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Lipid-induced mTOR activation in rat skeletal muscle reversed by exercise and 5′-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside

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Abstract

The serine/threonine protein kinase, mammalian target of rapamycin (mTOR) is regulated by insulin and nutrient availability and has been proposed to play a central role as a nutrient sensor in skeletal muscle. mTOR associates with its binding partners, raptor and rictor, to form two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) respectively. We have investigated the assembly of mTORC1/2 and the activation of their downstream substrates (i.e. Akt, S6K1) in response to known effectors of mTOR, excess lipid availability and AMP-activated protein kinase (AMPK) activation/exercise training in rat skeletal muscle. The *in vivo* formation of mTORC1 and 2 and the activation of their respective downstream substrates were increased in response to chronic (8 weeks) consumption of a high-fat diet. Diet-induced mTORC activation and skeletal muscle insulin resistance were reversed by 4 weeks of exercise training, which was associated with enhanced muscle AMPK activation. In order to determine whether AMPK activation reverses lipid-induced mTOR activation, L6 myotubes were exposed to 0·4 mM palmitate to activate mTORC1/2 in the absence or presence of 5′-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR). Palmitate exposure (4 h) increased insulin-stimulated S6K1 Thr389 phosphorylation by 60%, indicating activation of mTORC1. AMPK activation with 1 mM AICAR abolished lipid-induced mTOR activation *in vitro*. Our data implicates reductions in mTOR complex activation with the reversal of lipid-induced skeletal muscle insulin resistance in response to exercise training or AICAR and identifies mTOR as a potential target for the treatment of insulin resistance.

Introduction

The insulin signal transduction cascade regulates many processes in skeletal muscle including glucose and lipid metabolism, cellular growth and differentiation and protein

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Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

synthesis (Avruch 1998, Kimball *et al.* 2002, Taniguchi *et al.* 2006). Accordingly, impaired insulin signalling has been implicated in several disease states including type 2 diabetes (Zamboni *et al.* 2005, Taniguchi *et al.* 2006). Insulin action in muscle is a tightly regulated process with inputs from several diverse stimuli such as nutrient availability, hormonal milieu and exercise/muscle contraction (Hawley *et al.* 2006, Taniguchi *et al.* 2006). Specifically, chronic consumption of a high-fat (HF) diet increases the circulation of non-esterified fatty acids (NEFA) resulting in marked skeletal muscle insulin resistance in rodents and humans (Barnard & Youngren 1992, Boden 2002, Lessard *et al.* 2007). In contrast, one of the most potent means to improve insulin action in muscle is exercise training (Barnard & Youngren 1992, Hawley 2004, Hawley & Lessard 2008). We and others have previously demonstrated that exercise reverses the effects of an HF diet on skeletal muscle insulin resistance (Barnard & Youngren 1992, Lessard *et al.* 2007). However, the precise mechanism(s) by which lipids induce insulin resistance and exercise training reverses these defects in skeletal muscle is currently unknown.

The serine/threonine protein kinase, mammalian target of rapamycin (mTOR), is a member of the phosphatidylinositol 3 kinase (PI3K) family of enzymes. This kinase is regulated by insulin and nutrient availability and plays a central role as a nutrient sensor in skeletal muscle (Patti & Kahn 2004, Marshall 2006). mTOR associates with its binding partners, raptor and rictor, to form two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) respectively (Kim *et al.* 2002, Sarbassov *et al.* 2004, Bhaskar & Hay 2007). Activation of mTORC1 through a negative feedback loop inhibits insulin signalling via the activation of its downstream substrate ribosomal protein S6 kinase 1 (S6K1) and subsequent inhibitory serine phosphorylation of insulin receptor substrate 1 (IRS1; Tremblay & Marette 2001). In contrast, mTORC2 is a positive regulator of insulin signal transduction through its phosphorylation of protein kinase B/Akt on its serine 473 activation site (Hresko & Mueckler 2005, Sarbassov *et al.* 2005). Its ability to differentially modulate both proximal and distal components of the insulin signalling cascade raises the intriguing possibility that mTOR may be a critical regulator of insulin action in skeletal muscle.

Recent evidence demonstrates that in obese rodents or in rodents consuming an HF diet, overactivation of mTOR may be associated with impairments to skeletal muscle insulin action (Khamzina *et al.* 2005, Tremblay *et al.* 2007). In contrast, S6K1 null mice are protected from HF diet-induced insulin resistance (Um *et al.* 2004). Taken collectively, these results (Um *et al.* 2004, Khamzina *et al.* 2005, Tremblay *et al.* 2007) implicate mTORC1 activation as a possible mechanism for impaired insulin action in response to elevated lipid availability. However, it is unclear what role, if any, mTORC2 activation plays in lipid-induced insulin resistance. Activation of Akt by mTORC2 has several potential consequences for myocellular metabolism, as the Akt pathway is responsible for mediating most of the metabolic actions of insulin (Taniguchi *et al.* 2006). At present, it is unknown whether in skeletal muscle mTORC1 and 2 are differentially regulated in response to increased lipid availability.

We have previously demonstrated that endurance training increases the activation of 5′-AMP-activated protein kinase (AMPK) in skeletal muscle of HF-fed rodents (Lessard *et al.*

2007). AMPK, because of its role in suppressing energy-consuming processes, is a known physiological inhibitor of the energy consuming mTOR signalling pathway (Kimball 2006, Deshmukh *et al.* 2008). At present, the effect of endurance training on mTOR activation in insulin resistant muscle has not been investigated.

mTOR complexes can positively or negatively affect insulin action and are regulated by nutrients and AMPK. Therefore, we hypothesised that improvements in muscle insulin action following exercise training in HF-fed animals would be associated with altered activation and formation of mTOR complexes 1/2. Accordingly, we determined the effects of HF feeding and exercise training on the regulation of mTORC1 and 2 and the activation of their downstream substrates (i.e. Akt, S6K1/IRS1) in skeletal muscle. In addition, we determined whether acute palmitate exposure and the activation of AMPK by 5′-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) regulate the formation and activation of mTOR complexes 1/2.

Materials and Methods

Animals

Sprague–Dawley rats (~4-week-old) were given *ad libitum* access to either control chow diet (n=8; D12328, Research Diets Inc., New Bruns wick, NJ, USA, 73·1% carbohydrates, 10·5% fat and 16·4% protein) or an HF diet (n=16; D12330, Research Diets Inc., 25·5% carbohydrates, 58% fat and 16·4% protein). After a 4 week dietary period to induce insulin resistance, HF-fed animals were randomly assigned to either exercise training (HF EXT, n=8) or sedentary control (HF CON, n=8) groups. Exercise training consisted of treadmill running for 1 h/day, 5 days/week on a 15% incline at a speed that was progressively increased to 32 m/min during the first 5 days of training and maintained for subsequent 4 weeks. The third group of animals (CF CON) remained on the control chow diet for the duration of the study (8 wk). Following the experimental period animals were fasted for 8–12 h before undergoing hind-limb perfusion (described subsequently). Exercise trained animals undertook their last exercise bout 36–48 h prior to hind-limb perfusion. All experimental procedures were approved by the Institutional Animal Care and Use Committee at California State University, Northridge.

Hind-limb perfusion and 3-*O*-methylglucose transport

Rats were anaesthetised with an i.p. injection of sodium pentobarbital (6·5 mg/100 g body mass) and surgically prepared for hind-limb perfusion as previously described (Lessard *et al.* 2007). Briefly, cannulae were inserted into the abdominal aorta and vena cava, and the animals were sacrificed via an intracardiac injection of pentobarbital as the hind limbs were washed out with 30 ml Krebs–Henseleit buffer (KHB, pH 7·55). Immediately, the cannulae were placed in line with a non-recirculating perfusion system, and the hind limbs were allowed to stabilise during a 5 min washout period which consisted of a basic perfusate medium that contained 30% washed time-expired human erythrocytes (Ogden Medical Center, Ogden, UT, USA) KHB, 4% dialysed BSA (Fisher Scientific, Fair Lawn, NJ, USA), 0·2 mM pyruvate and was continuously gassed with a mixture of 95% O_2–5% CO_2 and warmed to 37 °C. Perfusions were performed at a flow rate of 7·5 ml/min and glucose

transport was measured over an 8 min period using 8 mM of the non-metabolised glucose analogue 3-*O*-methylglucose (3-MG; 32 μCi 3-[^{3}H] mg/mM, Perkin-Elmer Life Sciences, USA) in the presence (INSULIN) or absence (BASAL) of 500 μU/ml insulin. As an extracellular space marker 2 mM mannitol (60 μCi-[1-^{14}C] mannitol/mM, PerkinElmer Life Sciences) was used. Immediately following the perfusion, portions of the red gastrocnemius (RG), a muscle comprising 90–95% oxidative fibres (Wilson *et al.* 1998), were excised from both hind limbs, blotted on gauze dampened with cold KHB, freeze clamped in liquid N_2 and stored at −80 °C for later analysis. Rates of basal and insulin-stimulated skeletal muscle 3-MG transport were calculated as previously described (Lessard *et al.* 2007).

Muscle homogenisation

Portions of muscle were cut from basal and insulin-stimulated RG, weighed frozen and homogenised in an ice-cold homogenisation buffer (1:8 wt/vol) containing 50 mM Tris–HCl (pH 7·5), 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% glycerol (v/v), 1% Triton-X, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenyl-methylsulphonyl fluoride, 10 μg/ml trypsin inhibitor and 2 μg/ml aprotinin. Following centrifugation (21 000 ***g***, 4 °C) for 25 min the supernatant was collected and assayed for protein content.

Cell culture

Stock L6 myoblasts (American Type Culture Collection, Manassas, VA, USA) were maintained at 37 °C (95% O_2–5% $_2$ collagen-coated flasks in α-modified CO_2) on 75 cm Eagle's medium (Invitrogen) containing 10% foetal bovine serum (Sigma–Aldrich) culture medium, 1% penicillin–streptomycin (Sigma–Aldrich) and 5·5 mM glucose as previously described (Lessard *et al.* 2006). Differentiation was induced by switching to medium containing 2% horse serum (Sigma–Aldrich) when the myoblasts were ~90% confluent. Experimental treatments were started after 5 days, by which time nearly all of the myoblasts had fused to form myotubes. For experimental procedures the cells were maintained in 75 cm^2 flasks (co-immunoprecipitation experiments) or trypsinised and seeded in 6-well plates. All subsequent experiments were done after 4 h serum starvation. To determine the effect of acute palmitate treatment, cells were incubated with 0·4 mM palmitate in ethanol vehicle for 0, 2 or 4 h in 2% fatty-acid free BSA. The effect of AMPK activation was subsequently determined by incubating cells for 1 or 4 h with or without 1 mM AICAR (Sigma–Aldrich). A control group was maintained for each experiment by incubating in the presence of 2% fatty-acid free BSA (Sigma–Aldrich) and the appropriate vehicle (ethanol and/or PBS). For the co-immunoprecipitation experiments, cells were incubated with 0·4 mM palmitate for 4 h with or without 1 mM AICAR. For insulin-stimulated conditions 100 nM insulin was added to appropriate wells during the last 30 min of incubations. All experiments were run in triplicate.

Western blotting

Insulin-stimulated RG muscle lysates (60 μg) were solubilised in Laemmli buffer, separated by SDS-PAGE, and transferred to PVDF membranes. The membranes were then blocked (5% non-fat dry milk (NFDM)), and incubated overnight at 4 °C with primary antibodies specific for either mTOR (mTab1), phospho-Akt1 Ser473, phospho-Akt1 Thr308, (1:1000;

Upstate (Millipore) Biotechnology, Billerica, MA, USA), AMPKα, Akt1, Akt2, S6K1, Raptor, Rictor (mAb), TSC2, phospho-mTOR Ser2448, phospho-S6K1 Thr389, phospho-TSC2 Thr1462, phospho-IRS1 Ser636/639, phospho-Akt substrate (1:1000; Cell Signaling, Danvers, MA, USA) or phospho-AMPKα Thr172 which was raised against AMPKα peptide (KDGEFLRpTSCGAPNY) as described previously (Lessard *et al.* 2006). The immunoreactive proteins were detected with enhanced chemiluminescence (Amersham (GE Healthcare) Biosciences) and quantified by densitometry.

Akt2 immunoprecipitation

The Catch and Release v2.0 Reversible Immunoprecipitation System (Upstate (Millipore) Biotechnology) was used for detection of phospho-specific Akt2 sites as per manufacturer's instructions. Briefly, insulin-stimulated RG muscle lysates (600 μg) and Akt2 (rabbit) antibody (6 μg, Upstate (Millipore) Biotechnology) were rotated overnight at 4 °C, immunocomplexes were eluted and subjected to SDS-PAGE electrophoresis. Antibodies specific for phospho-Akt Ser 473 (mouse) or phospho-Akt Thr308 (mouse; Cell Signaling) were used to detect the immunoreactive proteins as described above.

Akt1 kinase activity assay

Akt1 Immunoprecipitation Kinase Assay Kit (Upstate (Millipore) Biotechnology) was used to determine Akt1 activation. Briefly, Akt1 antibody (4 μg/sample, Upstate (Millipore) Biotechnology) and insulin-stimulated RG muscle lystates (400 μg) were rotated overnight at 4 °C. Protein G (50 μl, Amersham (GE Healthcare) Biosciences) slurry was then added to the antibody–protein complex and rotated 120 min at 4 °C. The kinase activity assay was then performed as per manufacturer's instructions.

mTOR complex co-immunoprecipitation

The mTOR complexes have been previously been shown to be detergent sensitive (Sarbassov *et al.* 2004). Therefore, 0·3% CHAPS was substituted for 1% Triton-X in the homo-genisation buffer for the co-immunoprecipitation of the mTOR complexes. Basal and insulin-stimulated RG homogenates (5 mg) or L6 cell lysates (500 μg) and mTOR (mTab1) antibody (8 μl/sample, Upstate (Millipore) Bio-technology) were rotated overnight at 4 °C. Protein G (100 μl, Amersham (GE Healthcare) Biotechnology) slurry was then added to the antibody–protein complex and rotated 120 min at 4 °C. The beads were then washed five times with homogenisation buffer and aliquoted for western blotting. Aliquots of the bead–antibody–protein complex were resuspended in Laemmli buffer, subjected to SDS-PAGE and transferred to PVDF membranes. The membranes were then blocked (5% NFDM) and incubated overnight at 4 °C with primary antibodies specific for raptor, rictor (1:1000; Cell Signaling) and mTOR. Results are normalised to total mTOR.

Statistical analyses

All results are presented as mean±S.E.M. Differences between treatment groups were determined using a one-way ANOVA with a Student–Newman–Keuls *post hoc* test.

Results

Insulin-stimulated 3-MG transport in RG skeletal muscle

Basal rates of 3-MG transport were similar among groups (Fig. 1). The rates of insulin-stimulated glucose transport were decreased by the HF-diet ($P<0{\cdot}05$, versus CF CON; Fig. 1) but were normalised to control levels by exercise training ($P<0{\cdot}05$, versus HF CON; Fig. 1).

Chronically increased lipid availability enhances mTOR complex formation

The basal and insulin-stimulated formations of the mTORC1 and 2 complexes in response to increased chronic lipid availability and exercise were determined by immunoprecipitation of mTOR then probing for its binding partners (raptor and rictor, Fig. 2A). In the insulin-stimulated muscle, there was a 70% increase in co-immunoprecipitation of raptor and mTOR in HF CON ($P<0{\cdot}01$, versus CF CON; Fig. 2C), indicating higher mTORC1 complex formation in response to HF feeding. Exercise training completely abolished the HF diet-induced increase in mTORC1 formation such that values were restored to those observed in CF CON ($P<0{\cdot}01$, HF EXT versus HF CON; Fig. 2C). The co-immunoprecipitation of rictor and mTOR (i.e. mTORC2 complex formation), in the insulin-stimulated muscle, mirrored mTORC1 complex formation. HF feeding increased mTORC2 formation by 64% ($P<0{\cdot}05$, versus CF CON; Fig. 2B), while exercise training decreased mTORC2 formation by 79% ($P<0{\cdot}01$, HF CON versus HF EXT; Fig. 2B). These results demonstrate that in response to insulin both mTORC1 and mTORC2 formation are stimulated by chronically increased lipid availability, but these effects are reversed by exercise training. There were no significant differences in the formation of mTORC1 (Fig. 2C) and mTORC2 (Fig. 2B) in the basal muscle. Changes of mTOR complex formation occurred in the absence of changes in the total protein content of mTOR, raptor and rictor (Fig. 2D).

HF diet and exercise training differentially affects mTORC1 activity

In order to determine how mTORC1 complex formation translates to mTOR activation, the phosphorylation of its downstream substrates was measured under insulin-stimulated conditions. Increases in mTORC1 formation following an HF diet resulted in concomitant increases in mTOR activity, as demonstrated by phosphorylation of its downstream substrate S6K1 (Fig. 3A). In response to HF feeding phosphorylation of S6K1 was increased by 20% at Thr389 ($P<0{\cdot}001$, CF CON versus HF CON; Fig. 3A). Exercise training normalised S6K1 phosphorylation on Thr389 ($P<0{\cdot}001$, HF CON versus HF EXT; Fig. 3A). Increased S6K1 activation by HF feeding resulted in an 18% increase in inhibitory phosphorylation of IRS1 at Ser636/639 ($P<0{\cdot}05$, CF CON versus HF CON; Fig. 3B), which was reversed in HF EXT ($P<0{\cdot}05$, HF CON versus HF EXT; Fig. 3B). These results indicate that an HF diet increased mTORC1 activity, which induced a negative feedback loop on insulin signal transduction at the level of IRS1. Importantly, these diet-induced effects were reversed by exercise training. There were no changes in the protein concentration in total S6K1 or total IRS1 (Fig. 3A and B respectively).

Akt1 phosphorylation and activity mirrors the increase in mTORC2 complex formation

To assess mTORC2 activation in response to insulin stimulation, the isoform-specific phosphorylation of Akt1 and Akt2 at Ser473 was measured. In agreement with changes in mTORC2 formation, the phosphorylation of Akt1 was increased by 26% on the Ser473 site in HF CON ($P<0{\cdot}05$ versus CF CON) but was decreased by 30% in HF EXT ($P<0{\cdot}05$, versus HF CON; Fig. 4B). Changes in Akt1 Ser473 phosphorylation were accompanied by similar alterations in Akt1 kinase activity, which tended to increase in HF CON (12%, $P<0{\cdot}10$ versus CF CON, Fig. 4C) and was decreased in HF EXT ($P<0{\cdot}05$, HF EXT versus HF CON; Fig. 4C). Decreases in Akt1 Ser473 phosphorylation and activity following exercise training occurred despite an increase in total Akt1 protein ($P<0{\cdot}001$ CF CON versus HF EXT and $P<0{\cdot}001$ HF CON versus HF EXT; Fig. 4A). In contrast, following HF feeding or exercise training there were no changes in Ser473 phosphorylation or total protein content of the Akt2 isoform, or the phosphorylation of its downstream target the Akt substrate of 160 kDa (AS160; Fig. 4D). These results indicate that mTORC2 may preferentially phosphorylate and activate the Akt1 isoform. In addition, the phosphorylation of Akt1 and Akt2 on Thr308 were similar in all groups (Fig. 4B and D respectively), demonstrating that the phosphorylation of Thr308 is independent of mTORC2 formation.

Exercise training regulates the phosphorylation of mTOR and its physiological inhibitors AMPK and TSC2

AMPK and tuberous sclerosis complex 2 (TSC2) are known inhibitors of mTOR activation. Thus, their phosphorylation in response to HF feeding and exercise training was determined in response to insulin. The phosphorylation of AMPK on its Thr172 activation site was increased by 19% in HF EXT ($P<0{\cdot}05$, versus HF CON; Fig. 5B). No changes were noted in the total protein concentration of the AMPKα catalytic subunit (Fig. 5B). In contrast, exercise training decreased the phosphorylation of TSC2 on Thr1462 by 30% ($P<0{\cdot}01$, versus HF CON; Fig. 5C). As TSC2 is a downstream target of Akt, our observed decreases in TSC2 phosphorylation likely reflect reduced Akt1 Ser473 phosphorylation and activation following exercise training (Fig. 4A). There was no difference in the total protein concentration of TSC2 between the groups (Fig. 5C). Despite the increases in mTORC1 activation by HF feeding, the phosphorylation of mTOR on its Ser2448 site was increased in HF EXT by 37% ($P<0{\cdot}05$, versus HF CON) and 47% ($P<0{\cdot}01$, versus CF CON; Fig. 5A).

Acute palmitate and AICAR activation of mTOR complexes in L6 cell culture

It has been previously demonstrated that within 3–5 h circulating NEFA induce insulin resistance in skeletal muscle (Boden 2002). Treatment with the AMPK activator AICAR has been shown to enhance glucose transport and inhibit mTOR activation (Bolster *et al.* 2002, Ju *et al.* 2007). Therefore, we determined whether 4 h palmitate incubation could activate mTOR signalling *in vitro* and whether the stimulation of AMPK by AICAR could inhibit the formation of mTOR complexes 1/2 and the phosphorylation of their downstream substrates. We observed an incremental increase in palmitate-induced S6K1 and Akt1 phosphorylation after 2 and 4 h of palmitate exposure in L6 myotubes (Fig. 6A). AICAR decreased the phosphorylation of S6K1 after 1 and 4 h of treatment in both the absence (CON) and presence of palmitate (Fig. 6B). Similar to the results of the chronic *in vivo* experiments, 4 h

palmitate treatment increased the phosphorylation of S6K1 Thr389 and Akt1 Ser473 by 60 and 30% respectively ($P<0{\cdot}05$ versus CON; Fig. 6C). AICAR significantly increased the phosphorylation of AMPK on Thr172 ($P<0{\cdot}05$ versus CON, Fig. 6C). AICAR-induced AMPK activation decreased palmitate-induced S6K1 Thr389 phosphorylation by 63% ($P<0{\cdot}05$ versus Palm, Fig. 6C). There were no significant changes in the acute complex formation of either mTORC1 or mTORC2 in response to palmitate or AICAR (Fig. 6E and F).

Discussion

The aim of the present study was to determine the effect of increased lipid oversupply on the regulation of mTOR complexes, mTORC1 and mTORC2. Furthermore, we sought to establish whether mTOR complex formation is linked to the activation of downstream substrates involved in insulin signal transduction in skeletal muscle. We had previously demonstrated that exercise training can reverse impairments to skeletal muscle insulin action in response to chronic HF feeding (Lessard *et al.* 2007). Given the potential for mTOR complexes to both positively and negatively regulate insulin action in skeletal muscle, it is plausible that HF feeding and exercise training divergently regulate mTOR complex formation. Accordingly, we hypothesised that a potential mechanism by which exercise and HF feeding exert divergent effects on insulin action may involve the differential regulation of mTOR complex formation. For the first time, we demonstrate *in vivo* that both mTORC1 and mTORC2 formation are up-regulated by chronic lipid availability (HF diet) and that these changes are completely reversed by exercise training. Additionally, increased mTORC1 and mTORC2 formation in response to an HF diet was associated with activation of their respective downstream substrates and altered insulin signal transduction.

The hyperactivation of mTORC1 by nutrient excess (amino acids, lipids, obesity) has been implicated in decreased insulin signal transduction at the level of IRS1 (Patti *et al.* 1998, Tremblay & Marette 2001, Um *et al.* 2004, Khamzina *et al.* 2005, Krebs *et al.* 2007, Tremblay *et al.* 2007). It has been proposed that an S6K1-associated negative feedback loop results in the inhibitory serine phosphorylation of IRS1 in response to mTOR activation (Patti & Kahn 2004, Um *et al.* 2006). We have previously observed decreases in IRS1-associated PI3K activity in skeletal muscle after HF feeding (Lessard *et al.* 2007). However, the regulation of mTORC1 activation by acute and chronic lipid availability is not well described. Recent *in vitro* work in skeletal muscle cell culture has directly implicated palmitate with the inhibition of insulin stimulated glucose uptake (Pimenta *et al.* 2008) likely through the activation of mTORC1 and its inhibition of insulin signalling (Mordier & Iynedjian 2007). In support of a role for mTOR activation in insulin resistance, Miller *et al.* (2008) observed increased mTOR activation in the skeletal muscle of ob/ob mice (Miller *et al.* 2008). However, the authors did not observe improvements in whole-body insulin or glucose tolerance after acute treatment with rapamycin, a potent inhibitor of mTOR activation (Miller *et al.* 2008). In contrast, in the current study we observed an exercise-induced inhibition of mTOR signalling that corresponded with an increase in skeletal muscle specific insulin sensitivity (Fig. 1).

A novel finding of the present study was that an HF diet increased the activation of mTORC1 in skeletal muscle, as evidenced by an increase in Thr389 S6K1 phosphorylation (Figs 3A and 6C), which led to a concomitant increase in the serine phosphorylation of IRS1 (Fig. 3B). Our results support the contention that one potential mechanism for the inhibition of proximal insulin signalling following acute and chronic lipid oversupply may be increased mTORC1 activation leading to IRS1 serine phosphorylation. In contrast, we show that exercise training reverses the effects of chronic lipid oversupply on mTORC1 activation (Fig. 3A), as evidenced by normalisation of S6K1 phosphorylation and decreased serine phosphorylation of IRS1 (Fig. 3B). We and others have previously shown that exercise training increases AMPK activation (Atherton *et al.* 2005, Lessard *et al.* 2007, Hawley & Lessard 2008), suggesting that exercise training may reverse the effect of mTOR activation through increased AMPK activity. In agreement with this hypothesis, Ju *et al.* (2007) have shown in cell culture that AMPK regulates insulin action through the inhibition of IRS1 serine phosphorylation by mTOR/S6K1 (Ju *et al.* 2007). Here, we demonstrate that treating palmitate incubated cells with the AMPK activator AICAR inhibits phosphorylation of S6K1 at its Thr389 site (Fig. 6C).

Analogous to the effects of increased lipid availability on mTORC1, we demonstrate that an HF-diet increases the formation of mTORC2, as indicated by increased mTOR/rictor association (Fig. 1B). Again, exercise training reversed the effects of HF feeding on mTORC2 formation (Fig. 1B). The simultaneous activation/deactivation of mTORC1 and mTORC2 in skeletal muscle seems paradoxical, as these two complexes have opposing effects on insulin signal transduction (Bhaskar & Hay 2007). While mTORC1 activation inhibits IRS1 activation, it has recently been reported that mTORC2 is an upstream kinase of Akt at Ser473, which is required for its full activation in response to insulin (Hresko & Mueckler 2005, Sarbassov *et al.* 2005, Kumar *et al.* 2008). Our results indicate that in skeletal muscle, mTORC2 formation was coupled with Ser473 phosphorylation of the Akt1 isoform, which has primarily been studied with respect to its role in cell growth and differentiation (Cho *et al.* 2001, Wilson & Rotwein 2007). Similar to our *in vivo* findings we observed a significant increase in Ser473 Akt1 phosphorylation in L6 myotubes after 4 h palmitate incubation (Fig. 6C). In contrast, we observed no changes in Ser473 phosphorylation of the Akt2 isoform, which is an important regulator of glucose uptake in muscle (Fig. 3D). In addition, we could detect no mTORC2-associated changes in the phosphorylation of AS160, a downstream substrate of Akt, with a putative role in insulin-stimulated glucose uptake (Fig. 3D). The selective activation of Akt1 and the absence of Akt2 regulation by mTORC2 with HF feeding may explain how an HF diet impairs insulin stimulated glucose transport despite activation of mTORC2 in muscle. The converse argument would apply with respect to the ability of exercise training to enhance insulin-stimulated glucose uptake despite inhibiting mTORC2 formation.

The results of previous investigations that have examined the effects of endurance exercise on skeletal muscle mTOR activation are equivocal, with some studies demonstrating decreased (Williamson *et al.* 2006, Glynn *et al.* 2008, Miranda *et al.* 2008), no change (Atherton *et al.* 2005, Coffey *et al.* 2006, Mascher *et al.* 2007) or increased (Fujita *et al.* 2007) activation of mTOR following endurance exercise. In the present study, we observed an unexpected increase in the exercise-induced phosphorylation of Ser2448 on mTOR

despite decreased Akt activation (Fig. 4B and C). Our results are in agreement with recent studies that have demonstrated that the phosphorylation of mTOR on Ser2448 is independent of Akt activation (Parkington *et al.* 2003, Mothe-Satney *et al.* 2004, Chiang & Abraham 2005, Allemand *et al.* 2009). The results of the present investigation are also consistent with those of Miranda *et al.* (2008), who demonstrated that insulin-stimulated S6K1 activation was decreased following endurance training (Miranda *et al.* 2008). Thus, it appears that unlike resistance training, which is a potent activator of mTOR (Atherton *et al.* 2005, Coffey & Hawley 2007), endurance training may inhibit mTOR activation in skeletal muscle. Opposing roles for these two training modes on mTOR activation are not surprising given the highly contrasting nature of muscle adaptations resulting from endurance and resistance exercise (Atherton *et al.* 2005, Coffey & Hawley 2007).

One mechanism by which exercise training enhances insulin action in skeletal muscle is via the chronic activation of AMPK (Hawley & Lessard 2008). AMPK is a known physiological inhibitor of the energy consuming mTOR signalling pathway (Kimball 2006). Indeed, interventions that reduce intracellular ATP levels, or administration of the AMPK activator, AICAR, decreases mTOR activation as demonstrated by decreased phosphorylation of S6K1 (Kimball 2006, Deshmukh *et al.* 2008). In the present study, we observed an exercise-induced increase in AMPK Thr172 phosphorylation (Fig. 5B), suggesting that AMPK activation may be one possible mechanism to explain the inhibition of mTOR activation in response to exercise training (Fig. 5B). Indeed, when treating palmitate-incubated cells with the AMPK activator AICAR, we observed significant decreases in the phosphorylation of the mTORC1 substrate Thr389 S6K1 (Fig. 6C). Given that such opposing stimuli and cellular responses are associated with the AMPK and mTOR pathways it seems logical that AMPK activation results in the inhibition of mTOR.

It is known that the activation of mTORC1 and mTORC2 is sensitive to increased nutrient availability (Kim *et al.* 2002, Jacinto *et al.* 2004, Marshall 2006). Investigations using rapamycin, an mTOR inhibitor, have previously demonstrated that the formations of mTOR complexes are necessary for nutrient-induced activation of S6K1/IRS1 and Akt (Tremblay & Marette 2001, Sarbassov *et al.* 2006, Sipula *et al.* 2006, Krebs *et al.* 2007). Our results provide novel evidence that the *in vivo* formation of both mTOR complexes (mTORC1 and mTORC2) is responsive to both the chronic interventions of exercise training and an HF diet. Furthermore, we demonstrate that changes in mTOR complex formation are associated with the activation of their respective downstream substrates. In agreement with the results from the current study, Schieke *et al.* (2006) have shown that increases in mTOR-raptor association after immunoprecipitation correlated with the phosphorylation and activation of S6K1 (Schieke *et al.* 2006). In contrast, acute *in vitro* palmitate application in cell culture had no effect on the formation of the mTOR complexes (Fig. 6E and F) despite increased mTORC1/2 activation (Fig. 6C).

In conclusion, we demonstrate for the first time that an HF diet and exercise training have divergent *in vivo* effects on the formation and activation of mTORC1 and mTORC2. Our data also suggest that changes in the activation of the mTOR complexes may be one mechanism to explain the altered insulin signal transduction in response to lipid availability. The reversal of mTORC 1/2 activation by exercise training was associated with the

activation of AMPK in skeletal muscle. This observation is supported by our *in vitro* work demonstrating that AICAR abolishes the lipid-induced activation of S6K1. As impaired insulin action in skeletal muscle is pivotal at the onset of type 2 diabetes, these findings have implications for both the understanding of insulin signal transduction in skeletal muscle and the discovery of therapeutic targets for the treatment of its dysregulation.

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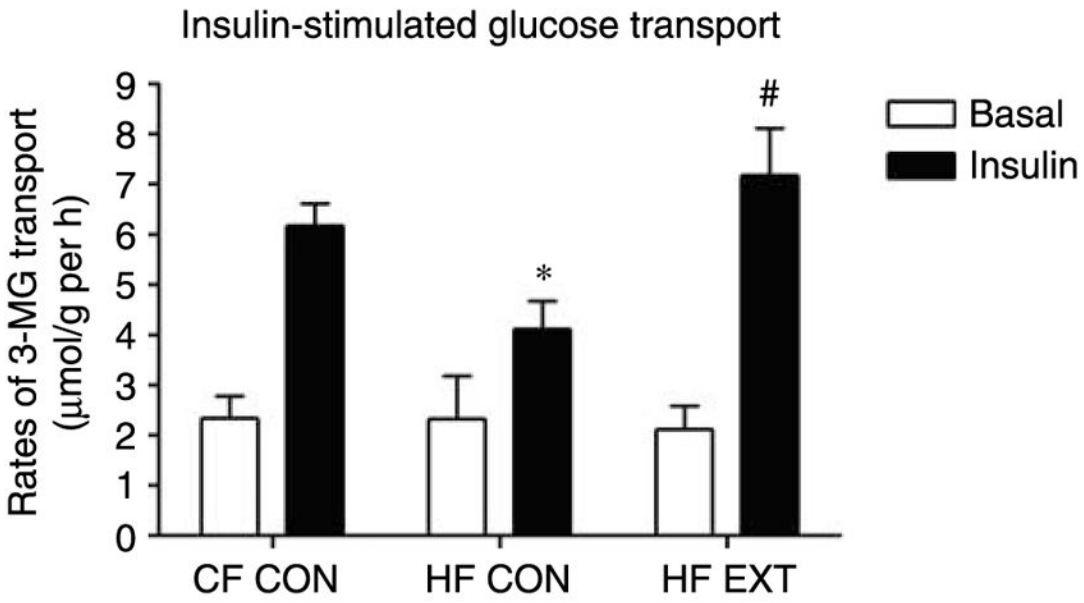

Figure 1.

Muscle glucose transport was measured in the red gastrocnemius muscle after an 8 min hind-limb perfusion with ^{3}H-3-*O*-methylglucose in the absence (basal) or presence (insulin) of insulin (500 µU/ml). Data expressed as rates µmol/g per hour (mean±S.E.M., *n*=6–8). Significant differences between groups **P*<0·05 versus CF CON, $^{\#}$*P*<0·05 versus HF CON.

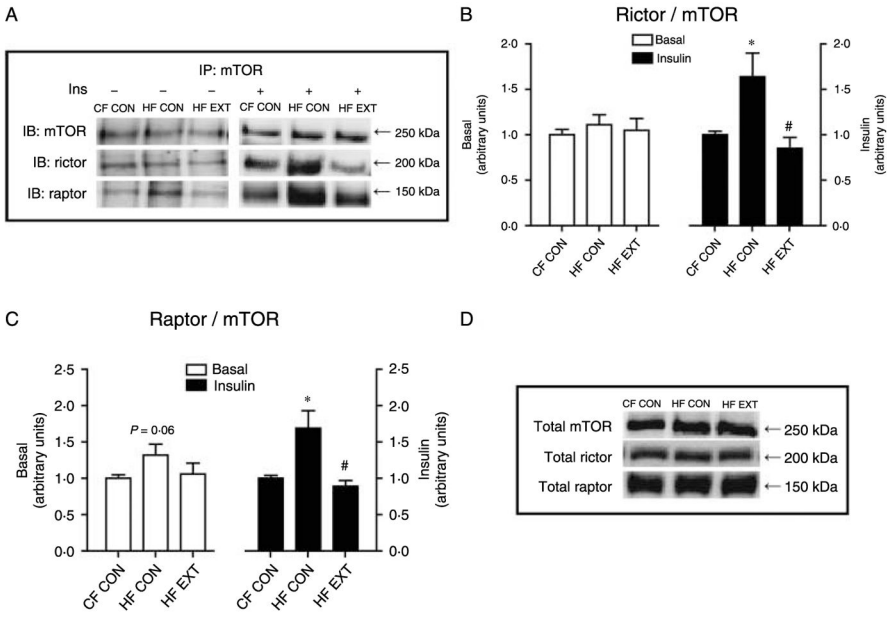

Figure 2.

The basal or insulin-stimulated formation of the mTOR complexes in response to high-fat feeding or after exercise training was determined by immunoprecipitation of mTOR followed by western blotting to detect its binding partners, (A) rictor and raptor, (B) rictor bound to mTOR, (C) raptor bound to mTOR are expressed as arbitrary units (mean±S.E.M., n=6–8). Relative protein levels of total mTOR, total rictor and total raptor (D) were quantified by densitometry. Representative blots are shown. Significant differences between groups $^{*}P<0{\cdot}05$ versus CF CON, $^{\#}P<0{\cdot}05$ versus HF CON.

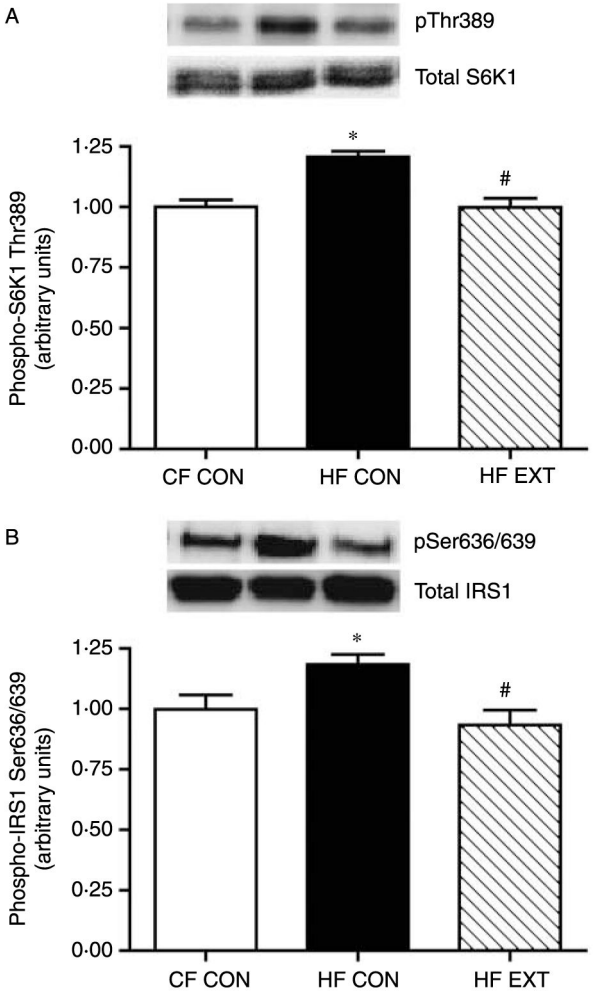

Figure 3.

The insulin-stimulated phosphorylation of S6K1 on Thr389 (A), IRS1 on Ser636/639 (B) were quantified using western blot analysis and densitometry. Representative immunoblots are shown on each figure. Values are expressed as arbitrary units (mean±S.E.M., n=8). Significant differences between groups $^{*}P<0{\cdot}05$ versus CF CON, $^{\#}P<0{\cdot}05$ versus HF CON.

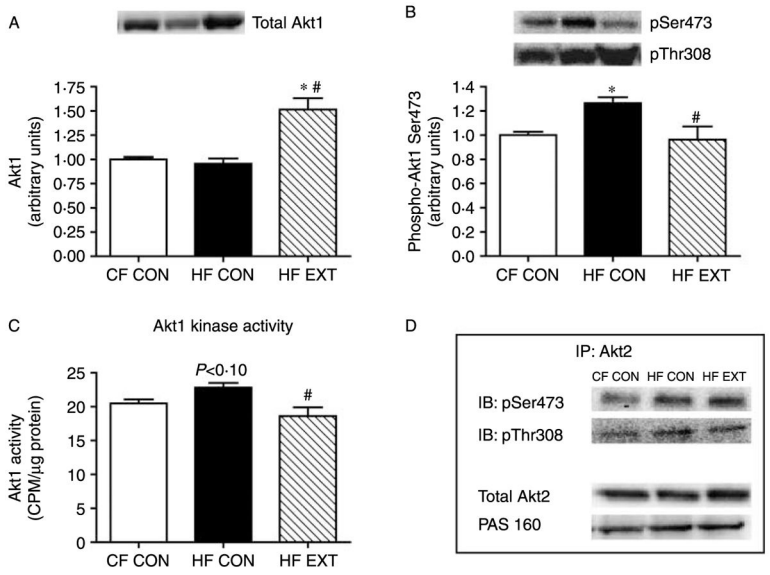

Figure 4.

Total Akt1 (A) and its insulin-stimulated phosphorylation on Ser473 and Thr308 (B) were quantified using western blot analysis and densitometry. Insulin-stimulated Akt1 kinase activity was measured using the immunoprecipitation of 400 µg of protein (C). Site-specific Akt2 phosphorylation was measured by immunoprecipitation of 600 µg of protein and western blot analysis using antibodies specific for Akt Ser473 and Akt Thr308 phosphorylation sites (D). Total Akt2 and the phosphorylation of AS160 (D) were quantified using western blot analysis and densitometry. Representative immunoblots are shown. Values are expressed as arbitrary units (mean±S.E.M., n=7–8). Significant differences between groups *P<0·05 versus CF CON, $^{\#}P$<0·05 versus HF CON.

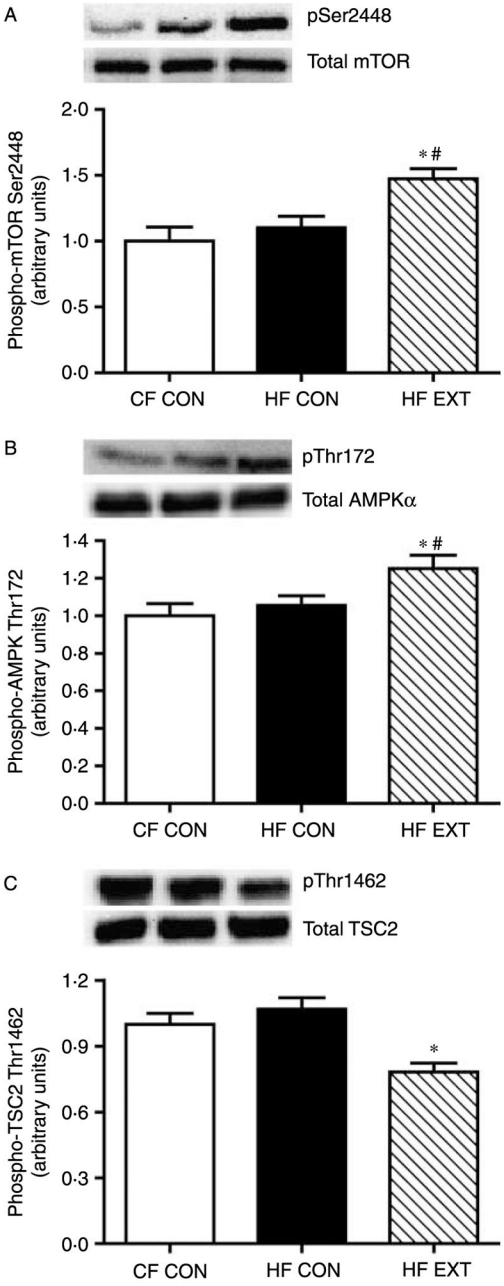

Figure 5.

Insulin-stimulated phosphorylation of mTOR and its inhibitors AMPK and TSC2. The phosphorylation of mTOR on Ser2448 (A) AMPKα on Thr172 (B) and TSC2 on Thr1462 (C) was quantified using western blot analysis and densitometry. Representative immunoblots are shown on each figure. Values are expressed as arbitrary units (mean ±S.E.M., n=7–8). Significant differences between groups *P<0·05 versus CF CON, $^{\#}P$<0·05 versus HF CON.

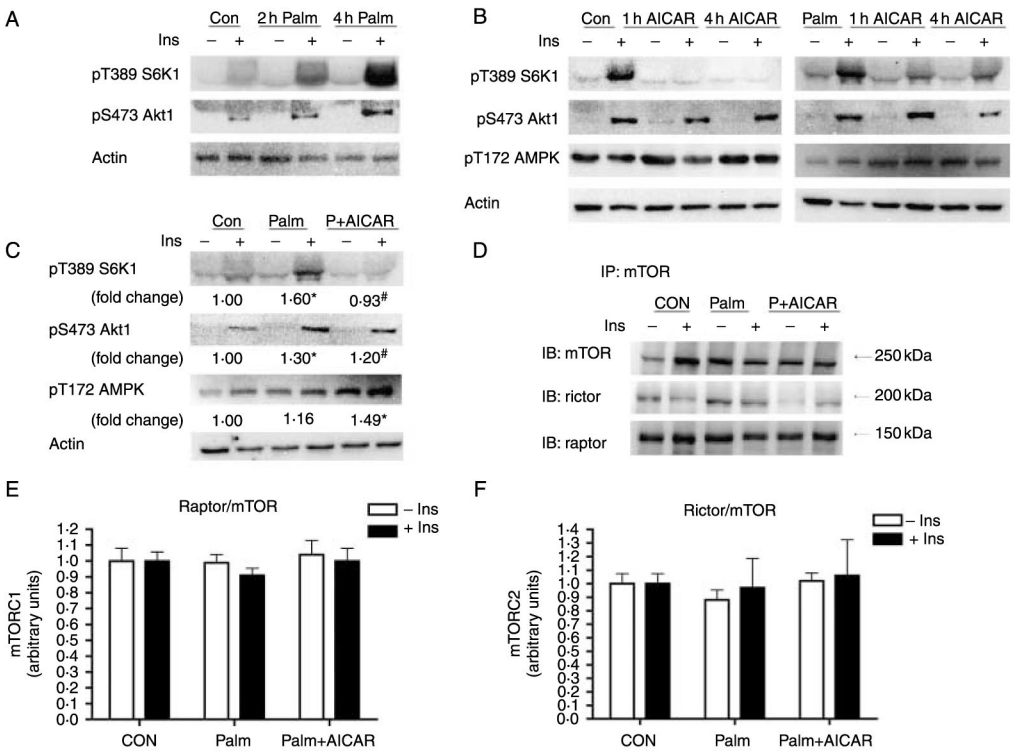

Figure 6.

The basal and insulin-stimulated activation of mTORC1/2 substrates in response to palmitate and/or AICAR treatment was determined in L6 myotubes. Phosphorylation of S6K1 on Thr389 and Akt1 on Ser 473 was measured in response to treatment with 0·4 mM palmitate for 0 (control), 2 (2 h Palm) or 4 (4 h Palm) hour (A). The effect of 1 mM AICAR on the phosphorylation of S6K1 (Thr389), Akt1 (Ser473) and AMPK (Thr172) was measured in cells treated with (Palm) or without (Con) 0·4 mM palmitate during the entire 4 h incubation (4 h AICAR) or during the last 1 h (1 h AICAR) (B). In order to associate changes in mTORC activation with complex formation, 75 mm^2 flasks of cells were incubated in 0·4 mM palmitate and 1 mM AICAR for 4 h to determine the phosphorylation of S6K1 on Thr389, Akt1 on Ser473 and AMPK on Thr172 (C). The formation of mTORC1/2 (D) was measured by immunoprecipitation of mTOR followed by western blot of mTOR binding partners' raptor (E) and rictor (F) which were quantified by densitometry. Insulin (100 nM) was added to the appropriate groups during the last 30 min of incubation. Representative blots are shown. Actin was run as a loading control for each sample. Significant differences between groups $^{*}P<0{\cdot}05$ versus CF CON, $^{\#}P<0{\cdot}05$ versus HF CON; $n=3$/treatment.