

Metformin Increases AMP-Activated Protein Kinase Activity in Skeletal Muscle of Subjects With Type 2 Diabetes

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Metformin is an effective hypoglycemic drug that lowers blood glucose concentrations by decreasing hepatic glucose production and increasing glucose disposal in skeletal muscle; however, the molecular site of metformin action is not well understood. AMP-activated protein kinase (AMPK) activity increases in response to depletion of cellular energy stores, and this enzyme has been implicated in the stimulation of glucose uptake into skeletal muscle and the inhibition of liver gluconeogenesis. We recently reported that AMPK is activated by metformin in cultured rat hepatocytes, mediating the inhibitory effects of the drug on hepatic glucose production. In the present study, we evaluated whether therapeutic doses of metformin increase AMPK activity in vivo in subjects with type 2 diabetes. Metformin treatment for 10 weeks significantly increased AMPK α 2 activity in the skeletal muscle, and this was associated with increased phosphorylation of AMPK on Thr172 and decreased acetyl-CoA carboxylase-2 activity. The increase in AMPK α 2 activity was likely due to a change in muscle energy status because ATP and phosphocreatine concentrations were lower after metformin treatment. Metformin-induced increases in AMPK activity were associated with higher rates of glucose disposal and muscle glycogen concentrations. These findings suggest that the metabolic effects of metformin in subjects with type 2 diabetes may be mediated by the activation of AMPK α 2. *Diabetes* 51:2074–2081, 2002

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ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; PCr, phosphocreatine.

Metformin is one of the most commonly used drugs for the treatment of type 2 diabetes. It is an effective hypoglycemic drug that also improves lipid profiles (1) and reduces cardiovascular risk (2). The Diabetes Prevention Program has recently shown that similar to diet and exercise, metformin treatment reduces the risk of developing diabetes in glucose-intolerant individuals (3). Whereas most studies have shown that the glucose-lowering effects of metformin are secondary to a decrease in hepatic glucose production (1,4–6), a significant body of data also suggest that this drug increases glucose disposal in skeletal muscle (1,7,8); however, its molecular site of action remains unclear.

AMP-activated protein kinase (AMPK) is a heterotrimeric enzyme composed of a catalytic subunit (α) and two regulatory subunits (β and γ) (9,10). There are two isoforms of the catalytic subunit: AMPK α 1, which is widely distributed, and AMPK α 2, which is expressed in skeletal muscle, heart, and liver (11). AMPK works as an intracellular fuel gauge that becomes activated by decreases in the ATP/ADP and phosphocreatine (PCr)/creatinine ratios through mechanisms involving phosphorylation by one or more upstream AMPK kinases, allosteric activation, and a decrease in the inhibitory action of phosphatases (9,12,13). The increase in AMPK activity results in the stimulation of glucose uptake in muscle, fatty acid oxidation in muscle and liver, and the inhibition of hepatic glucose production, cholesterol and triglyceride synthesis, and lipogenesis (14).

Chemical activation of AMPK with the compound 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) leads to increased glucose uptake (15–19) and enhanced insulin sensitivity in muscle (20,21). AICAR cannot stimulate muscle glucose uptake in mice carrying a kinase-dead AMPK mutant in muscle (19). In liver cells, activation of AMPK with AICAR causes inhibition of glucose production (22–24). Based on these similarities between the metabolic effects of AICAR and metformin in liver and muscle, we previously tested the possibility that AMPK could be involved in mediating the metabolic actions of metformin in animal tissue (25). Metformin significantly increased AMPK activity in cultured rat hepatocytes and isolated muscles, and, importantly, the activation of the enzyme was associated with diminished glucose production by the

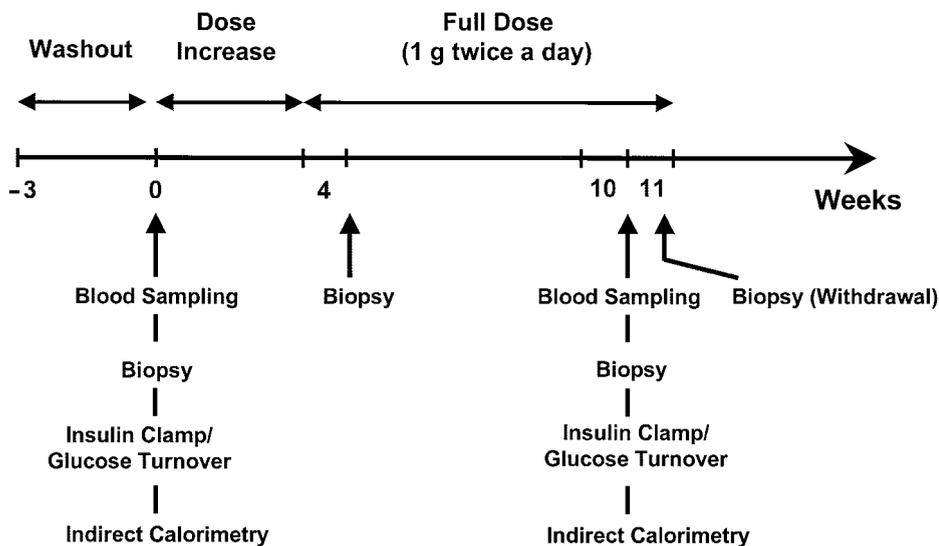


FIG. 1. Study protocol. Eight subjects (seven men and one woman) with type 2 diabetes entered the study. The subjects discontinued their antidiabetic therapy during a 3-week washout period. They underwent blood sampling for determination of glucose, insulin, HbA_{1c}, free fatty acids, lactate, and lipids. This was immediately followed by an initial vastus lateralis muscle biopsy as described in RESEARCH DESIGN AND METHODS. After the muscle biopsy, glucose turnover measurements and indirect calorimetry were performed during a hyperinsulinemic-euglycemic clamp as described in RESEARCH DESIGN AND METHODS. The subjects then started metformin orally at 500 mg/day, and the dose was increased weekly by 500 mg/day to a maximal of 1 g twice a day. After 4 weeks of treatment (1 week on maximal treatment), a second biopsy was done (4-week biopsy). Subjects continued on the maximal dose for 6 more weeks, and a third biopsy was done (10-week biopsy) immediately after blood sampling for the same metabolic parameters examined at the time of the first biopsy. Immediately after the 10-week biopsy, glucose turnover measurements and indirect calorimetry were performed during another clamp. Six subjects continued maximal dose treatment and underwent a final biopsy within 1 week of the 10-week biopsy but after withholding metformin overnight (withdrawal).

hepatocytes and higher muscle glucose uptake (25). To investigate whether the activation of AMPK by metformin is a likely mechanism for its metabolic effects in subjects with type 2 diabetes, in the present study, we examined if treatment with this drug modifies the activity of the AMPK $\alpha 1$ and $\alpha 2$ isoforms in skeletal muscle.

RESEARCH DESIGN AND METHODS

Subjects. Eight subjects with type 2 diabetes (seven men and one woman) were studied before, during, and after 10 weeks of treatment with metformin. The study was approved by the Ethical Committee at the Karolinska Institute. The subjects were informed of the purpose of the study and the potential side effects of the drug. Written consent was obtained from all the subjects. The diagnosis of type 2 diabetes was made by the subjects' physicians based on published criteria (26). Serum creatinine and amino transferases were measured at baseline to rule out ongoing kidney or liver disease. Three subjects were taking a sulfonylurea, two metformin, and three a combination of both drugs. Subjects were excluded if they had any history of systemic disease (other than diabetes) or were taking any other drug known to affect carbohydrate metabolism in muscle.

Study protocol. The subjects discontinued their antidiabetic therapy 3 weeks before the study. For 3 days before the study, they were given a diet containing 30–35 kcal/kg body wt composed of 55% carbohydrates, 30% fat, and 15% protein and were instructed to refrain from exercise and alcohol consumption. The experiments were done at the Clinical Research Center at Ersta Hospital after an overnight 12-h fast, starting between 8:00 and 9:00 A.M. Figure 1 shows the study protocol. After determination of total body weight and fat-free mass using bioimpedance (TBF-300; Tanita, Arlington Heights, Illinois), the subjects rested in the supine position for 30 min, followed by sampling of blood from an antecubital vein to determine glucose (27), insulin (Insulin RIA 100; Pharmacia, Uppsala, Sweden), lactate (28), free fatty acids (Wako Chemicals, Richmond, VA), HbA_{1c} (Abbott Laboratories, Solna, Sweden), and lipids (total cholesterol, HDL, LDL, and triglycerides, all using a kit from Abbott Laboratories). Immediately after blood sampling, an initial percutaneous needle biopsy of vastus lateralis muscle was done under local anesthesia as described previously (29). Approximately 100 mg wet wt muscle was obtained and rapidly frozen in liquid nitrogen. Immediately after the biopsy was done, glucose turnover (6,6-D₂-D-glucose) measurements and indirect calorimetry were done before and during a hyperinsulinemic-euglycemic clamp as described below. After the pretreatment studies were performed, the subjects were started on one 500-mg dose per day of metformin orally. This dose was continued for 1 week and then was increased weekly by 500 mg; hence, by the beginning of the fourth week, the dose was 1 g twice a day. After 1 week on 1 g twice a day (last day of week 4), the subjects came to the Research Center and a second muscle biopsy was done (4-week biopsy). Treatment was continued for 6 more weeks at this dose, and a third biopsy was done (10-week biopsy). Blood sampling was done immediately

before the 10-week biopsy for the same measurements done the day of the first biopsy. The 4- and 10-week biopsies were also done after overnight fasting and preceded by the subjects consuming the standardized diet described above for 3 days. These muscle biopsies were done ~3 h after the 1-g morning dose of metformin because that is the time when the concentrations of metformin in blood are expected to be the highest (t_{\max} 2.5–3.3 h) (1). Immediately after the 10-week biopsy was done, glucose turnover measurements and indirect calorimetry were performed again. To determine whether potential changes in AMPK activity would persist when blood metformin concentrations were low, six subjects continued treatment at 1 g twice a day and underwent a fourth biopsy within 7 days of the 10-week biopsy. For this biopsy, the morning dose of metformin was withheld; consequently, the biopsy was done ~12 h after the last dose ($t_{1/2}$ of metformin 2.4–4.7 h) (1). All the subjects tolerated the medication well, and they monitored their blood glucose concentrations at home throughout the study.

AMPK activity assay and immunoblotting. Muscle samples were homogenized as previously described (29), and lysates were used for determination of AMPK activity and immunoblotting. AMPK activity was determined after immunoprecipitating 200 μ g protein using specific antibodies made against the amino acid sequences 339–358 of AMPK $\alpha 1$ and 352–366 of $\alpha 2$ as previously described (29). The reaction was done using the synthetic peptide HMRSAMSGHLVKKRR as substrate (30). AMPK activity is expressed as incorporated ATP (picomoles) per milligram protein per minute. Immunoblotting was done as previously described (29). Briefly, proteins (60 μ g) from the muscle lysates were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated overnight at 4°C with the antibody against AMPK $\alpha 2$ described above (3 μ g/ml) or with anti-phospho-AMPK (Thr172) (3 μ g/ml; Cell Signaling, Beverly, MA). Bound antibodies were detected with anti-rabbit immunoglobulin-horseradish peroxidase-linked whole antibody and by using enhanced chemiluminescent (ECL) reagents (NEN Life Science Products, Boston, MA). The membranes were exposed to film, and the bands were quantified using ImageQuant software (Molecular Dynamics).

Acetyl-CoA carboxylase-2 activity. Proteins (200 μ g) from the muscle lysates were immunoprecipitated by overnight incubation at 4°C using a polyclonal anti-human acetyl-CoA carboxylase (ACC)-2 antibody raised against the sequence SRQKPPRNPLSSSD and protein A beads in buffer A (20 mmol/l Tris, pH 7.5, 1% Triton X-100, 50 mmol/l NaCl, 250 mmol/l sucrose, 50 mmol/l NaF, 5 mmol/l NaPPi, 2 mmol/l dithiothreitol, 4 mg/l leupeptin, 50 mg/l trypsin inhibitor, 0.1 mmol/l benzamide, and 0.5 mmol/l phenylmethylsulfonyl fluoride). The beads were then washed three times with buffer A and once with ACC assay buffer (60 mmol/l Tris, pH 7.5, 5 mmol/l MgCl₂, 1 mg/ml BSA, and 1.2 mmol/l β -mercaptoethanol) followed by determination of ACC activity using the ¹⁴C₂ fixation method in the presence of 10 mmol/l citrate and 1 mmol/l ATP (31).

ATP, PCr, and glycogen concentrations in muscle. Concentrations of ATP and PCr were determined in perchloric acid extracts of frozen muscle according to the method of Lowry and Passonneau (32). For measurements of glycogen, the muscles were hydrolyzed in 2 N HCl at 100°C for 2.5 h followed by neutralization with 2 N NaOH. Glycogen content was then measured by the

hexokinase method using the glucose HK reagent (Sigma, St. Louis, MO) and expressed per milligram protein.

Hyperinsulinemic-euglycemic clamp studies, tracer methodology, and indirect calorimetry. All subjects underwent a hyperinsulinemic-euglycemic clamp study before and after 10 weeks of metformin treatment as previously described (33). Briefly, 6,6-D₂-D-glucose (Cambridge Isotope Laboratories, Andover, MA) was given as a primed (3 mg/kg) continuous (2.4 mg · kg⁻¹ · h⁻¹) infusion starting 150 min before and through the clamps. All glucose infusates were enriched with the same isotope (molar excess 0.85% in the pretreatment and 1.27% in the posttreatment clamps) to minimize fluctuations in plasma tracer enrichment during clamps (34,35). Insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark) was infused intravenously at 1.0 mU · kg⁻¹ · min⁻¹ for 150 min. Glucose (20%) was simultaneously infused intravenously at a variable rate to maintain the blood glucose concentration at 5.0 mmol/l. Endogenous glucose release and whole-body glucose disposal rates were calculated using a modified Steele's equation taking into account the various tracer infusion rates (34). Whole-body glucose disposal was corrected by taking into account occasional differences in glucose concentrations between the beginning and the end of the steady-state period (34). Glucose clearance rates were calculated by normalizing whole-body glucose disposal rates to glucose concentrations to correct for any difference in the concentrations of glucose. Substrate oxidation rates were measured by indirect calorimetry (Deltatrac, Stockholm, Sweden) performed during 30-min periods as previously described (33), and nonoxidative glucose disposal was calculated by subtracting the glucose oxidation rate from whole-body glucose disposal.

Sampling and chemical analysis. Circulating glucose and 6,6-D₂-D-glucose concentrations were determined every 10 min during the time periods defined below. Data were collected for the 30-min periods immediately before the start of the insulin infusion (basal) and the last 30 min of the 150-min clamp. Blood glucose concentrations were determined immediately upon collection (27). Plasma was sampled every 10 min during 30-min steady-state periods for determination of 6,6-D₂-glucose enrichment. The trimethyl-*O*-methylxime derivative of plasma and glucose infusate was measured by gas chromatography mass spectroscopy (36). The 30-min averages were used for determination of glucose turnover measurements.

Statistical analysis. Data are presented as means ± SE. Analysis of the data were done using a one-way ANOVA with repeated measures (differences between means were determined with the Student-Newman-Keuls test) or using a paired *t* test. Correlation between AMPK α2 activity and whole-body glucose disposal was done using the Spearman rank-order correlation.

RESULTS

Clinical and metabolic characteristics of the subjects before and after treatment. The mean duration of type 2 diabetes was 3.9 years, ranging from 1 to 8 years (Table 1). The subjects had a small decrease in weight after 10 weeks of treatment (2.2%), whereas BMI and fat-free mass did not change significantly. After 10 weeks of treatment, blood glucose and serum insulin concentrations decreased by 14 and 28%, respectively (Table 1). Despite the improvement in glycemia, HbA_{1c} values did not decrease significantly (normal HbA_{1c}, <5.3%). Presumably, the 3-week washout period was not enough time for the HbA_{1c} to increase as a marker of the preexisting blood glucose concentrations. Serum HDL cholesterol concentrations increased 11%, and LDL cholesterol decreased 14% with treatment. There was a tendency for an increase in basal lactate concentrations, whereas lactate concentrations during the clamp were 21% higher after 10 weeks of treatment (Tables 1 and 3). Total cholesterol, triglycerides, and free fatty acids were unchanged.

AMPK α1 and α2 activity. To investigate if metformin increases the activity of AMPK α1 and α2 in subjects with type 2 diabetes, vastus lateralis biopsies were obtained before treatment began and after 4 and 10 weeks of treatment. Activity was measured in muscle lysates using specific antibodies to the α1 and α2 AMPK isoforms. As shown in Fig. 2A, AMPK α2 activity increased 52% after 4 weeks of treatment with metformin. The activity of AMPK α2 was further increased after 10 weeks of treatment by

TABLE 1

Clinical and metabolic characteristics of the subjects with type 2 diabetes

	Pretreatment	10 weeks
Age (years)	55 ± 1.5	—
Duration of diabetes (years)	3.9 ± 0.7	—
BMI (kg/m ²)	28.9 ± 1.4	28.6 ± 1.3
Weight (kg)	93.6 ± 5.3	91.5 ± 5.3*
Fat-free mass (kg)	65.7 ± 3	65.4 ± 3
HbA _{1c} (%)	6.2 ± 0.3	6.0 ± 0.4
Glucose (mmol/l)	8.3 ± 0.5	7.1 ± 0.4†
Insulin (pmol/l)	62.4 ± 7.2	44.4 ± 7.2*
Free fatty acids (mmol/l)	0.7 ± 0.1	0.7 ± 0.1
Cholesterol (mmol/l)	5.1 ± 0.4	4.7 ± 0.2
Triglycerides (mmol/l)	2.0 ± 0.4	1.9 ± 0.3
HDL cholesterol (mmol/l)	1.14 ± 0.1	1.26 ± 0.1‡
LDL cholesterol (mmol/l)	3.1 ± 0.3	2.7 ± 0.2‡
Lactate (mmol/l)	0.85 ± 0.07	0.96 ± 0.17

Data are means ± SE. *n* = 8 (7 men, 1 woman). All measurements were done after an overnight fast. **P* < 0.001, †*P* < 0.01, ‡*P* < 0.05 vs. pretreatment.

80% over baseline. This change in AMPK α2 activity was not associated with a change in the expression of AMPK α2 protein (Fig. 2B). Interestingly, the activation of AMPK α2 by metformin persisted after an overnight withdrawal (Fig. 3), when expected concentrations of the drug in blood would be low. In contrast, AMPK α1 activity did not change during treatment (data not shown). Figure 4 shows that the phosphorylation of AMPK-Thr172, a site known to activate AMPK (37), increased 80% after 10 weeks of treatment, indicating that this is the likely mechanism by which metformin increases AMPK activity.

ACC-2 activity. ACC is an enzyme that is phosphorylated and inactivated by AMPK and plays an important role in the regulation of fatty acid oxidation in muscle and liver (14). Consistent with the activation of AMPK, ACC-2 activity significantly decreased after 10 weeks of metformin treatment, and the inhibition of ACC-2 persisted after the overnight withdrawal period (Fig. 5). The protein content of ACC-2 in muscle did not change with metformin treatment (data not shown).

ATP, PCr, and glycogen content. Because AMPK is activated by depletion of high-energy-containing phosphates (9,12,13) and previous reports have suggested that metformin may change the energy status in hepatocytes (38,39), we measured ATP and PCr concentrations in the muscle. After 4 weeks of treatment, there was an 18% decrease in ATP, whereas concentrations of this metabolite were 23% lower after 10 weeks (Table 2). Similarly, there was a tendency for a decrease in muscle PCr after 4 weeks of treatment, and by 10 weeks, muscle PCr concentrations significantly decreased by 34%. Consistent with studies in rodents (40,41) and human cultured muscle cells (42), there was a 37% increase in muscle glycogen content after 10 weeks of treatment (Table 2).

Glucose turnover measurements and indirect calorimetry. Activation of AMPK has been associated with stimulation of muscle glucose uptake (15–19,43,44) and increased insulin sensitivity (20,21). Glucose infusion rates and whole-body glucose disposal during the clamp increased 18 and 21% after 10 weeks of metformin treatment, respectively (Table 3). A steady state was achieved in all

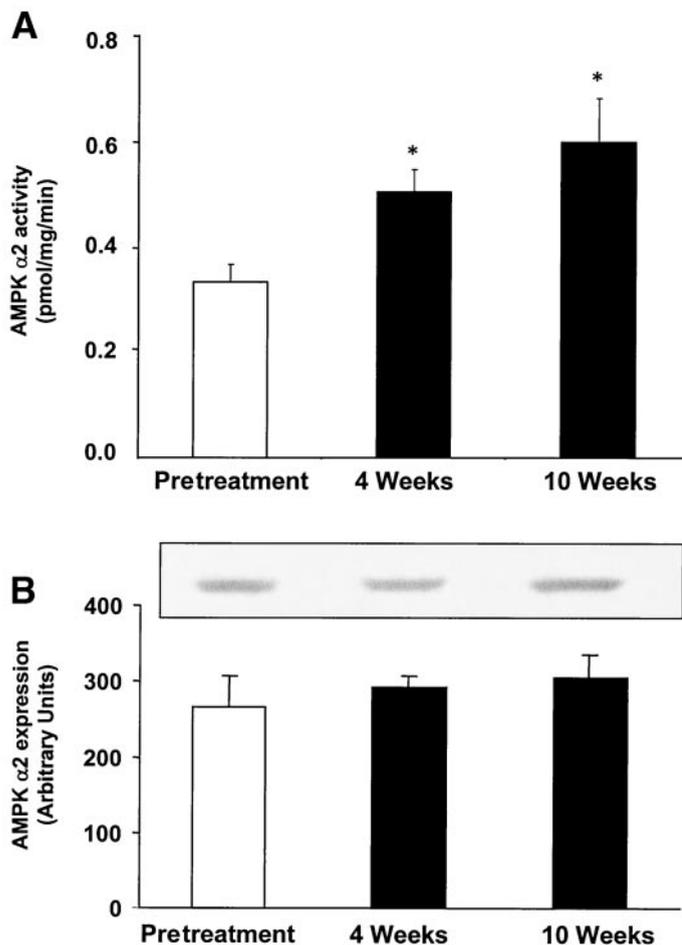


FIG. 2. Metformin increases AMPK $\alpha 2$ activity in muscle of subjects with type 2 diabetes. Biopsies of vastus lateralis muscle were obtained before and after 4 and 10 weeks of treatment. Muscle proteins were immunoprecipitated using specific antibodies against the $\alpha 2$ isoform of AMPK, and activity was assayed as described in RESEARCH DESIGN AND METHODS (A). Proteins (60 μ g) from muscle lysates were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was done with an anti-AMPK $\alpha 2$ -specific antibody as described in RESEARCH DESIGN AND METHODS (B). Graphical data are means \pm SE; $n = 8$; * $P < 0.05$ vs. pretreatment. B also shows a representative blot from one subject before and after 4 and 10 weeks of metformin treatment.

the subjects during the last 30 min of the clamp, both before and after metformin treatment. There was a significant rank-order correlation between whole-body glucose disposal and AMPK $\alpha 2$ activity after 10 weeks of treatment (correlation coefficient 0.8, $P < 0.05$). Because there was no change in glucose oxidation, the increase in glucose disposal during the clamp was mainly due to an enhancement in nonoxidative glucose disposal (Table 3). This increase in nonoxidative glucose disposal during treatment is consistent with our finding of increased muscle glycogen content. There was no change in basal endogenous glucose production after metformin treatment, whereas it was fully suppressed before and after metformin treatment during the clamp (Table 3). As determined by indirect calorimetry, there were no differences in fat and carbohydrate oxidation after 10 weeks of metformin treatment (Table 3). Blood glucose concentrations were successfully maintained close to 5 mmol/l during the clamp (Table 3). However, because the blood glucose

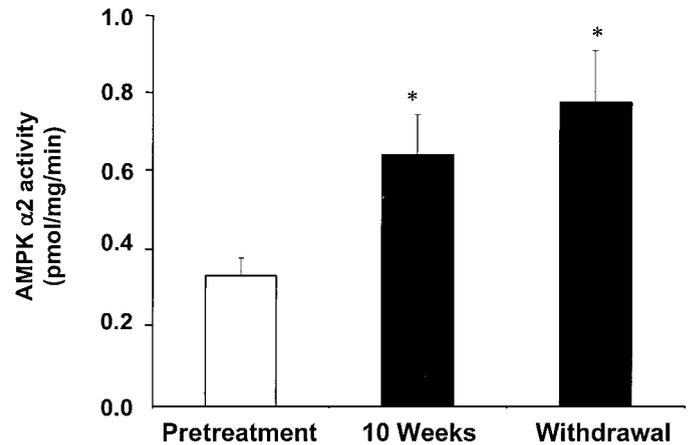


FIG. 3. Sustained activation of AMPK $\alpha 2$ in muscle of subjects with type 2 diabetes after an overnight withdrawal of metformin. Six subjects who were treated with metformin for 10 weeks underwent a fourth biopsy within 7 days of the 10-week biopsy but without the morning dose of metformin and after an overnight withdrawal period. AMPK $\alpha 2$ activity was measured in muscle lysates as described in RESEARCH DESIGN AND METHODS. Data are means \pm SE; $n = 6$; * $P < 0.05$ vs. pretreatment.

concentrations during the clamp were slightly lower before metformin treatment (Table 3), we also calculated glucose clearance rates by normalizing whole-body glucose disposal to the glucose concentrations. Glucose clearance increased significantly after 10 weeks of metformin treatment, both in the basal state and during the clamp (Table 3). Glucose clearance increased despite lower serum insulin concentrations after metformin treatment, in the basal state and during the clamp (Table 3).

DISCUSSION

Recently, we have shown that metformin increases AMPK activity in cultured rat hepatocytes and that the inhibition of glucose production in the hepatocytes was AMPK dependent (25). Metformin also increased AMPK activity and glucose uptake in isolated rat muscles incubated with the drug (25), suggesting that metformin may regulate systemic glycemia by stimulating AMPK in liver and skeletal muscle. If this hypothesis is correct, then therapeutic

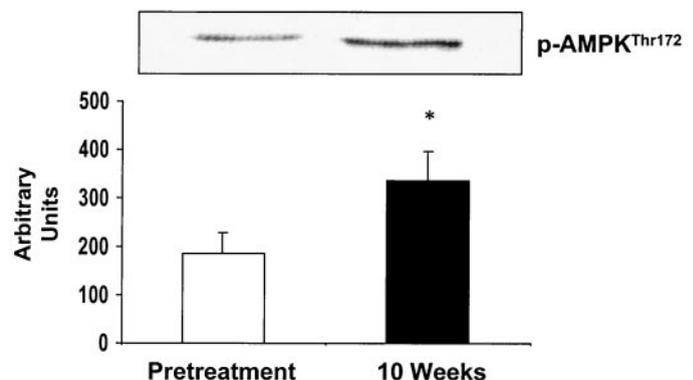


FIG. 4. Phosphorylation of AMPK-Thr172 before and after metformin treatment in muscle from subjects with type 2 diabetes. Proteins (60 μ g) from the muscle lysates were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. Blotting was done with the phospho-AMPK-Thr172 antibody described in RESEARCH DESIGN AND METHODS. A representative blot from one subject before and after 10 weeks of metformin treatment is shown. Data are means \pm SE; $n = 8$; * $P < 0.05$ vs. pretreatment.

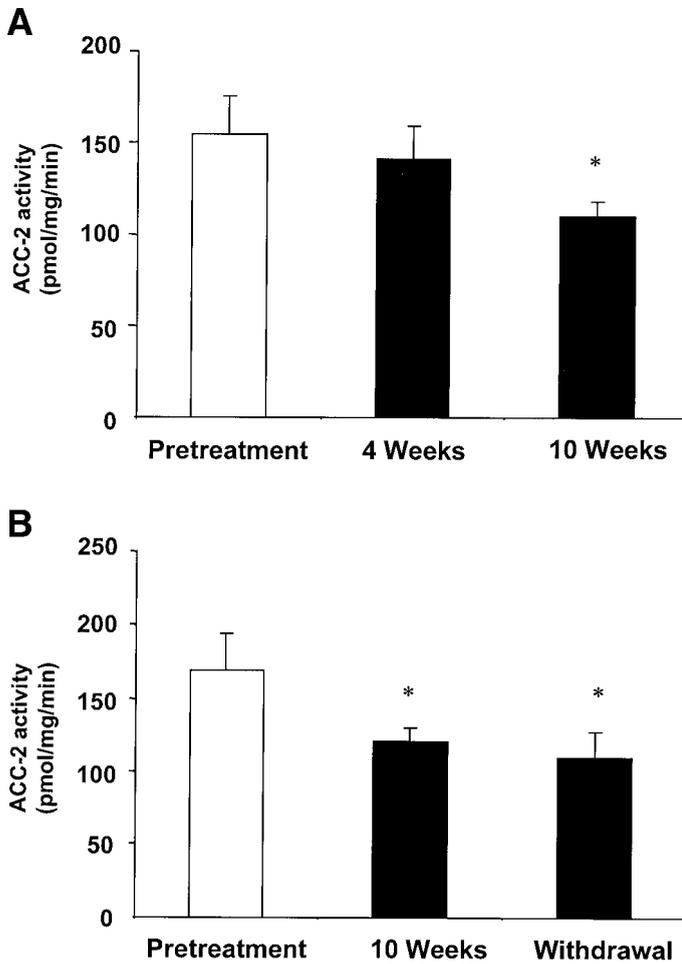


FIG. 5. Metformin decreases ACC-2 activity in muscle from subjects with type 2 diabetes. Biopsies of vastus lateralis muscle were obtained before and after 4 and 10 weeks of treatment. Muscle proteins were immunoprecipitated using a specific antibody raised against ACC-2, and activity was assayed as described in RESEARCH DESIGN AND METHODS (A) ($n = 8$). ACC-2 activity was measured in muscle from six subjects who were treated with metformin for 10 weeks and underwent a fourth biopsy within 7 days of the 10-week biopsy but without the morning dose of metformin and after an overnight withdrawal period (B) ($n = 6$). Data are means \pm SE; * $P < 0.05$ vs. pretreatment.

doses of metformin in human subjects should increase AMPK activity. In the current study, we found that metformin significantly increased AMPK α 2 activity in the skeletal muscle of subjects with type 2 diabetes and that this stimulation was accompanied by enhanced peripheral glucose disposal. This is the first report of a signaling molecule that is phosphorylated and activated in vivo by metformin in muscle tissue from subjects with type 2 diabetes. Many studies demonstrate that metformin functions mainly by inhibiting hepatic gluconeogenesis (1,4–6)

TABLE 2
Concentrations of ATP, PCr, and glycogen in muscle

	Pretreatment	4 weeks	10 weeks
ATP (nmol/mg muscle)	7.1 \pm 0.5	5.8 \pm 0.5*	5.5 \pm 0.3†
PCr (nmol/mg muscle)	11.9 \pm 0.9	10.4 \pm 1.2	7.9 \pm 0.8*
Glycogen (nmol/mg protein)	23.4 \pm 1.9	30.5 \pm 4.0	32.1 \pm 4.2‡

Data are means \pm SE. $n = 8$. * $P < 0.05$, † $P < 0.01$, ‡ $P = 0.05$ vs. pretreatment.

with little or no effect on glucose disposal (45), whereas other studies show a significant effect on glucose disposal (1,7,8). These discrepant findings are probably due to differences in patient selection, duration and dosing of metformin treatment, magnitude of reduction in hyperglycemia, and the methods used to measure glucose kinetics (1). Based on metformin's predominant effect on the liver, measuring hepatic AMPK activity during treatment would provide more direct evidence of the role of the enzyme on mediating the effects of the drug. However, obtaining liver biopsies would have proven impractical and probably unjustified. Obtaining skeletal muscle tissue carries a much lower risk, and based on our animal data, which show that metformin activates AMPK in both liver and muscle (25), we measured AMPK activity in the skeletal muscle. Together, these findings in animal tissue (25) and human skeletal muscle (present study) strongly support a role of AMPK in mediating the metabolic actions of metformin.

Despite years of research, the effects of metformin on glucose uptake in skeletal muscle remain controversial. Metformin increases glucose uptake in cultured human muscle cells (42), muscles from streptozotocin-treated rodents (40,46), and muscle strips from diabetic subjects (47). The increase in muscle glucose uptake by metformin has been associated with translocation of glucose transporters to the plasma membrane in L6 cells (48) and adipocytes of insulin-resistant Zucker rats (49). The mechanism leading to transporter translocation is not well understood. There is some evidence that metformin might increase glucose uptake by stimulating insulin receptor signaling. For example, in erythrocytes (50,51), monocytes (52), fat cells (53), and *Xenopus* oocytes (54,55), metformin increases insulin receptor binding (50,52,53) and tyrosine kinase activity (51,54,55). However, because these cells are not the main target of metformin's action, the clinical importance of these findings is uncertain. In streptozotocin-treated rats, metformin improves abnormal insulin receptor tyrosine kinase activity in muscle (40), although it is not clear whether this is a direct effect of the drug or secondary to amelioration of glucose toxicity during treatment. A recent study done in subjects with type 2 diabetes found that treatment with maximal therapeutic doses of metformin for 3–4 months increased insulin-stimulated whole-body glucose disposal by 22% but did not alter insulin receptor substrate-1-associated phosphatidylinositol 3-kinase or Akt activity in skeletal muscle (8). Thus, at least in skeletal muscle of subjects with type 2 diabetes, it is unlikely that metformin upregulates proximal steps in insulin receptor signaling.

In the present study, we found that during the clamp, endogenous glucose production was fully suppressed before as well as after metformin treatment. Presumably, the insulin concentrations achieved during the clamp were sufficient to fully suppress endogenous glucose production on both occasions. Basal and insulin-stimulated glucose clearance significantly increased after metformin treatment in the presence of lower serum insulin concentrations and completely suppressed endogenous glucose production, suggesting that metformin enhanced peripheral glucose disposal. There was a small but significant decrease in mean body weight after 10 weeks of treatment,

TABLE 3
Glucose turnover and indirect calorimetry data during a hyperinsulinemic-euglycemic clamp

	Basal		Clamp	
	Pretreatment	10 weeks	Pretreatment	10 weeks
Glucose infusion rate ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	—	—	4.29 ± 0.82	$5.05 \pm 0.87^*$
Whole-body glucose disposal ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	2.56 ± 0.16	2.62 ± 0.16	3.91 ± 0.69	$4.73 \pm 0.70^*$
Glucose clearance rate ($\text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	1.72 ± 0.13	$2.10 \pm 0.09^\dagger$	4.58 ± 0.89	$5.29 \pm 0.87^*$
Nonoxidative glucose disposal ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	1.12 ± 0.24	1.16 ± 0.20	1.50 ± 0.50	$2.48 \pm 0.45^*$
Endogenous glucose production ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	2.54 ± 0.20	2.66 ± 0.13	-0.49 ± 0.07	-0.20 ± 0.10
Glucose oxidation ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	1.43 ± 0.16	1.46 ± 0.10	2.40 ± 0.22	2.25 ± 0.35
Fat oxidation ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	0.86 ± 0.08	0.84 ± 0.06	0.57 ± 0.06	0.56 ± 0.08
Energy expenditure ($\text{kcal} \cdot \text{kg}^{-1} \cdot 24 \text{ h}^{-1}$)	22.01 ± 0.48	21.48 ± 0.36	21.36 ± 0.59	21.49 ± 0.68
Glucose (mmol/l)	8.3 ± 0.5	$7.1 \pm 0.4^\dagger$	4.8 ± 0.1	$5.0 \pm 0.1^*$
Insulin (pmol/l)	62.4 ± 7.2	$44.4 \pm 7.2^\S$	372.6 ± 14	$329 \pm 23^\ddagger$
Lactate (mmol/l)	0.85 ± 0.07	0.96 ± 0.17	0.67 ± 0.05	$0.80 \pm 0.04^\ddagger$

Data are means \pm SE. $n = 8$. $^*P < 0.05$ vs. pretreatment during the clamp. $^\dagger P < 0.05$ vs. pretreatment in the basal state, $^\ddagger P < 0.01$ vs. pretreatment during the clamp, $^\S P < 0.001$ vs. pretreatment in the basal state.

which could contribute to the increased glucose disposal. It is unlikely that the weight loss was involved in the increased AMPK $\alpha 2$ activity because, after 4 weeks of treatment, AMPK $\alpha 2$ activity had already increased significantly, whereas body weight had not changed (93.6 kg before treatment vs. 93.1 kg after 4 weeks).

We have shown that AMPK phosphorylation increases during treatment with metformin, both in muscle from diabetic subjects (current study) and in rat hepatocytes (25). Unlike AMP, metformin does not increase the activity of partially purified rat liver AMPK (25). Taken together, these findings indicate that phosphorylation rather than allosteric activation is the mechanism by which metformin enhances AMPK activity.

The effects of metformin on cellular energetics are unclear because of various results, depending on the experimental system used, concentrations of the drug, and duration of treatment. Studies recently demonstrated that, in hepatocytes, metformin decreases mitochondrial respiration through selective inhibition of complex 1 of the respiratory chain (38,39). The inhibition of complex 1 occurs even at therapeutic metformin concentrations (39). In these studies, there was a substantial decrease in the ATP/ADP ratio with metformin treatment, but in contrast to our findings, total ATP was unchanged. The finding of a greater decrease in ATP compared with PCr at 4 weeks was unexpected because, in situations such as acute exercise, the decrease in ATP is smaller than that for PCr. In rat hepatocytes, incubation with metformin for 1 h at therapeutic concentrations inhibited gluconeogenesis without altering NADH and NAD^+ in cytosolic and mitochondrial fractions, but metformin alone at higher concentrations did increase NADH/ NAD^+ (56). El Mir et al. (38) showed that the inhibition of complex 1 of the respiratory chain in hepatocytes is accompanied by increases in the lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios, suggesting that under certain conditions, metformin can alter the redox state. Further research is needed to determine whether the changes in muscle energy status and AMPK activity after chronic metformin treatment observed in this study are associated with alterations in mitochondrial respiration and the redox state.

Similar to the activation of AMPK by metformin in liver

cells (25), the inhibition of complex 1 in isolated mitochondria from metformin-treated hepatocytes increases with time (39). These findings are also consistent with a prolonged accumulation of metformin in different tissues during oral administration of the drug to mice (57) and could explain why in the present study the activation of AMPK $\alpha 2$ was sustained after metformin withdrawal. The $t_{1/2}$ of AMPK $\alpha 2$ in skeletal muscle is not known, but it is unlikely that the sustained activation of AMPK $\alpha 2$ observed in the study is related to the $t_{1/2}$ of the protein or a prolonged time for the enzyme activity to recover, because we (43) and others (58) have shown that AMPK activity comes back to baseline within 10 min after an acute stimulus, such as muscle contraction.

The effect of metformin on AMPK activity is isoform specific because only the $\alpha 2$ catalytic subunit was activated. In skeletal muscle from diabetic and nondiabetic subjects, AMPK $\alpha 2$ mRNA constitutes the majority (two-thirds) of α mRNA compared with $\alpha 1$ (29). Similar to the administration of metformin in humans, exercise studies have shown that AMPK $\alpha 2$ is the isoform activated during moderate-intensity treadmill running both in rats (43) and humans (29,59,60), whereas AMPK $\alpha 1$ remains unchanged. This result suggests that the $\alpha 2$ and not the $\alpha 1$ isoform is predominantly responsible for regulating the in vivo AMPK-mediated metabolic effects of both metformin and exercise in skeletal muscle.

It is unclear whether the stabilization or reduction of body weight produced by metformin is due to central (appetite suppression) or peripheral effects (1). Chronic treatment with both AICAR (61) and metformin (4) decreases fat mass. Moreover, ACC knockout mice maintain or lose weight despite increased food intake (62). These findings raise the possibility that AMPK may mediate, at least in part, the effects of metformin on body weight.

The specificity of AMPK as a molecular target of metformin is yet to be determined, and it will be interesting to examine if other insulin-sensitizing agents also alter AMPK activity in liver and peripheral tissues. The search for selective and more potent AMPK-activating agents designed for the treatment of type 2 diabetes will also be an important area for future investigations.

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