AMPK activity and isoform protein expression are similar in muscle of obese subjects with and without type 2 diabetes

Kurt Højlund,2,* Kirsty J. Mustard,3,* Peter Stehr,2 D. Grahame Hardie,3 Henning Beck-Nielsen,2 Erik A. Richter,1 and Jørgen F. P. Wojtaszewski1

1Department of Human Physiology, Copenhagen Muscle Research Centre, Institute of Exercise and Sport Sciences, University of Copenhagen, DK-2100 Copenhagen; 2Diabetes Research Centre, University of Southern Denmark and Department of Endocrinology, Odense University Hospital, DK-5000 Odense, Denmark; and 3Division of Molecular Physiology, School of Life Sciences, Wellcome Trust Biocentre, Dundee University, Dundee, DD1 5EH Scotland, United Kingdom

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Højlund, Kurt, Kirsty J. Mustard, Peter Stehr, D. Grahame Hardie, Henning Beck-Nielsen, Erik A. Richter, and Jørgen F. P. Wojtaszewski. AMPK activity and isoform protein expression are similar in muscle of obese subjects with and without type 2 diabetes. Am J Physiol Endocrinol Metab 286: E239–E244, 2004. First published October 7, 2003; 10.1152/ajpendo.00326.2003.—Acute or chronic activation of AMP-activated protein kinase (AMPK) increases insulin sensitivity. Conversely, reduced expression and/or function of AMPK might play a role in insulin resistance in type 2 diabetes. Thus protein expression of the seven subunit isoforms of AMPK and activities and/or phosphorylation of AMPK and acetyl-CoA carboxylase-β (ACCβ) was measured in skeletal muscle from obese type 2 diabetic and well-matched control subjects during euglycemic-hyperinsulinemic clamps. Protein expression of all AMPK subunit isoforms (α1, α2, β1, β2, γ1, γ2, and γ3) in muscle of obese type 2 diabetic subjects was similar to that of control subjects. In addition, α1- and α2-associated activities of AMPK, phosphorylation of α-AMPK subunits at Thr172, and phosphorylation of ACCβ at Ser221 showed no difference between the two groups and were not regulated by physiological concentrations of insulin. These data suggest that impaired insulin action on glycogen synthesis and lipid oxidation in skeletal muscle of obese type 2 diabetic subjects is unlikely to involve changes in AMPK activity and expression. AMPK is an αβγ heterotrimer that is activated by low cellular energy status, such as decreases in both the ATP/AMP ratio and the phosphocreatine content (16, 17, 23). Several isoforms of both the catalytic (α1, α2) and the two regulatory subunits (β1, β2, γ1, γ2, and γ3) have been identified in mammalian cells (7, 33, 34). Protein expression of AMPK subunit isoforms is changed in response to exercise training (8, 25, 30), which might contribute to the metabolic alterations induced by exercise training, e.g., enhanced peripheral insulin sensitivity. Mutations in γ2- and γ3-subunit isoforms cause glycogen storage disease in human heart (1) and glycogen accumulation in pig muscle (27), respectively, implying that these subunits have a role in glycogen synthesis. In addition, the whole body α2-deficient mouse is insulin resistant, possibly due to enhanced sympathetic nervous activity (35). Thus evidence exists to suggest that altered AMPK activity caused by changes in expression or function of different AMPK subunit isoforms might modulate the metabolic profile of specific tissues and might influence whole body metabolism.

In a recent study of the AMPK system in muscle of nonobese type 2 diabetic subjects (28), exercise-induced α2-associated AMPK activity and protein expression of the α1-, α2-, and β1-subunits were found to be normal. Furthermore, metformin treatment was reported to increase basal α2-associated AMPK activity in muscle of nonobese type 2 diabetic subjects (29). These findings argue against functional defects of the AMPK system in diabetic muscle, at least in the fasting and exercised states. However, we (18) have recently demonstrated that the mechanism responsible for impaired insulin-induced nonoxidative glucose metabolism and impaired activation of glycogen synthase in muscle of obese subjects with type 2 diabetes is likely to involve a hyperphosphorylation of glycogen synthase at the NH2-terminal sites Ser2 and Ser6 (18). AICAR treatment of isolated skeletal muscles and human myoblasts in vitro leads to inactivation of glycogen synthase (2, 14, 39), most likely induced by AMPK-mediated phosphorylation of glycogen synthase at Ser6 (6), which primes the protein for subsequent phosphorylation at Ser6 (8) by casein kinase I (10, 11). Thus the possibility exists that AMPK is involved in the hyperphosphorylation of glycogen synthase that occurs during insulin stimulation in obese type 2 diabetic subjects.

Another consistent metabolic feature of insulin resistance in type 2 diabetes is the failure of insulin to suppress lipid

5'-AMP-activated protein kinase (AMPK) is a signal intermediate in the metabolic regulation in mammalian cells. In skeletal muscle, AMPK has been implicated in the regulation of lipid oxidation, glucose transport, and glycogen synthase activity and, in liver, AMPK activation might lead to inhibition of gluconeogenesis, glycolysis, lipogenesis, and cholesterol formation (16, 17, 23, 36). On this background, AMPK has been suggested to play a role in the pathogenesis of type 2 diabetes, as well as to be a potential target for drug treatment of type 2 diabetes. Support for this concept comes from studies showing that acute and chronic treatment of insulin-resistant rodent models with the AMPK-activating agent 5-aminimidazole-4-carboxamide ribofuranoside (AICAR) improves glucose homeostasis and insulin sensitivity (5, 9, 15, 21, 32).
oxidation in skeletal muscle (22, 26). Although not consistent
(4), an inhibitory effect of insulin on AMPK activity has been
reported in hepatoma cells and in rat cardiac and skeletal
muscle (3, 13, 37, 38), and this appears to involve decreased
phosphorylation of Thr172 in α-AMPK (3). This effect has
been proposed to mediate activation of acetyl-CoA carboxylase
and, hence, suppression of lipid oxidation by insulin (13, 38).
Thus, despite normal activity of AMPK in the fasting and exercised
states in muscle of nonobese type 2 diabetic subjects (28), there
are data that could indicate that changes in the expression or
function of AMPK subunit isoforms other than α1, α2, and β1
could be involved in the failure of insulin to activate glycogen
synthase and to suppress lipid oxidation in the muscle of type
2 diabetic subjects.

To explore these possibilities, we measured basal AMPK
subunit expression of all of the seven recognized subunit
isoforms (α1, α2, β1, β2, γ1, γ2, and γ3) and the effect of
insulin on AMPK activity in resting skeletal muscle of obese
type 2 diabetic subjects and well-matched healthy control
subjects.

MATERIALS AND METHODS

Study subjects. Ten obese subjects with type 2 diabetes and 10
healthy, obese control male subjects, matched according to age
(51.1 ± 2.0 vs. 50.4 ± 1.6 yr) and body mass index (29.6 ± 0.9 vs.
31.1 ± 1.1 kg/m²), participated in the study (18). Subjects with type
2 diabetes were treated either by diet alone or by diet in combination
with sulfonylurea or metformin, which was withdrawn 1 wk before
the study. The patients were all negative for antibodies to glutamic
acid decarboxylase 65 (GAD65) and without signs of diabetic reti-
nopathy, nephropathy, neuropathy, or macrovascular complications.
The control subjects had normal glucose tolerance and no family
history of diabetes. All subjects had normal results on screening blood
tests of hepatic and renal function. All subjects were instructed to
refrain from strenuous physical activity for a period of 48 h before the
experiment. Informed consent was obtained from all subjects before
participation. The study was approved by the local ethics committee
and was performed in accord with the Helsinki Declaration.

Study design. All study subjects were admitted to the Diabetes
Research Centre at Odense University Hospital (Odense, Denmark).
After an overnight fast, all subjects underwent a euglycemic-hyper-
insulinemic clamp (4 h of insulin infusion, 40 μU·min⁻¹·kg⁻¹), as
described in detail previously (20). In type 2 diabetic subjects, plasma
glucose was allowed to decline to ~5.5 mmol/l before glucose
infusion was initiated. Total glucose disposal rates (GDR) were
described in detail previously (20). In type 2 diabetic subjects, plasma
fatty acid (FFA) concentrations were obtained in both groups. In
this latter case, a “pan-”γ antibody was generated in sheep to the peptide
CRAAPLWDSKKQSFVG (residues 69–83 of rat γ1, a sequence
highly conserved in human γ2 and γ3), coupled to keyhole limpet
hemocyanin, and affinity purified as described previously (41).

Calculations and statistical analysis. Control samples were added
to all activity assays and loaded on all gels in duplicates, and
assay-to-assay variation was accounted for by expressing data relative
to these samples. Data calculation and statistical analysis were
performed using the SigmaStat for MS Windows version 2.0 software.
Data are presented as means ± SE. Two-way ANOVA analysis for
repeated measures and Student’s t-test for unpaired data were used
as appropriate to detect any significant differences. Significance was
accepted at the \( P < 0.05 \) level.

RESULTS

Metabolic characteristics. The metabolic profile of the subjects
and glucose metabolism during basal conditions as well as during the clamp
were recently published in a paper describing the phosphorylation profile of muscle glycogen
synthase (18). Briefly, fasting levels of glycosylated hemoglo-
bin, plasma glucose, serum insulin, and C-peptide were signifi-
cantly higher in the diabetic group, whereas fasting levels of
serum FFA did not differ between the groups (18). During the
insulin-stimulated steady-state period (the 210- to 240-min
period), euglycemia at a plasma glucose concentration of ~5.5
mmol/l and physiological hyperinsulinemia at a serum insulin
concentration of ~400 pmol/l were obtained in both groups.
In this period, serum FFA concentrations were significantly
higher in the diabetic group vs. the control group (18). In
the basal state, GDR were significantly higher, whereas in the
insulin-stimulated state GDR were significantly lower in the
diabetic group compared with the control group (Table 1).

Indirect calorimetry data showed no significant differences in
glucose oxidation, nonoxidative glucose metabolism (glucose storage), or lipid oxidation in the basal state between the groups. During the insulin-stimulated state, both glucose oxidation and glucose storage were significantly lower, whereas lipid oxidation was significantly higher in type 2 diabetic subjects compared with control subjects (Table 1). The reduction in insulin-stimulated GDR in type 2 diabetic subjects was primarily accounted for by impaired glucose storage (75%).

AMPK activity and α-AMPK Thr\(^{72}\) and ACC\(β\) Ser\(^{221}\) phosphorylation. AMPK activity was measured in an isoform-specific assay after immunoprecipitation of either of the two α-isofoms. Neither α1- nor α2-associated activities displayed any regulation with insulin, and both were similar in magnitude between control and type 2 diabetic subjects (Fig. 1). In support of these in vitro measured activities, α-Thr\(^{72}\) phosphorylation was similar and not affected by insulin in either of the two groups (Fig. 2). Phosphorylation of ACC\(β\) on Ser\(^{221}\) has previously been used as an indicator of endogenous AMPK activity. As judged by Western blotting, the ACC\(β\) phosphorylation was also similar at rest and not affected by insulin in the two groups (Fig. 2). Several observations indicate that these findings are based on reliable assay conditions and usable muscle lysate samples. First, the absolute AMPK activities measured fell within the linear range of the assay and were similar to the levels previously reported by us and others using immunopurified AMPK from human muscle. Second, although AMPK activity or phosphorylation was not affected by insulin, the activity/phosphorylation of several other enzymes (measured in the same lysate) was regulated by insulin (18). Finally, the expected increase in AMPK activity/phosphorylation and ACC\(β\) phosphorylation was observed in control samples (added to all assays), representing muscle from the resting and contracted conditions, respectively.

AMPK subunit protein expression. We measured the protein content of all known AMPK subunit isoforms expressed in human muscle α1, α2, β1, β2, γ1, γ2, and γ3. In muscle biopsies from the basal state, all seven subunit isoforms were expressed to similar levels in the two groups (Fig. 3).

### Table 1. Euglycemic hyperinsulinemic clamp data

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control Subjects</th>
<th>Diabetic Subjects</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total glucose disposal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>2.45±0.08</td>
<td>2.92±0.10</td>
<td>0.001</td>
</tr>
<tr>
<td>Clamp</td>
<td>9.80±0.94\†</td>
<td>5.97±0.88\†</td>
<td>0.001</td>
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<tr>
<td>Glucose oxidation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1.49±0.11</td>
<td>1.71±0.29</td>
<td>NS</td>
</tr>
<tr>
<td>Clamp</td>
<td>3.53±0.21\†</td>
<td>2.57±0.29*</td>
<td>0.007</td>
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<tr>
<td>Lipid oxidation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1.62±0.04</td>
<td>1.66±0.16</td>
<td>NS</td>
</tr>
<tr>
<td>Clamp</td>
<td>0.76±0.07\†</td>
<td>1.21±0.11*</td>
<td>0.004</td>
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<tr>
<td>Nonoxidative glucose metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.93±0.12</td>
<td>1.20±0.26</td>
<td>NS</td>
</tr>
<tr>
<td>Clamp</td>
<td>6.68±0.79\†</td>
<td>3.89±0.78*</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Characteristics of type 2 diabetic and control subjects during basal steady-state period (Basal) and insulin-stimulated steady state (Clamp). Data represent means \(±\) SE expressed in mg·min\(^{-1}\)·kg FFM\(^{-1}\). Data for glucose and lipid oxidation and nonoxidative glucose metabolism are based on indirect calorimetry on 8 control and 9 diabetic subjects. NS, not significant. \(*P < 0.05\) and \(†P < 0.01\) vs. basal.

### DISCUSSION

Because of the apparent influence of AMPK on a variety of metabolic processes dysregulated in type 2 diabetic subjects, AMPK has been hypothesized to play a role in the pathogenesis of insulin resistance in patients with type 2 diabetes (16, 17, 36). In the present study, we found that muscle from obese type 2 diabetic subjects had a level of protein expression of all seven AMPK subunit isoforms that was similar to that of healthy but obese control subjects. We also observed similar AMPK activities associated with both the α1- and α2-catalytic isoforms, a similar degree of phosphorylation of Thr\(^{72}\) in the α-AMPK isoforms, and a similar phosphorylation of the AMPK target site on ACC\(β\) (Ser\(^{221}\)). This suggests that AMPK function is intact in muscle of obese type 2 diabetic subjects, at least in the fasted and insulin-stimulated states. These observations extend those previously reported in nonobese exercise-tolerant type 2 diabetic subjects (28).

The present findings, and the observation that nonobese type 2 diabetic subjects displayed normal AMPK activation during exercise (28) and showed increased AMPK activity in response to metformin treatment (29), argue against a functional defect in AMPK and suggest that pharmacological activation of the AMPK system is a feasible and attractive treatment of insulin resistance in type 2 diabetes. The finding that ACC\(β\) Ser\(^{221}\) is phosphorylated to a similar extent in muscle of type 2 diabetic subjects compared with control subjects (28), suggests that AMPK function is intact in muscle of obese type 2 diabetic subjects, at least in the fasted and insulin-stimulated states. These observations extend those previously reported in nonobese exercise-tolerant type 2 diabetic subjects (28).
and healthy control subjects in both the basal and insulin-stimulated states suggests that any regulation of lipid oxidation by AMPK (via ACCβ) might be intact and not regulated by insulin. However, studies of human muscle strips in vitro showed normal regulation of AMPK and ACCβ phosphorylation in response to AICAR treatment (24); however, despite this, AICAR-induced glucose transport was impaired, leading to the conclusion that downstream defects might still compromise actions of AMPK.

From a range of studies, it seems that altered AMPK activity might modify the metabolic profile of diabetic animals and might change insulin sensitivity of skeletal muscle (5, 9, 15, 21, 32). The data from our study and others of human skeletal muscle in vivo (28) indicate that type 2 diabetes per se is not associated with changes in AMPK expression or activity, but we cannot exclude the possibility that obesity-related insulin resistance might be associated with changes in AMPK expression. In addition, neither of these studies can exclude a role of altered AMPK function in other tissues in the pathogenesis of type 2 diabetes. From the present data, showing similar activity and expression of AMPK in muscle of type 2 diabetic and control subjects despite different insulin sensitivity, it appears that there is no simple relationship between these variables. This is in line with recent observations showing that lack of expression of the α2-AMPK isoform in knockout mice does not cause alterations in insulin sensitivity of isolated muscle, but rather causes insulin resistance due to central mechanisms (35).

We have recently published a subset of data from the present study showing that glycogen synthase in muscle from type 2 diabetic subjects becomes hyperphosphorylated on Ser7 and Ser10 in response to insulin stimulation and suggesting that this abnormality is involved in the impaired insulin activation of glycogen synthase found in this group of patients (18). Both biochemical and physiological studies in vitro suggest that AMPK is a glycogen synthase kinase, phosphorylating Ser7 at the NH2 terminus, which decreases glycogen synthase activity (2, 6, 14, 39). In cultured skeletal muscle cells, glucose deprivation has been shown to stimulate both of the catalytic subunit isoforms of AMPK, and this was associated with a significant decrease in the fractional velocity of glycogen synthase activity.

![Fig. 2.](image1.png)

**Fig. 2.** The Thr172 phosphorylation on α-AMPK (A) and Ser221 phosphorylation of acetyl-CoA carboxylase-β (ACCβ, B) were measured by Western blotting. Data are given as arbitrary scanning units. Open bars, basal conditions; solid bars, data obtained at end of steady-state period of euglycemic hyperinsulinemic clamp. Insets: representative immunoblots. Bas and Ins, basal and insulin-treated conditions. Specific bands were observed at 63 kDa for Thr172 phosphorylated α-AMPK and at ~260 kDa for Ser221 phosphorylated ACCβ. All data are expressed as means ± SE (n = 10).

![Fig. 3.](image2.png)

**Fig. 3.** Protein expression of AMPK subunit isoforms measured by Western blotting by use of muscle lysates from biopsies obtained during basal conditions. Data (means ± SE) are given as arbitrary scanning units (n = 10). The protein level in type 2 diabetic subjects was normalized to the level observed in the control subjects. Open bars, control subjects; solid bars, type 2 diabetic subjects. Specific bands were observed at (kDa) 63- α1, 63- α2, 34- β1, 30- β2, 35- γ1, 63- γ2, and 55- γ3.
MUSCLE AMPK EXPRESSION IN TYPE 2 DIABETIC SUBJECTS

(14). A regulatory role of AMPK in vivo is suggested from findings in patients with glycogen phosphorylase deficiency (McArdle’s disease) in whom the exercise-induced AMPK activation is correlated with a decreased glycogen synthase activity (31). However, the present data suggest that AMPK is not the kinase leading to the dysregulation of glycogen synthase, because AMPK activity in muscle of type 2 diabetic subjects is not increased in the basal state or in response to insulin.

In summary, the present data suggest that changes in AMPK activity or protein expression of AMPK subunit isoforms are not present in muscle of obese type 2 diabetic subjects compared with healthy obese controls. Therefore, AMPK does not appear to be a major contributor to the altered metabolic profile in type 2 diabetes. Importantly, the data also demonstrate that AMPK activity in resting human skeletal muscle is not regulated by physiological concentrations of insulin. Thus failure of insulin to activate glycogen synthesis and to suppress lipid oxidation in muscle of type 2 diabetic subjects is unlikely to involve changes in expression or activity of AMPK.

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