Exercise-induced TBC1D1 Ser237 phosphorylation and 14-3-3 protein binding capacity in human skeletal muscle

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TBC1D1 is a Rab-GTPase activating protein involved in regulation of GLUT4 translocation in skeletal muscle. We here evaluated exercise-induced regulation of TBC1D1 Ser237 phosphorylation and 14-3-3 protein binding capacity in human skeletal muscle. In separate experiments healthy men performed all-out cycle exercise lasting either 30 s, 2 min or 20 min. After all exercise protocols, TBC1D1 Ser237 phosphorylation increased (∼70–230%, $P < 0.005$), with the greatest response observed after 20 min of cycling. Interestingly, capacity of TBC1D1 to bind 14-3-3 protein showed a similar pattern of regulation, increasing 60–250% ($P < 0.001$). Furthermore, recombinant 5′AMP-activated protein kinase (AMPK) induced both Ser237 phosphorylation and 14-3-3 binding properties on human TBC1D1 when evaluated *in vitro*. To further characterize the role of AMPK as an upstream kinase regulating TBC1D1, extensor digitorum longus muscle (EDL) from whole body α1 or α2 AMPK knock-out and wild-type mice were stimulated to contract *in vitro*. In wild-type and α1 knock-out mice, contractions resulted in a similar ∼100% increase ($P < 0.001$) in Ser237 phosphorylation. Interestingly, muscle of α2 knock-out mice were characterized by reduced protein content of TBC1D1 (∼50%, $P < 0.001$) as well as in basal and contraction-stimulated (∼60%, $P < 0.001$) Ser237 phosphorylation, even after correction for the reduced TBC1D1 protein content. This study shows that TBC1D1 is Ser237 phosphorylated and 14-3-3 protein binding capacity is increased in response to exercise in human skeletal muscle. Furthermore, we show that the catalytic α2 AMPK subunit is the main (but probably not the only) donor of AMPK activity regulating TBC1D1 Ser237 phosphorylation in mouse EDL muscle.

(Received 16 June 2010; accepted after revision 7 September 2010; first published online 13 September 2010)
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Abbreviations AMPK, 5′AMP-activated protein kinase; BMI, body mass index; EDL, m. extensor digitorum longus; GAP, GTPase-activating protein; IP, immuno-precipitated; KD, kinase-dead; KO, knock-out; PAS, phospho-Akt-substrate.

Introduction

Insulin and muscle contraction are the two major physiological stimuli of glucose uptake in muscle. Despite distinct signalling pathways, a key event in this process for both stimuli is to increase sarcolemmal and T-tubule content of GLUT4 glucose transporters, thereby increasing muscle surface permeability for glucose (Hayashi *et al.* 1997; Richter *et al.* 2001). Within recent years significant progress has been made in understanding how intracellular signalling is converted to control of GLUT4 localization. This has involved the identification of TBC1D4 (also known as AS160) (Kane *et al.* 2002) and more recently TBC1D1 (Stone *et al.* 2006; Roach *et al.* 2007). Both these proteins contain a Rab-GTPase-activating protein (GAP) domain shown *in vitro* to regulate function of Rab proteins associated with GLUT4-containing vesicles (Miinea *et al.* 2005; Roach *et al.* 2007). In accordance with the current model of understanding, TBC1D1 and TBC1D4 act as brakes of GLUT4 translocation in the basal state, by converting target Rab proteins to an inactive GDP-bound form. In response to stimuli such as insulin and contraction GAP function is reduced, resulting in GTP loading and activation of target Rab proteins, and thus translocation of GLUT4.

It now seems to be substantiated that the function of TBC1D4 (Ramm *et al.* 2006; Sano *et al.* 2003; Kramer *et al.* 2006) but probably also TBC1D1 (Chen *et al.* 2008; An *et al.* 2010) is regulated by phosphorylation of Thr/Ser

 DOI: 10.1113/jphysiol.2010.194811

sites. This allows for binding of 14-3-3 proteins and inhibition of GAP domain function. Both TBC1D1 and TBC1D4 contain two clusters of possible phosphorylation sites and each cluster contains a potential 14-3-3 binding site (Ser237 and Thr596 on TBC1D1 and Ser341 and Thr642 on TBC1D4 in humans) (Chen *et al.* 2008; Ramm *et al.* 2006).

There is evidence supporting that phosphorylation of TBC1D4 in response to insulin stimulation is important for induction of glucose uptake in rodent muscle. Thus, overexpression of TBC1D4 mutated to prevent phosphorylation on four Akt sites (4P mutant) in skeletal muscle significantly reduces insulin-stimulated glucose uptake through a mechanism depending on a functional GAP domain of TBC1D4 (Kramer *et al.* 2006). Using this model also reduces *in situ* electrically stimulated glucose uptake, although this observation is debatable. Thus, in rat epitroclearis muscle, *in vitro* contraction-stimulated TBC1D4 phosphorylation detected with the phospho-Akt-substrate (PAS) antibody is completely prevented by PI3 kinase inhibition with wortmannin, whereas TBC1D1 phosphorylation and contraction-stimulated glucose uptake remain intact. In contrast, contraction-stimulated TBC1D1 (but not TBC1D4) phosphorylation and glucose uptake is markedly reduced by AMPK inhibition with compound C (Funai & Cartee, 2009). These observations indicate that TBC1D1 and TBC1D4 play functionally different roles in intact rodent muscle and suggest a role of TBC1D1 for contraction-stimulated glucose uptake. In support of this interpretation, it has been established that TBC1D1 phosphorylation increases in rodent muscle in response to exercise *in vivo* (Funai *et al.* 2009), and more recently that a 4P mutation of TBC1D1 in mouse muscle (including both sites corresponding to Ser237 and Thr596 in humans) significantly impairs contraction-induced but not insulin-stimulated glucose uptake (An *et al.* 2010).

A clue to understanding how functional differences between TBC1D1 and TBC1D4 may arise is derived from *in vitro* studies of the two proteins. Thus, pharmacological activation of AMPK results in robust TBC1D1 Ser237 phosphorylation and 14-3-3 binding in L6 myotubes as well as HEK-293 cells overexpressing human TBC1D1 (Chen *et al.* 2008). In contrast, in HEK-293 cells, the corresponding Ser341 on TBC1D4 mediates only a low-affinity basal interaction with 14-3-3 proteins and is weakly regulated in terms of Ser341 phosphorylation and 14-3-3 binding when AMPK is activated (Geraghty *et al.* 2007). These observations support the suggestion that TBC1D1 may be more sensitive than TBC1D4 for regulation by stimuli resulting in cellular energy perturbations activating AMPK, such as muscle contraction. In this context, we have recently shown that TBC1D1 is phosphorylated on Ser237 allowing for 14-3-3 binding in response to *in vitro* contractions (but not insulin stimulation) in mouse EDL muscle and that the contraction response is prevented in AMPK kinase-dead (KD) mice (Pehmoller *et al.* 2009).

The primary aim of the present investigation was to evaluate if phosphorylation of TBC1D1 on Ser237 and subsequent 14-3-3 protein binding is a physiological response to exercise in human skeletal muscle. Furthermore, in order to establish which catalytic subunit (α1 or α2) of AMPK might be responsible for TBC1D1 Ser237 phosphorylation, we also evaluated regulation of this site in response to *in vitro* contractions in whole body α1and α2 knock-out (KO) mice.

Methods

Human studies

Thirty young healthy men (age 27 ± 1 years, body weight 79 ± 2 kg, body mass index (BMI) 24 ± 0 kg m^{-2}) participated in the study. All subjects gave written informed consent prior to participation. The study was approved by the local ethics committee (no. KF1277313) and was in agreement with the *Declaration of Helsinki II*.

One to two weeks prior to the experimental day oxygen uptake was determined during incremental cycling on an ergometer ($\dot{V}_{O_2,peak}$ 52 ± 1 ml min^{-1} kg^{-1}) with an inclusion criteria of a $\dot{V}_{O_2,peak}$ value between 40 and 60 ml min^{-1} kg^{-1}. All subjects were then randomly assigned to one of three different cycle exercise protocols (see below) and performed one test trial of the assigned protocol. On the experimental day subjects arrived at the laboratory in the morning 3 h after a light breakfast. After 45 min of supine rest, a biopsy was obtained from the vastus lateralis muscle under local anaesthesia (2–3 ml of 2% Lidocaine). Next the subjects performed cycle exercise according to one of the following protocols, as previously described in more detail (Birk & Wojtaszewski, 2006).

Protocol 1. 20 min of cycle exercise at ∼80% $\dot{V}_{O_2,peak}$ (77 ± 3% $\dot{V}_{O_2,peak}$, work rate 222 ± 8 W), $N = 11$.

Protocol 2. 120 s cycle exercise at a work rate (376 ± 18 W) corresponding to ∼110% $\dot{V}_{O_2,peak}$, $N = 9$.

Protocol 3. 30 s 'all-out' sprint cycle exercise with an average work rate of 658 ± 26 W, $N = 10$.

Immediately after termination of exercise a biopsy was obtained from the leg not subjected to the pre-exercise biopsy. Generally, biopsies were randomized in regard to dominant and non-dominant leg. Post-exercise biopsies were obtained within 15 s of exercise termination and all biopsies were quickly frozen in liquid N_2 and stored (−80°C) for later analyses.

Animal studies

All animal experiments were approved by the Danish Animal Experimental Inspectorate in compliance with the European Convention for Protection of Vertebrate Animals Used for Scientific Purposes. The present experiments comply with the policies and regulations of *The Journal of Physiology* (Drummond, 2009).

The whole body α1 AMPK (Jorgensen *et al.* 2004) and α2 AMPK (Viollet *et al.* 2003) knock-out (KO) animals have previously been characterized in detail. With regard to muscle phenotype, muscles from α1 AMPK and α2 AMPK KO animals are completely devoid of the respective catalytic α-subunit and accordingly α-associated AMPK activity (Jorgensen *et al.* 2004). In this context, in α2 AMPK KO muscle the protein content of α1 AMPK is increased ∼2- to 3-fold, whereas in α1 AMPK KO muscle protein content of α2 AMPK is comparable to wild-type (WT) littermates (Jorgensen *et al.* 2004).

All animals used in this experiment were 17- to 23-week-old female littermates maintained on a C57BL/6 (α2 AMPK KO) or 129S6/sv (α1 AMPK KO) background. Animals were kept on a 10:14 h light–dark cycle with unlimited access to standard rodent diet and water. At the end of each experiment, the anaesthetized mouse was killed by complete cervical dislocation.

Muscle incubations

Animals were initially anaesthetized by intraperitoneal injection of pentobarbital sodium (6 mg (100 g body wt)$^{-1}$). EDL muscles were then quickly excised and suspended in incubation chambers (Multi Myograph system; Danish Myo-Technology, Aarhus, DK) at resting tension (3–4 mN). All muscles were incubated for 30 min in buffer (standard Krebs–Henseleit–Ringer buffer including 2 mM pyruvate, 8 mM mannitol and 0.1% BSA) at 30°C with continuous gas mixture oxygenation (95% O_2 and 5% CO_2). Subsequently, muscles were stimulated to contract with electrical stimulation using 10 s trains (100 Hz, 0.2 ms impulse, ∼30–40 V) per minute for 10 min. After stimulation muscles were quickly removed from the chambers and frozen in N_2 for later analyses.

Muscle lysate preparation

Human muscle tissue was freeze-dried and dissected free of visible fat, blood and connective tissue prior to homogenization in ice-cold buffer (10% glycerol, 20 mM sodium pyrophosphate, 1% NP-40, 2 mM PMSF, 150 mM sodium chloride, 50 mM Hepes, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 10 μg ml^{-1} aprotinin, 3 mM benzamidine, 10 μg ml^{-1} leupeptin and 2 mM sodium orthovanadate (pH 7.4)). Intact mouse muscles were homogenized in the same buffer. All homogenates were subsequently rotated end over end for 1 h at 4°C before being centrifuged at 17,500 *g* at 4°C for 25 min. Supernatants were collected and stored at −80°C for later analyses. Total protein concentrations were analysed by the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA).

SDS-PAGE and Western blot analyses

For analyses of TBC1D1 Ser237 phosphorylation and TBC1D1 14-3-3 overlay, total TBC1D1 protein was initially immuno-precipitated (IP) from muscle lysate using 0.8 μg of TBC1D1 antibody in a mix of protein G–agarose beads (Millipore, Glostrup, Denmark) and lysate (14-3-3: 300 μg; Ser237: 600 μg) overnight at 4°C. Subsequently the beads were washed twice in ice-cold PBS and heated in SDS sample buffer (5 min, 96°C). For analyses of AMPK Thr172 and acetyl-CoA carboxylase-β (ACC-β) Ser227 phosphorylation aliquots of muscle lysate (30 μg) were heated in SDS sample buffer (5 min, 96°C).

Muscle proteins were separated using 5% or 7.5% Tris-HCl gels (Bio-Rad Laboratories, Copenhagen, Denmark), and transferred (semi-dry) to PVDF membranes (Immobilon Transfer Membrane, Millipore, Glostrup, Denmark). After blocking (in TBS + Tween (TBST) + 2% skim milk) the membranes were incubated with primary antibodies (TBST + 2% skim milk) or 14-3-3 protein (TBST + 1% skim milk) followed by incubation in horseradish peroxidase-conjugated secondary antibodies (see Antibodies and 14-3-3 overlay). After detection (Kodak Image Station 2000MM) and quantification (Kodak MI) the signal was finally corrected for between-gel variation relative to a muscle lysate standard run on all gels. Membranes used for detection of TBC1D1 Ser237 phosphorylation and 14-3-3 overlay were subsequently stripped (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl) for 2 h at 50°C before being reprobed with total TBC1D1 primary antibody. This allowed for correction of the signal relative to the actual content of TBC1D1 in the immuno-precipitate.

Antibodies

The following commercial antibodies were used: anti-AMPK Thr172 (Cell Signaling Technologies, Danvers, MA, USA) and anti-ACC-β Ser227 (Upstate Biotechnologies, Waltham, MA, USA). All HRP-conjugated secondary antibodies were from Dako (Glostrup). Phosphorylation of TBC1D1 Ser237 was detected using an anti-TBC1D1 Ser237 antibody generated as previously described (Chen *et al.* 2008).

14-3-3 overlay assay

14-3-3 protein (a mix of the *Saccharomyces cerevisiae* BMH1 and BMH2 14-3-3 isoforms) were expressed in *E. coli* DH5α and purified as previously described (Moorhead *et al.* 1996). 14-3-3 proteins were subsequently labelled with the ester of digoxygenin-3-*O*-methylcarbonyl-ε-aminocaproic-acid-*N*-hydroxy-succinimide (DIG) according to the manufacturer's description (Roche, Basel, Switzerland). PVDF membranes containing TBC1D1 after IP and SDS-PAGE were blocked (TBS-T + 1% skim milk) and incubated in DIG–14-3-3 protein (TBS-T + 1% skim milk) overnight (4°C). Next, membranes were incubated in HRP-conjugated anti-DIG antibody (Roche, Basel, Switzerland) allowing for detection and quantification.

Dephosphorylation assay

In order to verify that the signal obtained with the TBC1D1 Ser237 antibody in human samples were phosphorylation dependent, muscle lysate proteins were resolved by SDS-PAGE and transferred (semi-dry) to a PVDF membrane. The membrane was then treated for 2 h at 37°C with a dephosphorylation buffer (50 mM Tris-HCl, 0.1 mM Na_2EDTA, 5 mM dithiothreitol, 0.01% Brij 35 and 2 mM $MnCl_2$; pH 7.5) either with or without 500 U ml^{-1} lambda protein phosphatase (λ-PPase, New England BioLabs, Hitchin, UK). After extensive washing (TBS-T) detection of total TBC1D1 and TBC1D1 Ser237 phosphorylation was performed as described above. Using the same procedure, we next evaluated if the capacity of TBC1D1 to bind 14-3-3 protein was likewise depending on phosphorylation of TBC1D1.

In vitro kinase assays

Since we were able to abolish TBC1D1 Ser237 phosphorylation and 14-3-3 protein binding capacity by dephosphorylation of TBC1D1 (Fig. 1*A*), we next evaluated if we subsequently could restore these TBC1D1 properties by AMPK mediated *in vitro* phosphorylation of TBC1D1. After IP of TBC1D1 (300 μg), the beads + protein were incubated in 100 μl of dephosphorylation buffer including λ-PPase (see Dephosphorylation assay) for 2 h at 37°C. After washing in ice-cold PBS the beads were next incubated in reaction buffer (10 mM Hepes, 5 mM $MgCl_2$, 1 mM EGTA, 200 μM ATP, 100 μM AMP) with or without constitutively active recombinant AMPK ($\alpha1\beta1\gamma1$, SignalChem, Richmond, British Columbia, Canada) for 30 min at 37°C. The reaction was stopped by heating the beads in SDS sample buffer. Subsequently, the samples were evaluated for TBC1D1 Ser237 phosphorylation by Western blotting as described above. After stripping (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl) for 2 h at 50°C, 14-3-3 binding capacity was ultimately evaluated as described above.

Statistical analysis

Data are expressed as means ± S.E.M. Means were compared by two-way analysis of variance (ANOVA). When analysis of variance revealed significant differences, a Tukey's *post hoc* test for multiple comparisons was performed. P values below 0.05 were considered statistically significant.

Results

Regulation of TBC1D1 in human skeletal muscle

In three separate experiments healthy men performed high-intensity cycle exercise lasting either 30 s, 2 min or 20 min. Muscle tissue was obtained before and immediately after termination of exercise in all trials. After immuno-precipitation, TBC1D1 Ser237 phosphorylation and 14-3-3 protein binding was measured by Western blotting and overlay analysis, respectively, and expressed relative to total TBC1D1 protein content in each precipitate. In response to all exercise trials, TBC1D1 Ser237 phosphorylation/protein increased (70–230%, $P < 0.005$), with the greatest response observed after 20 min (Fig. 1*A*). 14-3-3 protein binding to TBC1D1/protein displayed a similar pattern of regulation, increasing 60–250% ($P < 0.001$) in response to exercise (Fig. 1*B*) whereas total content of TBC1D1 did not change with exercise (data not shown). Interestingly, dephosphorylation of TBC1D1 ablating Ser237 phosphorylation (and probably other phosphorylations) led to a full loss of 14-3-3 binding capacity (Fig. 2*A*). Furthermore, subsequent AMPK-mediated phosphorylation of TBC1D1 *in vitro* could restore both Ser237 phosphorylation and 14-3-3 binding capacity (Fig. 2*B*). This demonstrates that 14-3-3 binding to TBC1D1 depends on phosphorylation of the protein and furthermore suggests that AMPK-mediated phosphorylation of TBC1D1 Ser237 may play a role in this context. Based on samples from the present study, we have previously reported that only $\alpha2\beta2\gamma3$ AMPK complexes are activated in response to the exercise regimes performed, in close correlation with increases in ACC-β Ser227 phosphorylation (Birk & Wojtaszewski, 2006). Since ACC-β Ser227 is a known downstream target of AMPK, this latter measurement is considered a crude marker of endogenous AMPK activity *in vivo*. Interestingly, in that study the pattern of AMPK

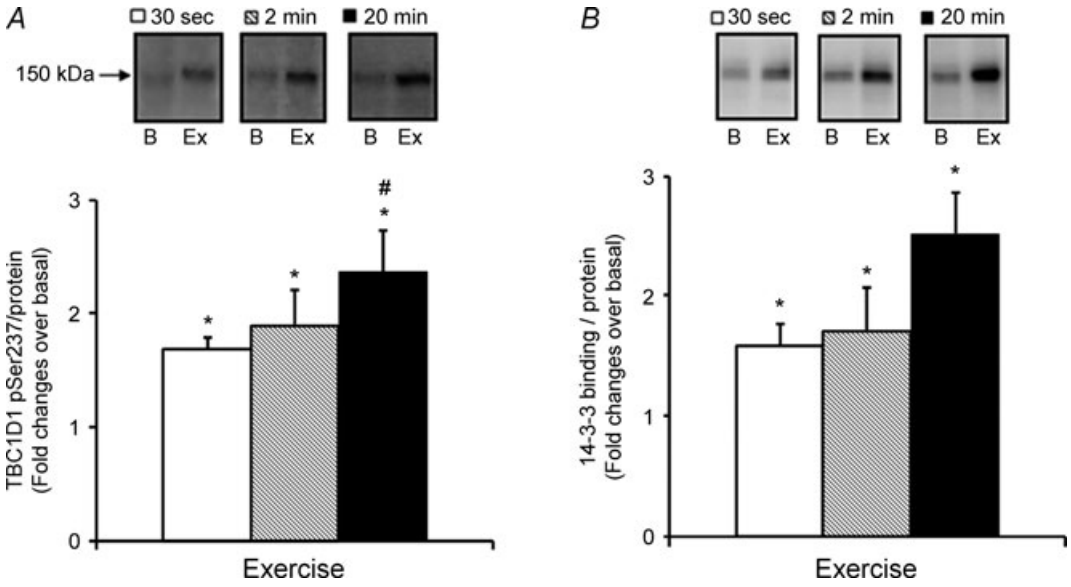

Figure 1. Effect of exercise on TBC1D1 Ser237 phosphorylation and 14-3-3 binding capacity in human skeletal muscle

TBC1D1 (*A*: 600 μg IP, *B*: 300 μg IP) was isolated by SDS-PAGE and Western blotting and subsequently evaluated for TBC1D1 Ser237 phosphorylation (*A*) and 14-3-3 binding capacity (*B*). After stripping of the membrane total TBC1D1 content was next evaluated allowing for expression of values relative to protein content in the sample. Values (30 s, white; 2 min, striated; or 20 min, black) are expressed as fold changes with exercise relative to basal values from the same subject. Representative blots demonstrate basal (B) and signals obtained in response to exercise (Ex). *Significantly different from basal values, $P < 0.005$. #Exercise effect significantly different from 30 s and 2 min values, $P < 0.05$. Values are expressed as means ± S.E.M. $N = 9$–11.

activation (either $\alpha2\beta2\gamma3$ AMPK activity or ACC-β Ser227 phosphorylation) mimics the observed regulation at the level of TBC1D1 Ser237 phosphorylation and 14-3-3 binding in the present study. Thus, activation is observed already after 30 s of exercise but the greatest response is observed after 20 min of cycling.

Regulation of TBC1D1 Ser237 phosphorylation in rodent muscle

In order to further examine the role of AMPK as an upstream kinase of TBC1D1 Ser237 phosphorylation we next evaluated regulation of TBC1D1 in isolated EDL muscle from whole body $\alpha1$ and $\alpha2$ AMPK KO

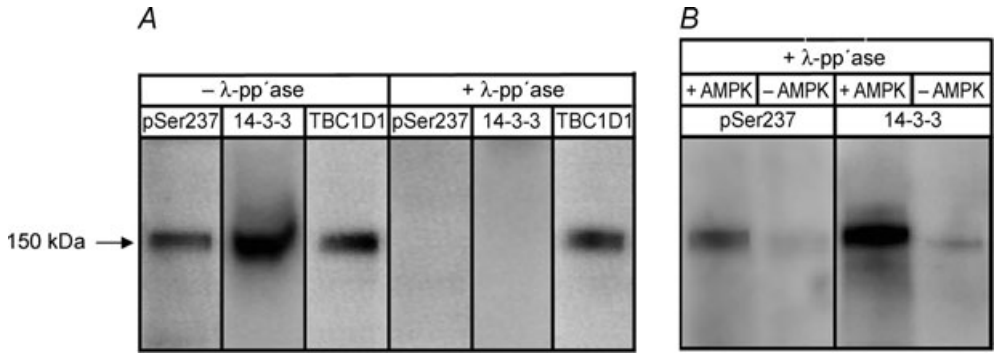

Figure 2. *In vitro* evaluation of TBC1D1 regulation

A, TBC1D1 (6 × 300 μg) was immuno-precipitated (IP) from exercised human skeletal muscle lysate and subjected to SDS-page and Western blotting. After subsequent phosphatase treatment of the membrane with (+) or without (–) λ-phosphatase (λ-pp'ase) individual membrane lanes were evaluated for TBC1D1 protein content, TBC1D1 Ser237 phosphorylation and 14-3-3 binding capacity. *B*, TBC1D1 (4 × 300 μg IP) from a pool of human muscle lysate was subjected to phosphatase treatment (+λ-pp'ase) *in vitro* and subsequent AMPK assay *in vitro* with (+) or without (–) the presence of recombinant active AMPK. Subsequently, the samples were subjected to SDS-PAGE and Western blotting and evaluated for TBC1D1 Ser237 phosphorylation and 14-3-3 binding capacity.

mice. Electrically induced contraction elicited Ser237 phosphorylation similarly in muscle of WT and α1 AMPK KO mice (+100%, $P < 0.001$) (Fig. 3*A* and *C*). However, muscle of α2 AMPK KO mice was characterized by a marked reduction in total protein content of TBC1D1 (−50%, $P < 0.001$) (Fig. 3*B*) as well as a reduction in basal and contraction-stimulated Ser237 phosphorylation (−80%, $P < 0.001$) (Fig. 3*A* and *C*). Still, in these mice, Ser237 phosphorylation did increase to a minor extent with contraction ($P < 0.05$) when phosphorylation was expressed relative to protein content (Fig. 3*C*). To validate that the contraction protocol did in fact result in activation of AMPK, we next measured AMPK Thr172 phosphorylation (covalent activation) (Fig. 4*A*) as well as ACC-β Ser227 phosphorylation (marker of *in vivo* AMPK activity) (Fig. 4*B*). In response to contraction, phosphorylation of AMPK and ACC-β increased (+300%, $P < 0.005$) similarly in WT and α1 AMPK KO mice. In contrast, muscle of α2 AMPK KO mice was characterized by a marked reduction (−80%, $P < 0.001$) in resting and contraction-stimulated AMPK and ACC-β phosphorylation, thus mimicking TBC1D1 Ser237 phosphorylation. Representative images of all animal Western blot analyses can be viewed in Fig. 5.

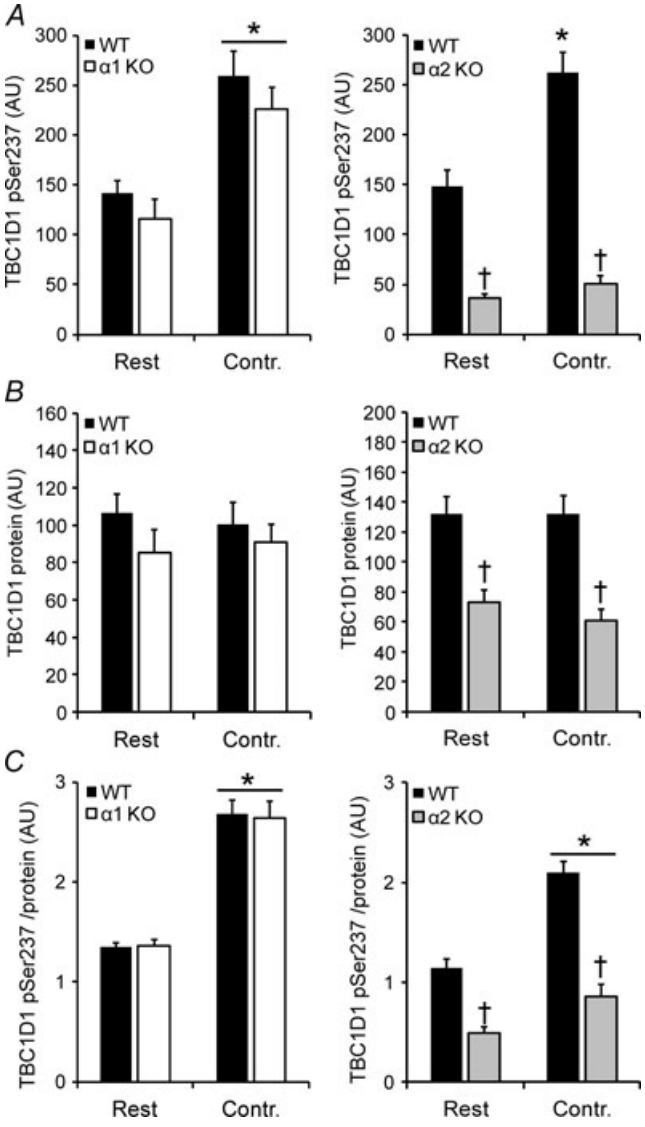

Figure 3. Role of AMPK for contraction-induced TBC1D1 Ser237 phosphorylation in rodents
The effect of *in vitro* contraction (Contr.) of wild type (WT) or whole body α1 or α2 AMPK knock-out (KO) mouse EDL muscle on TBC1D1 Ser237 phosphorylation was evaluated. TBC1D1 (300 μg IP) was isolated by SDS-PAGE and Western blotting and subsequently evaluated for TBC1D1 Ser237 phosphorylation (*A*) and after stripping total TBC1D1 protein content (*B*). Finally values were expressed as phosphorylation relative to total protein (*C*). *Significantly different from resting (Rest) values, $P < 0.001$. †Significantly different from WT, $P < 0.005$. Values are expressed in arbitrary units as means ± S.E.M. $N = 7$–12.

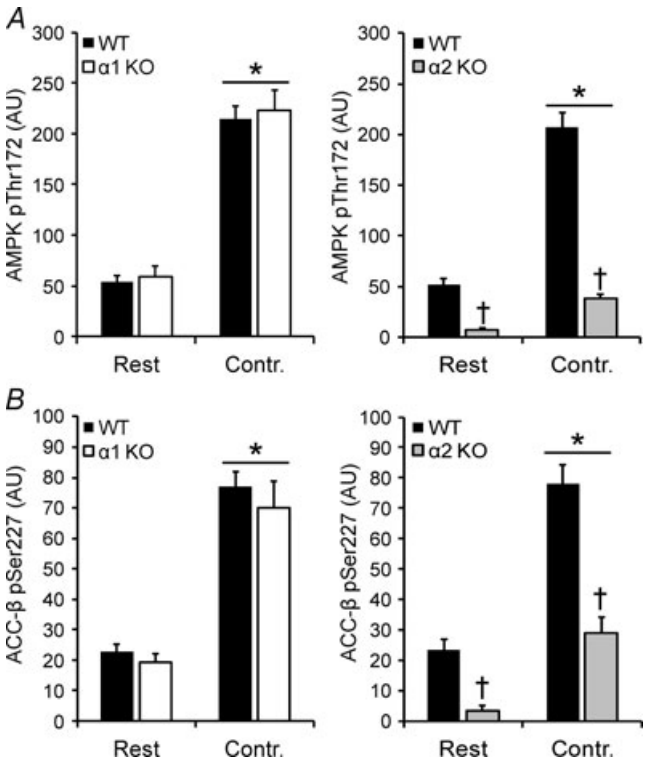

Figure 4. AMPK Thr172 and ACC-β Ser227 phosphorylation in rodents
Effect of *in vitro* contraction (Contr.) of wild type (WT) or whole body α1 or α2 knock-out (KO) mouse EDL muscle on AMPK Thr172 phosphorylation (*A*) and ACC-β Ser227 phosphorylation (*B*). *Significantly different from resting (Rest) values, $P < 0.001$. †Significantly different from WT, $P < 0.005$. Values are expressed in arbitrary units as means ± S.E.M. $N = 7$–12.

Discussion

The principal finding in this study is that acute high-intensity exercise of different duration results in

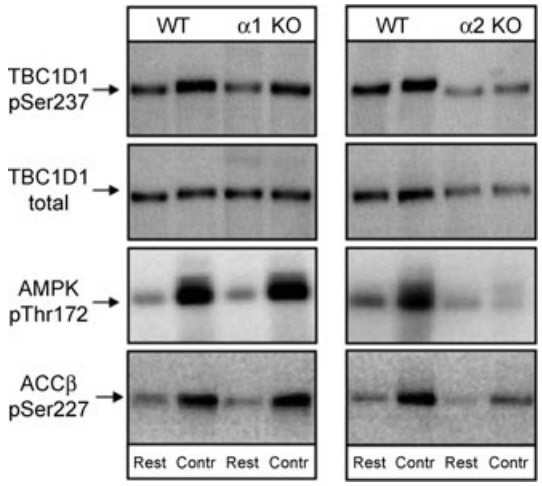

Figure 5. Representative immunoblots
The figure shows representative immunoblot images of TBC1D1 protein, TBC1D1 Ser237 phosphorylation, AMPK Thr172 phosphorylation and ACC-β Ser227 phosphorylation in whole body α1 and α2 knock-out (KO) and respective wild-type EDL muscle. Rest: resting muscle. Contr: contracted muscle.

increased Ser237 phosphorylation and 14-3-3 protein binding capacity of TBC1D1 in human skeletal muscle (Fig. 1). Interestingly, this exercise response is observed already after 30 s of 'all-out' cycle exercise; however, a more pronounced response is detected when high-intensity exercise is continued for 20 min (Fig. 1). In addition, using a genetic approach, we also demonstrate that TBC1D1 Ser237 phosphorylation in mouse muscle predominantly is attributable to activation of the AMPK α2 catalytic subunit but not the α1 catalytic subunit in response to muscle contraction (Fig. 3).

TBC1D1 is a recently discovered protein with Rab GTPase activating properties, believed to be involved in regulation of GLUT4 translocation in skeletal muscle. Here we provide the first evidence that TBC1D1 is regulated in response to an acute exercise bout in human skeletal muscle. Available evidence indicates a potent role of Ser237 phosphorylation of TBC1D1 for 14-3-3 protein binding to occur (Chen *et al.* 2008; Pehmoller *et al.* 2009). Consistent with this, we show that regulation of 14-3-3 binding capacity of TBC1D1 mimics the pattern of regulation of TBC1D1 Ser237 in human skeletal muscle (Fig. 1). Further strengthening the association, we also demonstrated *in vitro* that dephosphorylation of TBC1D1 extracted from intact human skeletal muscle disrupts 14-3-3 binding capacity (Fig. 2*A*) and interestingly, that subsequent AMPK-mediated phosphorylation of TBC1D1 *in vitro* restores 14-3-3 binding capacity (Fig. 2*B*). Collectively, these data suggest that in human skeletal muscle exercise leads to AMPK-mediated TBC1D1 Ser237 phosphorylation and subsequent 14-3-3 protein binding. Currently, the impact of phosphorylation and 14-3-3 binding on TBC1D1 function remains to be elucidated. However, it has been demonstrated that these modifications are critical events allowing for TBC1D4 to regulate GLUT4 traffic (Ramm *et al.* 2006; Stockli *et al.* 2008), and owing to the extent of homology between TBC1D1 and TBC1D4, it may also apply to TBC1D1. Based on studies in adipocytes, 14-3-3 protein binding to TBC1D4 in response to insulin stimulation appears to prevent the interaction of TBC1D4 with the GLUT4 vesicle-associated insulin-responsive amino peptidase (IRAP) and release of TBC1D4 to the cytosol (Larance *et al.* 2005; Ramm *et al.* 2006). Furthermore, inhibition of insulin-stimulated GLUT4 translocation to the cell membrane by the 4P mutant of TBC1D4 in adipocytes can be completely reversed by co-expression of a high-affinity 14-3-3 binding sequence (Ramm *et al.* 2006). These observations indicate that a key role of TBC1D4 phosphorylation in response to insulin stimulation is to induce 14-3-3 binding and subsequent dissociation of TBC1D4 from GLUT4 vesicles, thus allowing for GLUT4 translocation. To what extent phosphorylation also regulates GAP-domain activity or function remains to be elucidated.

In regard to TBC1D1, it was recently reported that a 4P mutant (including the corresponding site to Ser237 in humans) of TBC1D1 overexpressed in mouse tibialis anterior muscle significantly reduced contraction- (but not insulin-) induced glucose uptake (An *et al.* 2010). This substantiates the role of TBC1D1 in regulation of contraction-mediated glucose uptake and underscores an important regulatory impact of phosphorylation also on TBC1D1.

We have previously demonstrated that *in vitro* contraction leads to increased phosphorylation of TBC1D1 on Ser237 and Thr596 (the two potential 14-3-3 binding sites) in rodent muscle and that these responses are completely abolished in AMPK kinase-dead (KD) mice (Pehmoller *et al.* 2009). Since in the AMPK KD mice, contraction-induced activation of both catalytic (α1 and α2) subunits is inhibited, it has not been possible to attribute these contraction responses to one particular catalytic subunit isoform. Here we show that knock-out of α1 does not affect contraction-induced TBC1D1 Ser237 phosphorylation in rodents. In contrast, the lack of α2 markedly reduces both basal TBC1D1 Ser237 phosphorylation as well as the absolute contraction response coinciding with reduced total TBC1D1 protein. We have previously reported that TBC1D4 expression is normal in EDL muscle of these mice (Treebak *et al.* 2006) indicating that α2 AMPK selectively affects TBC1D1 protein content. Interestingly, and in contrast to observations in AMPK KD mice where both α1 and α2 activation is prevented, we are not able to completely abolish the contraction response in α2 KO animals. It can be speculated that this discrepancy relates to compensatory

regulation by $\alpha 1$ in $\alpha 2$ KO animals as a mechanism to sustain muscle function. Supporting this interpretation is the previous observation of a marked increase in total $\alpha 1$ protein in $\alpha 2$ KO muscle (Jorgensen *et al.* 2004). In this context, it is noteworthy that *in vitro* contraction-induced glucose uptake is normal in $\alpha 2$ KO mice (Jorgensen *et al.* 2004) whereas a significant ∼40% reduction is observed in AMPK KD mice (Mu *et al.* 2001; Jensen *et al.* 2008). Whether this discrepancy relates to the residual increase in TBC1D1 Ser237 phosphorylation in the a2 KO muscle during contraction remains to be investigated.

In the present study TBC1D1 Ser237 phosphorylation was detected using an antibody that has previously been verified to identify human TBC1D1 overexpressed in cells but not when Ser237 is mutated to alanine (Chen *et al.* 2008). We detect a clear signal at the expected molecular mass (∼150 kDa) in human skeletal muscle lysates after immuno-precipitation of TBC1D1. Furthermore, since the signal can be completely removed with λ-phosphatase treatment of the membrane (Fig. 2*A*), collectively we feel confident that our results represent genuine TBC1D1 Ser237 phosphorylation. As presented, $\alpha 2$ AMPK complexes appear to be dominantly responsible for TBC1D1 Ser237 phosphorylation during contractions in rodents. Using samples from the present human study, we have previously reported that only $\alpha 2/\beta 2/\gamma 3$ AMPK complexes are activated in response to the high-intensity exercise protocols performed (Birk & Wojtaszewski, 2006). Notably, and similar to regulation of TBC1D1 Ser237 phosphorylation, the most pronounced $\alpha 2/\beta 2/\gamma 3$ AMPK activation was observed after 20 min of high-intensity exercise in that investigation. Correlatively, these data allow for the scenario that in response to high-intensity exercise in human skeletal muscle $\alpha 2/\beta 2/\gamma 3$ AMPK trimers are activated leading to phosphorylation of TBC1D1 on Ser237 which subsequently increases 14-3-3 protein binding capacity.

In the present study we did not obtain measurements of glucose transport capacity in muscle. However, our results suggest that after 20 min of exercise at ∼80% $\dot{V}_{O_2,peak}$ a more potent stimulus of GLUT4 translocation is induced when compared to more intense exercise regimes of short duration (<120 s). Previously it has been demonstrated in rodent muscle, independent of stimulus intensity (high or low voltage), that GLUT4 translocation is only partial (<50%) after 5 min of *in situ* contraction when compared to 15 min (Lauritzen *et al.* 2010). In addition, during submaximal exercise (∼75% $\dot{V}_{O_2,peak}$) in humans, GLUT4 translocation to the sarcolemma is only partial (<50%) after 5 min of exercise compared to 40 min (Kristiansen *et al.* 1997). In agreement with our observations on TBC1D1 signalling and consistent with previous measurements of glucose uptake during exercise (Wahren *et al.* 1971; Katz *et al.* 1986; Wojtaszewski *et al.* 2003), this suggests a substantial response time for regulation of glucose transport capacity. Supporting this interpretation, both PAS phosphorylation of TBC1D1 as well as glucose transport has been shown to gradually increase within the first 20 min of *in vitro* muscle contraction in rodents (Funai & Cartee, 2008).

In rodent muscle, contraction leads to phosphorylation of both Ser237 and Thr596 (14-3-3 binding sites) on TBC1D1 (Pehmoller *et al.* 2009). Based on *in vitro* evaluation of bacterially expressed TBC1D1 (Chen *et al.* 2008) as well as on observations in AMPK KD animals (Pehmoller *et al.* 2009), it seems likely that AMPK is implicated in regulation of both sites. In contrast, in response to insulin stimulation only Thr596 phosphorylation is increased in a PI3 kinase-dependent manner (Pehmoller *et al.* 2009). This has been demonstrated both using a phospho-Thr596 site-specific antibody as well as by using the PAS antibody (Pehmoller *et al.* 2009) expected to reflect Thr596 phosphorylation (Chen *et al.* 2008). Curiously, in human vastus lateralis muscle known to express both TBC1D1 and TBC1D4, all detectable signal obtained with the PAS antibody in insulin-stimulated samples is associated with TBC1D4 (Treebak *et al.* 2007). We have tried to elaborate on this observation using the phospho-Thr596 site-specific antibody verified to detect phosphorylated human TBC1D1 overexpressed in HEK-293 cells (Chen *et al.* 2008). Consistent with the PAS data (Treebak *et al.* 2007), we have not been able to detect measurable Thr596 phosphorylation in human skeletal muscle preparations from either basal, exercised or maximally insulin-stimulated muscle after immuno-precipitation of TBC1D1 in as much as 1 mg of muscle lysate protein (data not shown). It cannot be ruled out that specificity of the antibody does not allow for detection of endogenous TBC1D1 Thr596 phosphorylation in human skeletal muscle. Alternatively, and in contrast to rodents, TBC1D1 Thr596 phosphorylation may be negligible under these physiological conditions in human skeletal muscle. Presumably, TBC1D1 can be phosphorylated on a range of other sites as indicated by observations in cultured cells (Chen *et al.* 2008; Chavez *et al.* 2008) and rodent muscle (Taylor *et al.* 2008; Funai & Cartee, 2009), with the potential of modulating 14-3-3 binding affinity. For instance, prior Akt-induced Ser235 phosphorylation of TBC1D1 in cultured cells has been demonstrated to negatively influence 14-3-3 binding to TBC1D1 Ser237 (Chen *et al.* 2008). Whether these observations can be extrapolated to human skeletal muscle remains to be determined. Furthermore, the exact role of 14-3-3 protein binding to TBC1D1 with regard to regulation of glucose uptake is still unresolved.

Collectively, this study provides evidence that an acute bout of high-intensity exercise results in increased Ser237 phosphorylation and 14-3-3 protein binding capacity of TBC1D1 in human skeletal muscle. Consistent with the

observation that $\alpha 2$ AMPK is the primary TBC1D1 Ser237 kinase in rodents, regulation of this site in humans mimics exercise-induced activation of $\alpha 2/\beta 2/\gamma 3$ AMPK complexes in the same muscle samples.

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Author contributions

All authors contributed to conception and design of the study, interpretation of data, and drafting or critically revising the manuscript. J.B., J.F.P.W. and E.A.R. conducted the human exercise experiments. C.P. optimized all animal TBC1D1 analyses. C.F. conducted the animal experiments and performed the experimental analyses. All authors approved the final version of the manuscript.

Acknowledgements

This work was supported by grants from the Lundbaek Foundation, Denmark, The Danish Medical Research Council, Denmark, The Novo Nordisk Fundation, and the Danish Diabetes Association. The Molecular Physiology group, Department of Exercise and Sport Sciences is part of the UNIK Project: Food, Fitness & Pharma for Health and Disease, supported by the Danish Ministry of Science, Technology and Innovation. C.F. was supported by a fellowship from The Danish Agency for Science, Technology and Innovation, Denmark. We thank Jonas T. Treebak for fruitful suggestions in general and expert technical information particularly regarding *in vitro* analyses. Furthermore, we kindly thank Professor Carol MacKintosh (University of Dundee, UK) for providing invaluable reagents used in this study as well as Dr. Benoit Viollet for providing founder $\alpha 1$ and $\alpha 2$ AMPK KO animals.