Reduced glycogen availability is associated with increased AMPKα2 activity, nuclear AMPKα2 protein abundance, and GLUT4 mRNA expression in contracting human skeletal muscle

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Abstract: Glycogen availability can influence glucose transporter 4 (GLUT4) expression in skeletal muscle through unknown mechanisms. The multisubstrate enzyme AMP-activated protein kinase (AMPK) has also been shown to play an important role in the regulation of GLUT4 expression in skeletal muscle. During contraction, AMPK α2 translocates to the nucleus and the activity of this AMPK isoform is enhanced when skeletal muscle glycogen is low. In this study, we investigated if decreased pre-exercise muscle glycogen levels and increased AMPK α2 activity reduced the association of AMPK with glycogen and increased AMPK α2 translocation to the nucleus and GLUT4 mRNA expression following exercise. Seven males performed 60 min of exercise at ~70% $VO_{2\ peak}$ on 2 occasions: either with normal (control) or low (LG) carbohydrate pre-exercise muscle glycogen content. Muscle samples were obtained by needle biopsy before and after exercise. Low muscle glycogen was associated with elevated AMPK α2 activity and acetyl-CoA carboxylase β phosphorylation, increased translocation of AMPK α2 to the nucleus, and increased GLUT4 mRNA. Transfection of primary human myotubes with a constitutively active AMPK adenovirus also stimulated GLUT4 mRNA, providing direct evidence of a role of AMPK in regulating GLUT4 expression. We suggest that increased activation of AMPK α2 under conditions of low muscle glycogen enhances AMPK α2 nuclear translocation and increases GLUT4 mRNA expression in response to exercise in human skeletal muscle.

Key words: exercise, subcellular localization, glycogen binding domain, AMP-activated protein kinase.

Résumé : La disponibilité du glycogène peut influencer par des mécanismes non encore connus l'expression de GLUT4 (« glucose transporter 4 ») dans le muscle squelettique. La protéine kinase dépendante de l'AMP (AMPK) jouerait, en présence de plusieurs substrats, un important rôle dans la régulation de l'expression de GLUT4 dans le muscle squelettique. Au cours de la contraction musculaire, l'AMPK α2 passe dans le noyau et l'activité de cette isoforme de l'AMPK est accrue quand le contenu en glycogène est faible. Dans la présente étude, nous vérifions si un faible contenu en glycogène avant l'effort associé à une activité accrue de l'AMPK α2 réduit l'association de l'AMPK au glycogène et augmente la translocation de l'AMPK α2 dans le noyau et l'expression de l'ARNm codant pour GLUT4. Sept hommes participent à 2 moments différents à une séance d'exercice d'une durée de 60 min et à une intensité proche de 70 % du VO_2 de crête : (*i*) contenu normal de glycogène (control) et (*ii*) faible contenu de glycogène (LG) avant l'effort. On prélève par biopsie à l'aiguille des échantillons de tissu musculaire avant et après la séance d'exercice. Un faible contenu en glycogène est associé à une augmentation de l'activité de l'AMPK α2 et de la phosphorylation de l'acétyl-CoA carboxylase, à une augmentation de la translocation de l'AMPK α2 dans le noyau et à une augmentation de l'ARNm codant pour GLUT4. La transfection de myotubes primaires humains comportant un adénovirus actif de l'AMPK stimule l'expression de l'ARNm codant pour GLUT4, ce qui constitue une preuve du rôle de l'AMPK

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dans la régulation de l'expression de GLUT4. Nous concluons que l'augmentation de l'activation de l'AMPK α2 en présence d'un faible contenu en glycogène améliore la translocation de l'AMPK α2 et accroît l'expression de l'ARNm codant pour GLUT4 à la suite d'un exercice musculaire chez l'humain.

Mots clés : exercice physique, localisation infracellulaire, affinité du glycogène, protéine kinase dépendante de l'AMP.

[Traduit par la Rédaction]

Introduction

Muscle glycogen availability during contraction can influence the expression of glucose transporter 4 (GLUT4), the major facilitative glucose transporter in skeletal muscle (Richter et al. 2001). In rodent skeletal muscle, the maintenance of low muscle glycogen levels after exercise stimulates GLUT4 expression to a greater extent than if muscle glycogen levels are restored after the exercise bout (Garcia Roves et al. 2003). In addition, the overexpression of glycogen phosphorylase in cultured human skeletal muscle cells, resulting in increased glycogen turnover, increased GLUT4 expression (Baque et al. 1998). The regulation of GLUT4 transcription by contraction and glycogen availability is not fully understood, but emerging evidence suggests that AMP-activated protein kinase (AMPK) may play a role in mediating these effects. Low levels of muscle glycogen correlate with greater activation of the AMPK α2 isoform in contracting skeletal muscle (Wojtaszewski et al. 2003; Derave et al. 2000), whereas activation of AMPK α2 by 5-aminoimidazole-4-carboxamide riboside (AICAR) is suppressed when glycogen levels are elevated (Aschenbach et al. 2002; Wojtaszewski et al. 2000).

AMPK is a multi-substrate enzyme regulating glucose transport, glycolysis, lipid metabolism, protein synthesis, and gene transcription in response to many metabolic stress signals, including exercise (Kemp et al. 2003). Recently, work from our group has shown that an acute bout of exercise results in translocation of AMPK α2 to the nucleus in human skeletal muscle (McGee et al. 2003), suggesting that, during contraction, a major role of AMPK may be to interact with nuclear targets and influence gene expression.

Although the classic pathway for AMPK activation involves an increase in AMP:ATP, the regulation of AMPK by AMP is complex, involving not only direct covalent activation by LKB1 (Woods et al. 2003; Hawley et al. 2003) to phosphorylate the α subunit at T172, but also allosteric regulation through changes in AMP:ATP (Kemp et al. 2003). Moreover, recent studies from our laboratory (Polekhina et al. 2003) and others (Hudson et al. 2003) have identified the presence of a glycogen-binding domain (GBD) within the β1 subunit of AMPK. In this study, we demonstrated that mutation of conserved residues within the GBD abolished binding to glycogen in vitro.

We have recently demonstrated that in a low-glycogen state, AMPK activation is enhanced (Watt et al. 2004). In the present study, muscle biopsies from a study by Watt et al. (2004) were used to determine whether enhanced activation of AMPK was associated with a reduced association of AMPK with glycogen and whether enhanced AMPK activation increased AMPK nuclear translocation and GLUT4 mRNA expression. We hypothesized that reduced glycogen availability would result in reduced binding of AMPK α2 to a glycogen-enriched fraction isolated from skeletal muscle, greater translocation of AMPK α2 to the nucleus, and enhanced GLUT-4 mRNA expression.

Materials and methods

The subjects' characteristics, pre-experimental, and experimental protocols have previously been described (Watt et al. 2004). Seven recreationally active men gave their written informed consent to participate in the study, which was approved by the RMIT Human Ethics Committee. The subjects' age, mass, and height averaged 24 ± 2 y, 77 ± 3 kg, and 1.81 ± 0.02 m, respectively. Subjects participated in 3–5 sessions of aerobic exercise weekly, but none were specifically endurance trained.

Pre-experimental protocol

Subjects visited the laboratory and performed an incremental exercise test on a cycle ergometer until they reached volitional exhaustion. Peak pulmonary oxygen uptake ($V\text{O}_{2\ peak}$) averaged 49.0 ± 3.7 mL·(kg·min)$^{-1}$. On subsequent visits, subjects were asked to refrain from alcohol, caffeine, and strenuous exercise for the 24 h before the experimental trial. Glycogen-depleting exercises (see below) were completed on 2 occasions between 4:00 and 5:00 pm the day before the experimental trial. Subjects then returned to the laboratory the following morning (8:00 am) to perform the experimental protocol (see below), following the consumption of a high- or low-carbohydrate diet that was provided randomly. The high-carbohydrate diet contained 6.2 MJ consisting of 79% carbohydrate, 3% fat, and 18% protein (denoted herein as control); the low-carbohydrate diet contained 6.4 MJ consisting of 4% carbohydrate, 59% fat, and 37% protein (denoted herein as LG). Subjects were instructed to consume all food before 10:00 pm and were permitted to consume water thereafter.

The glycogen-depleting exercise involved 2 alternating periods of cycling at 70% $V\text{O}_{2\ peak}$ for 20 min, followed by 20 min of intermittent exercise. The intermittent exercise consisted of 2 min cycling at 90% $V\text{O}_{2\ peak}$, followed by 2 min at 50% $V\text{O}_{2\ peak}$. Subjects then performed arm-cranking exercise (~50 W) for 20 min and recommenced intermittent cycle exercise until exhaustion. The subjects were then asked to complete further arm-cranking exercise until exhaustion. This regime was adopted to minimize any overnight glycogen resynthesis during LG in the lower limbs.

Experimental protocol

After consuming either the high- or low-carbohydrate meals, subjects arrived the following morning after an overnight fast. Subjects lay supine while a teflon catheter was

inserted into an antecubital vein and 1 leg was prepared for subsequent needle biopsy by making 2 incisions through the skin and fascia of the vastus lateralis under local anesthesia. A venous blood sample (8 mL) was obtained, and a muscle sample was obtained by needle biopsy immediately before exercise. The subject subsequently commenced cycling at ~70% $VO_{2\,peak}$. Respiratory gases were collected for 4 min at 20, 40, and 60 min of exercise and analyzed for VO_2 and carbon dioxide production using an on-line metabolic system (Medgraphics, St. Paul, Minn.). Venous blood samples were also obtained at these times. A second muscle sample was immediately obtained at 60 min with the subject remaining on the cycle ergometer. Muscle samples were obtained from the contralateral leg in the subsequent trial.

Blood analysis

Whole blood was mixed in a sodium–heparin collection tube and the plasma was obtained after centrifugation at 10 000*g* for 2 min. Plasma samples were frozen at –80 °C for later analysis of epinephrine (LDN, Nordhorn, Germany) by radioimmunoassay.

Muscle analysis

Glycogen contents and muscle adenine nucleotides were determined on freeze-dried samples, which were dissected free of all visible connective tissue and blood and assayed as we have described previously (Watt et al. 2002).

AMPK α1 and α2 activities and ACCβ phosphorylation

Frozen vastus lateralis muscle samples weighing ~25 mg were homogenized in 200 μL of ice-cold lysis buffer as described previously (Chen et al. 2000). Isoform-specific AMPK activity was measured in immunoprecipitates from 140 μL of muscle lysates with saturating concentrations of AMP (0.2 mmol/L) as previously described (Chen et al. 1999). The expression and phosphorylation of ACCβ was measured by Western blot of the same muscle lysate used to measure AMPK activity as previously described by Chen et al. (1999). ACCβ phosphorylation is a substrate of AMPK and is phosphorylated at Ser221 in human skeletal muscle. The phosphorylation status of ACCβ is considered an in vivo indication of AMPK activity taking into account both the allosteric and covalent regulation of AMPK. ACCβ phosphorylation was corrected for total ACCβ phosphorylation as previously described (Chen et al. 1999).

AMPK glycogen localization

Frozen tissue samples weighing ~50 mg were homogenized into 750 μL glycogen isolation buffer (50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, and protease inhibitors cocktail (Roche, Basel, Switzerland)), spun at 6000*g* for 10 min, and the resultant supernatant spun at 50 000*g* for 30 min. This procedure resulted in tightly packed translucent glycogen pellet underlying a well-defined looser microsomal pellet that was carefully removed. The resulting supernatant was called the cytosolic fraction. Protein concentration was determined using the BCA protein assay (Pierce). The glycogen pellet was resuspended into 250 μL glycogen isolation buffer. Glycogen levels were determined by digesting an aliquot of the glycogen pellet with amyloglucosidase at 50 °C for 30 min in a buffer containing 0.1 mol/L sodium acetate (pH 6.0). Following centrifugation at 14 000*g* for 2 min, an aliquot was removed and glucose levels determined using a 2-enzyme, color-based glucose assay (Sigma Chemicals, St. Louis, Miss.). As demonstrated in Fig. 1A, the relative glycogen concentration in the purified glycogen fraction was ~20-fold greater then the cytosolic fraction. We then compared 30 μg of protein from human muscle cytosolic and glycogen fractions by 12% self-denatured sodium polyacrylamide gele electrophoresis (SDS PAGE) stained with Coomassie blue dye as shown in Fig. 1B. Furthermore, we identified 2 of the major proteins present in rat liver glycogen by mass spectrometry (MS). Briefly, the proteins were excised and digested with protein sequencing grade trypsin overnight at 30 °C with the resulting peptides desalted by ZipTip™ (Millipore, Bedford, Mass.) and identified by tandem MS on a Q(q)TOF-type mass spectrometer API QSTAR Pulsar i (Applied Biosytems, Foster City, Calif.). The larger polypeptide was identified as glycogen-debranching enzyme with 4 peptides were identified by tandem MS and searching with the Mascot search engine (data not shown). The smaller polypeptide was positively identified as rat liver glycogen phosphorylase based on the masses of 14 peptides (data not shown). The 2 major polypeptides shown in the human glycogen fraction have not been positively identified by mass spectrometry, but appear to be glycogen phosphorylase and glycogen-debranching enzyme given their identical size and relative quantities when compared with the rat liver glycogen fraction. These proteins are clearly not visible in the cytosolic fraction shown in Fig. 1B.

After the completion of quality-control experiments, human muscle biopsies were extracted as described above into glycogen and cytosolic fractions. One hundred micrograms of protein were solubilized into glycogen-isolation buffer containing 1% NP-40. AMPK α2 antibody (3 μg) was added to each fraction together with 100 μL of 20% Protein A sepharose and incubated overnight at 4 °C with mixing. Immunoprecipitates were collected, washed twice with glycogen isolation buffer containing 1% NP-40, and analyzed by Western blot by probing with an AMPK α2 antibody. Densitometry was used to quantify the amount of AMPK α2 immunoreactivity in the glycogen fraction of each sample.

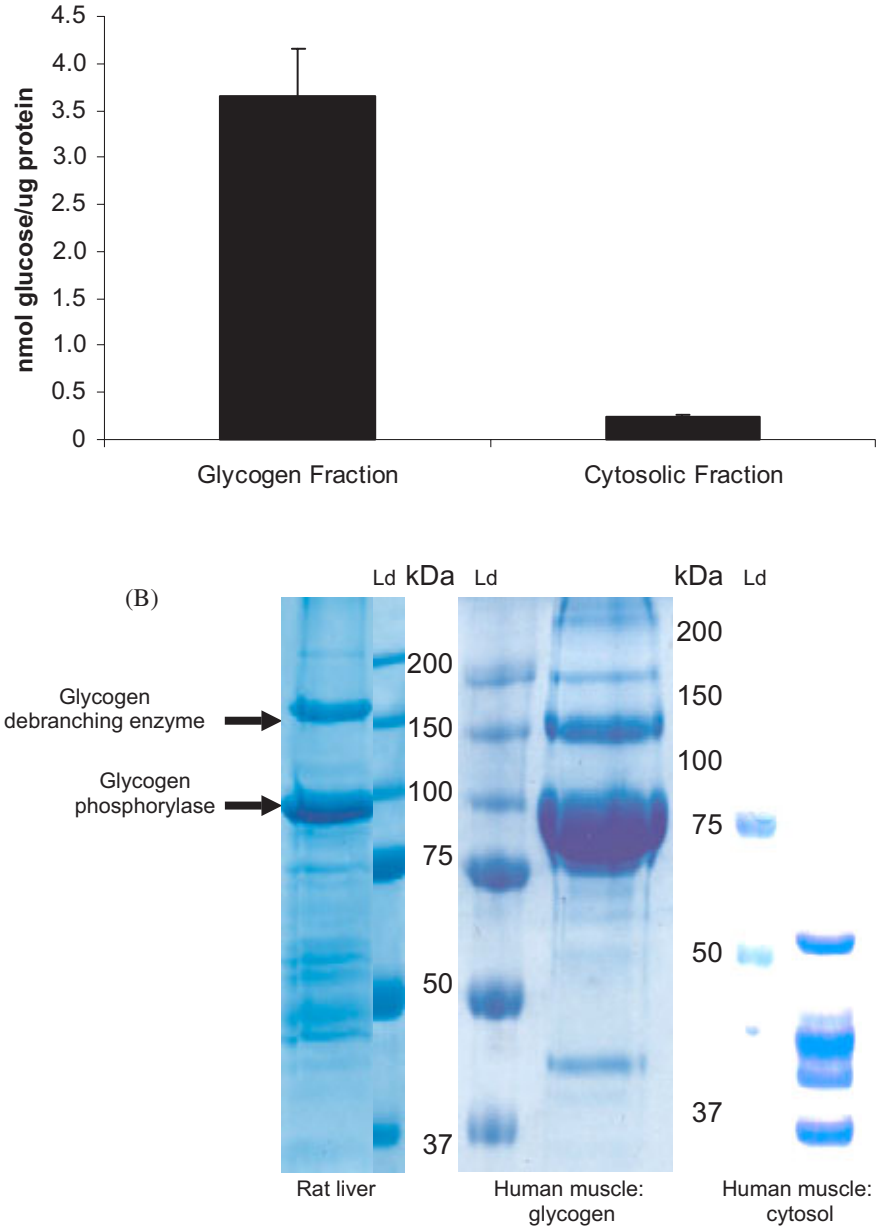
AMPK nuclear localization

Nuclear proteins were isolated and immunoblotted with antibodies for AMPK α1 and α2 as previously described (McGee et al. 2003). To further characterize the nuclear and cytosolic fractions and to ensure purification of the fractions, quality-control experiments were performed using antibodies against DHPR α1 (plasma membrane, t-tubules), SERCA2 (sarcoplasmic reticulum (SR)), uncoupling protein-3 (UCP3, mitochondria), and histone 1 (nucleus).

Quantification of GLUT4, and AMPK α1 and α2 mRNA using real-time RT-PCR

A portion of muscle (~30 mg) was extracted for total RNA using a modification of the acid guanidium thiocyanate – phenol chloroform extraction method described previously (Febbraio and Koukoulas 2000). The total RNA was subsequently quantified 2–3 more times before 1 μg of each total RNA sample was reverse transcribed in a 10 μL reaction

Fig. 1. Human glycogen was purified as described in Materials and methods. (A) The relative glycogen concentration in the purified glycogen fraction was ~20-fold greater than in the cytosolic fraction. (B) After purification, 30 μg of protein from human muscle cytosolic and glycogen fractions were stained with Coomassie blue dye as shown and major bands present in rat liver were identified by mass spectrometry. Ld, molecular mass marker.



containing 1× TaqMan RT buffer, 5.5 mmol/L $MgCl_2$, 500 mmol/L each 2′-deoxynucleoside 5′-triphosphate, 2.5 mmol/L random hexamers, 0.4 U/mL RNAse inhibitor, 1.25 U/mL Multiscribe reverse transcriptase (Applied Biosystems), and made up to volume with 0.05% DEPC-treated H_2O. Control samples were also analyzed, where all of the above reagents except the Multiscribe reverse transcriptase were added to RNA samples. The reverse-transcription reactions were performed using a GeneAmp PCR system 2400 (Perkin Elmer,Wellesley, Mass.) with conditions set at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. Two millilitres of 0.5 mol/L EDTA (pH 8.0) was added to each sample and stored at –20 °C until further analysis. Real-time PCR was employed to quantify human GLUT4 and AMPK α1 and α2 expression from the cDNA samples. Human probes and primers were designed (Primer Express version 1.0, Applied Biosystems) from the human gene sequence accessed from GenBank–EMBL and are presented in Table 1. Each TaqMan probe was labeled with the fluorescent tags FAM (6-carboxyfluorescein) at the 5′ end and TAMRA (6-carboxy-tetramethylrhodamine) at the 3′ end. We also amplified ribosomal 18S mRNA as our reference gene, since this gene is known to be constitutively expressed and was not altered by the dietary regime (data not shown). TaqMan probes and primers for 18S were supplied in a control reagent kit

Table 1. VE, VO_2, and RER in subjects during cycle exercise at 70% $VO_{2\ peak}$ with normal (control) or low (LG) pre-exercise muscle glycogen levels.

	VE (L/min)	VO_2 (mL(kg·min)$^{-1}$)	RER
Control			
Ex 20	70.3±3.8	36.1±2.0	0.93±0.01
Ex 40	66.4±4.6	34.2±2.7	0.90±0.01
Ex 60	62.3±3.5	33.6±2.7	0.88±0.01
LG			
Ex 20	73.2±5.7	39.2±2.2	0.85±0.01[a]
Ex 40	75.4±7.5	37.3±2.7	0.81±0.02[a]
Ex 60	73.3±7.3	36.1±2.5	0.81±0.01[a]

Note: VE, ventilatory exchange, VO_2, pulmonary oxygen uptake; RER, respiratory exchange ratio; Ex, exercise. Values are means ± SE; n = 7.

(Applied Biosystems). We quantified gene expression using a multiplex comparative critical threshold (C_T) method (Bio-Rad i Cycler IQ™, Hercules, Calif.) as previously described (Febbraio and Koukoulas 2000). PCRs were carried out in 25 μL reactions of TaqMan universal PCR master mix (1×), 50 nmol/L TaqMan 18S probe, 20 nmol/L 18S forward primer, 80 nmol/L 18S reverse primer, and probes and primers at specific concentrations ranging from 50 nmol/L to 150 nmol/L (probes) and from 50 nmol/L to 900 nmol/L (primers) for each gene of interest. The sequences of the forward (F) and reverse (R) primers and probes (P) are listed from 5′-3′ and are as follows: AMPKα1 (F) CAGGGACT GCTACTCCACAGAGA, (R) CCTTGAGCCTCAGCATCT GAA, (P) TCAGTTAGCAACTATCGATCTTGCCAAAG GAGT; AMPK α2 (F) CAACTGCAGAGAGCCATTCACTT, (R) GTGAAACTGAAGACAATGTGCTT, (P) CTGGCTC TCTCACTGGCTCTTTGACCG; and GLUT4 (F) CCTGCC AGAAAGAGTCTGAAGC, (R) ATCCTTCAGCTCAGCC AGCA, (P) CAGAAACATCGGCCCAGCCTGTCA. The specific concentrations for each gene were optimized in preliminary experiments. cDNA and control preparations not containing RT were amplified using the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Cell culture experiments

Primary skeletal muscle cell culture was established from muscle biopsies of 5 male subjects (24 ± 2 y, 75 ± 5 kg; $VO_{2\ peak}$ = 41 ± 3 mL·(kg·min)$^{-1}$) as described (Chen et al. 2005). Cells were grown to 80% confluence in α-MEM supplemented with 10% *v*/*v* FBS, 0.5% *v*/*v* penicillin, and 0.5% *v*/*v* Fungizone in a 37 °C incubator with 5% CO_2 before being differentiated for 4 d in α-MEM, 2% horse serum, and 1% each of penicillin, streptomycin, and amphotericin B (PSA). Cells were then infected with a constitutively active AMPK adenovirus (100 plaque-forming units (PFU)/plate) or control vector (AdGo, 100 PFU/plate) as described (Woods et al. 2000). Expression levels of both control and CA-AMPK infected cells was determined by visual examination of green fluorescence protein under ultraviolet light. Seventy-two hours post infection ~85% of all myotubes were infected in both control and CA-AMPK-infected cells (data not shown) and ~90% of myotubes were differentiated (data not shown). mRNA was then extracted according to manufacturer's instructions using the RNeasy mini kit (Qiagen, Doncaster, Australia), reverse transcribed using the thermoscript RT-PCR system (Invitrogen, Mt. Waverley, Australia), and GLUT4 mRNA was analysed using quantitative real-time PCR as previously described (Chen et al. 2005).

Calculations and statistics

Free ADP and AMP concentrations were calculated as described previously (McGee et al. 2003). Statistical analysis was performed by 2-way analysis of variance with repeated measures (time × trial) and specific differences were located using a Student–Newman–Keuls post hoc test. For cell culture experiments, a t test was performed. Statistical significance was set at $p \leq 0.05$. Data are expressed as the mean ± SEM.

Results

The exercise load (175 ± 18 W), oxygen uptake, and ventilation were not different at any point during exercise (Table 1), whereas the respiratory exchange ratio was lower in the low-glycogen trial. Plasma epinephrine was elevated with exercise in the low-glycogen condition relative to the control condition (60 min: 3.95 ± 0.5 nmol/L vs. 2.05 ± 0.3 nmol/L). The exercise–diet regimen was successful in producing normal (control) and low muscle glycogen (LG, $p < 0.05$) levels at the onset of exercise (Table 2). Muscle glycogen content was higher ($p < 0.05$) in Control at completion of exercise (Table 2) and glycogen use was greater ($p < 0.05$). Muscle nucleotide, creatine, and creatine phosphate concentrations were also unaltered by glycogen status (Table 2). Circulating insulin and glucose values have previously been reported (Watt et al. 2004).

AMPK α2 activity and ACCβ phosphorylation

AMPK activities and ACCβ phosphorylation have been reported previously (Watt et al. 2004) and are summarized in Table 3. AMPK α1 activity was unaltered by glycogen status or exercise. AMPK α2 activity was not different between control and LG groups at rest, but was greater (+134%, p = 0.05) in the LG compared with the control group at the cessation of exercise. Exercise increased ACCβ phosphorylation in the LG group (+112%, p = 0.05); however, in the control group, the increase in ACCβ did not achieve statistical significance.

AMPK association with human skeletal muscle glycogen

As shown in Fig. 2, AMPK α2 associates with glycogen in both control and LG, with less associated in the LG condition. At rest, glycogen-bound AMPK α1 and α2 immunoreactivity decreased by ~40% in the LG condition when compared with the control. However, after exercise, glycogen-bound AMPK α2 immunoreactivity did not change in the LG condition when compared with control (Fig. 2).

AMPK α2 nuclear translocation

The nuclear protein histone 1 was only observed within the nuclear fraction, suggesting significant purification of this fraction from skeletal muscle. However, there was some expression of DHPR α1 and SERCA2 in the nuclear

Table 2. Muscle glycogen, nucleotide (AMP), and creatine phosphate (PCr) concentrations before and immediately after 60 min cycle exercise at 70% $VO_{2\ peak}$ with normal (control) or low (LG) pre-exercise muscle glycogen levels.

	Glycogen (mmol/kg dm)	Free AMP (μmol/kg dm)	ATP (mmol/kg dm)	PCr (mmol/kg dm)
Control				
Rest	390±37	0.16±0.05	23.8±0.8	84.4±4.7
Exercise	111±35[a]	2.86±1.24[a]	24.0±1.6	42.4±9.8[a]
LG				
Rest	150±31[b]	0.10±0.04	24.3±1.0	89.3±4.8
Exercise	17±6[a,b]	4.42±1.61[a]	23.4±1.3	41.6±8.6[a]

Note: Values are means ± SE, n = 7. dm, dry mass.
[a]Significantly different from rest in the same trial.
[b]Significantly different from control at the same time ($p < 0.05$).

Table 3. AMPK α1 and α2 activities and ACCβ phosphorylation before and immediately after 60 min cycle exercise at 70% $VO_{2\ peak}$ with normal (control) or low (LG) pre-exercise muscle glycogen levels.

	AMPK α1 (pmol·(min·mg protein)$^{-1}$)	AMPK α2 (pmol·(min·mg protein)$^{-1}$))	PACCβ/ACCβ (arbitrary units)
Control			
Rest	0.25±0.06	0.30±0.06	0.39±12
Exercise	0.16±0.05	0.38±0.20	0.67±0.14[a]
LG			
Rest	0.17±0.03	0.23±0.04	0.38±0.10
Exercise	0.20±0.06	0.89±0.15[a,b]	1.11±0.25[a,b]

Note: Values are means ± SE, n = 7.
[a]Significantly different from rest in the same trial.
[b]Significantly different from control at the same time ($p < 0.05$).

fraction, albeit at much lower levels than in the cytosolic fraction, suggesting some contamination of the nuclear fraction with plasma membrane and (or) t-tubule and SR. No UCP3 was detected in the nuclear fraction. No difference was observed in the nuclear AMPK α2 content when comparing control with LG at rest. Exercise stimulated translocation of AMPK α2 to the nucleus in LG (Fig. 3), an effect that was not observed when skeletal muscle glycogen was elevated. Consequently the nuclear AMPK α2 concentration was greater ($p < 0.05$, power = 0.368) after exercise in LG compared with control. Cytosolic AMPK α1 and α2 protein expression was unaltered by exercise or glycogen levels (data not shown).

GLUT4 and AMPK α1 and α2 mRNA expression

GLUT4 mRNA expression was increased ($p < 0.05$, power = 0.697) after 60 min of exercise in the LG but not in the control group (Fig. 4A). AMPK α1 and α2 mRNA expression were not altered by exercise in either control or LG conditions (data not shown). Primary human myotubes infected with a CA-AMPK adenovirus had increased (p = 0.012) GLUT4 mRNA, relative to cells infected with a control vector (Fig. 4B).

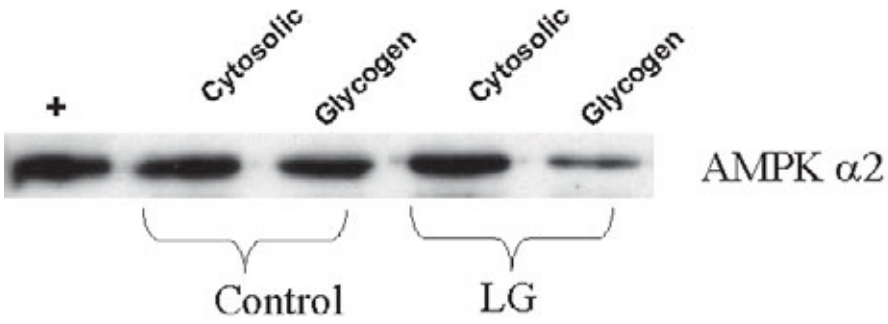
Discussion

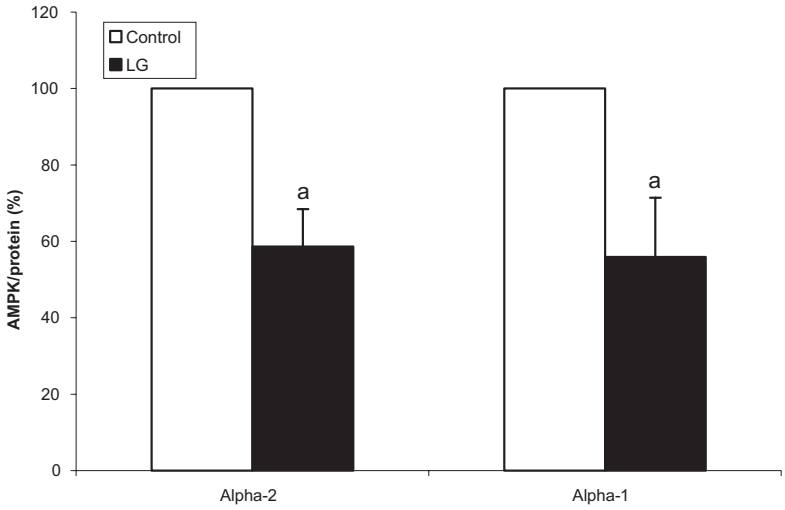
This study shows that increased activation of AMPK α2 when skeletal muscle glycogen is low is associated with increased AMPK α2 nuclear translocation and increased GLUT4 mRNA expression following acute exercise. These data suggest that skeletal muscle glycogen may be an important regulator of AMPK α2 activity, which in turn may play a role in the regulation of GLUT4 mRNA expression.

Several studies have reported enhanced AMPK α2 activation by contraction (Derave et al. 2000; Kawanaka et al. 2000) or AICAR (Aschenbach et al. 2002) (Wojtaszewski et al. 2002*a*) in skeletal muscle with low glycogen levels. We have previously reported (Watt et al. 2004) that the reduction of skeletal muscle glycogen by ~60% before exercise and by ~85% at the completion of exercise leads to increased AMPK α2 activation and ACCβ phosphorylation compared with individuals exercising with normal levels of muscle glycogen. The finding that AMPK α2 activity was not stimulated during moderate intensity exercise when muscle glycogen content was normal was unexpected; however, it should be noted that a recent report (Roepstorff et al. 2004) has also failed to demonstrate significant activation of AMPK α2 after exercise performed with similar glycogen levels. Despite the lack of change observed in AMPKα2 activity during the control condition, ACCβ phosphorylation was elevated. The finding of elevated ACCβ phosphorylation in control, despite no detectable increase in AMPK α2 activity is not the first time in which a dissociation between AMPK α2 activity and ACCβ phosphorylation has been observed (Wojtaszewski et al. 2002*b*).

The molecular basis for the relationship between glycogen and AMPK has only been recently elucidated by the identification of a glycogen-binding domain within the AMPK β subunit (Polekhina et al. 2003; Hudson et al. 2003). The regulation of AMPK by glycogen remains unclear; however, glycogen itself does not directly affect the activity of activated AMPK α1β1γ1 (Polekhina et al. 2003). In the present study, we hypothesized that the activation status of AMPK may be related to the amount of AMPK associated with glycogen at any time. To answer this question, we obtained a glycogen-rich fraction by differential centrifugation and isolated AMPK α1 and α2 by immunoprecipitation. We demonstrate that AMPK associates with the glycogen-rich fraction in vivo as shown in Fig. 2. Analysis of glycogen-associated AMPK α1 and α2 by immunoblot identified a 40% decrease in glycogen-associated AMPK α1 and α2 between control and low glycogen in the resting state (Fig. 2). Taken together, these data suggest that increased access of AMPK α2 to AMPKK may explain why AMPK α2 is more readily activated in individuals with low muscle glycogen. Despite the association between glycogen content and

Fig. 2. Representative Western blot of AMPK α2 (above) and densitometry/quantification (below) of AMPK α1 and α2 expression immunoprecipitated from cytosolic and glycogen fractions at rest. Values are means ± SE (n = 7). [a]Significantly different from control ($p < 0.05$).



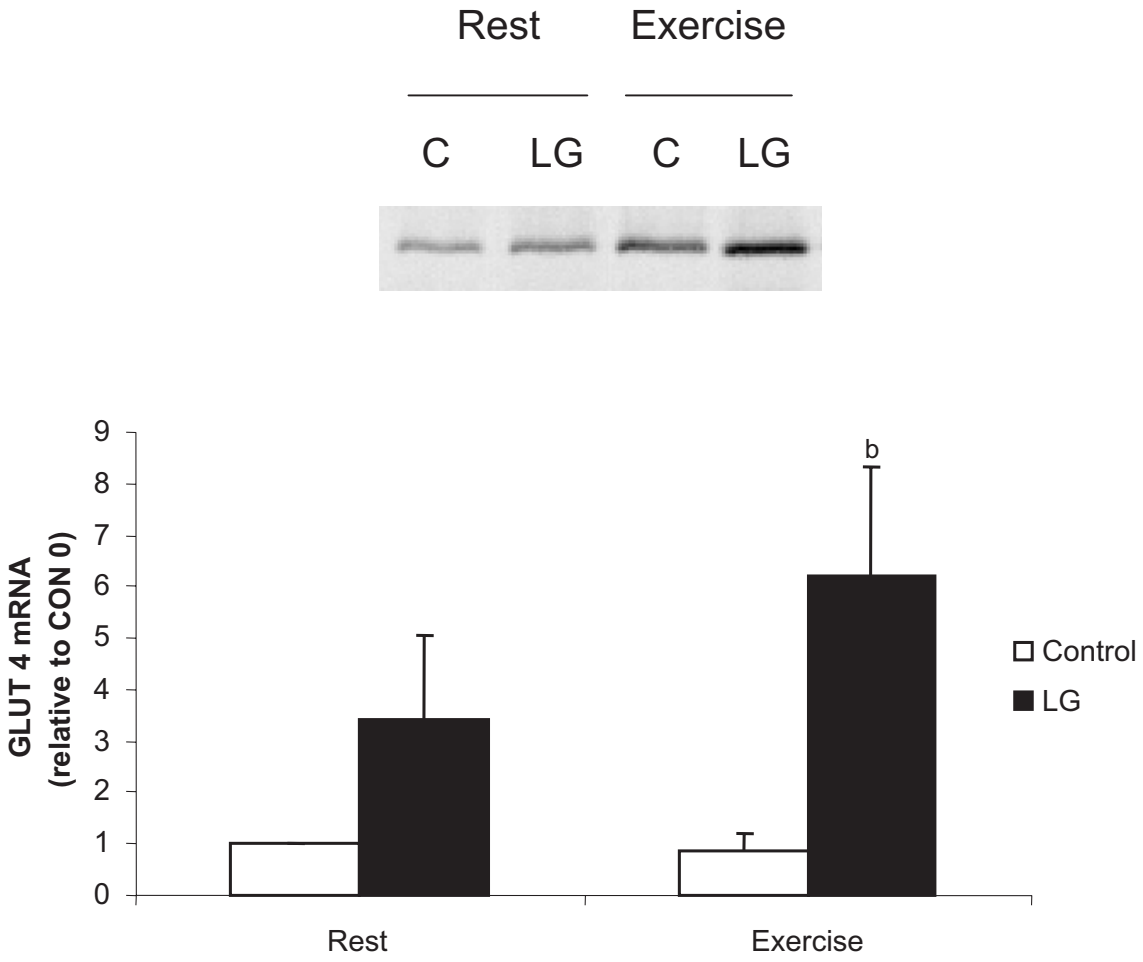


AMPK α2 expression and activity, we feel there are some difficulties in the current experimental design, which limit our interpretation of these results. Although we demonstrate a significant enrichment in glycogen content (~25-fold) and an association of this enriched glycogen fraction with AMPK α2 expression, we are unable to distinguish between non-specific trapping and (or) colocalization of AMPK within the glycogen-enriched fraction and direct binding. Although we (Polekhina et al. 2003) and others (Hudson et al. 2003) have demonstrated a direct interaction in mammalian cells the observation that AMPK α1 expression was also reduced despite no change in activity suggests that non-specific trapping of proteins within the glycogen pellet may have occurred. Owing to the limited size of human muscle biopsies, it was not possible to determine if the association of other proteins with glycogen was also reduced after exercise in the LG condition. These preliminary data suggest that future studies in human skeletal muscle in vivo examining the relationship between muscle glycogen and AMPK expression activity are warranted.

Despite the possibility of a direct effect of glycogen on AMPK activity, other explanations are plausible. Firstly, although we demonstrated increased AMPK α2 activity after exercise in LG in the absence of increased intramuscular concentrations of AMP, there is a possibility of a potential physiological effect of non-statistical, but biologically relevant, elevations in AMP due to the sensitivity of AMPK to small changes in AMP:ATP (Kemp et al. 2003). Secondly, the hormonal responses to altered glycogen levels in response to exercise could not be controlled in the present study. In rodents, pharmacological concentrations of isoprotenerol increase AMPK α2 activity, which suggests an important role of catecholamines in regulating AMPK (Minokoshi et al. 2002). In the present study, epinephrine levels were elevated during the low glycogen trial compared with the control trial (Watt et al. 2004), suggesting a possible stimulatory role for β-adernergic regulation of AMPK. Alternatively increased release of interleukin-6 (IL-6) under conditions of low muscle glycogen (Steensberg et al. 2001) may also be a contributing factor to the enhanced activation of AMPK (Kelly et al. 2004; MacDonald et al. 2003). Future studies investigating these possibilities are warranted.

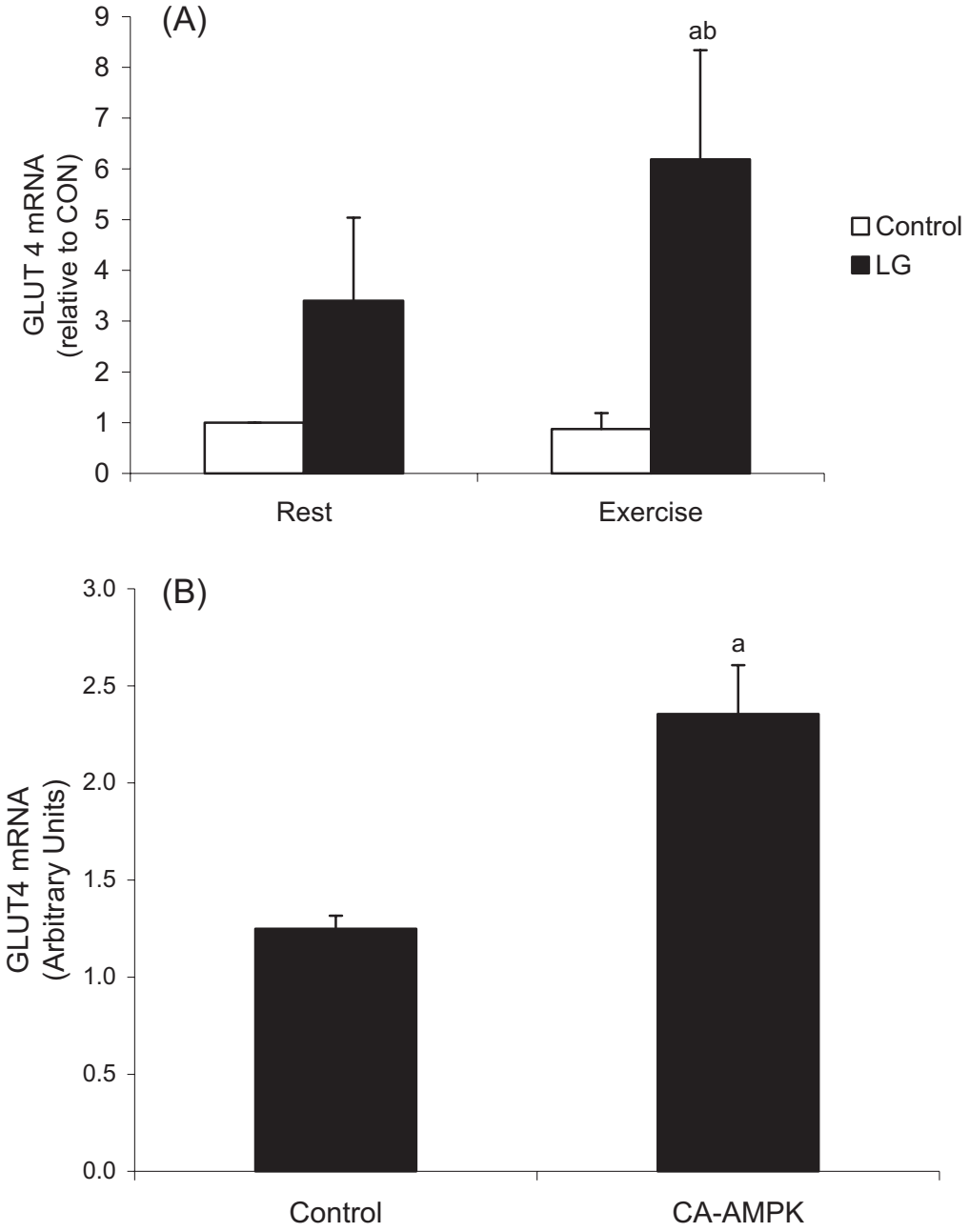
A single exercise bout enhances GLUT4 mRNA expression (Rodnick et al. 1990). Initial studies demonstrating that

Fig. 3. Representative Western blot (above) and densitometry/quantification (below) of nuclear localization of AMPK α2 before and immediately after 60 min cycle exercise at 70% $VO_{2\ peak}$ with normal (CON) or low (LG) pre-exercise muscle glycogen content. Values are means ± SE (n = 7). [b]Significantly different from 0 min of the same trial ($p < 0.05$).



exercise stimulated AMPK activity suggested that AMPK may be a potential regulator of GLUT4 transcription (Winder and Hardie 1996). In support of this idea are observations in rodent skeletal muscle demonstrating that the pharmacological activation of AMPK by AICAR increases GLUT4 expression to a similar degree as chronic exercise training (Holmes et al. 1999; Winder et al. 2000; Ojuka et al. 2000). Similarly, infection of rodent skeletal muscle cells (Fryer et al. 2002) with a constitutively active AMPK adenovirus also increases GLUT4 expression demonstrating a direct effect of AMPK on GLUT4 transcription, an effect which we have replicated in primary human myotubes (Fig. 4B). Taken together these data support the concept that AMPK can influence GLUT4 transcription. It should be noted that these studies do not indicate that AMPK is the only regulator of GLUT4 gene transcription. Indeed, recent data by Holmes et al. (Holmes et al. 2004) elegantly demonstrate in a AMPK dominant negative mouse that AMPK is not obligatory for the effects of exercise on GLUT4 expression. Furthermore, there was trend for resting GLUT4 mRNA to be higher in the low glycogen condition, in the absence of any difference in AMPK α2 activity. Other factors, such as the calcium calmodulin dependent protein kinase (Ojuka et al. 2002) may also contribute to exercise induced GLUT4 biogenesis and may be capable of compensating for reduced AMPK activity in the AMPK dominant negative model. The mechanisms by which AMPK activation may increase GLUT4 expression are unknown, but an important role of myocyte enhancer factor (MEF) 2A, MEF2D, and peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) has been demonstrated following AMPK activation by either exercise (Baar et al. 2002; Pilegaard et al. 2003) or AICAR treatment (Ojuka et al. 2002). In support of this concept, we recently demonstrated translocation of AMPK α2 to the nucleus with exercise in skeletal muscle (McGee et al. 2003), and increased phosphorylation of MEF-2 (McGee and Hargreaves 2004). In this study, nuclear AMPK α2 translocation after exercise occurred in the low glycogen trial only. Furthermore, the enhanced AMPK α2 nuclear translocation was associated with elevated GLUT4 mRNA following exercise. The mechanism(s) contributing to the translocation of AMPK α2 to the nucleus are unknown. Exercise in the Control trial did not significantly increase α2 nuclear translocation, consistent with the lack of change in AMPK α2 activity in this group, which was unexpected (Table 3). Future studies are needed to discern whether different degrees of AMPK activation result in altered translocation of AMPKα2 to the nucleus.

Fig. 4. (A) GLUT4 mRNA expression before and immediately after 60 min cycle exercise at 70% VO_2 peak with normal (CON) or low (LG) pre-exercise muscle glycogen content. Values are means ± SE (n = 7). (B) GLUT4 mRNA expression in primary human myotubes infected with a control vector (Ad-Go) or constitutively active AMPK (CA-AMPK). Values are means ± SE (n = 5). [a]Significantly different from control ($p < 0.05$). [b]Significantly different from 0 min of the same trial ($p < 0.05$).



In conclusion, our results suggest that reduced AMPK α2 association with glycogen may mediate the observed increase in AMPK α2 activity and α2 nuclear translocation observed following exercise when muscle glycogen content is reduced. Furthermore, our data suggest a role for elevated AMPK activity in the regulation of GLUT-4 expression in human skeletal muscle.

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