Two weeks of metformin treatment induces AMPK-dependent enhancement of insulin-stimulated glucose uptake in mouse soleus muscle

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Two weeks of metformin treatment induces AMPK-dependent enhancement of insulin-stimulated glucose uptake in mouse soleus muscle. Metformin-induced activation of AMPK has been associated with enhanced glucose uptake in skeletal muscle, but so far no direct causality has been examined. We hypothesized that an effect of in vivo metformin treatment on glucose uptake in mouse skeletal muscles is dependent on AMPK signaling. Oral doses of metformin or saline treatment were given to muscle-specific kinase dead (KD) AMPKα2 mice and wild-type (WT) littermates either once or chronically for 2 wk. Soleus and extensor digitorum longus muscles were used for measurements of glucose transport and Western blot analyses. Chronic treatment with metformin enhanced insulin-stimulated glucose uptake in soleus muscles of WT (~45%, P < 0.01) but not of AMPK KD mice. Insulin signaling at the level of Akt protein expression or Thr308/Ser473 phosphorylation was not changed by metformin treatment. Insulin signaling at the level of Akt and TBC1D4 protein expression as well as Akt Thr308/Ser473 and TBC1D4 Thr642/Ser711 phosphorylation were not changed by metformin treatment. Also, protein expressions of Rab4, GLUT4, and hexokinase II were unaltered after treatment. The acute metformin treatment did not affect glucose uptake in muscle of either of the genotypes. In conclusion, we provide novel evidence for a role of AMPK in potentiating the effect of insulin on glucose uptake in soleus muscle in response to chronic metformin treatment.

metformin; AMPK; skeletal muscle; glucose uptake; insulin

METFORMIN IS WORLDWIDE the most prescribed drug used to treat insulin resistance in type 2 diabetic patients and is also utilized in other patient groups with decreased insulin sensitivity. Most attention on the beneficial effects of metformin in regard to amelioration of insulin sensitivity has been in the liver. Both acute and chronic metformin treatments reduce hepatic glucose production by reducing gluconeogenesis and glucose output (73). Several in vitro (9, 30, 60, 77) and in vivo (6, 42, 58) studies in rodents have documented that metformin both acutely (77) and chronically (6, 10, 42) are also able to stimulate peripheral glucose disposal by increasing glucose uptake in skeletal muscle. The clinical relevance of these latter findings are supported by human clamp studies showing increased peripheral glucose disposal after metformin treatment (45, 52, 57), although this is not a totally consistent finding (15).

The molecular mechanisms underlying the effects of metformin are still not fully described, but inhibition of complex I in the mitochondria respiration chain has been reported (19, 54). Metformin treatment also induces activation of AMPK in muscle cells (9, 23, 40, 60), intact rodent muscles (60, 66, 77), and human diabetic skeletal muscles (45, 52). AMPK is activated under conditions of cellular stress, e.g., exercise, muscle contractions, ischemia, and hypoxia (31, 46, 75), where it catalyzes changes in the cellular metabolism to reduce ATP consumption as well as raising ATP production by an inhibitory effect on the anabolic and a stimulatory effect on the catabolic processes in the cell (69). Hence, AMPK activation by metformin has been suggested to be linked to complex I inhibition, which may lead to disturbance of the cell energy balance and thus AMPK activation (29).

Genetic evidence supports a role for AMPK in processes involved in glucose uptake and GLUT4 regulation in relation to acute and chronic treatment with the pharmacological activator AICAR (33, 34). Although metformin treatment has been coupled to both AMPK activation and upregulation of glucose uptake in skeletal muscle, only a few studies have measured both AMPK activation and glucose uptake (42, 45, 52, 60, 77). In fact, to our knowledge, no studies have investigated any causality between metformin, AMPK activation, and glucose uptake in skeletal muscle.

We hypothesized that potential effects of metformin treatment in vivo on glucose uptake in skeletal muscles would be dependent on an intact AMPK signaling system. Therefore, we investigated the role of AMPK in the acute and chronic effects of metformin to stimulate glucose transport in skeletal muscle by using muscle-specific kinase dead (KD) AMPKα2 mice (50).

METHODS

Animals

Experiments were approved by the Danish Animal Experiments Inspectorate and complied with the European convention for protection of vertebrate animals used for scientific purposes. The mice used in these experiments were muscle-specific kinase dead (KD) AMPKα2 mice, described previously (50). Female mice 22–30-wk old on a C57BL/6J background were studied and wild-type (WT) littermates were used as controls.
Treatment Models

All animals were kept on a 10:14-h light-dark cycle with unlimited access to standard rodent chow food and water. 

Acute metformin time course experiment. WT mice were given one dose of metformin (150 mg/kg, 1,1-dimethylbiguanide HCl; Sigma-Aldrich, Germany) or saline by gavage. Muscles were removed from anesthetized animals 2, 4, 6, 8, and 10 h after treatment and immediately frozen in liquid N2.

Acute (6-h) metformin treatment experiment. WT and KD mice were divided into two groups each, one treated with metformin and the other given saline. Metformin (150 mg/kg) and saline solutions were administered once by gavage 6 h before dissection and isolation of soleus and EDL muscles for incubations ex vivo (see Muscle Incubation Procedure).

Chronic metformin treatment experiment. WT and KD mice were divided into two groups each, one treated with metformin and the other given saline. Metformin (150 mg/kg) and saline solutions were administered by gavage twice daily in the morning (around 8–10 AM) and afternoon (around 4–6 PM) for 2 wk. The last dose of metformin/saline was given the afternoon before the experimental day.

Acute (16-h) metformin control experiment. A control experiment was conducted to differentiate the outcome in the chronic metformin treatment from possible effects related to the last dose of metformin administered the day before. WT mice were treated once with metformin (150 mg/kg) or saline by gavage the afternoon before the experimental day, i.e., 16–18 h before isolation of muscles.

Muscle Incubation Procedure

Fed mice were anesthetized by intraperitoneal injection of pentobarbital sodium (0.1 mg/g body wt). Soleus and extensor digitorum longus (EDL) muscles were removed from the animal and suspended at resting tension in incubation chambers (Multi Myograph System; Danish Myo-Technology) with basal buffer (Krebs-Henseleit buffer, pH 7.4: 117 mM NaCl, 4.7 mM KCl, 2.5 mM Ca2Cl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 24.6 mM NaHCO3, 5 mM HEPES, 0.1% BSA, 8 mM mannitol and 2 mM sodium pyruvate) at 30°C oxygenated with 95% O2 and 5% CO2 gas. For maximal insulin stimulation, insulin (10,000 μU/ml Actrapid; Novo Nordisk, Denmark) was added to the basal buffer. Basal and insulin-stimulated conditions were paired muscles from each leg of the same mice. All muscles were incubated for 40 min. For the assessment of glucose uptake, radioactively labeled tracers were added to the medium during the last 10 min of incubation (see next section). Following the 40-min incubation muscles were harvested and washed in ice-cold buffer, dried on filter paper and quickly frozen in liquid nitrogen. Muscles used for Western blot analyzes (see next section) were treated in the same way but without measuring glucose uptake during the last 10-min incubation. Muscles were stored at −80°C.

Muscle Preparation Used for Glucose Uptake

Glucose uptake was measured as accumulation of 2-deoxy[3H]-glucose (1 mM; Perkin Elmer), [14C]mannitol (8 mM) (PerkinElmer) was used as a marker of the extracellular space. For measurement of the accumulated 2-deoxy[3H]glucose and [14C]mannitol, muscles were heated and shaken at 80°C for 10 min in 1 M NaOH, neutralized with 1 M HCl, and centrifuged for 2 min at 13,000 g. Subsequently, radioactivity in the supernatants was measured by liquid scintillation counting (Ultima gold LSC-cocktail and Liquid Scintillation Analyzer Tri-Carb 2910 TR, PerkinElmer) and related to the specific activity of the incubation buffer.

Muscle Lysate Preparation Used for Western Blot Analyzes

Whole muscles were homogenized in ice-cold buffer (10% glycerol, 20 mM sodium pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na3VO4, 3 mM benzamidine, pH 7.5) by a tissue lyser for 2 × 1 min at 30 Hz (Tissue Lyser II; Qiagen Retch, Germany). Muscle homogenates were rotated end over end at 4°C for 1 h, after which they were centrifuged for 20 min at 15,000 g. The supernatants were harvested as the muscle lysate and stored at −80°C. Total protein content in supernatants was determined by the bicinchoninic acid method (Pierce Biotechnology).

Fig. 1. Effect of acute (2–10 h before isolation of muscles) metformin treatment on AMPK activation in soleus and EDL muscles from WT mice. Gray bars without and with hatches show results from saline- and metformin-treated mice, respectively. A: phosphorylated AMPK (AMPKp) in soleus muscles 2–10 h after a single dose metformin/saline. B: phosphorylated acetyl-CoA carboxylase (ACCP) in soleus muscles 2–10 h after a single dose metformin/saline. C: AMPKp in EDL muscles 2–10 h after a single dose metformin/saline. D: ACCP in EDL muscles 2–10 h after single dose metformin/saline. †Significant difference between saline and metformin treatment (main effect, P < 0.05); †P = 0.009; n = 6–8. Values are means ± SE.
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SDS-Page and Western Blot Analyses

Lyase proteins were separated by SDS-PAGE on self-cast Tris-HCl (7–12%) PAGE gels and by semidry blotting transferred to a PVDF membrane (Immobilon Transfer Membranes; Millipore, Denmark). The membrane was blocked in a washing buffer (10 mM Tris base, 150 mM NaCl, and 0.25% Tween 20, pH 7.4) containing low-fat milk protein (2–5%) or BSA (3%) solution and afterward probed with primary antibodies and appropriate secondary antibodies (see next section). Protein bands were visualized using a Kodak Image Station (2000 MM; Kodak, Rochester, NY) after probing with enhanced chemiluminescence (ECL, Millipore). Bands were quantified using Kodak 1D 3.6 software (Kodak). Membranes used for detecting phosphorylations of AMPK pThr172, Akt pSer473 and TBC1D1/D4 pThr642 were stripped (buffer containing 100 mM 2-mercaptoethanol, 0.5% SDS, and 62.5 mM Tris-HCl). After checking for successful removal of the primary antibody, the membrane were reprobed with

Fig. 2. Effect of acute (6-h) metformin treatment on glucose uptake in soleus and EDL muscles from WT and AMPK kinase dead (KD) mice. Filled and open bars show results from muscles incubated with or without insulin (10,000 µU/ml), respectively (A and C). Gray bars without and with hatches show results from saline and metformin treated mice respectively (B). A: basal and insulin-stimulated glucose uptake in soleus muscles from metformin- or saline-treated WT and AMPK KD mice. B: Δ values (insulin-basal) of insulin-stimulated glucose uptake in soleus muscles from metformin- or saline-treated WT and AMPK KD mice. C: basal and insulin-stimulated glucose uptake in EDL muscles from metformin- or saline-treated WT and AMPK KD mice. *Significant difference between basal and insulin-stimulated glucose uptake (main effect, P < 0.01); ‡significant difference between genotypes (main effect, P < 0.01); #significant interaction between insulin and genotype (P < 0.01); n = 12. Values are means ± SE.

Fig. 3. Effect of chronic metformin treatment on glucose uptake in soleus and EDL muscles from WT and AMPK KD mice. Filled and open bars represent results from muscles incubated with or without insulin (10,000 µU/ml), respectively (A and C). Gray bars without and with hatches show results from saline- and metformin-treated mice respectively (B). A: basal and insulin-stimulated glucose uptake in soleus muscles from metformin- or saline-treated WT and AMPK KD mice. B: Δ values (insulin-basal) of insulin-stimulated glucose uptake in soleus muscles from metformin- or saline-treated WT and AMPK KD mice. C: basal and insulin-stimulated glucose uptake in EDL muscles from metformin- or saline-treated WT and AMPK KD mice. *Significant difference between basal and insulin-stimulated glucose uptake (main effect, P < 0.01); ‡significant difference on insulin-stimulated glucose uptake between interventions in WT mice (P < 0.01); ‡significant difference between genotypes treated with metformin (P < 0.01); ‡significant interaction between genotype and treatment (P < 0.01); ‡significant interaction between insulin, genotype, and treatment (P < 0.01); n = 12–13. Values are means ± SE.
the corresponding total protein antibody. The phosphorylation results are not related to the total protein blots.

Antibodies Used for Detection of Specific Phosphorylation Sites and Protein Expressions

Antibodies used were as follows: acetyl-CoA carboxylase (ACC2) phosphorylation at Ser\(^{271}\); anti-phospho-ACC2 (Ser\(^{79}\); Upstate Biotechnology, #07-303); Akt2 protein expression: anti-Akt2 (D6G4) antibody (Cell Signaling Technology #3063); Akt protein phosphorylation at Thr\(^{308}\); anti-phospho-Akt Thr\(^{308}\) antibody (Cell Signaling Technology #9275); Akt protein phosphorylation at Ser\(^{473}\); anti-phospho-Akt Ser\(^{473}\) antibody (Cell Signaling Technology #2167); TBC1 D1/D4 (AS160) phosphorylation at Thr\(^{642}\); anti-AS160 phospho-Thr\(^{642}\) antibody (Symansis, NZ #3028 P1); and TBC1 D1/D4 (AS160) protein expression: anti-AS160 protein (Rab-GAP) antibody (Milli-antibody (Cell Signaling Technology #9271); AMPK\(\alpha\)2 protein expression: anti-AMPK\(\alpha\)2-specific antibody raised in sheep as previously described (76), kindly donated by D. G. Hardie, University of Dundee, Scotland, UK; AMPK\(\alpha\) subunit phosphorylation at Thr\(^{72}\); anti-phospho-AMPK\(\alpha\) antibody Thr\(^{72}\) antibody, Cell Signaling Technology #2531; GLUT4 protein expression: anti-GLUT4 antibody (Fierce Biotechnology #PA1-3031065); hexokinase (HK)II protein expression: anti-hexokinase II (HXK2/HK2) antibody (Alpha Diagnostics HXK23-A); Rab4 protein expression: anti-Rab4 antibody (Cell Signaling Technology #2167); TBC1 D1/D4 (AS160) protein expression: anti-AS160 protein (Rab-GAP) antibody (Millipore #07-741); TBC1 D1/D4 (AS160) phosphorylation at Thr\(^{642}\); anti-AS160 phospho-Thr\(^{642}\) antibody (Symansis, NZ #3028 P1); and TBC1 D1/D4 (AS160) phosphorylation at Ser\(^{711}\) (71). Secondary HRP-conjugated antibodies used were from Dako (Glostrup, Denmark).

Statistics

To enable comparison of both genotype and metformin treatment effect on the insulin stimulation, three-way repeated-measures ANOVA was performed in SPSS, without post hoc testing. Two-way ANOVA in SigmaStat 3.5 was used for testing genotype and metformin effect on insulin-stimulated increments when interaction was obtained with the three-way repeated-measures ANOVA. Differences between groups were considered statistically significant when \(P < 0.05\). All data are expressed as means \(\pm\) SE. Specific differences were localized using Student-Newman-Keuls post hoc test.

RESULTS

One Oral Dose of Metformin Treatment Activates AMPK in Mouse Skeletal Muscles

An acute time course experiment was conducted to deduce the time point at which AMPK activation is most pronounced after acute metformin treatment. One dose of metformin (150 mg/kg) or saline (control) was administered by oral gavage to WT mice. The metformin treatment induced a significant AMPK activation 2–10 h (main effect) after the metformin treatment in soleus muscles (Figs. 1, A and B, and 9A) and a borderline (\(P = 0.069\); power = 0.32) significant activation in EDL muscles (Figs. 1, C and D, and 9A) evaluated by AMPK phosphorylation and ACC phosphorylation. The power of the statistics test is not strong enough to select a given time point (with \(n = 6\) in each group) at which AMPK and ACC phosphorylation is significant increased. Consequently, the statistics test does not present a given time point at which p-AMPK and p-ACC were maximally activated after metformin treatment. Nevertheless, based on the seemingly greatest difference between saline and metformin on p-AMPK and p-ACC at 6 h, we chose the 6-h time point for the acute study as described below.

Acute (6-h) Metformin Treatment Does Not Potentiate Basal or Insulin-Stimulated Glucose Uptake in Soleus and EDL Muscles

To test whether a potential acute effect of in vivo metformin treatment on glucose uptake is dependent on AMPK signaling, WT and AMPK KD littermate mice were treated once orally with metformin or saline. Six hours after treatment, soleus and EDL muscles were isolated for the measurement of ex vivo glucose uptake. Neither basal nor maximal insulin-stimulated glucose uptake in soleus (Fig. 2, A and B) and EDL (Fig. 2C) were affected by metformin treatment. Soleus muscles of the AMPK KD mice had a significantly lower insulin-stimulated increments in glucose uptake compared with WT soleus muscles (Fig. 2B, \(P < 0.01\)) independently of the metformin treatment.

Chronic Metformin Treatment Induces AMPK-Dependent Enhancement of Insulin-Stimulated Glucose Uptake in Soleus Muscles

To investigate the role of AMPK in relation to potential chronic effects of metformin treatment on glucose uptake, WT

![Fig. 4. Effect of acute metformin treatment (~16 h before isolation of muscles) on glucose uptake in soleus and EDL muscles from WT mice. Filled and open bars represent results from muscles incubated with or without insulin (10,000 µU/ml) respectively. A: basal and insulin-stimulated glucose uptake in soleus muscles from metformin- or saline-treated WT mice. B: basal and insulin-stimulated glucose uptake in EDL muscles from metformin- or saline-treated WT mice. *Significant difference between basal and insulin-stimulated glucose uptake (main effect, \(P < 0.01\)); \(n = 12\). Values are means \(\pm\) SE.](http://ajpendo.physiology.org/Downloadedfrom)
and AMPK KD littermate mice were treated with metformin for 2 wk. The last dose of metformin was administered in the afternoon of the day before soleus and EDL muscles were isolated for measurement of glucose uptake. Maximal insulin-stimulated increments in glucose uptake were enhanced by ~45% in soleus of metformin-treated mice compared with saline-treated WT mice (Fig. 3, A and B, *P < 0.01) and by ~60% compared with metformin-treated AMPK KD mice (Fig. 3B, *P < 0.01). Basal glucose uptake was not affected by metformin treatment in soleus (Fig. 3A). In EDL muscle there was no effect of metformin treatment or genotype on either basal or insulin-stimulated glucose uptake (Fig. 3C). We performed an acute (16-h) control experiment to differentiate outcomes in the chronic metformin experiment from possible effects related to the last dose of metformin administered the afternoon before. As shown in Fig. 4, A and B, the last dose of metformin did not significantly enhance basal or insulin-stimulated glucose uptake in either soleus or EDL, although there was a tendency toward a main effect of metformin in soleus (*P = 0.089; power = 0.29).

AMPK and Insulin Signaling Marker Proteins Are Not Affected by Chronic Metformin Treatment

To explore further the metformin-induced AMPK-dependent enhancement of insulin-stimulated glucose uptake, we investigated AMPK activation and proteins related to the insulin signaling cascade and glucose uptake. The chronic treatment did not affect AMPKα2 protein expression (data not shown) and based on measurement of p-AMPK Thr172 and p-AMPK Thr172 expression was not significantly affected in the AMPK KD mice. Filled and open bars represent results from muscles incubated with or without insulin (10,000 μU/ml) respectively.

We then investigated insulin signaling at the level of Akt Thr308 and Ser473 phosphorylation as indicators of Akt activation. Both sites have increased phosphorylation upon insulin stimulation but were not influenced by genotype or metformin treatment (Figs. 5, A–D, and 9B). Akt protein expression was not regulated by genotype or treatment (data not shown).

Downstream of Akt, the AS160 (TBC1D4) protein is a potential convergence point for insulin- and AMPK-regulated glucose uptake. TBC1D4 Thr442 and Ser711 phosphorylations were both up regulated by insulin (Figs. 6, A–D, and 9B). Phosphorylation of Ser711 was also dependent on AMPK, where the insulin-stimulated response was significantly decreased in KD compared with WT muscles (Fig. 6, B, D, and E, *P < 0.01). Metformin treatment did not influence basal or insulin-stimulated phosphorylation status of TBC1D4. TBC1D4 protein expression in soleus and EDL was not affected by genotype or metformin treatment (data not shown).

Metformin has recently been associated with AMPK-TBC1D4-dependent GLUT4 translocation via the Rab4 protein (40). We investigated the Rab4 protein expression, but as shown in Figs. 7, A and B, and 9B, the protein is not downregulated in AMPK KD mice, and the chronic metformin treatment did not induce upregulation of the Rab4 protein expression.

GLUT4 and HKII Protein Expressions Are Not Regulated by Chronic Metformin Treatment

As shown in Figs. 8, A and C, and 9B, GLUT4 protein expression was not significantly affected in the AMPK KD mice, and metformin did not induce changes in the expression. The HKII protein was downregulated in soleus muscles of the AMPK KD mice, but metformin treatment did not affect the protein expression (Fig. 8, B and D and 9B).

DISCUSSION

The main finding in the present study was an increased insulin response in soleus muscles after chronic metformin...
treatment of WT mice that is absent in AMPKα2 KD mice. Although it is impossible to exclude other secondary effects of the AMPK KD construct, our observation strongly indicates that the enhanced insulin-stimulated glucose uptake in skeletal muscles observed after oral metformin treatment for a longer period of time indeed is dependent on AMPK. To our knowledge, it is the first time that such a causal relationship to AMPK has been investigated and reported.

Increased insulin-stimulated glucose uptake in soleus muscle after chronic treatment has been shown earlier, but these studies have primarily been done in insulin-resistant animals (6, 10, 42), and one study failed to detect an effect in soleus (59). Basal glucose uptake was not affected by metformin in the present study. This is in accord with findings in other in vivo rodent studies (6, 10), is supported by clinical observations (52), and is in line with the general
view that the effect of metformin is dependent on the presence of insulin (73).

The majority of studies have examined soleus in regard to metformin and glucose uptake, but a single study also used other muscles and observed an enhancing effect on glucose uptake in rat epitrochlearis and extensor carpi muscles (10). Based on this, it is difficult to deduce whether fiber type distribution plays a role for the lack of a metformin effect in EDL in the present study, since EDL and epitrochlearis both contain primarily glycolytic fibers, whereas soleus is dominated by oxidative fibers (1, 3). However, a species-specific difference could also influence the results, as is the case in regard to the effect of chronic AICAR treatment on glucose uptake in rats vs. mice (12, 62), where AICAR enhances insulin-stimulated glucose uptake in EDL muscles of rats, whereas an effect on insulin-stimulated glucose uptake has been shown to be absent in both muscles in mice (62). Clearly, we do not have an explanation for the different results in soleus and EDL muscles in the present study. However, we speculate that it could be related to the different AMPK trimer complex composition in the two muscles (70). It might be that some complexes are more prone to be activated by metformin than other complexes, as shown for another AMPK activating compound (74). Also, metformin uptake and accumulation (74) might differ between the muscles due to different capillarization (17) and possible different membrane metformin transporter content and/or composition (37).

We believe that the metformin effect observed in the soleus muscle after 2 wk of metformin treatment might have physiological relevance. The glycolytic EDL muscle is more representative for the mouse muscles (4) than soleus. Based on this, the physiological significance of our observations might be questioned. However, it is the more oxidative soleus and not the highly glycolytic EDL muscle in mice that in regard to muscle fiber type distribution is most like human skeletal muscle (4, 32). Consequently, the soleus muscle, more than the EDL, may reflect regulation in human skeletal muscle. In that respect, the physiological importance might be acknowledged given that several human studies have demonstrated enhanced insulin-stimulated glucose disposal by use of hyperinsulinemic euglycemic clamp after chronic metformin treatment in T2D patients (45, 52, 57). Although adipose tissue (21) and the intestine (7) may play a role on the effect of metformin on glucose metabolism during hyperinsulinemic euglycemic clamp conditions, skeletal muscle represents the major site of insulin-stimulated glucose uptake (18, 20). Therefore, the effect of metformin on the muscle tissue is very likely to be of greatest importance during the clamp (64, 73). In T2D patients, the impact on glucose disposal has been reported to be between ~15 and 40% (64), which is comparable with our observations on metformin-induced enhancement of insulin stimulated glucose uptake in the soleus muscle. Although we are aware that our reflections regarding application of our observations in mouse muscle to humans are only speculative, we believe our finding may shed further light into the molecular mechanism by which metformin regulates glucose uptake in human skeletal muscle. In that respect, we think these speculations are supported by the fact that AMPK activation has been shown after chronic metformin treatment in T2D patients (45, 52).

The metformin dose used in the present study and in many other in vivo rodent studies evaluating glucose uptake is about 200–300 mg·kg⁻¹·day⁻¹ (6, 10). The recommended dose used in the clinic to treat T2D patients is 2–3 g/day, corresponding to 30–40 mg·kg⁻¹·day⁻¹ for a 70-kg person (14, 25). However, comparable plasma levels of metformin in humans and mice with the denoted treatment models have been reported (6, 14).

It is a consistent finding that metformin does not influence β-cell secretion of insulin or the insulin receptor binding in skeletal muscle (43, 58), whereas one study reported increased...
The effect of chronic metformin treatment on the proximal steps of the intracellular insulin signaling is equivocal, since one study showed enhancement of the insulin receptor tyrosine kinase activity (58) whereas other studies reported no effect on PI 3-kinase activity and Akt activity (35, 45) and unaltered IRS-1 and Akt expression (35). In the present study, we observed an insulin effect on Akt phosphorylation but did not detect any changes with metformin treatment. Downstream of Akt, TBC1D4 is thought to be involved in GLUT4 vesicle recruitment to the plasma membrane via regulation of Rab proteins (47, 65). We found an AMPK-dependent enhancement of insulin-stimulated glucose uptake after chronic metformin treatment. Therefore, we measured Thr\(^{642}\) and Ser\(^{711}\) phosphorylation sites as representing Akt and AMPK targets, respectively. Although Ser\(^{711}\) is also shown to be regulated by insulin, it is in an Akt-independent manner (38, 71). We did not detect an effect of the chronic metformin treatment on phosphorylation of these sites. A recent study by Lee et al. (40) reported enhanced AS160 phosphorylation at site Thr\(^{642}\) in an AMPK-dependent manner upon acute metformin treatment of mouse C\(_2\)C\(_12\) cells. However, due to very high metformin concentrations (10 mM) and a long incubation period (6–12 h), it is difficult to compare such in vitro studies with in vivo treatment. Nevertheless, the study by Lee et al. also showed that metformin increases Rab4 expression in these cells via AMPK-TBC1D4 signaling (40). A role for Rab4 in insulin-stimulated GLUT4 vesicle translocation has also been suggested in rat skeletal muscle (2, 61), but, as is evident from our results, we did not see an AMPK dependence or metformin effect on the Rab4 protein content in mouse soleus muscles. The absence of a metformin effect on GLUT4 protein expression in the present study is in accord with the majority of chronic in vivo studies (28, 59, 66, 68), besides studies done with STZ (T1D) and obese fat-fed rats where reduced GLUT4 expression was partially rescued after metformin treatment (13, 42). The glucose phosphorylation step and thus hexokinase can be rate limiting for glucose uptake (24), and metformin treatment has been shown to upregulate hexokinase activity/expression (6, 16, 66); thus, a role for hexokinase in metformin-enhanced glucose uptake is possible. The reason for the discrepancy between the studies by Bailey et al. (6) and Da et al. (16) and our study, showing no metformin effect on hexokinase expression, could be due to the T1D model used in those two studies showing metformin-induced rescue of a depressed expression (6, 16). On the other hand, Suwa et al. (66) examined the consequences of metformin emplying normal rats and actually did not detect a change in soleus and only a moderate increase in white gastrocnemious.

It has been suggested that reduced mitochondrial content as well as protein expressions and activities observed in diabetic muscles may contribute to skeletal muscle insulin resistance (44, 48). However, this is a debatable topic (67), and other studies have not confirmed such a relationship (11, 27). We recently showed that 2-wk metformin treatment did not regulate mitochondrial respiration in tibialis anterior muscles from WT mice but in AMPK KD mice only (39). In that respect, our observations of an AMPK-dependent enhancement of insulin-stimulated glucose uptake after 2-wk metformin treatment could not be explained by regulation of mitochondria function,
although caution should be taken, as mitochondria function and glucose transport were measured in different muscles.

Alternatively, we speculate that the lack of changes in protein activation or expression related to insulin signaling at the level of Akt, TBC1D4, Rab4, GLUT4, or HKII in the present study may suggest that AMPK and metformin affect steps further distal related to fusion or docking of GLUT4 (36), which might be affected by membrane fluidity and cholesterol content. The latter is supported by cell studies indicating that both AMPK (26) and metformin (51, 72) play a role in changes in membrane composition that may lead to enhanced insulin action and GLUT4 translocation.

To our knowledge, we are the first to describe the acute effect of oral in vivo metformin treatment on skeletal muscle glucose uptake in rodents. The absence of an effect with acute metformin treatment in the present study is in line with Bailey et al. (5), showing no effect on glucose uptake in soleus measured with an intravenous glucose tolerance test after acute intravenous administration of metformin (250 mg/kg) in rats, and a clinical study by Perriello et al. (56) reporting only an effect on glucose production but not on glucose uptake measured with a hyperinsulinemic euglycemic clamp after acute metformin treatment of T2D patients. However, these results are in contrast to recent observations by Sajan et al. (60), showing markedly increased basal glucose uptake in vastus lateralis already 30 min after metformin injection in mice. The dissimilarity could be due to a combination of possible muscle type differences and a higher metformin dose used in the latter study (250 mg/kg), probably resulting in higher peak plasma metformin concentration due to injection instead of oral treatment (25, 55). An unexpected finding in our acute study was decreased insulin-stimulated glucose uptake in soleus muscle from AMPK KD compared with WT mice. We have not previously observed such an AMPK effect in our AMPK KD strain (22), and to our knowledge it has not been demonstrated in similar AMPK mouse strains (41, 49) or in other transgenic AMPK mouse models (8, 53, 63). Furthermore, we did not rediscover this genotype defect in our chronic metformin treated mice, so it is not a consistent characteristic for the AMPK KD model.

We conclude that the potentiating effect of chronic metformin treatment on insulin-stimulated glucose uptake in soleus muscle is dependent on AMPK signaling. At present, we are unable to explain this phenomenon, but it seems to be unrelated to changes in the insulin signaling pathway at the level of Akt and TBC1D4 as well as protein expression of GLUT4 and hexokinase.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

METFORMIN ENHANCES INSULIN-STIMULATED GLUCOSE UPTAKE


27. Hardie DG. Neither LKB1 nor AMPK are the direct targets of metformin. Gastroenterology 131: 973, 2006.


