal muscle responses to lactate, either separately or in relation to each other. Although our previous study demonstrated that administration of lactate caused muscle hypertrophy in mice [20], it is not clear whether MCTs or GPR81 are involved in lactate-induced muscle hypertrophy in animals. Therefore, we tried to investigate the potential role of GPR81 in the lactate-induced muscle hypertrophy by evaluating the expression of GPR81 in fast- and slow-twitch muscles.

Methods

Animals and grouping

All animal experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health (Bethesda, MD, USA). The Animal Use Committee of Toyohashi SOZO University approved the procedures of animal experiments in this study (A2016003, A2017002, A2018002). Male 8-week-old C57BL/6J mice (n=52) were used. To investigate the effect of GPR81 activation on skeletal muscle mass (muscle weight and protein content), mice were randomly divided into control (Con;

n=26) and GPR81 agonist-administered (AG; n=26) groups. All mice were housed in a clean room maintained at approximately 23 °C with a 12/12 hours light-dark cycle. Solid diet and water were provided ad libitum.

Administration of GPR81 agonist

In the present study, a 3-chloro-5-hydroxy-benzoic acid (CHBA, Tokyo Chemical Industry, Tokyo, Japan), was used as the GPR81 agonist [31,32]. In the AG group, CHBA (50 mg/kg body weight) was administered to the mice with an oral sonde 5 days a week for 3 weeks, according to the previously reported method [20]. To avoid the side effects of CHBA on mice, its dose was selected with consideration for the study in mice [31]. A stock solution of CHBA (100 mg/ml) was prepared in dimethylsulfoxide (DMSO) and diluted with water to the final DMSO concentration of 5 %. The same amount of 5 % DMSO in water was administered to the Con group.

Sample preparation and evaluation of muscle mass

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and then sacrificed by cervical dislocation 1, 2, and 3 weeks after the initiation of CHBA administration (n=8-9 mice per group in each time point). The left plantaris and soleus muscles were dissected from the hindlimb of mice immediately after sacrifice. Isolated muscle tissues were rapidly weighed, frozen in liquid nitrogen, and stored at −80 °C until later analysis.

Frozen muscles were homogenized with a tissue lysis reagent (CelLyticTM-MT, 100 µl/mg muscle weight, Sigma-Aldrich, St. Louis, MO, USA) containing 1 % (v/v) Protease/Phosphatase Inhibitor Cocktail (5872, Cell Signaling Technology, Danvers, MA, USA) on ice. To measure the muscle protein content, part of each tissue lysate (50 µl) was mixed with the equal amount of 2 N NaOH and completely solubilized at 37 °C for 1 h [33]. The protein content of the lysate was determined using the Bradford technique (protein Assay kit, Bio-Rad, Hercules, CA, USA) and bovine serum albumin (Sigma-Aldrich) as the standard protein. And then the absolute muscle protein content in each muscle sample was calculated.

Western blot analysis

Western blot analysis was performed with some modifications to previously reported methods [19,33]. The rest of each tissue lysate was sonicated to extract proteins, centrifuged at 20,000 g at 4 °C for 10 min, and the supernatant was collected. After measuring the protein content in the supernatant using the Bradford technique, the supernatant solutions were mixed with sodium-dodecylsulfate (SDS) sample buffer (62.5 mM Tris-HCl (pH 6.8), 2.3 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 30 % (v/v) glycerol, 0.05 % (w/v) bromophenol blue) and heated at 95 °C for 5 min. The SDS-polyacrylamide gel electrophoresis (PAGE) was performed in a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad) at 200 V. Equal amounts of protein (3 µg) were loaded into the wells on each gel (Mini-PROTEAN TGX precast gels, Bio-Rad). Molecular weight markers (Prestained Protein Standards, Bio-Rad) were applied to the gels to check the electrophoresis and transfer process.

Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Trans-Blot Turbo Mini 0.2 µm PVDF Transfer Packs, Bio-Rad) using Trans-Blot Turbo Transfer System (Bio-Rad) at 1.3 A and 25 V for 7 min. After the transfer, the membranes were blocked for 20 min using Blocking One-P (Nacalai Tesque, Kyoto, Japan). The membranes were then incubated with a primary antibody: phosphorylated ERK1/2 Thr^{202}/Tyr^{204} (p-ERK1/2: 9101, Cell Signaling), total ERK1/2 (t-ERK1/2: 9102, Cell Signaling), phosphorylated p90RSK Ser^{380} (p-p90RSK: 11989, Cell Signaling), total p90RSK (t-p90RSK: 9355, Cell Signaling) overnight at 4 °C, followed by a secondary antibody: anti-rabbit immunoglobulin G conjugate to horseradish peroxidase (Cell Signaling) for 2 h at room temperature. After the final wash, protein bands were visualized with chemiluminescence (ImmunoStar LD, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), were captured using LumiCube (Liponics, Tokyo, Japan), and were analyzed using JustTLC software (Sweday, Sodra Sandby, Sweden).

The protein expression level of GPR81 in mouse skeletal muscles (n=7) was also evaluated by western blotting using a primary antibody: GPR81 (sc-32647, Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (4967, Cell Signaling Technology) [19].

Statistical analysis

All values were expressed as means ± SEM. The statistically significant level of GPR81 expression was assessed using F-test followed by unpaired Student's t-test. Other significant levels in animal experiments were tested using two-way (agonist administration and time) ANOVA. The significance level was set at 0.05.

Results

GPR81 expression in mouse skeletal muscle

Protein expression of GPR81 was observed in both fast-twitch plantaris and slow-twitch soleus muscles of mice. There was no significant difference in the expression level of GPR81 between the two types of muscles (Fig. 1).

Effects of lactate receptor agonist on muscle mass and intracellular signals in mouse skeletal muscle

Figs. 2 and 3 show the body weight, plantaris and soleus muscle mass (muscle weight and protein content) of mice in response to oral administration of CHBA, a GPR81 agonist.

There was no significant difference in mouse body weight between Con and AG groups during the experimental period (Fig. 2A). In the plantaris and soleus muscles, a significant main effect of CHBA administration and time was observed on the muscle weight (Fig. 2B). In the AG group, the absolute muscle weight and the muscle weight relative to body weight was significantly higher than that in the Con group ($p < 0.05$).

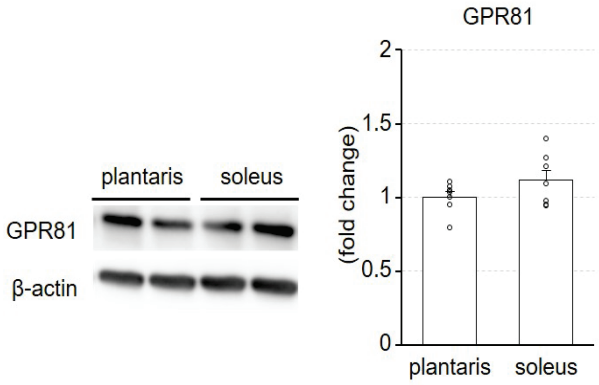

Fig. 1. Representative expression patterns and mean expression of GPR81 in mouse plantaris and soleus muscles. GPR81: G-protein-coupled receptor 81. Values are expressed relative to the value of plantaris muscle. Values are means ± SEM. n=7 per group.

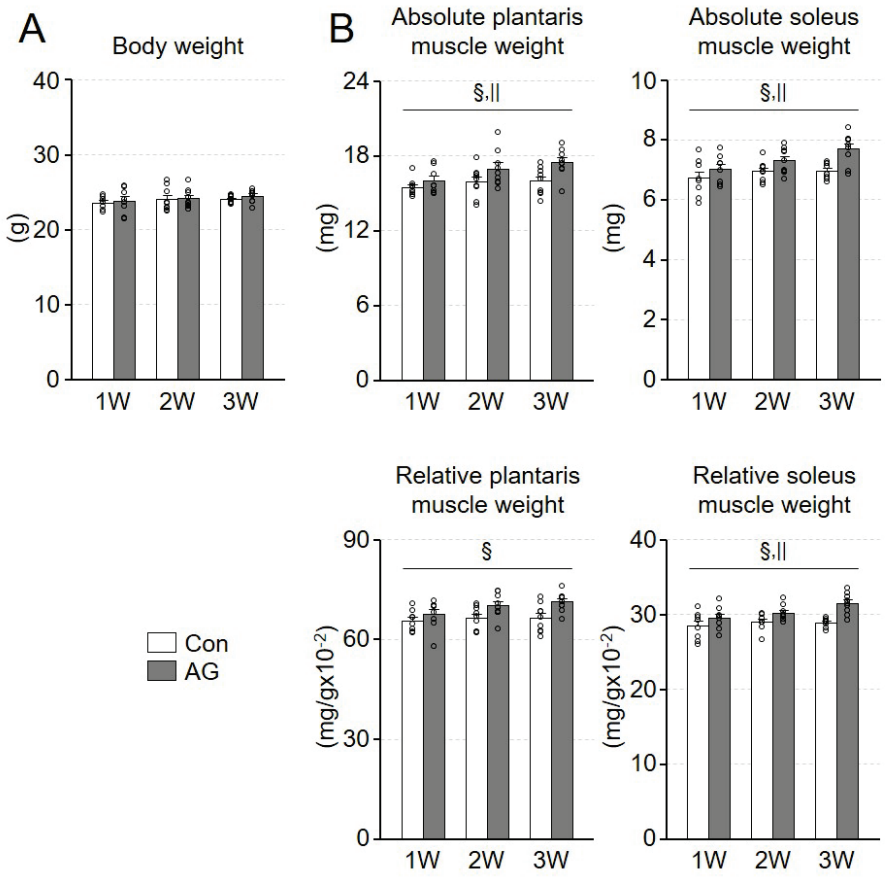

Fig. 2. Effects of oral CHBA administration on mouse body weight (**A**), absolute and relative muscle weight of plantaris and soleus muscles (**B**). Relative muscle weight: relative muscle weight to body weight; Con: control group; AG: 3-Chloro-5-hydroxybenzoic acid (CHBA)-administered group; 1W, 2W, and 3W: CHBA administration for 1, 2, and 3 weeks, respectively. Values are means ± SEM. n=8-9 per group in each time point. §: Significant main effect of CHBA administration, $p < 0.05$. ||: Significant main effect of time, $p < 0.05$.

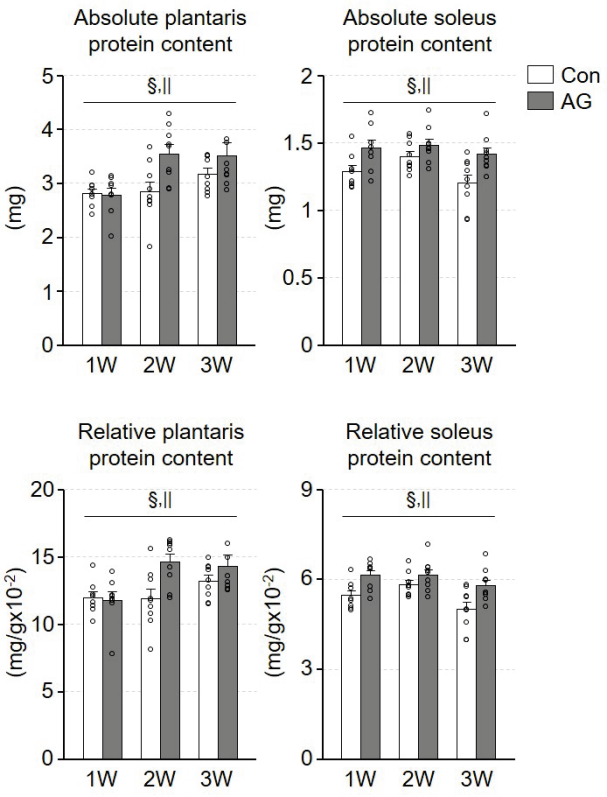

Fig. 3. Effects of oral CHBA administration on absolute and relative protein content of plantaris and soleus muscles. Relative protein content: relative protein content to body weight. See Figure 2 for other abbreviations, statistics and symbols. Values are means ± SEM. n=8-9 per group in each time point.

A significant main effect of CHBA administration and time was also found on the protein content (Fig. 3). Administration of CHBA significantly increased absolute and relative muscle protein content in both plantaris and soleus muscles ($p < 0.05$).

The changes in phosphorylation levels of ERK1/2 and p90RSK in response to CHBA administration were shown in Figure 4. There was a significant main effect of CHBA administration on the relative expression level of phosphorylated ERK1/2 (p-ERK1/2) and p-p90RSK in mouse plantaris and soleus muscles. CHBA administration significantly upregulated the relative expression level of p-ERK1/2 and p-p90RSK ($p < 0.05$). A significant main effect of time was observed on the relative expression level of p-p90RSK.

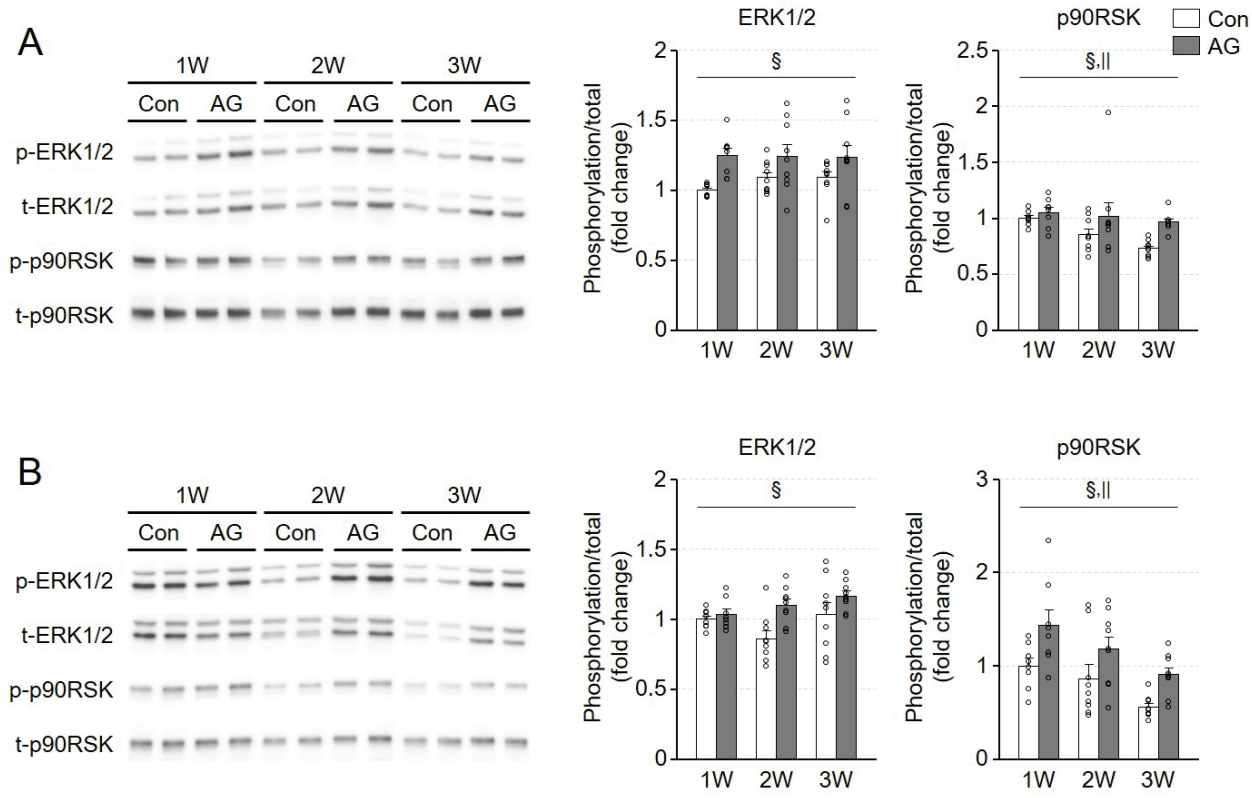

Fig. 4. Representative expression patterns and mean expressions of p-ERK1/2/t-ERK1/2 and p-p90RSK/t-p90RSK in plantaris (**A**) and soleus (**B**) muscles in response to CHBA. p-ERK1/2: phosphorylated p42/44 extracellular signal-regulated kinase-1/2; t-ERK1/2: total ERK1/2; p-p90RSK: p90 ribosomal S6 kinase; t-p90RSK: total p90RSK. See Figure 2 for other abbreviations, statistics and symbols. Values are expressed relative to the value of Con at 1W. Values are means ± SEM. n=8-9 per group in each time point.

Discussion

The present study demonstrated that no significant difference in the expression level of GPR81 was observed between the fast-twitch plantaris and slow-twitch soleus muscles of mice. Oral administration of CHBA to mice increased the muscle weight and protein content in both plantaris and soleus muscles. Furthermore, in both muscles, there was also CHBA-associated increase in the phosphorylation level of ERK1/2 and p90RSK.

Effect of GPR81 agonist on skeletal muscle mass

The present study confirmed GPR81 expression in mouse skeletal muscles, such as fast-twitch plantaris and slow-twitch soleus muscles (Figure 1). This result is consistent with the report using mice [23]. In the present study, the oral administration of CHBA increased the absolute and relative muscle weight and protein content in both plantaris and soleus muscles of mice. This is the first report showing administration of GPR81 agonist increased skeletal muscle mass in mice *in vivo*. Additionally, there was no significant difference in the expression level of GPR81 between fast-twitch plantaris and slow-twitch soleus muscles, suggesting that GPR81 agonist increased muscle mass similarly in both types. Taken together with the previous observation that GPR81 agonists increased the diameter of C2C12 myotubes [19], the findings from the present study demonstrate that activation of GPR81 stimulates increases in muscle mass at both cellular and tissue levels of skeletal muscle.

GPR81-associated intracellular signals

The present study demonstrated that phosphorylation level of ERK1/2 and p90RSK was significantly increased following administration of CHBA to mice. This result is supported by the studies showing that ERK pathway was activated by the GPR81 agonist 3,5-DHBA in rat L6 [25] and mouse C2C12 skeletal muscle cells [19]. Therefore, it was suggested that the ERK/p90RSK pathway exists downstream of GPR81 in mouse skeletal muscle tissue. It is well known that the ERK/p90RSK pathway is an anabolic signal involved in protein synthesis, leading to muscle hypertrophy [3-5]. Moreover, the previous study showed that inhibition of the ERK/p90RSK pathway attenuated myotube hypertrophy induced by the GPR81 ligand lactate in C2C12 cells [19]. There is a possibility that GPR81-mediated activation of the ERK/p90RSK pathway contribute to the increase in skeletal muscle mass.

GPR81 stimulation and skeletal muscle hypertrophy

It has been suggested that extracellular lactate is one of the regulators of skeletal muscle plasticity [19,20,34,35]. In skeletal muscle cells, extracellular lactate positively affected the myotube hypertrophy and myotube formation [19,20,34]. The previous study reported that oral lactate administration to mice increased blood lactate concentrations and enhanced skeletal muscle hypertrophy and regeneration [20]. Similarly, the study in mice has demonstrated that lactate promotes glycerol-induced muscle regeneration [34]. Recently, lactate has been shown to inhibit the loss of muscle mass due to caloric restriction [35]. On the other hand, it has been reported that lactate administration has no additional hypertrophic effect in synergist ablation-induced mechanical overload in mouse skeletal muscle [36]. Judging from the gain of muscle mass by synergist ablation and lactate administration [36], the hypertrophic effect of lactate is not necessarily strong, but rather small, compared to exercise-associated mechanical loading.

Although the mechanisms of lactate-induced increase in skeletal muscle mass have not been fully elucidated, as shown in the present study, lactate receptor GPR81 in skeletal muscle may play a role in the mechanism of increasing skeletal muscle mass. If GPR81 activation induces skeletal muscle hypertrophy in pathological as well as healthy human skeletal muscle, CHBA and lactate might be a possible countermeasure to muscle atrophy due to reduced physical activity. Further studies including human experiments are needed to elucidate this issue.

Numerous studies have been performed using young mice on the effects of various external stimuli on skeletal muscle hypertrophy and atrophy [7,8,37,38]. Furthermore, the age-related suppression of muscle hypertrophy in response to various stimuli is also reported [39-42]. In the present study, therefore, young mice were used to investigate the effects of GPR81 agonist on skeletal muscle mass. Time-related changes in skeletal muscle mass and intracellular signal were observed in the present study, but the reasons for these changes cannot be explained at present. The mice used in this study were in the growth phase, which may have influenced the time-related changes.

In conclusion, the present study demonstrated GPR81 agonist-associated increase in muscle mass and

activation of ERK pathway in mouse skeletal muscles. Stimulation of lactate receptor GPR81 may have a positive impact on skeletal muscle mass.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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