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Ablation of AMP-Activated Protein Kinase α2 Activity Exacerba tes Insulin Resistance Induced by High-Fat Feeding of Mice

Nobuharu Fuji i, Richard C. Ho, Yasuko Manabe, Niels Jessen, Taro Toyoda, William L. Holland, Scott A. Summers, Michael F. Hirshman, and Laurie J. Goodyear

OBJECTIVE—We determined whether muscle AMP-activated protein kinase (AMPK) has a role in the development of insulin resistance.

RESEARCH DESIGN AND METHODS—Muscle-specific transgenic mice expressing an inactive form of the AMPK α2 catalytic subunit (α2i TG) and their wild-type littermates were fed either a high-fat (60% kcal fat) or a control (10% kcal fat) diet for 30 weeks.

RESULTS—Compared with wild-type mice, glucose tolerance in α2i TG mice was slightly impaired on the control diet and significantly impaired on the high-fat diet. To determine whether the whole-body glucose intolerance was associated with impaired insulin sensitivity in skeletal muscle, glucose transport in response to submaximal insulin (450 μU/ml) was measured in isolated soleus muscles. On the control diet, insulin-stimulated glucose transport was reduced by ~50% in α2i TG mice compared with wild-type mice. High-fat feeding partially decreased insulin-stimulated glucose transport in wild-type mice, while high-fat feeding resulted in a full blunting of insulin-stimulated glucose transport in the α2i TG mice. High-fat feeding in α2i TG mice was accompanied by decreased expression of insulin signaling proteins in gastrocnemius muscle.

CONCLUSIONS—The lack of skeletal muscle AMPK α2 activity exacerba tes the development of glucose intolerance and insulin resistance caused by high-fat feeding and supports the thesis that AMPK α2 is an important target for the prevention/amelioration of skeletal muscle insulin resistance through lifestyle (exercise) and pharmacologic (e.g., metformin) treatments. Diabetes 57: 2958–2966, 2008

The development of insulin resistance in skeletal muscle precedes the onset of type 2 diabetes by decades (1). Although the underlying mechanism is not fully understood, in recent years there has been growing interest in AMP-activated protein kinase (AMPK) as a potential target to attenuate skeletal muscle insulin resistance. As examples, two well-known therapies for type 2 diabetes, physical exercise and metformin, both activate AMPK in skeletal muscle (2). Despite this emphasis on AMPK, there is little direct evidence showing that AMPK is in fact critical in the development of skeletal muscle insulin resistance.

AMPK is an energy-sensing enzyme that is activated by acute increases in the cellular AMP-to-ATP ratio. In skeletal muscle, AMPK activity is increased by stimuli such as exercise, hypoxia, ischemia, and osmotic stress, all of which reduce cellular energy level (2). When intracellular ATP decreases, AMPK acts to switch off ATP-consuming pathways, such as glycogen, fatty acid, and protein synthesis pathways, and acts to switch on alternative pathways for ATP regeneration, such as glucose transport, glycolysis, and fatty acid oxidation. AMPK may also play a role in enhancing insulin sensitivity and/or responsiveness for glucose transport (3–6) in skeletal muscle.

AMPK is a heterotrimeric serine/threonine kinase that consists of a catalytic α-subunit and regulatory β- and γ-subunits (7–11). In skeletal muscle, α2 (12,13) is the major catalytic isoform expressed. To examine a possible role of AMPK in the development of insulin resistance, muscle-specific transgenic mice expressing an inactive form of the AMPK α2 catalytic subunit isoform (α2i TG mice) (14) and their wild-type littermates were subjected to a high-fat diet. Here, we show that ablation of muscle AMPK α2 activity worsens the glucose intolerance induced by high-fat feeding. This exacerbated glucose intolerance is associated with a marked decrease in muscle glucose transport in response to insulin, measured in vitro in isolated muscles. These results demonstrate that AMPK α2 activity is an important factor contributing to insulin action on glucose transport in skeletal muscle. Therefore, activation of muscle AMPK α2 by exercise and/or pharmacological stimulation can be a significant strategy to improve insulin resistance in patients with type 2 diabetes.
buffer containing the same concentration of insulin and 1 mmol/l 2-[3H]-deoxy-

Immediately after muscle incubation, the muscles were transferred to KRB

St. Louis, MO) and serum free fatty acids using the NEFA C kit (Wako,

St. Charles, MO). Serum triglycerides were determined by kit assay (Sigma,

concentrations were assessed using a rat/mouse insulin enzyme-linked

immunosorbent assay kit with a mouse insulin standard (Linco Research,

Dallas, TX).

Muscle incubation and glucose transport. The mice were fasted overnight and killed. The soleus muscles were

used for these measurements.

Muscle incubation and glucose transport. After 30 weeks of dietary treatment, the mice were fasted overnight and killed. The soleus muscles were rapidly removed and treated as previously described (14). Briefly, each soleus muscle was mounted on an incubation apparatus and preincubated in Krebs-Ringer bicarbonate (KRB) buffer containing 2 mmol/l pyruvate for 20

min. The muscles were then incubated in KRB buffer in the absence or

presence of 450 Ci/ml [3H]-glucose (1.5

Ci/ml) and 7 mmol/lD-[14C]-mannitol (0.3

Ci/ml), and glucose

transport was measured as described (14).

Immunoblotting. Immunoblotting was done using standard procedures as previously described (18). Antibodies were from commercial sources and consisted of insulin receptor β-subunit (Cell Signaling), insulin receptor substrate-1 (IRS-1), Akt (Upstate Biotechnology, Lake Placid, NY), and GLUT1 and GLUT4 (Chemicon, Temecula, CA). Gastrocnemius muscles were used for immunoblotting.

Measurement of isoform-specific AMPK activity. AMPK activity was

measured as previously described (14). Briefly, muscle lysates (150 μg protein) were immunoprecipitated with specific antibodies against the α1 or α2 catalytic subunits and protein A beads. The kinase reaction was carried out

in 40 mmol/l HEPES, pH 7.0; 0.1 mmol/l synthetic SAMS peptide; 0.2 mmol/l AMP; 80 mmol/l NaCl; 0.8 mmol/l dithiothreitol; 5 mmol/l MgCl2 and 0.2

mmol/l ATP (2 μCi of [γ-32P]ATP) for 20 min at 30°C. Reaction products were spotted on Whatman P81 filter paper, the papers were extensively washed in

1% phosphoric acid, and radioactivity was assessed with a scintillation counter. Kinase activity was assessed by incorporated ATP (pmol) per

immunoprecipitated protein (mg) per min.

Noninvasive physiological and behavioral characterization. Wild-type and α2i TG mice were subjected to noninvasive physiological and behavioral characterization using the Comprehensive Lab Animal Monitoring System

TABLE 1

<table>
<thead>
<tr>
<th>Body weight (g)</th>
<th>Control diet</th>
<th>High-fat diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>26.5 ± 0.7</td>
<td>24.8 ± 0.7</td>
</tr>
<tr>
<td>α2i TG</td>
<td>25.0 ± 0.6</td>
<td>24.9 ± 0.5</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>190.6 ± 10.8</td>
<td>171.8 ± 16.6</td>
</tr>
<tr>
<td>α2i TG</td>
<td>165.4 ± 11.4</td>
<td>182.5 ± 12.3</td>
</tr>
</tbody>
</table>

Data are means ± SE. n = 5–6 for all points. *P < 0.01 vs. control diet in same genotype. ‡P < 0.05 vs. control diet in same genotype.
(CLAMS; Columbus Instruments, Columbus, OH) at the Physiology Core Laboratory of the Joslin Diabetes Center. The mice were monitored for 24 h and assessed for oxygen consumption ($VO_2$; mL/kg/h), carbon dioxide generation ($VCO_2$; mL/kg/h), heat generation calculated from gas exchange data (kcal/h), respiratory exchange ratio calculated from gas exchange data, food consumption (g/24 h), water consumption (ml/24 h), and locomotive activity evaluated by three-dimensional fixed-point observation (counts/h). Monitoring started at 1000 h, and CLAMS assessment was made during both the light cycle (0700–1900 h) and dark cycle (1900–0700 h).

**RESULTS**

**Body weight and blood glucose concentrations.** Body weights and blood glucose concentrations are summarized in Table 1. The effects of the high-fat diet on body weight emerged by week 4 and remained for the remaining experimental period in both wild-type and α2i TG mice. The high-fat diet–induced increase in body weight was similar between wild-type and α2i TG mice. Blood glucose concentrations in the fasted state tended to be higher with the high-fat diet compared with the control diet in both wild-type and α2i TG mice (main effect of the diet, $P < 0.05$ at week 26 and $P = 0.07$ at week 29). The relatively minor effect of the high-fat diet on blood glucose may be due to the mouse strain used in this study. It has been shown that in FVB mice, the background strain used in the current study are resistant to high-fat diets compared with other mouse strains (19,20). No significant differences in blood glucose concentrations were observed between genotypes in both control and high-fat diet–treated mice. There was a tendency for blood glucose concentrations to decline from week 0 to week 4, which is likely due to acclimation of the mice to handling during blood collection.

**AMPK activity.** To confirm that muscle AMPK α2 activity was inhibited in muscles of α2i TG mice, isoform-specific AMPK activities were measured in gastrocnemius muscle at the end of the experimental period. As expected, α2 activity was blunted in α2i TG mice, and there was no compensatory increase in α1 activity in the α2i TG mice (Fig. 1). Interestingly, there was a tendency for AMPK α2 activity to diminish with long-term high-fat feeding in wild-type mice, whereas there was no effect of high-fat feeding on AMPK α1 activity (Fig. 1).

**Glucose tolerance.** To test whether the lack of muscle AMPK α2 activity induces glucose intolerance in vivo, wild-type and α2i TG mice fed the control or high-fat diet...
Glucose transport in isolated muscle. We next examined muscle insulin sensitivity for glucose transport using the in vitro muscle incubation system (Fig. 3). At week 30, soleus muscles were isolated from the mice and incubated in the absence or presence of a submaximal dose of insulin (450 µU/ml). In mice fed the control diet, insulin increased glucose transport by twofold in wild-type mice. This increase in insulin-stimulated glucose transport tended to be diminished in α2i TG compared with wild-type mice (P = 0.07). Insulin significantly increased glucose transport in wild-type mice fed both the standard and high-fat diet, but this increase was blunted by 40% with the high-fat feeding. In α2i TG mice, insulin-stimulated glucose transport was totally abolished on the high-fat diet, showing that a lack of AMPK activity further worsens high-fat diet–induced muscle insulin resistance. These results suggest that impaired insulin-stimulated glucose transport in α2i TG mice on the high-fat diet contributes to the deterioration of glucose intolerance observed in these animals. Isolated extensor digitorum longus muscles were also used to determine glucose transport in vitro (data not shown). However, the increase in insulin-stimulated glucose transport was very small even in control diet–fed wild-type mice, making it difficult to compare the difference between genotype and fed conditions and demonstrating that extensor digitorum longus muscles become insulin resistant with age.

Expression of insulin signaling molecules. Because α2i TG mice showed decreased insulin sensitivity for glucose transport, we also determined the effects of diet and genotype on protein expression of key insulin signaling molecules. Since use of the entire soleus muscle was necessary to measure glucose transport, we used gastrocnemius muscles for these analyses. The decrease in insulin-stimulated glucose transport in the α2i TG mice was accompanied by reductions in insulin receptor-β subunit, IRS-1, and Akt compared with wild-type mice fed the high-fat diet (Fig. 4). Expression of these molecules was not significantly different between α2i TG and wild-type mice fed the control diet. Therefore, lack of AMPK α2 activity itself does not seem to be a trigger for the downregulation of insulin signaling molecules. Instead, there may be synergic effects of high-fat feeding and a lack of AMPK α2 activity on the expression of these proteins. Interestingly, GLUT4 expression was not different among the four groups (Fig. 4). Although it has been reported that activation of AMPK by 5-aminimidazole-4-carboxamide-1β-4-ribofuranoside (AICAR) increases GLUT4 expression in skeletal muscle (21,22), our results show that ablation of AMPK activity does not affect GLUT4 protein expression.

Muscle lipid and glycogen content. Muscle lipid concentrations are considered to be an important determinant of insulin resistance in skeletal muscle (23). Therefore, we measured muscle triglyceride, diacylglycerol, and ceramide content in tibialis anterior muscles (Fig. 5). Muscle triglyceride concentrations tended to be higher in α2i TG mice compared with wild-type mice on the control diet,
although the difference did not reach statistical significance \((P = 0.09)\). The high-fat diet also tended to increase triglyceride content in wild-type mice compared with the control diet \((P = 0.08)\). There was no additive effect of the high-fat diet and lack of AMPK \(\alpha_2i\) activity on triglyceride content. Muscle diacylglycerol concentrations were not different between wild-type and \(\alpha_2i\) TG mice on the control diet. The high-fat diet increased diacylglycerol content, and this increase was statistically significant only in the \(\alpha_2i\) TG mice. There were no differences in muscle ceramide concentrations among the four groups. Muscle glycogen concentrations are also considered to be an important factor in the regulation of insulin-stimulated glucose transport \((24)\). Muscle glycogen content in the \(\alpha_2i\) TG mice was lower compared with wild-type mice under both the standard and high-fat diet conditions. The high-fat diet did not significantly affect muscle glycogen content in both wild-type and \(\alpha_2i\) TG mice.

**Serum insulin, free fatty acid, and triglyceride concentrations.** As shown in Fig. 6, the high-fat diet significantly increased serum insulin in both the wild-type and \(\alpha_2i\) TG mice, with no difference between genotypes. Both serum free fatty acids and triglycerides tended to decrease with the high-fat diet compared with the control diet in both wild-type and \(\alpha_2i\) TG mice, although these changes did not reach statistical significance.

**Respiratory exchange ratio.** As expected, respiratory exchange ratio was significantly reduced \((P < 0.05)\) with the high-fat diet in both wild-type \((\text{control } 0.86 \pm 0.03; \text{high fat } 0.76 \pm 0.03)\) and \(\alpha_2i\) TG \((\text{control } 0.84 \pm 0.03; \text{high fat } 0.75 \pm 0.01)\) mice. There were no differences of respiratory exchange ratio between genotypes in both control and high-fat diet conditions.

**DISCUSSION**

AMPK has been proposed to be a key regulator of glucose metabolism in skeletal muscle and a therapeutic target for the treatment of type 2 diabetes \((10,25)\). Although direct evidence for a role of AMPK in the long-term regulation of skeletal muscle insulin sensitivity is lacking, there are several lines of evidence to support the concept that AMPK may be important for normal insulin action in this tissue. As examples, acute activation of AMPK is followed...
by an increase in insulin action on glucose transport in skeletal muscle (3, 4, 6, 26, 27). Prior incubation of isolated rat epitrochlearis muscles with the AMPK activator AICAR enhanced insulin-stimulated glucose transport by twofold (4). It was reported that the insulin-sensitizing effects of both AICAR and hyperosmolarity were diminished by inhibition of AMPK activity with compound C in C2C12 myotubes (6). The effects of chronic AMPK activation via long-term AICAR treatment have also been investigated. AICAR treatment of Zucker diabetic fatty rats for 8 weeks improved insulin sensitivity measured by a hyperinsuline-mic-euglycemic clamp, an affect due mainly to increased glucose transport in skeletal muscle (28). In contrast, there was no effect of 1 h of AICAR pretreatment on insulin-stimulated glucose transport in primary human muscle cells (29), and administration of AICAR for 1 week did not increase insulin-stimulated glucose transport in isolated muscles from KKAy-CEPT (30) and db/db (31) mice. The differences between the studies might come from the distinct types of muscles used for each of the investigations, since chronic AICAR administration was shown to improve insulin-stimulated glucose transport in rat skeletal muscle in a fiber-type–specific manner (5). Although current observations are not entirely consistent, studies using AICAR generally suggest that AMPK has a role in insulin sensitivity for glucose transport in skeletal muscle.

AICAR is taken up into skeletal muscle and metabolized to ZMP, an analog of AMP. Therefore, AICAR is not completely specific for AMPK because ZMP regulates other AMP-sensitive enzymes such as fructose-1,6-bisphosphates (32) and muscle glycogen phosphorylase (33). Given the nonspecificity of AICAR to AMPK activation and the inconsistent findings discussed above, it is important to use an animal model in which AMPK activity is eliminated to determine the role of AMPK in insulin sensitivity. In the current study, we showed that ablation of skeletal muscle AMPK α2 activity aggravates the development of whole-body glucose intolerance caused by high-fat feeding. Although we cannot fully rule out the possibility that glucose intolerance in high-fat–fed α2i TG mice was due to defects in insulin secretion or hepatic glucose production, this seems unlikely because the ablation of AMPK α2 activity blunted insulin-stimulated glucose transport measured in isolated muscles from high-fat–fed mice. These results demonstrate that AMPK is a necessary factor in order to maintain normal insulin action in skeletal muscle. Our results also suggest that a lack of muscle AMPK activity may increase the risks of insulin resistance and type 2 diabetes. Since glucose tolerance in α2i TG mice was not significantly different compared with wild-type mice on the control diet, ablation of AMPK α2 activity itself may not be a direct trigger in the development of insulin resistance. Rather, lack of AMPK activity may work as a precipitating factor for the development of insulin resistance. Importantly,

![FIG. 5. Effect of lack of AMPK α2 activity and high-fat diet on muscle lipid and glycogen content. Thirty weeks after the dietary treatments, the mice were fasted overnight. Triglyceride, diacylglycerol, ceramide, and glycogen content were analyzed in extracts from tibialis anterior muscles. Data are represented as means ± SE. n = 5–6 for all groups.](image-url)
AMPK activity in skeletal muscle from patients with type 2 diabetes appears to be intact. Basal activity is similar to matched controls (13,34), and the enzyme can be activated normally by acute exercise (13) and metformin in vivo (13,35) and by AICAR in vitro (36). These reports also suggest that skeletal muscle insulin resistance is unlikely to be initiated by dysfunction of the AMPK trimer.

Other studies have shown that obese insulin-resistant Zucker rats have reduced AMPK activity and related signaling abnormalities in skeletal muscle (37–39). These reports indicate that skeletal muscle AMPK activity can be altered if the obesity and/or insulin resistance is severe, and, in this situation, lower AMPK activity may contribute to the obese and insulin-resistant phenotype (39). Importantly, exercise training can reverse abnormalities of impaired AMPK signaling in obese Zucker rats, which may contribute to the beneficial effect of exercise on improvement of insulin resistance (39). Interestingly, it has also been reported that suppression of AMPK signaling is involved in tumor necrosis factor–induced skeletal muscle insulin resistance (40). Furthermore, the antiobesity effects of ciliary neurotrophic factor have been recently shown to be mediated by AMPK in skeletal muscle, and these effects are not suppressed by diet-induced obesity. Thus, AMPK may be a significant contributing factor to the development of insulin resistance in obesity.

Recently, it has been reported that there are age-associated reductions in AMPK activity in skeletal muscle (41). In this report, acute activation of AMPK α2 by AICAR infusion or exercise observed in young rats (3 months old) was blunted in skeletal muscle of old rats (28 months old). This blunted AMPK α2 activation was associated with reduced mitochondrial biogenesis, suggesting that aging-associated reductions in muscle AMPK activity may be an important contributing factor in the reduced mitochondrial function and resultant insulin resistance (41).

Interestingly, treatment with the antidiabetes agent rosiglitazone for 1 week enhanced AICAR-induced AMPK α2 activation and glucose uptake in insulin-resistant high-fat–fed rats, suggesting that rosiglitazone potentiates AMPK α2 activity (27). Since rosiglitazone acutely activates AMPK (42), chronic or repetitive activation of AMPK, such as exercise training, may potentiating its function/activity. It has been reported that basal AMPK activity is elevated with endurance training in human skeletal muscle (43). Therefore, activation of muscle AMPK by exercise and/or pharmacological stimulation can be an important strategy to improve insulin resistance (44).

Accumulation of muscle triglyceride is recognized as a consistent marker of insulin resistance (45). Interestingly, in our study, muscle triglyceride content tended to increase in the α2i TG mice fed the standard diet, and, in conjunction, insulin-stimulated glucose transport also tended to be lower in the α2i TG mice. One of the major roles of AMPK in skeletal muscle is fatty acid oxidation (8,46). Therefore, it is possible that ablation of AMPK activity inhibited fatty acid oxidation and facilitated accumulation of triglycerides in α2i TG mice. The high-fat diet increased muscle triglyceride content in both wild-type and α2i TG mice. The high-fat diet and AMPK activity ablation, however, did not have an additive effect on triglyceride content. Since insulin-stimulated glucose transport was further impaired by a high-fat diet in the α2i TG mice, triglyceride content is not the factor that exacerbates muscle insulin resistance in this study. Diacylglycerol has been shown to accumulate in the insulin-resistant muscle of obese animal models, including high-fat–fed rats (23). Since the diacylglycerol-sensitive novel protein kinase Cε, ε and/or θ, are associated with reduced insulin action in skeletal muscle (47,48), intracellular accumulation of diacylglycerol may be a factor contributing to insulin resistance. Consistent with previous reports, the high-fat diet used in this study increased diacylglycerol content in both wild-type and α2i TG mice. No difference, however, was observed between wild-type and α2i TG mice, similar to the triglyceride finding. Ceramide, a lipid implicated in reduced insulin signaling (23), did not change in response to the high-fat diet or lack of AMPK activity.

We do not know the exact mechanism by which ablation of AMPK activity exacerbates insulin resistance induced by a high-fat diet. However, the reduced expression of insulin receptor β-subunit, IRS-1, and Akt may be an essential component for the blunted insulin action on glucose transport. Interestingly, high fat feeding and ablation of AMPK activity per se did not alter expression of...
those molecules. In future studies, it will be important to investigate the signaling mechanisms that induce the synergistic effects of combining a high-fat diet and ablation of AMPK.

It has been reported that muscle glycogen content is inversely correlated with insulin-stimulated glucose transport (49–51). However, in the current study, glycogen content in the gastrocnemius muscles was lowered by ablation of AMPK activity in α2i TG mice compared with wild-type mice in both control and high-fat diet (Fig. 4). Consistent with our results, decreased muscle glycogen content was also observed in AMPK α2 knockout (soleus muscle) (52) and AMPK dominant-negative TG mice (gastrocnemius muscle) (53). Therefore, glycogen cannot be a major factor responsible for the impaired insulin-stimulated glucose transport in α2i TG mice (Fig. 2).

The greater impairment of glucose tolerance in the high-fat–fed α2iTG mice compared with wild-type mice did not occur immediately upon commencement of high-fat feeding. This may be explained by the findings that FVB mice have a higher degree of resistance to high-fat diets compared with other mouse strains (19,20). It is possible that the effects of blunted AMPK activity on glucose tolerance, glucose transport, and expression of insulin signaling proteins would have been more pronounced if the animals were on a different background strain.

In summary, the present study shows for the first time that ablation of AMPK α2 activity specifically in skeletal muscle exacerbates the development of glucose intolerance and insulin resistance caused by high-fat feeding. We conclude that AMPK functions in the regulation of insulin-stimulated glucose transport in skeletal muscle. Keeping AMPK activity intact by regular exercise or using an AMPK activator such as rosiglitazone or metformin may be important in the prevention and treatment of skeletal muscle insulin resistance.

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