Predominant $\alpha2/\beta2/\gamma3$ AMPK activation during exercise in human skeletal muscle

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5′AMP-activated protein kinase (AMPK) is a key regulator of cellular metabolism and is regulated in muscle during exercise. We have previously established that only three of 12 possible AMPK $\alpha/\beta/\gamma$-heterotrimers are present in human skeletal muscle. Previous studies describe discrepancies between total AMPK activity and regulation of its target acetyl-CoA-carboxylase (ACC)β. Also, exercise training decreases expression of the regulatory $\gamma3$ AMPK subunit and attenuates $\alpha2$ AMPK activity during exercise. We hypothesize that these observations reflect a differential regulation of the AMPK heterotrimers. We provide evidence here that only the $\alpha2/\beta2/\gamma3$ subunit is phosphorylated and activated during high-intensity exercise *in vivo*. The activity associated with the remaining two AMPK heterotrimers, $\alpha1/\beta2/\gamma1$ and $\alpha2/\beta2/\gamma1$, is either unchanged (20 min, 80% maximal oxygen uptake ($\dot{V}_{O_2,peak}$)) or decreased (30 or 120 s sprint-exercise). The differential activity of the heterotrimers leads to a total α-AMPK activity, that is decreased (30 s trial), unchanged (120 s trial) and increased (20 min trial). AMPK activity associated with the $\alpha2/\beta2/\gamma3$ heterotrimer was strongly correlated to $\gamma3$-associated α-Thr-172 AMPK phosphorylation ($r^2 = 0.84$, $P < 0.001$) and to ACCβ Ser-221 phosphorylation ($r^2 = 0.65$, $P < 0.001$). These data single out the $\alpha2/\beta2/\gamma3$ heterotrimer as an important actor in exercise-regulated AMPK signalling in human skeletal muscle, probably mediating phosphorylation of ACCβ.

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5′AMP-activated protein kinase (AMPK) is activated in response to numerous cellular stresses including metabolic poisons (e.g. arsenite, azide, dinitrophenol), pharmacological agents (e.g. metformin, thiazolidinediones) and pathological stressors (e.g. glucose deprivation, ischaemia and hyperosmolarity) (Kahn *et al.* 2005). Exercise is another well-established physiological stimulus for AMPK activation in skeletal muscle. During exercise the AMPK system is activated in an intensity- and time-related manner in both rodent and human skeletal muscle (Winder & Hardie, 1996; Rasmussen & Winder, 1997; Chen *et al.* 2000, 2003; Fujii *et al.* 2000; Wojtaszewski *et al.* 2000). In general, studies in human muscle have reported that $\alpha2$- rather than $\alpha1$-associated activity is more prone to be increased during exercise *in vivo* (Chen *et al.* 2000; Fujii *et al.* 2000; Wojtaszewski *et al.* 2000; McConell *et al.* 2005).

Causal links between AMPK and cellular biological effects in skeletal muscle have been reported. Thus, AMPK seems to regulate several aspects of carbohydrate and fat metabolism either directly by interfering with key enzymes, or indirectly via regulation of gene transcription in resting muscle (Kahn *et al.* 2005). It is currently hypothesized that some of the beneficial effects of exercise on insulin action, and prevention of type 2 diabetes, are mediated by AMPK (Fisher *et al.* 2002; Iglesias *et al.* 2002), a premise that is supported by the observation that the antidiabetic drug metformin also acts through AMPK (Zhou *et al.* 2001; Musi *et al.* 2002).

AMPK is a member of the Snf1/AMPK serine/threonine protein kinase family, and exists as heterotrimeric complexes comprising a catalytic α subunit and regulatory β and γ subunits, each of which is expressed as two or more isoforms encoded by distinct genes (Hardie *et al.* 2003). The catalytic activity of the α subunits ($\alpha1$ and $\alpha2$) is increased after phosphorylation at the Thr-172 residue within the activation loop (Hawley *et al.* 1996), by upstream kinases (e.g. LKB1 and CaMKKα/β)(Hawley *et al.* 2003; Woods *et al.* 2003; Hawley *et al.* 2005; Hurley *et al.* 2005; Sakamoto *et al.* 2005). The β subunit of AMPK ($\beta1$ and $\beta2$) contains two conserved domains, one required for assembly of

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the $\alpha/\beta/\gamma$ heterotrimer, and a glycogen-binding domain that may target AMPK to glycogen particles (Hudson *et al.* 2003; Polekhina *et al.* 2003). The γ subunit ($\gamma1$, $\gamma2$ and $\gamma3$) contains two 'Bateman domains' (Bateman, 1997; Adams *et al.* 2004) that bind the regulatory nucleotides AMP and ATP. Binding of AMP activates the kinase allosterically and promotes the AMPK α-Thr-172 phosphorylation by upstream kinases as well as by inhibiting dephosphorylation by phosphatases (Hardie *et al.* 1999; Adams *et al.* 2004).

Although we have been able to detect all seven subunit isoforms of AMPK in human skeletal muscle (Frosig *et al.* 2004; Wojtaszewski *et al.* 2005), only three ($\alpha1/\beta2/\gamma1$, $\alpha2/\beta2/\gamma1$ and $\alpha2/\beta2/\gamma3$) of the 12 possible AMPK heterotrimeric complexes are present to a significant extent, and a marked difference in abundance among these is apparent ($\alpha2/\beta2/\gamma1 >> \alpha2/\beta2/\gamma3 \geq \alpha1/\beta2/\gamma1$) (Wojtaszewski *et al.* 2005). Until now, AMPK signalling in skeletal muscle in response to exercise has only been reported either as total AMPK activity, total α-Thr-172 phosphorylation, or as the activity associated with either of the two catalytic α isoforms, $\alpha1$ and $\alpha2$.

As mentioned, differential regulation between the two catalytic α subunits has been observed during conditions in which global AMPK activity is increased (Wojtaszewski *et al.* 2002; Nielsen *et al.* 2003). Disassociation between total AMPK activity and regulation of its downstream target acetyl-CoA-carboxylase (ACC)β has also been reported (Wojtaszewski *et al.* 2002). In addition, different AMPK heterotrimeric complexes display different sensitivities to allosteric activation by AMP *in vitro* (Salt *et al.* 1998). Thus, it is likely that also between the two different $\alpha2$ AMPK heterotrimers ($\gamma1$ *versus* $\gamma3$) differential regulation occurs in response to various stimuli. In fact, exercise training attenuates exercise-induced AMPK $\alpha2$ activity in skeletal muscle (Nielsen *et al.* 2003; Yu *et al.* 2003; McConell *et al.* 2005) and reduces the expression of AMPK $\gamma3$ mRNA and protein, whereas it increases expression of $\alpha1$, $\beta2$ and $\gamma1$ (Langfort *et al.* 2003; Nielsen *et al.* 2003; Frosig *et al.* 2004; Wojtaszewski *et al.* 2005). Extending on these observations, we hypothesize that exercise in human skeletal muscle primarily activates the $\alpha2/\beta2/\gamma3$ AMPK heterotrimer rather than $\alpha1/\beta2/\gamma1$ and $\alpha2/\beta2/\gamma1$, even though $\alpha2/\beta2/\gamma3$ only accounts for one-fifth of all heterotrimers.

In the present study, AMPK signalling in skeletal muscle was investigated *in vivo* during different exercise modalities in humans. We report that the three AMPK heterotrimers present in human skeletal muscle undergo differential regulation during exercise, an observation that is highly relevant in elucidating the physiological role of AMPK in skeletal muscle and in the exploration of muscular AMPK as a potential drug target in diseases associated with insulin resistance.

Methods

Thirty healthy young men (age 27 ± 1 years, body weight 79 ± 2 kg, body mass index (BMI) $24 \pm 0\,\mathrm{kg\,m^{-2}}$) gave their written informed consent to participate in this study, which was approved by the Copenhagen Ethics Committee (no. KF01277313) and was in agreement with the *Declaration of Helsinki II*.

One to two weeks prior to the experimental day the maximal oxygen uptake was determined during incremental cycling on an ergometer ($\dot{V}_{O_2,peak}$ $52 \pm 1\,\mathrm{ml\,min^{-1}\,kg^{-1}}$). Subjects with a $\dot{V}_{O_2,peak}$ below 40 or above 60 $\mathrm{ml\,min^{-1}\,kg^{-1}}$ were excluded from this study. Subjects were randomly assigned to one of three exercise interventions, and they all performed one test trial prior to the experiment. The subjects were instructed not to perform moderate-heavy physical activity the day before the experiment. On the experimental day, the subjects arrived at the laboratory in the morning 3 h after a light breakfast, with the use of minimal physical effort. After 45 min of rest, a needle biopsy from the vastus lateralis muscle was obtained under local anaesthesia (2–3 ml of 2% lidocaine). The subject then performed bicycle exercise in accordance to one of the following three protocols:

(1) Eleven subjects performed 20 min of bicycling at 80% $\dot{V}_{O_2,peak}$ ($77 \pm 3\%$ $\dot{V}_{O_2,peak}$, work rate $= 222 \pm 8$ W, total work performed 266 ± 9 kJ).

(2) Nine subjects performed a 120 s bicycle test at a work rate (376 ± 18 W) corresponding to 110% of peak work rate, which was defined as the highest work intensity maintained for a whole minute during the incremental $\dot{V}_{O_2,peak}$ test. Within the first 30 s, the subject increased the pedal frequency to the range of 100–120 $\mathrm{min^{-1}}$ before resistance was applied to the bike. Exercise duration was slightly variable (115 ± 4 s) due to the onset of fatigue at somewhat different time points (total work performed 43 ± 2 kJ). When occurring, fatigue quickly developed into a state where pedal frequency dropped markedly, at which time the test was terminated.

(3) Ten subjects performed a 30 s 'all out' sprint exercise trial. Without resistance on the bike, the subject increased the pedal frequency to $\sim 140\,\mathrm{min^{-1}}$ and after 10 s a workload corresponding to 7.5 N (kg body weight)$^{-1}$ was applied. On average the test lasted 30.5 ± 0.5 s, and in this period the average work rate was 658 ± 26 W (total work performed 21 ± 1 kJ).

Independent of exercise protocol, the subject was placed in the supine position immediately after exercise, and a second biopsy was obtained from the vastus lateralis muscle. One insertion was made in each leg, and the pre- and post-exercise biopsies were randomly taken in the dominant and the non-dominant leg. The biopsies were frozen in liquid nitrogen within 15 s after the termination of the exercise. The biopsies were stored at -80°C.

Muscle lysate preparation

Homogenates and lysates were prepared from 70 mg (w/w) muscle, that was freeze-dried, dissected free of visible fat, blood and connective tissue and homogenized in 50 mM Hepes (pH 7.5), 10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 10 μg ml^{-1} aprotenin, 10 μg ml^{-1} leupeptin, 2 mM Na_3VO_4, 3 mM benzamidine. Homogenates rotated end over end at 4°C for one hour. Lysates were prepared from the homogenates by centrifuging 25 min at 17 500 *g* and 4°C. Total homogenate and lysate protein content were analysed by the bicinchoninic acid method (Pierce Biotechnology, Inc., Rockford, IL, USA). Unless stated specifically, all chemicals were of analytic grade from Sigma-Aldrich (Denmark).

Muscle glycogen

Muscle glycogen content was measured in muscle homogenates (150 μg of protein) as glycosyl units after acid hydrolysis determined by a fluorometric method (Lowry & Passonneau, 1972).

Muscle lactate, adenosine triphosphate (ATP), creatine (Cr), and phosphocreatine (PCr)

Freeze-dried muscle biopsy specimens were extracted with perchloric acid, neutralized, and analysed for lactate, ATP, Cr and PCr as previously described (Lowry & Passonneau, 1972). The estimations of free concentrations of ADP and AMP were based on the near-equilibrium nature of the creatine phosphokinase and adenylate kinase reactions, respectively. Free ADP was estimated from the measured ATP, Cr and PCr contents, and the H^+ concentration was estimated using the measured muscle lactate content according to the formula presented by Mannion *et al.* (1993) for dry muscle. The equilibrium constant (K_{obs}) value employed for creatine phosphokinase was 1.66×10^9 M^{-1} (Lawson & Veech, 1979). Free AMP was estimated from the measured ATP and the estimated free ADP using a K_{obs} for adenylate kinase of 1.05 (Lawson & Veech, 1979).

SDS-PAGE and Western blotting

Muscle lysate proteins were separated using 10% Tris-HCl gels (Biorad, Denmark), and transferred (semidry) to PVDF-membranes (Immobilion Tranfer Membrane, Millipore A/S, Denmark). After blocking (Tris-buffered saline + 0.05% Tween-20 (TBST) + 2% skimmed milk), the membranes were incubated with primary antibodies (TBST + 2% skimmed milk) followed by incubation in horseradish peroxidase-conjugated secondary antibody (TBST + 2% skimmed milk) (DAKO, Denmark). Following detection and quantification using a CCD-image sensor and 1D software (Kodak Image Station, 2000MM, Kodak, Denmark), the protein content was expressed in arbitrary units relative to a human skeletal muscle control sample. By loading a control sample in different amounts it was ensured that the quantification was within the linear response range for each particular protein probed for.

Antibodies used for AMPK subunit isoform detection

The primary antibodies used for detection of the AMPK subunit isoforms α1, α2, β1, β2, γ1 and γ3 were as previously described (Wojtaszewski *et al.* 2005). Phosphorylation of AMPK α subunits (Thr-172) and acetyl-CoA-carboxylase-β (ACCβ) (Ser-221) was detected using phospho-specific antibodies (Cell Signalling Technology Inc., MA, USA and Upstate Biotechnology, MA, USA, respectively).

Detection of the AMPK heterotrimeric composition

The subunit isoforms (α1, α2, γ3 and α-Thr-172) was immunoprecipitated (IP) from 400 μg of muscle lysate using specific antibodies and sepharose-coupled G-protein overnight at 4°C in IP-buffer (50 mM NaCl, 1% Triton X-100, 50 mM NaF, 5 mM Na-pyrophosphate, 20 mM Tris-base (pH 7.5), 500 μM PMSF, 2 mM DTT, 4 μg ml^{-1} leupeptin, 50 μg ml^{-1} soybean trypsin inhibitor, 6 mM benzamidine and 250 mM sucrose). Samples of the IP, the post-IP lysate and the pre-IP lysate were prepared with Laemmli buffer and boiled for 3 min at 96°C, and analysed by SDS-PAGE and Western blotting using each of the seven (α1, α2, β1, β2, γ1, γ3 and α-Thr-172) antibodies recognizing the various AMPK subunits and the phosphorylated α subunits. The precipitation efficiency and the degree of co-immunoprecipitation were evaluated by comparing the signal in the pre- to that of the post-IP.

AMPK activity

Isoform-specific AMPK activity was measured on IPs from 200 μg of muscle lysate protein as described above. After an overnight incubation at 4°C, the IP was washed once in IP-buffer, once in 480 mM Hepes (pH 7.0) and 240 mM NaCl, and twice in 240 mM Hepes (pH 7.0) and 120 mM NaCl leaving 10 μl of buffer with the Sepharose after the last wash. The reaction ran for 30 min at 30°C in a total volume of 30 μl containing 833 μM DTT, 200 μM AMP, 100 μM AMARA-peptide, 5 mM $MgCl_2$, 200 μM ATP and 2 μCi of [γ-^{32}P]-ATP. The reaction was stopped by

spotting 25 μl onto a piece of P81 filter paper, which was then washed for four times 15 min in 1% phosphoric acid. The dried filter paper was analysed for activity using liquid scintillation counting.

The $\alpha2/\beta2/\gamma1$ activity was analysed by immunodepleting lysates for $\alpha2/\beta2/\gamma3$ heterotrimers by an overnight $\gamma3$ IP, followed by yet another overnight $\alpha2$ IP on which the $\alpha2/\beta2/\gamma1$ activity was measured. Neither one nor two overnight incubations at 4°C had any influence of the phosphorylation state of α-AMPK subunits (data not shown). Also the activity associated with each of the three complexes was unaffected by either one or two overnight IPs compared to a 4 h IP (data not shown).

Statistical analyses

Results are presented as mean ± S.E.M. Statistical evaluation was performed where appropriate by paired Student's *t* test or by one- or two-way ANOVA for repeated measurements using the Tukey's *post hoc* test. Differences between groups were considered statistically significant for $P < 0.05$.

Results

The heterotrimeric composition using co-immunoprecipitation (co-IP) analyses extend and confirm our previous findings of the presence of only three heterotrimers in human skeletal muscle; $\alpha1/\beta2/\gamma1$, $\alpha2/\beta2/\gamma1$ and $\alpha2/\beta2/\gamma3$. The present analyses were performed on biopsies from 11 subjects, allowing for interpersonal variation compared to the earlier study using a pool of biopsy material from different subjects (Wojtaszewski *et al.* 2005). Because the $\beta1$ isoform was not co-immunoprecipitated with either of the two α isoforms (data not shown) and all $\alpha1$ and $\alpha2$ co-immunoprecipitated with $\beta2$ (Wojtaszewski *et al.* 2005), the relative contribution to total AMPK heterotrimers of either of the two α isoforms can be estimated by comparing the amount of $\beta2$ co-immunoprecipitated with $\alpha1$ and $\alpha2$. Doing so, the present study reveals that complexes containing the $\alpha1/\beta2$ isoforms contribute with the minority (15 ± 9%, $n = 11$) of all complexes (Fig. 1*A* and *B*), whereas the $\alpha2/\beta2$ isoforms contribute with the majority (99 ± 1%, $n = 11$). Acknowledging the limitation of using the multi-step IP procedure and the semiquantitative nature of the Western blotting technique, in particular when comparing very strong to very low signals (as in the case of the $\alpha2/\beta2$ analysis (Fig. 1*B*)), we consider that the contribution of the $\alpha2/\beta2$ complexes is overestimated rather than that of the $\alpha1/\beta2$ complexes being underestimated.

No measurable $\gamma3$ was associated with $\alpha1$ (Fig. 1*A* and *D*). Comparing the amount of $\alpha2$ co-immunoprecipitated with $\gamma3$, it was evident that 17 ± 4% ($n = 11$) of $\alpha2/\beta2$ was associated with $\gamma3$ (Fig. 1*D*). Based on our previous observation that $\gamma1$, but not $\gamma2$, co-immunoprecipitated with $\alpha1$, $\alpha2$ or $\beta2$ (Wojtaszewski *et al.* 2005), we anticipate that the remaining trimeric complexes are $\alpha1/\beta2/\gamma1$ and $\alpha2/\beta2/\gamma1$. However, the anti-$\gamma1$ antibodies available do not fully immunoprecipitate all $\gamma1$ protein, making measurements of the relative amount of $\alpha1/\beta2/\gamma1$ and $\alpha2/\beta2/\gamma1$ complexes difficult. Together with our previous results (Wojtaszewski

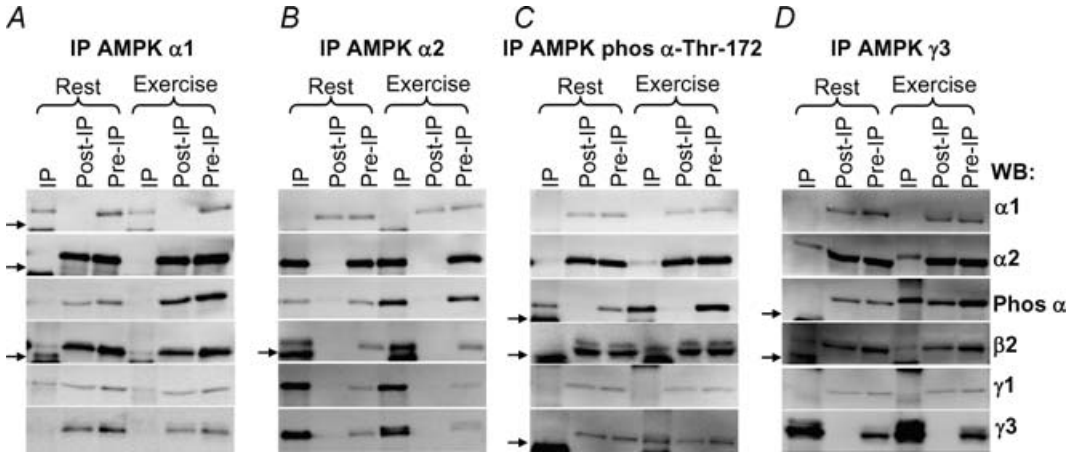

Figure 1. AMPK heterotrimer composition and phosphorylation in human skeletal muscle
Lysates were prepared from biopsies taken in the vastus lateralis muscle before and after 20 min of exercise at 80% $\dot{V}_{O_2,peak}$ ($n = 11$). From 400 μg of lysate AMPK $\alpha1$ (*A*), $\alpha2$ (*B*), phospho α-Thr-172 (*C*) or $\gamma3$ (*D*) was immunoprecipitated (IP). The figure shows representative blots of the IP, post-IP and pre-IP (lysate) in the rested and exercised state. One-eighth of the IP corresponding to 50 μg was loaded together with 20 μg of the post- and pre-IPs. The blotted membranes were analysed with anti-$\alpha1$, -$\alpha2$, -phospho α-Thr-172, -$\beta2$, -$\gamma1$ and -$\gamma3$ as indicated to the far right. The small arrow indicates IgG light and heavy chains on the blots.

et al. 2005) indicating a minor existence of $\alpha1/\beta2$ and $\alpha2/\beta2$ dimers, the approximate distribution of the three AMPK heterotrimers can however, be estimated to be ~15% $\alpha1/\beta2/\gamma1$, ~65% $\alpha2/\beta2/\gamma1$ and ~20% $\alpha2/\beta2/\gamma3$.

Comparing muscle biopsies obtained before and after exercise (20 min, 80% $\dot{V}_{O_2,peak}$), no differences were apparent in the subunit isoform expression or the heterotrimeric subunit composition when analysed by co-IP using the anti-$\alpha1$, $\alpha2$ and $\gamma3$ antibodies (Fig. 1*A*, *B* and *D*).

Limited to only three major heterotrimers, we aimed to evaluate the activation pattern among these during exercise. First we investigated the Thr-172 phosphorylation at α AMPK (p-AMPK) using a phospho-specific antibody (no signal observed using this antibody after phosphatase treatment of lysate (data not shown)). All phosphorylated α subunits were precipitated with the AMPK α-Thr-172 antibody (Fig. 1*C*). Interestingly, the changes associated with exercise (20 min, 80% $\dot{V}_{O_2,peak}$) were largely confined to $\alpha2$ (Fig. 1*B* and *C*) rather than to $\alpha1$ complexes (Fig. 1*A* and *C*). Surprisingly, only ~10% of all $\alpha2$ protein was phosphorylated during exercise (Fig. 1*C*). When measured by direct blotting, the increase compared to rest in p-AMPK with exercise corresponded to $251 \pm 28\%$ ($n = 11$), i.e. the increase (exercise minus rest) corresponded to $55 \pm 5\%$ ($n = 11$) of the phosphorylation seen during exercise (Fig. 2). In agreement, in the exercised state $59 \pm 6\%$ ($n = 11$) of all phosphorylated α subunits was associated with $\gamma3$ (Fig. 1*D*). Because p-AMPK associated with $\gamma3$ at rest was hardly detectable (Fig. 1*D*), and because the changes in total p-AMPK corresponded to the changes in p-AMPK associated with $\gamma3$ (55% *versus* 59%), these data suggest that the $\gamma3$-containing heterotrimers are retaining the majority of the Thr-172 α-AMPK phosphorylation during this type of exercise. In line with this, no apparent difference in the $\gamma1$-associated p-AMPK was observed between rest and exercise (Fig. 1*C*). These data also suggest that in resting muscle most or all of the phosphorylated AMPK heterotrimers must be $\alpha1/\beta2/\gamma1$ and $\alpha2/\beta2/\gamma1$. Finally, the phosphorylated $\gamma3$ heterotrimers in the exercised state accounted only for $32 \pm 5\%$ ($n = 11$) of all the $\gamma3$ heterotrimers, still leaving the majority of these unphosphorylated and available for activation (Fig. 1*C*).

The present data on p-AMPK strongly indicate a differential regulation of the $\alpha2$-associated activity depending on the γ isoform present in the complex; at least during activation of the AMPK system by a high-intensity exercise regime. To confirm this, activity assays were performed using IP of either $\alpha1$, $\alpha2$ or $\beta2$, in principle representing the trimers $\alpha1/\beta2/\gamma1$, $\alpha2/\beta2/\gamma1 + \alpha2/\beta2/\gamma3$ and $\alpha1/\beta2/\gamma1 + \alpha2/\beta2/\gamma1 + \alpha2/\beta2/\gamma3$, respectively. In agreement with the increased total p-AMPK (Fig. 2), total AMPK activity (as measured by $\beta2$ IP) increased (Fig. 3*A*). This was fully accounted for by the increase in $\alpha2$ AMPK activity as the $\alpha1$ AMPK activity remained unchanged by exercise (Fig. 3*A*). To fully decipher which of the two $\alpha2$ complexes are regulated under these conditions, we performed analysis using first IP of $\gamma3$ (representing the $\alpha2/\beta2/\gamma3$ heterotrimer). From the post-$\gamma3$ IP supernatant, $\alpha2$ was subsequently immunopurified (representing the $\alpha2/\beta2/\gamma1$ heterotrimer). This $\gamma3$ immunodepletion procedure was necessary to measure the $\alpha2/\beta2/\gamma1$ activity as we have not been able to immunoprecipitate $\gamma1$ completely from human muscle. The activity associated with $\alpha2/\beta2/\gamma3$ increased in response to exercise, and in fact the absolute increase was largely similar to that seen in total $\alpha2$-associated activity (i.e. $\alpha2/\beta2/\gamma1 + \alpha2/\beta2/\gamma3$) (Fig. 3*A*), suggesting that only the activity of the $\alpha2/\beta2/\gamma3$ heterotrimer was increased (Fig. 4*A*). This was substantiated by the observation that the $\alpha2$-associated activity remaining after $\gamma3$ immunodepletion (i.e. $\alpha2/\beta2/\gamma1$ activity) was not regulated by exercise (Fig. 4*A*). Thus, of the three AMPK trimers, only the $\alpha2/\beta2/\gamma3$ was phosphorylated and activated by this exercise intervention (20 min, 80% $\dot{V}_{O_2,peak}$).

We were then intrigued to investigate the AMPK regulation during more severe exercise during which cellular energy status is more markedly disturbed. Previously is has been shown by Chen *et al.* (2000) that a 30 s bicycle sprint activates both AMPK $\alpha1$ and $\alpha2$, whereas more moderate exercise normally only activates $\alpha2$ (Fujii *et al.* 2000; Wojtaszewski *et al.* 2000, 2003; Nielsen *et al.* 2003), as seen in the 20 min trial. Thus, subjects performed an exercise regime leading to exhaustion within either 120 or 30 s. In accordance with the marked differences

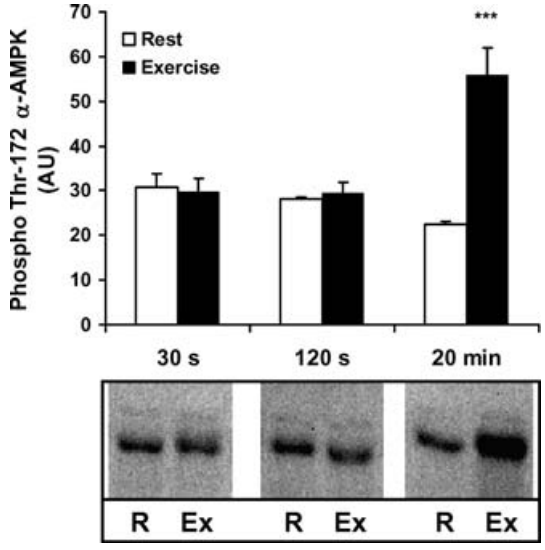

Figure 2. Thr-172 α-AMPK phosphorylation
Lysates are from the vastus lateralis muscle before and after exercise for 30 s ($n = 10$), 120 s ($n = 9$) and 20 min ($n = 11$), respectively; 25 μg of lysate were run on SDS-PAGE and blotted for phosphorylation of Thr-172 α-AMPK. Results are means ± S.E.M., $^{***}P < 0.001$, difference between rest and exercise.

in exercise intensity (222 ± 8, 376 ± 18 and 658 ± 26 W for the 20 min, 120 s and 30 s trial, respectively), the rate of glycogen degradation was markedly different (8 ± 1, 49 ± 8 and 172 ± 22 mmol (kg dry weight)$^{-1}$ min^{-1} for the 20 min, 120 s and 30 s trial, respectively). Compared to the 20 min protocol, the two high-intensity exercise regimes lead to significantly higher accumulation of muscle lactate and a higher PCr degradation (Table 1). No significant differences were observed between the two short-term high-intensity exercise regimes regarding

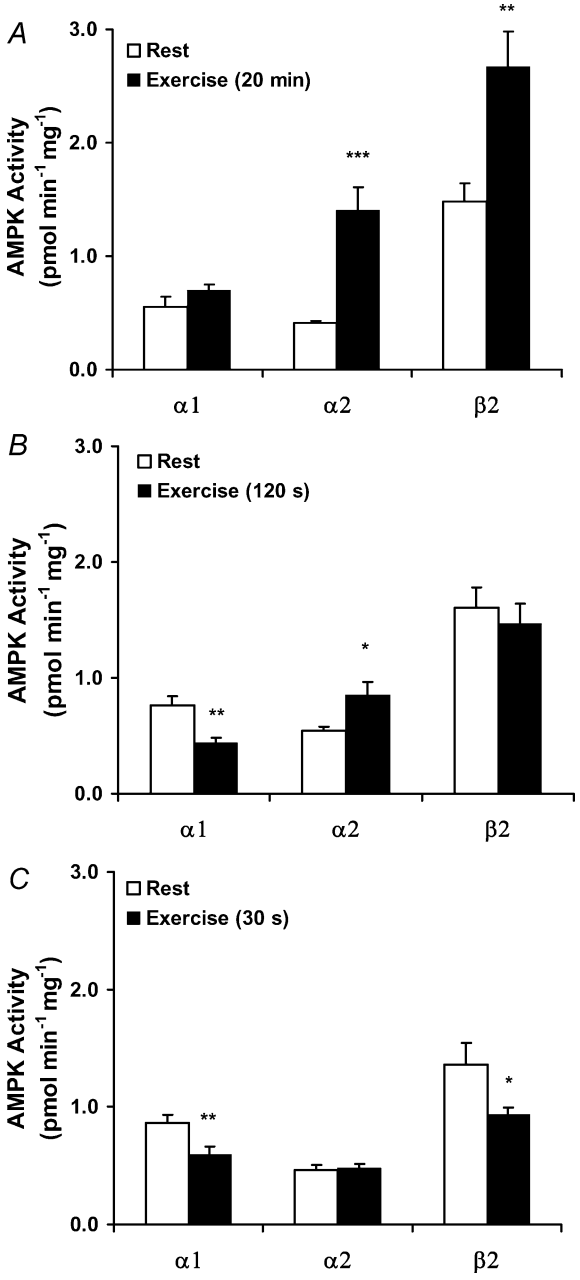

Figure 3. AMPK subunit-associated activity in response to exercise
Lysates are from the vastus lateralis muscle before and after exercise for *A*, 20 min ($n = 11$); *B*, 120 s ($n = 9$) and *C*, 30 s ($n = 10$). AMPK $\alpha1$ ($\alpha1/\beta2/\gamma1$), $\alpha2$ ($\alpha2/\beta2/\gamma1 + \alpha2/\beta2/\gamma3$) or $\beta2$ $\alpha1/\beta2/\gamma1 + \alpha2/\beta2/\gamma1 + \alpha2/\beta2/\gamma3$) were immunoprecipitated from 200 μg of lysate, and activity against the AMARA peptide was measured in the presence of 200 μM AMP. Results are means ± S.E.M., $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, significant differences between rest and exercise.

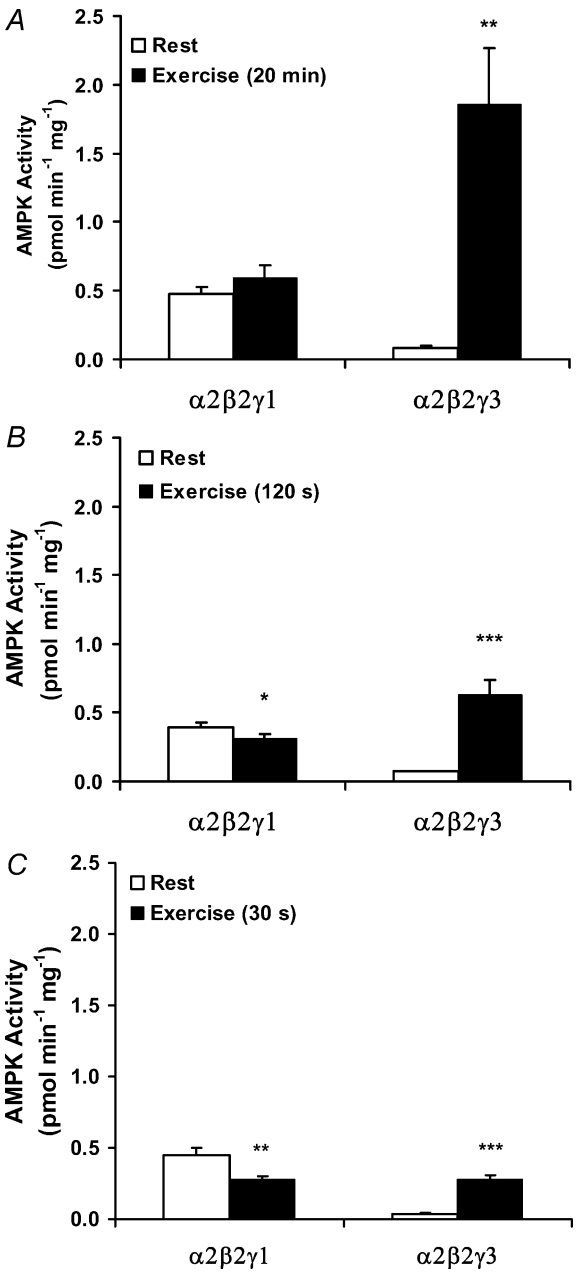

Figure 4. AMPK subunit-associated activity in response to exercise
Lysates are from the vastus lateralis muscle before and after exercise for *A*, 20 min ($n = 11$); *B*, 120 s ($n = 9$) and *C*, 30 s ($n = 10$). AMPK $\gamma3$ ($\alpha2/\beta2/\gamma3$) was immunoprecipitated from 200 μg of lysate, and activity against the AMARA peptide was measured in the presence of 200 μM AMP. The $\alpha2/\beta2/\gamma1$-associated activity was determined by IP of $\alpha2$ after immunodepleting the lysate for $\gamma3$. Results are means ± S.E.M., $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, significant differences between rest and exercise.

Table 1. Parameters of cellular energy status

	30 s ($n = 10$)			120 s ($n = 9$)			20 min ($n = 11$)		
	Rest	Exercise	Delta	Rest	Exercise	Delta	Rest	Exercise	Delta
Lactate, mmol (kg dry weight)$^{-1}$	7 ± 2	89 ± 6***†††	82 ± 6†††	5 ± 1	86 ± 3***†††	81 ± 3†††	5 ± 1	21 ± 3***	16 ± 3
Cr, mmol (kg dry weight)$^{-1}$	48 ± 4	103 ± 5***†††	55 ± 3†††	59 ± 7	110 ± 6***†††	51 ± 6†††	43 ± 4	62 ± 7***	19 ± 6
PCr, mmol (kg dry weight)$^{-1}$	81 ± 5	41 ± 3***†††	−41 ± 5††	90 ± 7	36 ± 4***†††	−54 ± 9†††	81 ± 5	76 ± 6	5 ± 9
PCr/(Cr + PCr)(× 100)	63 ± 1	29 ± 0***†††	−34 ± 1†††	61 ± 1	24 ± 2***†††	−36 ± 3†††	66 ± 1	56 ± 4**	− 10 ± 4
ATP, mmol (kg dry weight)$^{-1}$	25 ± 1	21 ± 1	5 ± 1	26 ± 1	20 ± 1	6 ± 2	26 ± 2	26 ± 2	− 1 ± 3
AMP_{free}, μmol (kg dry weight)$^{-1}$	0.8 ± 0.1	1.4 ± 0.2‡‡‡	0.7 ± 0.2	1.1 ± 0.2	2.6 ± 0.7‡‡‡	1.5 ± 0.6	0.7 ± 0.1	1.5 ± 0.5‡‡‡	0.9 ± 0.5
AMP_{free}/ATP ($\times 10^3$)	32 ± 2	60 ± 8‡‡‡	27 ± 8	41 ± 5	129 ± 35‡‡‡	88 ± 35	27 ± 3	70 ± 28‡‡‡	43 ± 27
Glycogen, mmol (kg dry weight)$^{-1}$	436 ± 45	350 ± 39***	−86 ± 11†††	425 ± 24	340 ± 28***	− 98 ± 15††	454 ± 30	282 ± 27***	−157 ± 13

Values are mean ± S.E.M. Cr, creatine; PCr, phosphocreatine; AMP_{free}, free AMP calculated as described in Methods. Delta: exercise minus rest value, calculated as mean of the individual differences. Significantly different from Rest (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Significantly different from 20 min (††$P < 0.01$, †††$P < 0.001$). Significant main effect of exercise (‡‡‡$P < 0.001$).

lactate accumulation, glycogen degradation or changes in PCr and ATP concentrations (Table 1).

In response to both high-intensity exercise protocols, there was, surprisingly, no detectable increase in p-AMPK (Fig. 2). In line with this, the total AMPK activity ($\beta 2$ IP) was unchanged (120 s trial) or slightly decreased (30 s trial) (Fig. 3*B* and *C*). These outcomes were again the result of highly differential regulation of the activity associated with the $\alpha 1$ and $\alpha 2$ complexes. Thus, in the 120 s trial, $\alpha 1$-associated activity decreased significantly, whereas $\alpha 2$ activity increased significantly. The magnitude of the changes was approximately equal, giving rise to the unchanged total AMPK activity (Fig. 3*B*). In the 30 s trial, $\alpha 1$-associated activity also decreased significantly, whereas the $\alpha 2$ activity was unchanged. In agreement, total AMPK activity slightly decreased in this trial (Fig. 3*C*). Between the two $\alpha 2$ complexes, only the $\alpha 2/\beta 2/\gamma 3$ activity increased in response to exercise in both trials, whereas a minor but significant decrease in $\alpha 2/\beta 2/\gamma 1$ activity was observed (Fig. 4*B* and *C*). Thus, even during short-term high-intensity exercise, only the $\alpha 2/\beta 2/\gamma 3$ was activated.

We measured the amount of phosphorylated α subunits associated with $\gamma 3$ in all three exercise trials (Fig. 5*A*). The analyses reveal a similar pattern of regulation as for the $\gamma 3$-associated AMPK activity (Fig. 4*A*–*C*). In line with this, a strong significant correlation was seen between these two measures ($r^2 = 0.84$, $P < 0.001$), suggesting that differences in $\gamma 3$ activity between trials indeed were related to changes in phosphorylated α subunits associated with $\gamma 3$.

ACCβ is a target for AMPK in muscle. As expected, during exercise in all three trials Ser-221 phosphorylation of ACCβ increased (Fig. 6*A*). Like the $\gamma 3$-associated AMPK activity, the changes in ACCβ phosphorylation were regulated in a workload- and/or time-dependent manner, and a strong and significant correlation was observed between these measures ($r^2 = 0.65$, $P < 0.001$)(Fig. 6*B*). Although this does not prove a causal relationship, it indicates that phosphorylation of ACCβ may be a good endogenous predictor of $\alpha 2/\beta 2/\gamma 3$, but not of total AMPK activity during exercise.

Discussion

The three AMPK heterotrimeric complexes present in human skeletal muscle display differential regulation during exercise. Previous studies have only divided the heterotrimers into two groups, the $\alpha 1$- and $\alpha 2$-containing heterotrimers. Doing so, it has been shown that AMPK $\alpha 2$ activity is activated during many exercise regimes with different work intensities and lengths (Chen *et al.* 2000, 2003; Fujii *et al.* 2000; Wojtaszewski *et al.* 2000, 2003; Nielsen *et al.* 2003; McConell *et al.* 2005; Dreyer *et al.* 2006). It seems that $\alpha 2$ is activated in a workload-dependent manner, and only at exercise intensities above 50% $\dot{V}_{O_2,peak}$ (Fujii *et al.* 2000; Wojtaszewski *et al.* 2000; Chen *et al.* 2003), or if low-intensity exercise continues to exhaustion (Wojtaszewski *et al.* 2002). Activation of $\alpha 1$ is somewhat more controversial, as several studies have been unable to show $\alpha 1$ activation (Fujii *et al.* 2000; Wojtaszewski *et al.* 2000, 2003; Nielsen *et al.* 2003; Yu *et al.* 2003; Akerstrom *et al.* 2006), whereas others have shown an activation at various work intensities (Chen *et al.* 2000, 2003; McConell *et al.* 2005; Lee-Young *et al.* 2006).

To get a better understanding of the activation of AMPK, we have analysed the specific heterotrimers separately. Our data predict a unique role of the $\alpha 2/\beta 2/\gamma 3$ heterotrimer in human skeletal muscle, as only this heterotrimer is phosphorylated and activated during the three high-intensity exercise regimes investigated. The $\alpha 2/\beta 2/\gamma 3$ heterotrimer only constitutes one-fifth of all AMPK heterotrimers, and only one-third of these heterotrimers contain phosphorylated $\alpha 2$ protein, indicating a large pool of spare AMPK signalling within the cell even during high-intensity exercise. Accordingly, only a small fraction (10%) of the total $\alpha 2$ protein pool is phosphorylated during exercise. AMPK can be activated through various mechanisms and elicits a number of different events in the cell depending on the stress situation. Our data support the idea that the whole pool of AMPK heterotrimers in the cell is divided into different subsets reacting to various stimuli and acting on various targets. Part of this diversity seems to be related

to expression of different heterotrimers which create a basis for different AMP sensitivities but which may also localize the heterotrimers at different cellular locations. The question remains as to whether an exercise regime different from the three investigated, or other stimuli of AMPK may lead to another activation pattern of the three AMPK heterotrimers.

Total AMPK activity and total α-AMPK phosphorylation was regulated differently during the three exercise regimes, and did not covary with the $\gamma3$-associated AMPK activity. Surprisingly, this was due to a different regulation of the activity associated with $\gamma1$. Thus, in the 30 and 120 s trials, the increase in $\alpha2/\beta2/\gamma3$ activity was counterbalanced by decreases in $\alpha1/\beta2/\gamma1$ and $\alpha2/\beta2/\gamma1$ activities. In contrast, the $\alpha1/\beta2/\gamma1$ and $\alpha2/\beta2/\gamma1$ activities were largely unchanged in the 20 min 80% $\dot{V}_{O_2,peak}$ exercise trial, resulting in a coordinated regulation of the total AMPK activity/phosphorylation and the $\alpha2/\beta2/\gamma3$ activity/phosphorylation. It is interesting that both $\gamma1$ heterotrimers respond similarly in all three exercise trials regardless of α isoform in the complex. It is evident that $\alpha1$ and $\alpha2$ have different intrinsic activities (Michell *et al.* 1996), but our observations show that regulation of their activity is highly dependent on the γ isoform in the complex. This isoform-specific regulation of the heterotrimers suggests that the action of the upstream kinases or phosphatases is highly specific. The nature and regulation of the upstream

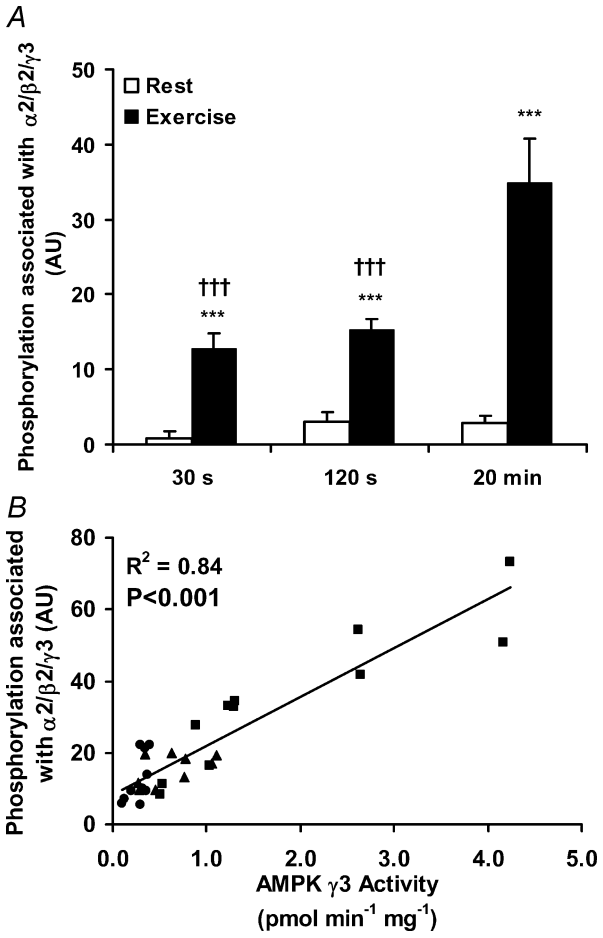

Figure 5. AMPK phosphorylation in relation to AMPK $\gamma3$-associated activity
Lysates are from the vastus lateralis muscle before and after exercise for 30 s ($n = 10$), 120 s ($n = 9$) and 20 min ($n = 11$), respectively. *A*, 25 μg of lysate were run on SDS-PAGE and blotted for Thr-172 phosphorylation of α-AMPK. These signal intensities were multiplied with the amount of Thr-172 phosphorylated α-AMPK co-immunoprecipitated with $\gamma3$ to get the level of phosphorylation associated with the $\gamma3$ trimers. *B*, relationship between the $\gamma3$-associated level of Thr-172 α-AMPK phosphorylation and the AMPK activity of $\alpha2/\beta2/\gamma3$. ● = 30 s trial ($n = 10$); ▲ = 120 s trial ($n = 9$) and ■ = 20 min trial ($n = 11$). A significant coefficient was found with linear regression analysis ($r^2 = 0.84$, $P < 0.001$). Results are means ± S.E.M., ***$P < 0.001$, difference between rest and exercise, †††$P < 0.001$, significantly different from the 20 min trial.

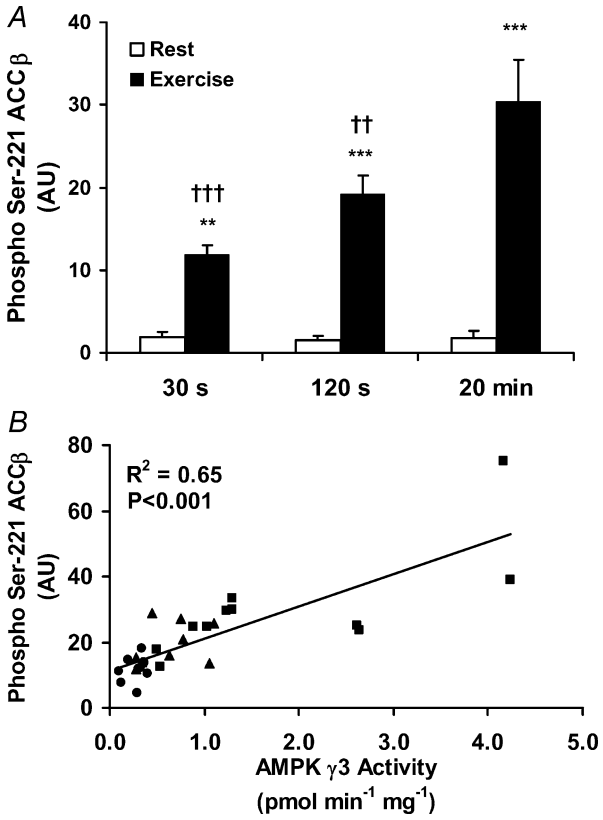

Figure 6. ACCβ phosphorylation in relation to AMPK $\gamma3$-associated activity
Lysates are from the vastus lateralis muscle before and after exercise for 30 s ($n = 10$), 120 s ($n = 9$) and 20 min ($n = 11$), respectively. *A*, 25 μg of lysate were run on SDS-PAGE and blotted for phospho Ser-221 ACCβ. *B*, relationship between the level of ACCβ phosphorylation and the AMPK activity of $\alpha2/\beta2/\gamma3$. ● = 30 s ($n = 10$), ▲ = 120 s ($n = 9$) and ■ = 20 min ($n = 11$). A significant coefficient was found with linear regression analysis ($r^2 = 0.65$, $P < 0.001$). Results are means ± S.E.M., ***$P < 0.001$, difference between rest and exercise, †$P < 0.05$ and †††$P < 0.001$, significantly different from the 20 min trial.

kinase in human skeletal muscle is largely unknown, but a minor increase in activity of an unidentified AMPKK enzyme with exercise has been reported in human skeletal muscle (Chen *et al.* 2003). Recently, both CaMKKα/β and LKB1 have been identified as upstream kinases (Woods *et al.* 2003; Hurley *et al.* 2005; Hawley *et al.* 2003, 2005). Whereas the role for CaMKKα/β in muscle is unknown, LKB1 seems to be an important upstream kinase for AMPK during contractions, although LKB1 activity *per se* is not regulated during contractile activity in rodent muscle (Sakamoto *et al.* 2004; Taylor *et al.* 2004). Thus, the highly selective activation of the $\alpha2/\beta2/\gamma3$ heterotrimer could be depending on colocalization of this complex with the upstream regulators as has been seen for other kinases in the same kinase family (Hook & Means, 2001). Since AMP binding facilitates the phosphorylation of liver AMPK by LKB1, another explanatory scenario is that such an effect is particular favourable for LKB1 acting on the $\alpha2/\beta2/\gamma3$ heterotrimer in human skeletal muscle.

Surprisingly, we were not able to reproduce the findings by Chen and coworkers of an increase in $\alpha1$-associated AMPK activity during either of the two high-intensity exercise regimes of which one, the 30 s trial, was similar to the one used by Chen *et al.* (2000). A range of methodological differences may explain this discrepancy, e.g. the metabolic fitness of the subjects studied and perhaps the different antibodies used. In this regard our co-IP experiments suggest a high degree of α-isoform specificity of the two antibodies used in the present study. Furthermore, the total AMPK activity pulled down by these two antibodies adds up to the total activity measured in the $\beta2$ IP, indicating consistency within the activity data. Still, although we and others have not previously reported significant increases in $\alpha1$-associated AMPK activity, the present data do not exclude that such activation may take place during different exercise regimes or in a different group of subjects.

Although, $\alpha2/\beta2/\gamma3$ phosphorylation/activation may be related to total work performed in the present study, neither the rate of energy turnover, rate of glycogen degradation nor parameters of cellular energy balance (PCr, AMP_{free} or the PCr/(PCr + Cr) and AMP_{free}/ATP ratios) associates positively with $\alpha2/\beta2/\gamma3$ phosphorylation/activity. This could indicate that the degree of phosphorylation/activation at any given moment is dependent on multiple signals that may have cumulative effects over time.

One immediate and important observation in this study is that although p-AMPK may reflect the total AMPK activity within a sample, such measurements may cover highly different regulation among the heterotrimers. In fact, this may bring some explanation to previous observations that regulation of AMPK (measured as p-AMPK or α-AMPK-associated activity) is not in accordance with regulation of ACCβ (Ser-221 phosphorylation) (Wojtaszewski *et al.* 2002, 2003; Roepstorff *et al.* 2005). For future reference, interpretation of such data should be done with caution. Interestingly, under the conditions applied here, ACCβ phosphorylation is correlated to, and may be considered a good marker of, $\alpha2/\beta2/\gamma3$ activity in human skeletal muscle. Also this may imply that the $\alpha2/\beta2/\gamma3$ heterotrimer regulates ACCβ phosphorylation during exercise and thus may be an actor in regulating fatty acid oxidation. It might seem contradictory to start regulating fatty acid oxidation in the high-intensity exercise regimens that require anaerobic energy turnover, primarily via glycogenolysis. However, even though fatty acid oxidation decreases with exercise intensity, there is an increase compared to rest even at high exercise intensities (Romijn *et al.* 1993; Dean *et al.* 2000). In addition, fatty acid oxidation is important in the post-exercise recovery period where restoration of the glycogen storage is highly prioritized. Phosphorylation of ACCβ may therefore increase the capacity for free fatty acid (FFA) oxidation if the muscle is continuously being used for work (although at a lower intensity) or for an optimal post-exercise recovery sparing carbohydrate oxidation.

In the present study we have performed analyses of the AMPK system in an *in vivo* setting using IP on whole muscle tissue. We do acknowledge that this method has some limitations. The use of tissue preparation opens up the possibility that some of the changes observed may have occurred in cell types other than muscle fibres present within the tissue. Also, we cannot exclude that the antibodies used for immunoprecipitation may, to a small extent, influence the activity measured *in vitro* and, although the results from Western blotting do indicate total immunoprecipitation efficiency, this may vary to a small extent between antibodies, giving rise to some variability.

Northern blot analyses of human mRNA reveal that the $\gamma3$ isoform has a highly tissue-specific expression, and is solely found in skeletal muscle (Cheung *et al.* 2000; Milan *et al.* 2000). In human vastus lateralis muscle, the $\alpha2/\beta2/\gamma3$ heterotrimer only accounts for approximately one-fifth of all AMPK heterotrimers when measured in co-IP experiments (Wojtaszewski *et al.* 2005 and current study). This is further supported by the present observation that the $\alpha2/\beta2/\gamma3$ heterotrimer contributes very little to total AMPK activity in resting muscle, similarly to observations made in rat muscle (Cheung *et al.* 2000; Durante *et al.* 2002). Interestingly, in human skeletal muscle exercise training has been shown to reduce mRNA and $\gamma3$ protein expression (Nielsen *et al.* 2003; Frosig *et al.* 2004; Wojtaszewski *et al.* 2005). As hypothesized, the present finding, that only $\gamma3$ heterotrimers are activated in response to exercise, is in accordance with the observation of attenuated AMPK activation during acute exercise after a period of exercise training, even when exercise is performed at the same relative intensity (Nielsen *et al.*

2003; Yu *et al.* 2003; Frosig *et al.* 2004; McConell *et al.* 2005).

The regulatory $\gamma 3$ protein has received special interest, as naturally occurring mutations result in pronounced pathological alterations that may be linked with changes in glycogen metabolism (Milan *et al.* 2000; Barnes *et al.* 2004). In muscle lacking catalytic or regulatory AMPK isoforms, glycogen metabolism is also affected (Mu *et al.* 2003; Barnes *et al.* 2004; Jorgensen *et al.* 2004). In addition, AMPK activity in muscle is influenced by glucose and/or glycogen availability (Derave *et al.* 2000; Wojtaszewski *et al.* 2003; Jorgensen *et al.* 2004). Thus, although we do not fully understand the mechanisms, there seems to be a delicate association between AMPK and many aspects of glucose/glycogen metabolism in skeletal muscle. Whether the selective activation of $\alpha 2/\beta 2/\gamma 3$ during exercise is caused by alteration in glucose/glycogen metabolism or is a regulator of such metabolic processes remains to be seen in future studies.

AMPK in both liver and muscle is hypothesized to be a potential target for pharmacological treatment of diseases associated with insulin resistance, e.g. type 2 diabetes mellitus (Kahn *et al.* 2005). Pharmacological AMPK activation in resting muscle improves insulin action similarly to prior exercise or exercise training (Fisher *et al.* 2002; Iglesias *et al.* 2002). Thus, the present findings do not only relate to exercise, because drug refinement specifically targeting the $\gamma 3$ complex may be a suitable way of improving drug-action specificity and perhaps decreasing adverse effects, since $\gamma 3$ is only expressed in skeletal muscle.

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