Metformin increases the PGC-1α protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo

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Suwa, Masataka, Toru Egashira, Hiroshi Nakano, Haruka Sasaki, and Shuzo Kumagai. Metformin increases the PGC-1α protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo. J Appl Physiol 101: 1685–1692, 2006.—AMP-activated protein kinase (AMPK), which was activated by an antihyperglycemic drug metformin, has been hypothesized to mediate metabolic adaptations. The purposes of the present study were 1) to confirm whether acute metformin administration induced AMPK phosphorylation and 2) to determine whether chronic metformin treatment increased the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) expression, glycolytic and oxidative enzyme activities, andcytochrome c and glucose transporter-4 (GLUT4) protein expression in the rat soleus and red and white gastrocnemius muscles. The single oral administration of metformin (300 mg/kg body wt) enhanced the AMPK phosphorylation at 5 and/or 6 h after treatment. In the chronic study, rats were fed either normal chow or chow containing 1% metformin for 14 days. Metformin treatment resulted in a mean daily glucose output and insulin-stimulated glucose uptake in skeletal muscle and adipocytes (7, 23). Furthermore, both experimental and epidemiological research suggests that metformin might have additional cardiovascular protective actions beyond its antihyperglycemic properties (7, 23). Despite more than 40 yr of clinical use, their mechanisms of action still remain obscure. Recent studies have demonstrated that the metformin activates the skeletal muscle 5′-AMP-activated protein kinase (AMPK) both in vivo (32) and in vitro (10, 56). AMPK is composed of the catalytic kinase α-subunit and regulatory subunits β and γ (50). The phosphorylation of threonine 172 in α subunit is strongly associated with the AMPK activity (34, 43). The AMPK is activated by energy deprivation such as a decreased intracellular ATP concentration and/or an increased AMP concentration (50). The AMPK phosphorylation can be regulated by upstream kinases including LKB1 (40, 53) and Ca2+/calmodulin-dependent protein kinase kinases (16, 20, 52). In skeletal muscle, AMPK is mainly regulated by LKB1 (39). Although the mechanism whereby metformin activates AMPK is not fully understood, effects of metformin in controlling the blood glucose level are mainly due to hepatic LKB1-AMPK pathway (41). Metformin treatment to the patients with Type 2 diabetes decreases ATP level in human skeletal muscle (32). On the other hand, in vitro treatment of metformin increases AMPK activity without any changes of AMP-to-ATP ratio in skeletal muscle cells (10). Therefore, metformin would activate skeletal muscle AMPK through both AMP/ATP level-dependent and -independent mechanisms. Skeletal muscle AMPK is activated by exercise (6, 11), leptin (30), and adiponectin (55), as well as metformin (10, 32, 56). The activation of AMPK by its specific activator 5-aminoimidazole-4-carboxamide-1β-d-ribofuranoside (AICAR) stimulates the glucose uptake and fatty acid oxidation in skeletal muscle cells (17, 29). In addition, chronic AICAR-treatment increases insulin-stimulated glucose uptake, insulin signaling such as phosphatidylinositol 3-kinase and protein kinase B activities, glucose transporter 4 (GLUT4) protein expression, hexokinase (HK) activity, and mitochondrial oxidative enzyme activities in skeletal muscle (5, 19, 21, 33, 38, 44, 51).

The activation of AMPK by AICAR also increases the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) expression in skeletal muscle (24, 44, 45). PGC-1α is a nuclear-encoded transcriptional coactivator, and it plays an important role in mitochondrial biogenesis, glucose metabo-
lism, muscle fiber pattern, and adaptive thermogenesis (25, 26, 37, 54). PGC-1α is associated with insulin sensitivity and Type 2 diabetes (8, 15, 27, 31, 35). The inhibition of PGC-1α by small interfering RNA prevents AICAR-induced fatty acid oxidation in C2C12 muscle cells (24). It is therefore plausible that the increased skeletal muscle mitochondrial oxidative enzyme activity by activation of AMPK (38, 44, 51) is at least partially due to an increased PGC-1α expression.

Metformin treatment has been hypothesized to increase PGC-1α protein expression and oxidative enzyme activities as well as HK activity and GLUT4 expression via AMPK phosphorylation in skeletal muscle. The present study was conducted 1) to confirm whether oral metformin treatment in rats induced AMPK phosphorylation in skeletal muscle and 2) to determine whether chronic metformin treatment increased the PGC-1α protein expression and metabolic components, including the activity of glycolytic and oxidative enzymes and GLUT4 protein expression in rat skeletal muscle.

**MATERIALS AND METHODS**

**Animals.** Eight-week-old male Wistar rats with a body weight of 250–280 g (Kyudo, Tosu, Saga, Japan) were used for the present study. All rats were handled daily for at least 5 days before beginning their treatment regimen. All rats were housed in a temperature- (22 ± 2°C) and humidity-controlled (60 ± 5%) room with a 12-h light (0700 to 1900) and 12-h dark (1900 to 0700) cycle. All experimental procedures were strictly conducted in accordance with the Nakamura Gakuin University guidelines for the Care and Use of Laboratory Animals and were approved by the University Animal Experiment Committee.

**Protocol for acute treatment study.** To determine both the AMPK and acetyl-CoA carboxylase (ACC) phosphorylation with acute metformin treatment, metformin hydrochloride (Dainippon Sumitomo Pharma, Osaka, Japan) (300 mg/kg body wt) in saline was orally administered in the metformin-treated group (n = 24). This dose of metformin was sufficient to decrease blood glucose level and activate peripheral AMPK (3, 14, 28, 57). In the control group (n = 24), a comparable volume of saline was orally administered. Six rats per group at each point of time were anesthetized with pentobarbital sodium (60 mg/kg body wt ip), 4, 5, or 6 h after injection of saline or metformin. These procedures were performed in a fed state. The soleus and gastrocnemius muscles were rapidly dissected, and then the red and white parts of the gastrocnemius muscle were carefully isolated. The muscles were immediately frozen in liquid nitrogen, and they were stored until analysis.

To inhibit phosphatases, following lysis buffer was used to determine phospho-AMPK and ACC protein levels as well as total AMPK and ACC. [50 mM HEPES, 0.1% Triton X-100, 4 mM EDTA, 10 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 25 mM NaF, 5 mM Na3VO4, and 1 tablet/50 ml Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Tokyo, Japan)] for either 1 h at room temperature (PGC-1α, 10% (phospho- and total ACC), 7.5% (PGC-1α), 15% (cytochrome c), and 15% (cytochrome c) resolving gels. The proteins separated by SDS-PAGE were transferred onto the polyvinylidene difluoride membrane electrophoretically. The membrane was incubated with a blocking buffer of casein solution (SP-5020, Vector Laboratories, Burlingame, CA) for either 1 h at room temperature or overnight at 4°C. The membrane was reacted with affinity-purified rabbit polyclonal antibody to phospho-AMPK-α (Thr172) (1:500 dilution, no. 2531S, Cell Signaling, Beverly, MA), total AMPKα (1:1,000 dilution, no. 2532, Cell Signaling), phospho-ACC (Ser79) (1:500 dilution, no. 3661, Cell Signaling), total ACC (1:500 dilution, no. 3662, Cell Signaling), PGC-1α (1:500 dilution, AB3242, Chemicon International, Temecula, CA), or GLUT4 (1:8,000 dilution, AB1346, Chemicon International) or mouse monoclonal antibody to cytochrome c (1:500 dilution, ABN0012, clone 7H8.2C12, Biosource, Camarillo, CA) for either 1 h at room temperature (PGC-1α and GLUT4) or overnight (others), and then was incubated with biotinylated anti-rabbit IgG (1:1,000 dilution, Vector Laboratories), except for cytochrome c, or with biotinylated anti-mouse IgG (1:1,000 dilution, Vector Laboratories) to determine the cytochrome c level for 30 min. The band on the membrane was visualized by avidin and biotinylated horseradish peroxidase macromolecular complex technique (PK-6100, Vector Laboratories). The band densities were determined using the NIH Image 1.62 software package (National Institutes of Health, Bethesda, MD).

**Enzyme assay.** The enzyme activities were measured spectrophotometrically. All enzymatic assays were carried out at 30°C using saturating concentrations of substrates and cofactors as determined in

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preliminary analyses. Citrate synthase (CS; tricarboxylic acid cycle) activities were measured at 412 nm to detect the transfer of sulfhydryl groups to DTNB. HK (mobilization of blood glucose), pyruvate kinase (glycolysis), lactate dehydrogenase (LDH; anaerobic glycolysis), and β-hydroxyacyl-CoA dehydrogenase (β-HAD; β oxidation) activities were measured at 340 nm by following the production or disappearance of NADH or NADPH.

For the HK (EC 2.7.1.1) assay, 100 mM Tris–HCl, 0.4 mM NADP, 5 mM MgCl2, 700 U/ml glucose-6-phosphate dehydrogenase, 1 mM glucose, and 5 mM ATP, pH 7.0, were used.

For the pyruvate kinase (EC 2.7.1.40) assay, 50 mM Tris–HCl, 0.1 mM MgCl2, 10 mM MgCl2, 0.28 mM NADH, 1.5 mM ADP, 6 U/ml LDH, and 5 mM phosphoenolpyruvate, pH 7.6, were used.

For the LDH (EC 1.1.1.27) assay, 50 mM Tris–HCl, 0.28 mM NADH, and 2.4 mM pyruvic acid, pH 7.6, were used.

For the CS (EC 4.1.3.7) assay, 100 mM Tris–HCl, 0.1 mM DTNB, 0.3 mM acetyl-CoA, 3.33 mM K2HPO4, and 0.5 mM oxalacetate, pH 8.0, were used.

For the β-HAD (EC 1.1.1.35) assay, 100 mM Tris–HCl, 0.28 mM NADH, 5 mM EDTA, and 0.1 mM acetacetyl-CoA, pH 6.9, were used.

Statistical analysis. All data are expressed as means ± SE. The unpaired t-test was used to compare between the control and metformin-treated groups. A value of P < 0.05 was considered to be significant.

RESULTS

Western blotting for AMPK and ACC in the acute study. Figure 1 shows the Western blot detections of phospho-AMPK protein in the muscles. The densitometric data indicated that the AMPK phosphorylation levels of the metformin-treated group 5 h after administration of metformin in the white gastrocnemius muscle was significantly higher than that of the saline-treated control group (+18%; P = 0.046). In addition, 6 h after metformin administration, the AMPK phosphorylation levels were significantly higher than those of the control group in the soleus (+35%; P = 0.030) and red (+54%; P = 0.005) and white (+46%; P = 0.017) gastrocnemius muscles. On the other hand, at 1 or 4 h after metformin administration in any muscles or at 5 h after the injection in the soleus or red gastrocnemius muscle, the AMPK phosphorylation levels were not altered.

Total AMPK, phospho-ACC, and total ACC protein expressions 6 h after a single administration of metformin were also determined (Table 1). The ACC phosphorylation levels were significantly increased in all three muscles investigated. On the other hand, the total AMPK and ACC protein levels were not altered by metformin administration.

Animal characteristics in the chronic study. Body, muscle, and epididymal fat tissue weights in metformin-treated group were identical with control group (Table 2). The blood glucose and lactate levels in metformin-treated group were not different from those in control group (Table 2). On the other hand, serum insulin level in metformin-treated group was significantly lower than that in the control group (P = 0.006, respectively).

Figure 2 indicates the daily absolute and relative metformin intake. The mean absolute and relative metformin intake per day was 186.5 mg and 631 mg/kg body wt, respectively.

AMPK and ACC protein expressions in the chronic study. Phospho- and total AMPK and ACC protein expressions in the soleus and white gastrocnemius muscles with chronic metformin treatment were determined. The phospho- or total AMPK protein levels were not altered by metformin treatment (data not shown). The phospho-ACC protein levels in the metformin-treated group were significantly higher in the soleus (+55%; P = 0.021) and white gastrocnemius muscles (+32%; P = 0.015). The total ACC protein levels were not altered by metformin treatment (data not shown). Because ACC is a downstream target of AMPK, it was speculated that AMPK activity was in fact elevated by metformin in vivo.

PGC-1α protein expression. Figure 3A shows representative Western blot detections of PGC-1α protein in the muscles. An
immunoreactive band of ~120 kDa was detected on Western blotting. This molecular mass of the PGC-1α protein seems to be identical with the previous studies (47). Other studies demonstrated that the molecular mass of PGC-1α protein was ~90 kDa (49). The reason for this difference is unknown at present. Figure 3B shows the mean values of PGC-1α protein content in the muscles. The densitometric data indicated that the PGC-1α protein levels of the metformin-treated group were significantly higher than those of the control group in the soleus (+49%; \( P = 0.003 \)) and red (+35%; \( P = 0.006 \)) and white (+79%; \( P = 0.011 \)) gastrocnemius muscles.

**Enzyme activities.** Figure 4 indicates the muscle enzyme activities. The HK activity of the metformin-treated group in the white gastrocnemius muscle was significantly higher than that of the control group (+24%; \( P = 0.021 \)), whereas no differences were observed in the soleus or red gastrocnemius muscles (Fig. 4A). The pyruvate kinase activity of the metformin-treated group in the white gastrocnemius muscle was significantly higher than that of the control group (+11%; \( P = 0.019 \)), whereas such differences were not observed in the soleus or red gastrocnemius muscles (Fig. 4B). The LDH activities in any muscles were not altered by the metformin treatment (Fig. 4C). The CS activities of metformin treatment group were significantly higher than in the control group in the soleus (+20%; \( P = 0.013 \)) and red (+19%; \( P = 0.006 \)) and white (+14%; \( P = 0.016 \)) gastrocnemius muscles (Fig. 4D). The 8-HAD activity of metformin-treated group in the soleus muscle was significantly higher than that of the control group (+20%; \( P = 0.013 \)), whereas no differences were observed in the red or white gastrocnemius muscles (Fig. 4E).

**Cytochrome c and GLUT4 protein expressions.** Figure 5A shows the Western blot detections of the cytochrome c protein in the muscles. The cytochrome c protein content of the metformin-treated group in the soleus muscle was significantly higher than that of control group (+35%; \( P = 0.046 \)). In the white gastrocnemius muscle, cytochrome c protein content tended to be high (+18%; \( P = 0.078 \)). No significant difference was observed in the red gastrocnemius muscle. Figure 5B shows the Western blot detections of the GLUT4 protein in the muscles. No significant differences were observed in any muscles between the groups.

**DISCUSSION**

The present study is the first to demonstrate that 5–6 h are required for AMPK phosphorylation to be enhanced in skeletal muscle after the acute oral administration of metformin. In addition, chronic metformin treatment increases the skeletal

### Table 1. Effects of 6 h after a single metformin administration on total AMPK-α, phospho ACC (Ser79), and total ACC protein expressions

<table>
<thead>
<tr>
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<th>Control (n = 6)</th>
<th>Metformin (n = 6)</th>
<th>Control (n = 6)</th>
<th>Metformin (n = 6)</th>
<th>Control (n = 6)</th>
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<tbody>
<tr>
<td><strong>Soleus</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total AMPK-α</td>
<td>100±9</td>
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<td>100±3</td>
<td>100±8</td>
<td>92±6</td>
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<td>Phospho-ACC</td>
<td>100±9</td>
<td>141±10*</td>
<td>100±13</td>
<td>152±9*</td>
<td>100±12</td>
<td>190±17*</td>
</tr>
<tr>
<td>Total ACC</td>
<td>100±8</td>
<td>100±10</td>
<td>100±5</td>
<td>98±4</td>
<td>100±17</td>
<td>102±8</td>
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<td><strong>Red Gastronemius</strong></td>
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<tr>
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Values are means ± SE. *\( P < 0.01 \) vs. control.

### Table 2. Characteristics of the control and metformin-treated rats

<table>
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<th>Metformin (n = 8)</th>
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<tr>
<td>Pre body weight, g</td>
<td>267±3</td>
<td>267±2</td>
</tr>
<tr>
<td>Post body weight, g</td>
<td>329±8</td>
<td>332±5</td>
</tr>
<tr>
<td>Soleus weight, mg</td>
<td>120±6</td>
<td>127±5</td>
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<tr>
<td>Gastrocnemius weight, mg</td>
<td>1578±79</td>
<td>1659±35</td>
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<tr>
<td>Epididymal fat tissue weight, mg</td>
<td>3167±196</td>
<td>2911±132</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>127±3</td>
<td>124±3</td>
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<tr>
<td>Blood lactate, mM</td>
<td>1.5±0.1</td>
<td>1.7±0.2</td>
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<tr>
<td>Serum insulin, ng/ml</td>
<td>5.835±0.358</td>
<td>4.485±0.231*</td>
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</table>

Values are means ± SE. Pre, before treatment; Post, after treatment. *\( P < 0.05 \) vs. control.

Fig. 2. Daily absolute (A) and relative (B) metformin intake of the rats fed a chow containing 1% of metformin for 14 days. Data are means ± SE; \( n = 8 \) rats.
muscle PGC-1α protein expression, HK activity, mitochondrial oxidative enzyme activities, and cytochrome c protein expression in vivo. Although we did not indicate any direct evidence, it is possible that such increments may at least partially be attributed to AMPK phosphorylation. On the other hand, chronic metformin treatment does not affect the GLUT4 protein expression, whereas AMPK activator AICAR treatment does enhance the GLUT4 expression (5, 21, 51).

Previous studies demonstrated that the activation of AMPK by AICAR enhanced the PGC-1α expression in skeletal muscle (24, 44, 45). These results are consistent with the findings of the present study showing that the metformin treatment induces the AMPK phosphorylation and the following increase of the PGC-1α protein expression in the skeletal muscle. A recent in vitro experiment also indicated that exposure to metformin enhanced the PGC-1α mRNA expression in cultured human skeletal myotubes (1), thus suggesting that the increased skeletal muscle PGC-1α protein content shown in this experiment may be directly induced by metformin. Collectively, these findings raise the possibility that the metformin treatment increases the PGC-1α expression at least in part via AMPK phosphorylation. Further experimentation to indicate direct evidence that AMPK mediated the increase of PGC-1α expression by metformin are thus called for.

There are many observations that PGC-1 is associated with Type 2 diabetes and insulin sensitivity. The genetic polymor-
The present study demonstrated that the metformin treatment increased the skeletal muscle mitochondrial CS activity in the soleus and red and white gastrocnemius muscles. In addition, the β-HAD activity also significantly increased in the soleus muscle with metformin treatment. This increment of mitochondrial oxidative enzyme activities with metformin would at least partially be expected to be due to the increased PGC-1α protein expression. A previous study demonstrated the forced expression of PGC-1α to stimulate mitochondrial proliferation, while increasing the mtDNA copy number, and upregulating the transcriptional factors involving mitochondrial biogenesis such as nuclear respiratory factor 1 and 2 and mitochondrial transcription factor A in C2C12 skeletal muscle cells (54). In addition, mice overexpressing PGC-1α demonstrate an upregulation of mitochondrial proteins in skeletal muscle (26), whereas PGC-1α-null mice show a diminished skeletal muscle mitochondrial density and expression of mitochondrial metabolic components (25). Such a role of PGC-1α for improving the mitochondrial capacity is one of the possible mechanisms for the metformin action.

It has been postulated that an impaired mitochondrial oxidative capacity could be a direct cause of insulin resistance (12). The offspring of Type 2 diabetic parents, considered to possess the highest risk for developing Type 2 diabetes (48), show reduced mitochondrial activity in skeletal muscle as well as insulin resistance (36). Furthermore, a decreased skeletal muscle mitochondrial size or oxidative enzyme activity is related to insulin resistance and Type 2 diabetes (4, 18, 22, 42). Taken together, a decreased mitochondrial oxidative enzyme activity in skeletal muscle can be one of the primary candidates for developing Type 2 diabetes. Therefore, the enhancement of the mitochondrial capacity is potentially effective for ameliorating or preventing the insulin resistance or Type 2 diabetes. It is speculated that the effectiveness of metformin for improving insulin sensitivity by the insulin-stimulated glucose uptake in skeletal muscle, as described elsewhere (23), may therefore be at least in part due to the enhanced skeletal muscle mitochondrial capacity.

In this study, we demonstrated that metformin treatment to normal rats for 14 days increased the HK activity of white gastrocnemius muscle. A previous study also demonstrated that the skeletal muscle HK activity increased by metformin treatment (250 mg·kg⁻¹·day⁻¹ for 3 wk) in the streptozotocin-induced diabetic mice (3). Because activation of AMPK by AICAR treatment increased HK activity of skeletal muscle (19, 33, 44, 51), it is suggested that metformin enhances the HK activity via AMPK activation in skeletal muscle. Previous studies demonstrated the HK activity to be strongly associated with insulin sensitivity and insulin-stimulated skeletal muscle glucose uptake (13), suggesting that an increased HK activity as well as mitochondrial oxidative enzymes in skeletal muscle may also contribute to the blood glucose-lowering effect of metformin in the patients with insulin-resistant Type 2 diabetes.

It is important to note that 5–6 h are required to phosphorylate AMPK with an oral single injection of metformin to rats in the present study. In contrast, intraperitoneal injection of AICAR to rats increased AMPK phosphorylation at 30 min after injection (2). Collectively, these data imply that the action of metformin regarding AMPK phosphorylation is much milder than that of AICAR. The blood metformin concentration is expected to be highest ~3 h after the oral metformin treatment (7). In addition, 3 h of incubation are needed to enhance the AMPK activity of skeletal muscle when the isolated rat epitrochlearis muscle is incubated with metformin.
Therefore, it seems to be reasonable that the AMPK phosphorylation in skeletal muscle occurs at 5 and/or 6 h after oral metformin injection. Furthermore, the AMPK phosphorylation was not increased, whereas ACC phosphorylation was increased after 2 wk of chronic metformin treatment by containing metformin in powder chow in the present study. This strategy of metformin treatment may therefore be another cause for the milder effect of metformin. In line with such a milder effect, the level of increased PGC-1α protein expression by 14-day treatment of metformin in this experiment (35–79% of control) seems to be lower than that of AICAR (87–114% of control) (44). Although the reason why metformin does not increase the β-HAD activity or cytochrome c protein expression in red or white gastrocnemius muscles or the HK activity in the soleus or red gastrocnemius muscles is unknown at present, it is possible that such a mild effect of metformin is related to the nonuniform results of the enzyme activities among muscles.

Previous studies demonstrated that the activation of AMPK increased the GLUT4 expression in fast-twitch muscles in vivo (5, 21, 51) and in vitro (33). Furthermore, an in vitro experiment demonstrated that metformin treatment for 8 days increases the GLUT4 expression in cultured human myotubes (1). On the contrary, in this study, we demonstrated that the in vivo treatment of metformin for 14 days did not affect the GLUT4 protein expression in any muscles, whereas AMPK phosphorylation was observed. Another study also indicated that oral metformin treatment in obese diabetic Zucker (fa/fa) rats for 24 days decreased the plasma glucose and insulin concentrations without any change in GLUT4 content (14). In addition, metformin treatment to their lean littermates (Fa/−/Fa) decreased the GLUT4 content in the red gastrocnemius muscle (14). Metformin treatment to dexamethazone-induced insulin-resistant mice for 10 days ameliorated the hyperglycemia and insulin resistance without any change in the GLUT4 expression (46). These results suggest that it might be difficult to enhance the GLUT4 expression with metformin treatment in vivo. Although the reason why metformin does not increase the GLUT4 in vivo is unclear at present, it is possible that some unknown mechanisms that inhibit the GLUT4 expression or promote degradation might exist. Such mechanisms might prevent the enhancement of GLUT4 expression via the AMPK signaling in normal Wistar rats of this study or overcome the AMPK signaling in Fa/−/Fa rats of the previous study (14). Further studies are called for to clarify the difference between the action of metformin and AICAR for skeletal muscle GLUT4 expression in vivo.

In conclusion, single oral metformin administration to rats increases the AMPK phosphorylation in skeletal muscle 5–6 h after administration. In addition, chronic metformin treatment for 14 days significantly increases the PGC-1α and cytochrome c protein expressions and HK, CS, and β-HAD activities. Although no direct evidences that AMPK mediates such effects of metformin have yet been indicated, it is possible that metformin may enhance oxidative potential possibly via AMPK phosphorylation. These effects are considered to be associated with the metformin action as ameliorating insulin resistance in the patients with Type 2 diabetes. On the other hand, metformin treatment in vivo does not affect the skeletal muscle GLUT4 expression.

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