Skeletal Muscle AMP-activated Protein Kinase Is Essential for the Metabolic Response to Exercise in Vivo

AMP-activated protein kinase (AMPK) has been postulated as a super-metabolic regulator, thought to exert numerous effects on skeletal muscle function, metabolism, and enzymatic signaling. Despite these assertions, little is known regarding the direct role(s) of AMPK in vivo, and results obtained in vitro or in situ are conflicting. Using a chronically catheterized mouse model (carotid artery and jugular vein), we show that AMPK regulates skeletal muscle metabolism in vivo at several levels, with the result that a deficit in AMPK activity markedly impairs exercise tolerance. Compared with wild-type littermates at the same relative exercise capacity, vascular glucose delivery and skeletal muscle glucose uptake were impaired; skeletal muscle ATP degradation was accelerated, and arterial lactate concentrations and in vivo muscle glucose uptake were impaired; skeletal muscle ATP degradation was accelerated, and arterial lactate concentrations were increased in mice expressing a kinase-dead AMPKα2 subunit (α2-KD) in skeletal muscle. Nitric-oxide synthase (NOS) activity was significantly impaired at rest and in response to exercise in α2-KD mice; expression of neuronal NOS (NOSμ) was also reduced. Moreover, complex I and IV activities of the electron transport chain were impaired 32 ± 8 and 50 ± 7%, respectively, in skeletal muscle of α2-KD mice (p < 0.05 versus wild type), indicative of impaired mitochondrial function. Thus, AMPK regulates neuronal NOS expression, NOS activity, and mitochondrial function in skeletal muscle. In addition, these results clarify the role of AMPK in the control of muscle glucose uptake during exercise. Collectively, these findings demonstrate that AMPK is central to substrate metabolism in vivo, which has important implications for exercise tolerance in health and certain disease states characterized by impaired AMPK activation in skeletal muscle.

The ubiquitously expressed serine/threonine AMP-activated protein kinase (AMPK) is an αβγ heterotrimer postulated to play a key role in the response to energetic stress (1, 2), because of its sensitivity to increased cellular AMP levels (3). Pharmacological activation of AMPK (primarily via the AMP analogue ZMP) increases catabolic processes such as GLUT4 translocation (4, 5), glucose uptake (6, 7), long chain fatty acid (LCFA) uptake (8), and substrate oxidation (6). Concomitantly, pharmacological activation of AMPK inhibits anabolic processes, and in skeletal muscle genetic reduction of the catalytic AMPKα2 subunit eliminates these pharmacological effects (9–12). Thus, AMPK has been proposed to act as a metabolic master switch (2, 13, 14). Physiologically, exercise at intensities sufficient to increase free cytosolic AMP (AMPfree) levels is a potent stimulus of AMPK, preferentially activating AMPKα2 in skeletal muscle (15–17). The metabolic profile of skeletal muscle during moderate to high intensity exercise is remarkably similar to skeletal muscle in which AMPK has been pharmacologically activated (i.e. increases in catabolic processes). This is consistent with the hypothesis that AMPK activation is required for the metabolic response to increased cellular stress. Given this, it is surprising that the direct role(s) of skeletal muscle AMPK during exercise under physiological in vivo conditions is unknown.

A number of studies have tried to attribute causality to the AMPK and metabolic responses to exercise using transgenic models. In mouse models in which AMPKα2 protein expression and/or activity has been impaired, contractions performed in isolated skeletal muscle in vitro, ex vivo, or in situ have demonstrated that skeletal muscle glucose uptake (MGU) is normal (9, 10), partially impaired (11, 18), or ablated (19). Furthermore, ex vivo skeletal muscle LCFA uptake and oxidation in response to contraction appears to be AMPK-independent (20, 21). A key limitation of these studies is that the experimental models were not physiological. Under in vivo conditions, mice expressing a kinase-dead (18) or inactive (22) AMPKα2 subunit in cardiac and skeletal muscle have impaired voluntary and maximal physical activity, respectively, indicative of a physiological role for AMPK during exercise. In this context, obese non-diabetic and diabetic individuals have impaired skeletal muscle AMPK activation during moderate intensity exercise (23) as well as during the post-exercise period (24), yet the contribution of this impairment to the disease state is unclear. Thus, in vivo studies...
Physiological Role of AMPK in Skeletal Muscle

are essential to define the role of AMPK in skeletal muscle during exercise.

Physical exercise of a moderate intensity is an effective adjunct treatment for chronic metabolic diseases such as obesity and type 2 diabetes (25). Given the importance of elucidating the molecular mechanism(s) regulating skeletal muscle substrate metabolism during exercise and the putative role of AMPK as a critical mediator in this process, we tested the hypothesis that AMPKα2 is functionally linked to substrate metabolism in vivo.

EXPERIMENTAL PROCEDURES

Animal Maintenance—All procedures were approved by the Vanderbilt University Animal Care and Use Committee. Male and female C57BL/6J mice expressing a kinase-dead AMPKα2 subunit (α2-KD) in cardiac and skeletal muscle (18) and wild-type (WT) littermate mice were studied. Twenty one days after birth, litters were separated by gender, maintained in microisolator cages, fed a standard chow diet (5.5% fat by weight; 5001 Laboratory Rodent Diet, Purina), and had access to water ad libitum. All mice were studied at 16 weeks of age.

Exercise Stress Test—Peak oxygen consumption (VO$_{2}$peak) was assessed using an exercise stress test protocol. Two days prior to the exercise stress test, all mice were acclimatized to treadmill running by performing 10 min of exercise at a speed of 10 m.min$^{-1}$ (0% incline). To determine VO$_{2}$peak, mice were placed in an enclosed single lane treadmill connected to Oxymax oxygen (O$_2$) and carbon dioxide (CO$_2$) sensors (Columbus Instruments, Columbus, OH). Following a 30-min basal period, mice commenced running at 10 m.min$^{-1}$ on a 0% incline. Running speed was increased by 4 m.min$^{-1}$ every 3 min until mice reached exhaustion, defined as the time point whereby mice remained at the back of the treadmill on a shock grid for >5 s. O$_2$ consumption and CO$_2$ production were assessed at 30-s intervals throughout the basal and exercise periods. Basal values are representative of the final 10 min of the basal period. Prior to the VO$_{2}$peak test, body weight was measured, and body composition was assessed using an mq10 NMR analyzer (Bruker Optics, The Woodlands, TX). Given that changes in whole body VO$_2$ during exercise closely reflect changes occurring within exercising muscle (26), all oxygen consumption measurements were expressed per kg of lean body mass (kgLBM).

Metabolic Experiments—Following the exercise stress test, surgical procedures were performed as described previously (27) to catheterize the left common carotid artery and right jugular vein for sampling and infusions, respectively. The catheters were exteriorized, sealed with stainless steel plugs, and kept patent with saline containing 200 units.ml$^{-1}$ heparin and 5 mg.ml$^{-1}$ ampicillin. Mice were housed individually post-surgery, and body weight was recorded daily. Five days following surgery, all mice performed a 10-min bout of exercise at their pre-determined experimental running speed (see below). Experiments were performed 2 days later.

Approximately 1 h prior to the experiment, Micro-Renathane tubing was connected to the exteriorized catheters, and all mice were placed in the enclosed treadmill to acclimate to the environment. At $t = 0$ min, a base-line arterial blood sample was taken for the measurement of arterial glucose, plasma insulin, plasma nonesterified fatty acids (NEFA), plasma lactate, and hematocrit. Mice then remained sedentary or performed a single bout of exercise. Sedentary mice were allowed to move freely in the stationary treadmill for 30 min. Mice that exercised were divided into three groups as follows: 1) α2-KD mice performed a maximum of 30 min of treadmill exercise at 70% of their maximum running speed; 2) WT mice ran at the same absolute running speed as α2-KD mice; 3) WT mice ran at the same relative intensity as α2-KD mice. Running time was matched between groups.

In all mice, a bolus containing 13 μCi of 2-[14C]deoxyglucose (2-[14C]DG) and 26 μCi of [9,10-3H]-[R]-2-bromopalmitate (3H-R-BrP) was injected into the jugular vein at $t = 5$ min to provide an index of tissue-specific glucose and LCFA uptake and clearance, respectively. At $t = 7, 10, 15, and 20$ min, arterial blood was sampled to determine blood glucose, plasma NEFA, plasma lactate, and plasma 2-[14C]DG and 3H-R-BrP. Hematocrit was measured at $t = 20$ min, and at $t = 30$ min or exhaustion, arterial blood was taken for the measurement of blood glucose, plasma insulin, plasma NEFA, plasma lactate, plasma 2-[14C]DG, and 3H-R-BrP. Following the final arterial blood sample, 50 μl of yellow DYE-TRAK® microspheres (15 μm; Triton Technology Inc., San Diego) were injected into the carotid artery, followed by a small flush of saline, to assess the percentage of cardiac output to gastrocnemius (%QG) and the left and right kidney. Mice were then anesthetized with an arterial infusion of sodium pentobarbital (3 mg). The soleus, right gastrocnemius, superficial vastus lateralis (SVL), heart, and brain were rapidly excised, frozen in liquid nitrogen, and stored at $-70^\circ$C. The left gastrocnemius and left and right kidney were placed into 15-ml polypropylene tubes and stored at 4°C prior to microsphere analysis.

Echocardiography—Transthoracic echocardiograms were performed as described previously (28). Mice were acclimated to the procedure over 3 days. Immediately following treadmill exercise, two-dimensional targeted M-mode echocardiographic images were obtained at the level of the papillary muscles from the parasternal short axis view and recorded at a speed of 150 cm/s for the measurement of heart rate. Echocardiograms were completed within 72 ± 13 s after exercise. Left ventricular wall thickness, end diastolic measurements, and left ventricular end systolic dimensions were determined as described previously (28) and are the average of three to five consecutive selective sinus beats using the leading edge technique. Heart rate was determined from the cardiac cycles recorded on the M-mode tracing.

Plasma and Tissue Radioactivity—Plasma 2-[14C]DG radioactivity was assessed by liquid scintillation counting following deproteinization with 0.3 N Ba(OH)$_2$ and 0.3 N ZnSO$_4$ as described previously (29). Plasma 3H-R-BrP radioactivity was determined directly from the plasma via liquid scintillation counting. Tissue 2-[14C]DG and 3H-R-BrP were determined using a modified method of Folch et al. (30). Chloroform:methanol (2:1) was added to a portion of tissue that had been crushed in liquid nitrogen using a mortar and pestle, homogenized on ice, and stored at 4°C for 60 min. KCl (0.1 M) was then added to the homogenate, and samples were centrifuged at 3500 × g for
AMPK and NOS Activity Assays—AMPKa2 and \( \alpha \)-1 were sequentially immunoprecipitated using 200 \( \mu \)g of protein, 2 \( \mu \)g of a rabbit AMPKa2 polyclonal antibody (Abcam), 2 \( \mu \)l of a rabbit AMPKa1 monoclonal antibody (Abcam), and immobilized Recomb protein A beads (Pierce). AMPK activity in the immune complexes was measured for 24 min at 30 °C (within the pre-determined linear range) in the presence of 200 \( \mu \)M AMP and calculated as picomoles of phosphate incorporated into the SAMSpeptide (100 \( \mu \)M; GenWay Biotech) per min per mg of protein subjected to immunoprecipitation.

NOS activity was measured on gastrocnemius and SVL muscle. Samples were homogenized in lysis buffer, and 5 \( \mu \)l of sample (~70 \( \mu \)g of protein) was added to pre-heated assay buffer (1.15 mM NADPH, 4 \( \mu \)M BH4, 100 mM calmodulin, 0.7 mM CaCl, 0.63 \( \mu \)M FAD, 3 \( \mu \)M L-[\(^{3}H\)]arginine). The assay was performed for 7 min at 37 °C (within the linear range), and NOS activity was measured with or without the NOS inhibitor \( N^{\omega} \)-nitro-L-arginine methyl ester (1 mM). NOS activity is the difference between samples incubated with or without \( N^{\omega} \)-nitro-L-arginine methyl ester and was calculated as picomoles of L-[\(^{3}H\)]arginine converted to picomoles of L-[\(^{3}H\)]citrulline per min per mg of protein.

OXPHOS Activity Assays—Post-600 \( \times \) g supernatants of gastrocnemius muscle were prepared as described previously (35). Briefly, frozen samples were homogenized in 120 mM KCl, 20 mM HEPES (pH 7.4), 2 mM MgCl, 1 mM EGTA, and 5 mg/ml bovine serum albumin and centrifuged twice at 600 \( \times \) g for 10 min at 4°C. The second supernatant was stored in 2 \( \mu \)g/ml aliquots at −70 °C. All assays were performed at 30 °C in a final volume of 1 ml using a SpectraMax Plus 384 spectrophotometer (Molecular Devices). Prior to measurement of complex I, I + II, and II + III activity, samples were diluted 1:1 in hypotonic media (final concentration of 25 mM potassium phosphate (pH 7.2), 5 mM MgCl) and freeze-thawed three times.

Complex I activity (NADH:ubiquinone oxidoreductase; EC 1.6.5.3) was measured by following the decrease in absorbance due to the oxidation of NADH at 340 nm, with 425 nm as the reference wavelength (\( \epsilon = 6.81 \text{ mM}^{-1} \text{cm}^{-1} \)) (35). The reaction was initiated by adding 30 \( \mu \)g of protein to the assay buffer (25 mM potassium phosphate (pH 7.2), 5 mM MgCl, 2 mM KCN, 2.5 mg/ml bovine serum albumin (fraction V), 130 mM NADH, 65 \( \mu \)M decylubiquinone, 2 \( \mu \)g/ml antimycin A) and monitored for 5 min. Rotenone (2 \( \mu \)g/ml) was added, and the reaction was monitored for 3 min. Complex I activity is the difference between total enzymatic rates and rates obtained in the presence of rotenone. Complex I + II (NADH-cytochrome c oxidoreductase) activity was determined as described previously (36) with minor modifications. The reaction was initiated by adding 30 \( \mu \)g of protein to the assay buffer (50 mM potassium phosphate (pH 7.2), 80 \( \mu \)M cytochrome c (bovine heart), 130 mM NADH, 2 mM KCN, 5 mM MgCl). The increase in absorbance due to the reduction of ferriyochrome c (\( \epsilon = 19 \text{ mM}^{-1} \text{cm}^{-1} \)) was monitored for 3 min at 550 nm with 580 nm as the reference wavelength. Rotenone (2 \( \mu \)g/ml) was added, and the reaction was monitored for a further 3 min. Complex I + II activity is the rotenone-sensitive rate.

Complex II activity (succinate:ubiquinone oxidoreductase; EC 1.3.5.1) was measured by following the reduction of 2,6-

15 min. The upper aqueous phase (containing 2-[\(^{14}\)C]DG) was used to determine 2-[\(^{14}\)C]DG-P as described previously (29). A portion of the lower lipid phase (containing \(^{3}\)H-R-BrP) was used to determine tissue \(^{3}\)H-R-BrP content (31).

**Plasma Hormones and Metabolites**—Immunoreactive plasma insulin was assayed with a double antibody method (32), and plasma NEFA were measured spectrophotometrically using an enzymatic colorimetric assay (NEFA C kit, Wako Chemicals Inc.). Plasma lactate was determined enzymatically (33). Glucose units were determined with 0.667M NaOH. Glucose levels were determined directly from ~5 \( \mu \)l of arterial blood samples using an ACCU-CHEK® Advantage monitor (Roche Diagnostics).

**Muscle Metabolites**—For muscle glycogen determination, 2 mM HCl was added to a portion (~10 mg) of crushed tissue samples, which were then incubated at 100 °C for 2 h and neutralized with 0.667 mM NaOH. Glucose units were determined using an enzymatic fluorometric method (33). Muscle lactate, P Cr, Cr, and ATP were analyzed from ~20 mg of crushed tissue using enzymatic fluorometric techniques (33). ADP\(_{\text{free}}\) and AMP\(_{\text{free}}\) were calculated as described previously (34).

**Microsphere Isolation**—Tissues were digested overnight in 1 M KOH at 60 °C. Following sonication with Triton X-100, microspheres were suspended in ethanol containing 0.2% (v/v) HCl, followed by ethanol. The microsphere:ethanol solution was evaporated at room temperature, and 200 \( \mu \)l of N,N-dimethylformamide (Sigma) was added to elute the fluorescent dye from the microspheres. The absorbance of the N,N-dimethylformamide solution was determined at 450 nm.

**Immunoblotting**—Muscle samples were homogenized in lysis buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 \( \mu \)g/ml trypsin inhibitor, 5 \( \mu \)g/ml protease inhibitor mixture, 50 mM NaF, and 5 mM sodium pyrophosphate). Samples were centrifuged at 10,000 \( \times \) g, and protein content in the supernatant was determined using the Bradford method. Protein expression of AMPK\(_{\text{a1}}\) and \( \alpha \)-2, acetyl-CoA carboxylase-\( \beta \), neuronal (n) nitric-oxide synthase (NOS), and endothelial (e) NOS was determined from 75 \( \mu \)g of whole cell lysate. Inducible NOS was immunoprecipitated using 200 \( \mu \)g of protein in conjunction with immobilized Recomb protein A beads (Pierce) and an anti-inducible NOS mouse monoclonal antibody (BD Biosciences). Proteins were separated using NuPAGE 4–12% BisTris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. Blots were probed with anti-AMPK\(_{\text{a1}}\) rabbit monoclonal antibody (1:500; Abcam, Cambridge, MA), anti-AMPK\(_{\text{a2}}\) goat polyclonal antibody (1:100; Santa Cruz Biotechnology), anti-\( \alpha \)-NOS mouse monoclonal antibody (1:500; BD Biosciences), anti-eNOS rabbit polyclonal antibody (1:100; Abcam, MA), and anti-inducible NOS mouse monoclonal antibody (1:100; BD Biosciences). Antibody binding was detected with either IRDye\(_{\text{TM}}\) 800-conjugated anti-rabbit IgG (1:10,000), IRDye\(_{\text{TM}}\) 700-conjugated anti-mouse IgG (1:10,000), or IRDye\(_{\text{TM}}\) 800-conjugated antigoat IgG secondary antibodies (Rockland Immunochemicals, Inc., Gilbertsville, PA). Acetyl-CoA carboxylase-\( \beta \) protein expression was detected using IRDye\(_{\text{TM}}\) 800-labeled streptavidin (1:5,000; Rockland).
Physiological Role of AMPK in Skeletal Muscle

dichlorophenolindophenol at 600 nm with 750 nm as the reference wavelength ($\epsilon = 19.1 \text{ mM}^{-1}\text{cm}^{-1}$) (35). Samples (30 $\mu$g) were incubated in 25 mM potassium phosphate (pH 7.2), 5 mM MgCl, and 20 mM succinate (pH 7.2) for 10 min at 30 °C. Antimycin A (2 $\mu$g/ml), rotenone (2 $\mu$g/ml), KCN (2 mM), and 2,6-dichlorophenolindophenol (50 $\mu$M) were added, and the reaction was monitored for 3 min. Decylubiquinone (65 $\mu$M) was added, and the reaction was monitored for a further 3 min. For the measurement of complex II + III activity (succinate-cytochrome c oxidoreductase), 30 $\mu$g of protein was added to 25 mM potassium phosphate (pH 7.2), 2 mM KCN, 20 mM succinate (pH 7.2), 2 $\mu$g/µl rotenone and incubated at 30 °C for 10 min. Ferricytochrome c was added (37.5 $\mu$M), and the increase in absorbance due to the reduction of ferricytochrome c was measured for 3 min at 550 nm with 580 nm as the reference wavelength.

Complex IV activity (cytochrome c oxidase; EC 1.9.3.1) was measured by following the decrease in absorbance at 550 nm due to the oxidation of ferrocytochrome c, with 580 nm as the reference wavelength ($\epsilon = 19.1 \text{ mM}^{-1}\text{cm}^{-1}$) (35). Samples (10 $\mu$g) were added to 20 mM potassium phosphate, 15 $\mu$M ferrocytochrome c, and 450 $\mu$M n-dodecyl β-D-maltoside, and the reaction was monitored for 30 s. Complex IV activity was calculated from the initial rate. Ferricytochrome c was prepared by adding 5 $\mu$M dithiothreitol to 200 $\mu$M ferricytochrome c. After 20 min, the 550 nm/565 nm ratio was determined, and ferricytochrome c was considered reduced if the ratio was between 10 and 20. Citrate synthase was measured on 10 $\mu$g of sample as described by Barrientos (37).

Calculations—The tissue-specific clearance of 2-[^14]C]DG and 3H-R-BP ($K_6$ and $K_7$, respectively) and the metabolic index for glucose and LCFA uptake ($R_g$ and $R_l$) were calculated as described previously (38). $K_g$ and $K_l$ are used as concentration-independent indices of muscle glucose and LCFA uptake, respectively. $R_g$ and $R_l$ are concentration-dependent indices of muscle glucose and LCFA uptake, respectively.

Percent cardiac output was calculated from fluorescent intensity as described previously (39) and is expressed as percent cardiac output to the tissue (%Q$_T$, where $%Q_T = (f_T/f_{\text{Ref}})(\text{tissue average}/\text{tissue mouse})$, $f_T$ and $f_{\text{Ref}}$ are the fluorescent intensity of the tissue and reference sample, respectively. Adequacy of microsphere mixing was assumed if %Q to the left and right kidney was within 10%. Of the 43 mice infused with microspheres, 34 met the inclusion criteria for analysis.

The amount of 2-[^14]C]DG-P present in the gastrocnemius muscle as well as the amount of microspheres trapped within the gastrocnemius muscle were used to determine the glucose tissue extraction index (TEI). The glucose TEI was calculated by expressing the percentage of 2-[^14]C]DG-P (expressed relative to the amount infused) relative to the percentage of microspheres (expressed relative to the amount infused). For the echocardiography experiments, an index linearly related to cardiac output was calculated as heart rate $\times$ (diastolic left ventricular internal dimension$^3$ – systolic left ventricular internal dimension$^3$) (28).

Statistical Analyses—Data are means ± S.E. Statistical analysis was performed using a Student’s t test, one-way analysis of variance (ANOVA), one-way repeated measures ANOVA, or two-way repeated measures ANOVA where appropriate with the statistical software package SigmaStat. If the ANOVA was significant ($p < 0.05$), specific differences were located using Fisher’s least significant difference test.

RESULTS

Exercise Capacity and Oxygen Consumption in Vivo Are Impaired in α2-KD mice during an Exercise Stress Test—At 16 weeks of age no significant differences were observed between α2-KD mice and WT littermates with respect to body weight (24 ± 2 versus 25 ± 1 g for WT and α2-KD, respectively), muscle mass (77 ± 1 versus 78 ± 2% body weight), or fat mass (8.5 ± 1.4 versus 9.4 ± 0.4% body weight). Basal VO$_2$ was similar between genotypes (78 ± 5 versus 79 ± 6 ml/kg$_{\text{LBM}}$·min$^{-1}$·kg$^{-1}$ for WT and α2-KD, respectively) as was the respiratory exchange ratio (0.77 ± 0.02 versus 0.77 ± 0.01). During an exercise stress test, α2-KD mice displayed marked exercise intolerance as seen by impairments in maximum running speed (38 ± 1 versus 21 ± 1 m·min$^{-1}$ for WT and α2-KD, respectively; $p < 0.001$) and running time (23 ± 1 versus 10 ± 1 min; $p < 0.001$). VO$_2$ during the stress test increased at a similar rate in WT and α2-KD mice (Fig. 1A); however, VO$_2$peak was reduced in α2-KD mice (142 ± 2 versus 113 ± 4 ml/kg$_{\text{LBM}}$·min$^{-1}$·kg$^{-1}$; $p < 0.001$). As a result, α2-KD mice were exercising at a greater percentage of VO$_2$peak compared with WT mice at any given absolute work rate (supplemental Table S1). Respiratory exchange ratio was similar between genotypes at exhaustion (0.89 ± 0.03 versus 0.90 ± 0.03). At a VO$_2$ of ~90 ml/kg$_{\text{LBM}}$·min$^{-1}$·kg$^{-1}$, VCO$_2$ increased disproportionately compared with VO$_2$ in WT mice (Fig. 1B), reflecting a change in either substrates utilized or acidosis. This effect was not apparent in α2-KD mice (Fig. 1C).

Acute Exercise Experiment, Controlling for Relative and Absolute Exercise Intensity—To examine the role of AMPKα2 in the regulation of skeletal muscle metabolic flux in vivo, α2-KD mice performed a single bout of treadmill exercise at 70% of their maximum running speed (α2-KD$_{70\%}$). Because of the difference in maximum running speed between the genotypes, WT mice that exercised at the same absolute speed as α2-KD$_{70\%}$ did so at ~45% of their maximum running speed (WT$_{45\%}$; supplemental Table S2). To best equate results to α2-KD$_{70\%}$, a second group of WT mice was exercised at 70% of their maximum running speed (WT$_{70\%}$; supplemental Table S2). As demonstrated in the results that follow, controlling for absolute and relative exercise intensity is essential for interpretation of the physiological and metabolic responses to exercise in vivo.

AMPKα Protein Expression and AMPK Activity Is Impaired in Skeletal Muscle of α2-KD Mice—Similar to other muscle groups (11), expression of the α2-KD subunit in gastrocnemius muscle was increased relative to native AMPKα2 (98 ± 8% higher in α2-KD compared with WT, $p < 0.01$; supplemental Fig. S1A). A concomitant decrease in AMPKα1 expression was observed in the gastrocnemius of α2-KD mice (51 ± 11% lower in α2-KD compared with WT, $p < 0.02$; supplemental Fig. S1A). Similar findings for AMPKα2 and α1 expression were observed in SVL muscle (data not shown). In gastrocnemius muscle of WT mice, AMPKα2 (supplemental Fig. S1B) and AMPKα1 activities (supplemental Fig. S1C) increased in an
intensity-dependent manner. AMPKα2 and -α1 activities were barely detectable in the gastrocnemius of α2-KD mice under sedentary conditions and did not change in response to exercise. Gastrocnemius acetyl-CoA carboxylase-β Ser221 phosphorylation was similar between genotypes at rest and increased to a similar extent in all groups in response to exercise (supplemental Fig. S1D).

**Skeletal Muscle ATP Concentrations Decrease in α2-KD Mice during Exercise in Vivo**—In response to exercise, no significant changes in ATP were observed in the gastrocnemius of WT_{45%} or WT_{70%} (Table 1). In contrast, exercise significantly decreased gastrocnemius ATP levels in α2-KD_{70%}. Lactate and creatine (Cr) significantly increased, whereas phosphocreatine (PCr), PCr:(PCr + Cr), and glycogen significantly decreased during exercise in all groups (Table 1). In α2-KD_{70%} and WT_{70%}, AMP_{free}, AMP_{total}, and AMP_{free:ATP} all increased in response to exercise (Table 1). The similar increase in AMP_{free} and AMP_{free:ATP} observed between α2-KD_{70%} and WT_{70%} shows that, by this criteria, cellular stress was equally elevated in these groups compared with WT_{45%}. This finding emphasizes the need to exercise mice at the same relative work intensity to obtain comparable energetic responses in vivo.

**Arterial Metabolites and Hormones Are Altered in α2-KD Mice at Rest and during Steady State Exercise in Vivo**—An increase in exercise intensity resulted in significantly lower arterial glucose levels in WT mice (Fig. 2A). Compared with WT_{70%}, arterial glucose levels during exercise were significantly greater in α2-KD_{70%}. Arterial NEFAs (Fig. 2B) and insulin (Fig. 2C) decreased to similar concentrations in all groups during exercise. Although no differences in basal insulin levels were observed between individual groups, basal insulin levels were greater in α2-KD_{70%} compared with the combined average of all WT mice (98 ± 6 versus 71 ± 7 pm, p < 0.05). Arterial lactate increased over time in all exercise groups (Fig. 2D), and a significant group effect was observed with α2-KD_{70%} > WT_{70%} > WT_{45%} (p < 0.01).

**Indices of Glucose Uptake, but Not LCFA Uptake, Are Impaired in Skeletal Muscle of α2-KD Mice during Exercise in Vivo**—In WT mice, an increase in exercise intensity increased the plasma disappearance of 2-[14C]DG at 7 and 10 min (Fig. 3A). Gastrocnemius K_{s} (Fig. 3B) and R_{e} (Fig. 3C) also increased in WT_{70%} compared with WT_{45%}. At the same relative exercise intensity, the disappearance of plasma 2-[14C]DG was attenuated at 7 min in α2-KD_{70%} mice when compared with WT_{70%}. In α2-KD_{70%} mice, gastrocnemius K_{s} was impaired by ∼60% when compared with WT_{70%} mice (Fig. 3B). Gastrocnemius R_{e}

**TABLE 1**

| Metabolite | Sedentary | Exercise |
|------------|-----------|----------|
|           | WT_{45%}  | WT_{70%} | α2-KD_{70%} |
| ATP (μmol·100 g⁻¹) | 29.1 ± 2.2 | 30.5 ± 1.6 | 25.7 ± 1.1 |
| Lactate (μmol·100 g⁻¹) | 12.3 ± 2.2 | 12.3 ± 1.7 | 57.7 ± 14.1 a |
| PCr (μmol·100 g⁻¹) | 62.4 ± 3.1 | 57.8 ± 3.3 | 28.5 ± 8.3 |
| Cr (μmol·100 g⁻¹) | 37.6 ± 3.1 | 42.2 ± 3.3 | 71.5 ± 8.3 a |
| PCr:(PCr + Cr) | 0.60 ± 0.03 | 0.58 ± 0.03 | 0.28 ± 0.08 a |
| ADP_{free} (μmol·100 g⁻¹) | 154 ± 19 | 178 ± 22 | 3.2 ± 1.3 |
| AMP_{total} (μmol·100 g⁻¹) | 0.9 ± 0.2 | 1.2 ± 0.2 | 0.13 ± 0.06 |
| AMP_{free:ATP} | 0.05 ± 0.01 | 0.03 ± 0.00 | 0.30 ± 0.15 a |
| Glycogen (μmol·100 g⁻¹) | 901 ± 174 | 630 ± 101 | 390 ± 106 a |

* a p < 0.05 versus corresponding basal.

**FIGURE 1.** Oxygen consumption is impaired in 16-week-old chow-fed C57BL/6J mice expressing a kinase-dead form of AMP-activated protein kinase α2 (α2-KD) in cardiac and skeletal muscle. Compared with WT littermates, the increase in oxygen consumption (ΔVO₂) during an exercise stress test is attenuated in α2-KD mice (A). B and C, carbon dioxide production (VCO₂) during an exercise stress test was plotted against VO₂ for WT and α2-KD mice, respectively. Note the change of slope of VCO₂ (supplemental Fig. S1) that is not present in α2-KD mice, respectively. Note the change of slope of VCO₂ during exercise in all groups (Table 1). In α2-KD_{70%} and WT_{70%}, ADP_{free}, AMP_{free}, and AMP_{free:ATP} all increased in response to exercise (Table 1). The similar increase in AMP_{free} and AMP_{free:ATP} observed between α2-KD_{70%} and WT_{70%} shows that, by this criteria, cellular stress was equally elevated in these groups compared with WT_{45%}. This finding emphasizes the need to exercise mice at the same relative work intensity to obtain comparable energetic responses in vivo.
during exercise was also impaired ~35% in α2-KD70% mice when compared with WT70% (Fig. 3C).

An increase in exercise intensity tended to increase $K_g$ in the soleus of WT mice ($p = 0.07$ for WT45% versus WT45%; supplemental Fig. S2A), whereas $K_g$ in WT70% was significantly greater than WT45% in SVL (supplemental Fig. S2B). These results paralleled findings observed for $R_i$ in soleus (supplemental Fig. S2C) and SVL (supplemental Fig. S2D). As with the gastrocnemius, $K_g$ in soleus and SVL was impaired ~30% in α2-KD70% mice when compared with WT70%, however, soleus and SVL $R_i$ was similar between α2-KD70% and WT70%.

Taken together, these findings show that glucose concentration-dependent ($R_i$) and -independent ($K_g$) indices of MGU are impaired in α2-KD mice during exercise in vivo compared with WT mice exercising at the same relative intensity. The finding that MGU was greater in WT70% compared with WT45% shows for the first time that the 2-[14C]DG method (38) can be used to determine the effect of different exercise intensities on multiple muscle groups in vivo.

Indices of LCFA clearance ($K_j$) and uptake ($R_i$) are shown in supplemental Fig. S3. $K_j$ increased to similar rates during exercise in soleus, gastrocnemius, and SVL of α2-KD70% and WT70%. In WT45%, $K_j$ responses were generally reduced. $R_i$ significantly increased in soleus, gastrocnemius, and SVL of α2-KD70%. In WT70%, $R_i$ significantly increased in soleus and gastrocnemius, whereas $R_i$ was elevated in gastrocnemius of WT45%. Given that $K_j$ and $R_i$ increased normally in response to exercise in α2-KD mice, it can be concluded that AMPKα2 is not essential for skeletal muscle LCFA uptake during exercise in vivo. This is in agreement with previous studies performed ex vivo (20, 21).

**Percent Cardiac Output to Skeletal Muscle Is Altered in α2-KD Mice at Rest and during Exercise in Vivo**—Under basal conditions %QG was ~2.5-fold greater in α2-KD mice compared with WT mice (Fig. 3D). Exercise increased %QG in WT45% (~4.5-fold) and WT70% (~4-fold). Exercise did not alter %QG in α2-KD70%. The glucose TEI did not differ between α2-KD and WT mice at rest (Fig. 3E). Exercise increased the glucose TEI to a similar extent in α2-KD70% and WT70%, demonstrating that the impairment in MGU seen in the gastrocnemius of α2-KD70% compared with WT70% during exercise was likely due to reduced substrate delivery (i.e. %QG). The TEI did not increase in WT45%, demonstrating that in WT mice the extraction of glucose by skeletal muscle is accelerated as exercise intensity increases.

**Cardiac Fuel Uptake and Function during Exercise in Vivo Are Not Impaired in α2-KD Mice**—Cardiac $K_g$ was similar between genotypes at rest, and exercise did not significantly increase $K_g$ in any group (supplemental Fig. S4A). Cardiac $R_i$ was also similar between genotypes at rest, and exercise significantly increased cardiac $R_i$ in α2-KD70% and WT70% (supplemental Fig. S4B). Cardiac $R_i$ did not increase during exercise in WT45%, and was significantly less than cardiac $R_i$ in α2-KD70% and WT70%. No significant differences were observed with respect to $K_g$ or $R_i$ in cardiac muscle between any of the three groups.
NOS Expression and Activity Are Reduced in Skeletal Muscle of 16-week-old Chow-fed C57BL/6J mice expressing a WT or kinase-dead (KD) form of AMP-activated protein kinase α2 (α2-KD) in cardiac and skeletal muscle.

Data are mean ± S.E. for n = 5–6 per group. Activities are expressed as nmol min⁻¹ mg⁻¹.

| Complex  | Enzyme                                      | WT   | α2-KD |
|----------|---------------------------------------------|------|-------|
| I        | NADH:ubiquinone oxidoreductase              | 74.9 ± 7.4 | 51.0 ± 5.9* |
| II       | NADH:cytochrome c oxidoreductase            | 11.1 ± 3.7  | 6.6 ± 2.6   |
| III      | Succinate:ubiquinone oxidoreductase         | 10.7 ± 2.1  | 9.5 ± 2.7   |
| IV       | Cytochrome c oxidoreductase                 | 13.4 ± 3.0  | 8.0 ± 1.7   |

* p < 0.05 versus corresponding WT.

NOS activity was also impaired in SVL muscle of α2-KD mice (supplemental Fig. S5); however, exercise did not alter NOS activity in any group, a finding that may be related to less recruitment of this muscle (i.e. attenuated Rє and Kє when compared with gastrocnemius muscle). Thus, AMPK is required for full expression of nNOSμ, as well as NOS activity at rest and in response to exercise. The observation that NOS activity increased in gastrocnemius of WT 70% but not WT 45% shows that NOS activity is sensitive to exercise intensity.

Activities of Specific Electron Transport Chain (ETC) Complexes Are Reduced in Skeletal Muscle of α2-KD Mice—The finding that exercise capacity, VO2peak, and ATP generation are impaired, although changes in arterial lactate levels are accelerated in α2-KD mice during exercise despite normal extraction of glucose in skeletal muscle, led us to hypothesize that mitochondrial function is impaired in these mice. Support for this hypothesis comes from the finding that a reduction in nNOSμ protein expression, such as seen in the present study, is associated with impaired activity of enzymes involved in skeletal muscle OXPHOS (41, 42). As shown in Table 2, complex I and complex IV activities of the ETC were significantly impaired in sedentary α2-KD mice when compared with WT mice, whereas no changes were observed for complex I + III, II, or II + III activities. Identical findings were observed if complex activities were normalized to citrate synthase levels, which did not differ between genotypes (50 ± 7 versus 52 ± 11 μmol min⁻¹ mg⁻¹ for WT and α2-KD, respectively). Thus, the impairment in skeletal muscle ETC complexes in α2-KD mice was not because of a nonspecific reduction in mitochondrial content, a finding that is in agreement with previous observations demonstrating no alteration in mitochondrial density, DNA, and other markers of mitochondrial content and biogenesis in gastrocnemius muscle of untrained α2-KD mice (43).

DISCUSSION

This study supports for the first time in vivo the hypothesis that AMPK is a critical mediator of the metabolic response to exercise. We demonstrate that AMPK regulates skeletal muscle metabolism in vivo at multiple levels, with the overall result being that a defect in AMPKα2 subunit activity in skeletal muscle grossly impairs exercise tolerance. Without a functionally active AMPKα2 subunit, glucose uptake during exercise in vivo is impaired in different skeletal muscle groups of α2-KD mice compared with WT littermate mice exercising at the same relative intensity. This may be due in part to impaired substrate...
Physiological Role of AMPK in Skeletal Muscle

**EXERCISE IN C57BL/6J MICE**

| WT | α2-KD |
|----|-------|
| GLUCOSE | GLUCOSE |
| | |
| CONTRACTION | CONTRACTION |
| | |
| G-6-P | G-6-P |
| | |
| ATP | ATP |
| | |
| LACTATE | LACTATE |
| | |
| AMPK | AMPK |
| | |
| nNOS | nNOS |
| | |
| NOS activity | NOS activity |
| | |
| NO | NO |
| | |
| vascular relaxation | vascular relaxation |

**FIGURE 5. Proposed model describing the role of skeletal muscle AMPKα2 during exercise *in vivo*.** Our results show that skeletal MGU during exercise is dependent on AMPKα2 activation, as mice expressing α2-KD have impaired MGU when compared with WT mice at the same relative exercise intensity. The impaired MGU in α2-KD mice is at least partially because of reduced vasodilation, which arises from an inability of AMPK to activate NOS and thus stimulate NO production. The impairment in AMPKα2 activation and/or reductions in the skeletal muscle isoform of neuronal NOS (nNOS) also attenuate mitochondrial function. This reduces mitochondrial ATP generation and diverts glucose toward anaerobic ATP generation, resulting in elevated plasma lactate levels. The whole body phenotype of these impairments is a reduction in exercise tolerance.

The novel finding that AMPK activity and expression is impaired in α2-KD mice reveals new insight regarding the role of AMPK in skeletal muscle, and it provides a mechanism that could account for or contribute to the exercise intolerance observed in the α2-KD mouse. Complex I and complex IV represent the proximal and distal ETC complexes, respectively, and thus play an integral role in OXPHOS and the generation of ATP. A deficiency in complex I activity will lead to excess levels of NADH and a lack of NAD⁺, resulting in impaired Krebs cycle function and elevated blood lactate (44), the latter being observed in α2-KD mice during exercise in this study. A deficiency in complex IV activity would impair the proton gradient required for subsequent ATP synthesis (45), explaining the accelerated net ATP degradation observed in skeletal muscle of α2-KD mice during exercise *in vivo*. Importantly, the changes in complex I and complex IV activities in α2-KD mice occurred despite similar levels of citrate synthase activity when compared with WT mice. This agrees with previous findings showing that mitochondrial density, mitochondrial DNA, cytochrome c protein expression, δ-aminolevulinate synthase mRNA expression, and peroxisome proliferator-activated receptor γ coactivator-1α mRNA expression are similar in gastrocnemius muscle of untrained α2-KD and WT mice (43). Thus, a functionally inactive AMPKα2 subunit is sufficient to impair mitochondrial function, without adversely altering markers of muscle mitochondrial content.

Although OXPHOS capacity was impaired in skeletal muscle of α2-KD mice, it is unclear whether the α2-KD subunit per se was directly responsible for this phenomenon. A novel finding with important implications was that nNOS protein expression was impaired in skeletal muscle of α2-KD mice. This finding is supported by the close association between AMPKα2 and nNOS (40). A decrease in nNOS protein expression has been associated with impairments in OXPHOS. Indeed, in skeletal muscle of patients with amyotrophic lateral sclerosis, reduced nNOS expression is highly associated with impaired ETC complex activities (42). Similarly, in skeletal muscle of nNOS−/− mice, ETC complex activities are reduced (41). Thus, the impairments in OXPHOS within skeletal muscle of α2-KD mice may be due to a direct impairment of AMPK or indirect effects mediated by reductions in nNOS protein expression.

The reduced nNOS expression may have also caused an impairment in muscle blood flow, as %QG did not increase in response to exercise in α2-KD mice, whereas an ~4-fold increase was observed in WT 70% and WT 45%. It has been shown that vasodilation in response to mild exercise is significantly impaired in animal models where nNOSα is partially impaired or ablated in skeletal muscle (46). Likewise, Lau et al. (47) have showed that ~50% of contraction-induced arteriolar dilation *in vitro* is dependent on nNOS. Conversely, restoring NOSα at the sarcolemma of skeletal muscle significantly improves the exercise-induced increase in skeletal muscle perfusion (48). It is well known that contracting muscle releases nitric oxide (NO) (49, 50). Given that NOS activity in gastrocnemius of α2-KD mice was impaired in response to intense exercise, it is a plausible hypothesis that NO efflux from α2-KD muscle was also impaired. NO is a potent stimulator of vasodilation (51), and as such impaired NOS activity during exercise may have also suppressed arteriolar relaxation in α2-KD mice. Aside from nNOS, it has been shown that the gastrocnemius of α2-KD mice contains significantly fewer capillaries compared with WT mice (52). Given that exercise normally causes a redistribution of blood flow toward contracting muscle (53), fewer capillaries in the gastrocnemius of α2-KD mice might have also resulted in less blood flow to this tissue during exercise.

We found for the first time that suppressed activation of AMPKα2 in skeletal muscle during exercise *in vivo* was associated with ~60 and ~35% reductions in concentration-inde-
pendent and -dependent indices of MGU, respectively. These impairments became apparent when work intensity was normalized to the same relative work rate in WT and 2-KD mice. As mentioned above, a novel observation in 2-KD mice was that %QG did not increase in response to exercise. As a result, the impairment in MGU in 2-KD mice could at least partially be ascribed to a reduction in vascular glucose delivery to the contracting muscle. In line with this theory, we demonstrated that the glucose TEI in gastrocnemius of 2-KD mice was similar to WT mice exercising at the same relative intensity, suggesting that the muscle had adequate capacity to extract glucose from the blood.

A key aspect of this study was utilizing WT mice exercising at the same relative and absolute levels as 2-KD mice. To date, we are unaware of any study that has controlled for this variable when utilizing exercise as a means to amplify metabolic signals in rodents. Dzamko et al. (21) recently reported that VO2 and whole body substrate oxidation (assessed via indirect calorimetry) was similar between 2-KD and WT mice exercising at the same absolute running speed. This agrees with findings from this present study; however, the findings of Dzamko et al. (21) do not account for the difference in relative exercise intensity. Our exercise stress test demonstrated that at the same absolute running speed 2-KD mice were exercising at a greater percentage of VO2peak. This is evidenced by the elevated cellular stress in skeletal muscle of 2-KD mice (i.e. AMPfree and AMPfree/ATP) compared with WT mice at the same absolute running speed, observations also observed in AMPKα2−/− and WT mice exercising at the same absolute speed (54). Furthermore, in the present study KF and RF were elevated in SVL of 2-KD mice compared with WT mice demonstrating that this muscle group, comprised primarily of fast glycolytic fibers, was recruited to a greater extent in 2-KD mice. Based on these observations, we propose that the relative exercise intensity should be compared in rodent exercise studies, as is generally done in human exercise studies. Performing an exercise stress test in rodents provides valuable data pertaining to maximum running speed and running time and facilitates interpretation of subsequent studies examining physiological responses to an acute bout of exercise in vivo.

Aside from examining the role of AMPKα2 in skeletal muscle during exercise, we also addressed the role of AMPKα2 in cardiac muscle. Expression of the 2-KD transgene is driven by the muscle creatine kinase promoter (18), which is present in cardiac and skeletal muscle (55). The muscle creatine kinase promoter activity, and thus expression of the α2-KD transgene, is much lower in cardiac muscle (18). Nevertheless, cardiac function of α2-KD mice has been shown to be significantly impaired in metabolically challenged states such as ischemia (56), and it has been suggested that the exercise intolerance of α2-KD mice may be due to impairments in cardiac function as opposed to skeletal muscle defects (21, 57). In this study changes in cardiac glucose and LCFA uptake, heart rate, and cardiac output in α2-KD mice were similar to WT mice exercising at the same relative intensity. Thus, a functionally inactive AMPKα2 subunit in cardiac muscle does not appear to impair substrate uptake or cardiac function during physiological exercise conditions.

In conclusion, we show for the first time that exercise performed in vivo by α2-KD mice elicits a phenotype characterized by impaired VO2peak exercise intolerance, enhanced ATP degradation in skeletal muscle, and lactic acidosis. At the same relative exercise intensity, MGU is impaired in α2-KD mice compared with WT mice. This is not because of attenuation in the fractional extraction of glucose by skeletal muscle but likely to impaired vascular glucose delivery to skeletal muscle. We also show that AMPK regulates nNOS protein expression and NOS activity, as well as mitochondrial function in skeletal muscle. Based on existing literature (46), it is likely that the effects of impaired AMPK activation on vascular and mitochondrial function are to an extent mediated by changes in nNOS. Thus, our findings demonstrate novel roles for AMPK in skeletal muscle and provide new insight into the role of AMPK during physiological exercise. Our findings have implications for chronic metabolic disease states such as obesity and type 2 diabetes, which are characterized by suppressed skeletal muscle AMPKα2 activity during exercise.

Acknowledgments—We thank Prof. Morris Birnbaum (University of Pennsylvania) for kindly supplying the α2-KD mice used for breeding. We thank Drs. Zhi Zhang Wang and Jeffrey Rottman of the Vanderbilt Mouse Metabolic Phenotyping Center, Cardiovascular Pathophysiology Core, for performing the echocardiography. We also thank Associate Professor Rodney Snow (Deakin University, Victoria, Australia) for helpful discussions regarding the metabolite analyses.

REFERENCES

1. Jørgensen, S. B., and Rose, A. J. (2008) Front. Biosci. 13, 5589–5604
2. Hardie, D. G. (2007) Nat. Rev. Mol. Cell Biol. 8, 774–785
3. Corton, J. M., Gillespie, J. G., and Hardie, D. G. (1994) Curr. Biol. 4, 315–324
4. Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J., and Winder, W. W. (1999) Diabetics 48, 1667–1671
5. Koistinen, H. A., Galuska, D., Chibalin, A. V., Yang, J., Zierath, J. R., Holman, G. D., and Wallberg-Henriksson, H. (2003) Diabetics 52, 1066–1072
6. Merrill, G. F., Kurth, E. J., Hardie, D. G., and Winder, W. W. (1997) Am. J. Physiol. 273, E1107–E1112
7. Bergeron, R., Russell, R. R., 3rd, Young, L. H., Ren, J. M.,Marcucci, M., Lee, A., and Shulman, G. I. (1999) Am. J. Physiol. 276, E938–E944
8. Shearer, J., Fueger, P. T., Vondrick, B., Bracy, D. P., Rottman, J. N., Clanton, J. A., and Wasserman, D. H. (2004) Diabetes 53, 1429–1435
9. Jørgensen, S. B., Viollet, B., Andreelli, F., Fressig, C., Birk, J. L., Schjerling, P., Vaulont, S., Richter, E. A., and Woitaszewski, J. F. (2004) J. Biol. Chem. 279, 1070–1079
10. Fuji, N., Hirshman, M. F., Kane, E. M., Ho, R. C., Peter, L. E., Seifert, M. M., and Goodyear, L. J. (2005) J. Biol. Chem. 280, 39033–39041
11. Lefort, N., St-Amand, E., Morasse, S., Côté, C. H., and Marette, A. (2008) Am. J. Physiol. Endocrinol. Metab. 295, E1447–E1454
12. Jørgensen, S. B., Treebak, J. T., Viollet, B., Schjerling, P., Vaulont, S., Woitaszewski, J. F., and Richter, E. A. (2007) Am. J. Physiol. Endocrinol. Metab. 292, E331–E339
13. Kemp, B. E., Stapleton, D., Campbell, D. J., Chen, Z. P., Murthy, S., Walter, M., Gupta, A., Adams, J. J., Katsis, F., Van Denderen, B., Jennings, I. G., Iseli, T., Michell, B. J., and Witters, L. A. (2003) Biochem. Soc. Trans. 31, 162–168
14. Winder, W. W., and Hardie, D. G. (1999) Am. J. Physiol. 277, E1–E10
15. Chen, Z. P., Stephens, T. J., Murthy, S., Canny, B. J., Hargreaves, M., Witters, L. A., Kemp, B. E., and McConell, G. K. (2003) Diabetes 52, 2205–2212
16. Musi, N., Hayashi, T., Fuji, N., Hirshman, M. F., Witters, L. A., and Good-
