

# Dihydrotestosterone stimulates amino acid uptake and the expression of LAT2 in mouse skeletal muscle fibres through an ERK1/2-dependent mechanism

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**Non technical summary** In a recent study we showed that dihydrotestosterone (DHT), but not testosterone, increases force production in fast contracting muscles and decreases it in slow contracting ones. These findings led us to suggest that DHT may be a better muscle building hormone than testosterone. In the present study, we have examined the effects of these hormones on amino acid transport in mouse fast and slow contracting skeletal muscle fibre bundles. Our results show that DHT increases protein synthesis and the transport of essential amino acids into fast contracting muscle fibre bundles, whereas testosterone does not. These findings confirm our previous proposition and reinforce our suggestion that DHT may be a better hormone for the treatment of muscle wasting conditions than testosterone.

**Abstract** Dihydrotestosterone (DHT) has acute/non-genomic actions in adult mammalian skeletal muscles whose physiological functions are still poorly understood. Therefore, the primary aim of this study was to investigate the acute/non-genomic effects of DHT on amino acid uptake as well as the cellular signal transduction events underlying these actions in mouse fast- and slow-twitch skeletal muscle fibre bundles. <sup>14</sup>C-Labelled amino acids were used to investigate the effects of DHT and testosterone (T) on amino acid uptake and pharmacological interventions were used to determine the cellular signal transduction events mediating these actions. While T had no effect on the uptake of isoleucine (Ile) and  $\alpha$ -methylaminoisobutyric acid (MeAIB) in both fibre types, DHT increased their uptake in the fast-twitch fibre bundles. This effect was reversed by inhibitors of protein translation, the epidermal growth factor receptor (EGFR), system A, system L, mTOR and MEK. However, it was relatively insensitive to inhibitors of transcription, androgen receptors and PI3K/Akt. Additionally, DHT treatment increased the expression of LAT2 and the phosphorylation of the EGFR in the fast-twitch fibre bundles and that of ERK1/2, RSK1/2 and ATF2 in both fibre types. Also, it decreased the phosphorylation of eEF2 and increased the incorporation of Ile into proteins in both fibre types. Most of these effects were reversed by EGFR and MEK inhibitors. From these findings we suggest that another physiological function of the acute/non-genomic actions of DHT in isolated mammalian skeletal muscle fibres is to stimulate amino acid uptake. This effect is mediated through the EGFR and involves the activation of the MAPK pathway and an increase in LAT2 expression.

(Received 9 February 2011; accepted after revision 19 May 2011; first published online 23 May 2011)

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**Abbreviations** AAS, anabolic-androgenic steroid; ATF2, activating transcription 2; Akt, protein kinase B; BCH, 2-aminobicyclo-(2,2,1)heptane-2-carboxylic acid; DHT, dihydrotestosterone; EDL, extensor digitorum longus; eEF2, eukaryotic elongation factor 2; EGFR, epidermal growth factor receptor; ERK1/2, extracellular-signal regulated kinases 1/2; Ile, isoleucine; JNK, c-Jun N-terminal kinase; LAT2, L-type amino acid transporter 2; MAPK pathway, mitogen-activated protein kinase pathway; MeAIB,  $\alpha$ -methylaminoisobutyric acid; MEK, mitogen-activated protein kinase/ERK kinase; MSK1/2, mitogen- and stress-activated protein kinases 1/2; mTOR, mammalian target of rapamycin; RSK1/2, 90 kDa ribosomal s6 kinase; SNAT2, sodium-coupled neutral amino acid transporter 2; T, testosterone.

## Introduction

Dihydrotestosterone (DHT) is a metabolite of the male sex hormone testosterone (T) that is produced in many tissues following the rapid and irreversible reduction of T by  $5\alpha$ -reductase (Bruchovsky & Wilson, 1968). Although the free plasma concentration of DHT and T (normal range  $1.5\text{--}20\text{ ng dl}^{-1}$  and  $4\text{--}57.5\text{ ng dl}^{-1}$  for testosterone and DHT, respectively) in healthy young adult men is similar, it is generally accepted that DHT is the more potent hormone because of its receptor binding kinetics (Saartok *et al.* 1984). However, as the expression of  $5\alpha$ -reductase in adult mammalian skeletal muscles is still controversial, it has been suggested that DHT may not have any physiological functions in skeletal muscle (Thigpen *et al.* 1993). Nevertheless, in a recent study we showed that DHT, but not T, modulates force production in isolated intact mouse skeletal muscle fibres. These effects were mediated through the EGFR and involved the activation of ERK1/2. From these findings we suggested that DHT may be the more potent anabolic-androgenic steroid (AAS) in adult mammalian skeletal muscle fibres (Hamdi & Mutungi, 2010). However, whether DHT has any other acute/non-genomic physiological functions in mammalian skeletal muscles is uncertain.

In adult males, AASs are important determinants of body composition (Wilson *et al.* 1988; Bhasin *et al.* 2001). For example, treating adult female laboratory animals (Exner *et al.* 1973a; Salmons, 1992), hypogonadal men (Bhasin *et al.* 1997), elderly men with low T concentrations (Ferrando *et al.* 2002), and men suffering from AIDS (Bhasin *et al.* 2000) with T or any of its many derivatives (commonly referred to as anabolic-androgenic steroids; AASs) for several weeks has been shown to increase lean body mass. Although the mechanisms underlying the changes in lean body mass are still poorly understood, it is generally believed that AAS administration increases protein synthesis (Kochakian, 1950; Ferrando *et al.* 2002) while at the same time decreasing its breakdown (Ferrando *et al.* 2002).

Skeletal muscle has the largest pool of amino acids in the body with the branched chain amino acids leucine, isoleucine and valine forming 35% of total skeletal muscle proteins (Harper *et al.* 1984). Therefore, for skeletal muscle mass to increase, as occurs following the chronic administration of AASs, it must have an adequate supply of essential amino acids. However, the few studies that have investigated amino acid uptake in human skeletal muscles, following AAS administration, were unable to demonstrate any changes in their uptake (Ferrando *et al.* 2002), thereby leaving the source of the new/extra amino acids unresolved. Therefore, it is still uncertain whether DHT and T have any effects on amino acid uptake in adult mammalian skeletal muscle fibres.

Amino acids enter and leave cells through specialized proteins known as amino acid transporters (Christensen, 1990). Although all cells express a variety of these transporters, the combinations found in each cell type seem to depend on the physiological functions of the cell and the location of the transporters, suggesting that their (the transporters) expression and activity is carefully regulated (McGivan & Pastor-Anglada, 1994; Wagner *et al.* 2001). While some of the physiological factors regulating the activity and expression of amino acid transporters belonging to system A in mammalian cells have been the subject of a number of previous studies (Sugawara *et al.* 2000; Hyde *et al.* 2002; Franchi-Gazzola *et al.* 2004; Palii *et al.* 2004; Kashiwagi *et al.* 2009), little is known about the physiological factors regulating the expression and activity of L-type amino acid transporters, especially in adult mammalian skeletal muscle fibres.

Therefore, the primary aims of this study were to investigate the acute effects of DHT and T on (1) amino acid uptake, (2) the expression of the amino acid transporters SNAT2 and LAT2, and (3) the cellular signalling pathway(s) mediating these actions in fast- and slow-twitch muscle fibre bundles isolated from adult female mice. Our results show that DHT, but not T, increases the uptake of essential amino acids and their incorporation into proteins in the fast-twitch skeletal muscle fibre bundles. These effects are mediated through the EGFR and involved activation of the MAPK pathway and an increase in the expression of LAT2. From these results we suggest that another physiological function of the non-genomic actions of DHT in adult mammalian skeletal muscle fibres is to regulate the transport of essential amino acids and their incorporation into proteins.

## Methods

### Preparation of the small intact skeletal muscle fibre bundles

All the experiments reported in this study were performed at room temperature ( $\sim 20^\circ\text{C}$ ) on small skeletal muscle fibre bundles isolated from either the extensor digitorum longus (EDL, a mainly fast-twitch muscle in adult mice) or the soleus (a predominantly slow-twitch muscle in adult mice) of adult female CD1 mice aged  $57.4 \pm 2.3$  days ( $n = 34$ ; range 45–79 days). The mice were killed by cervical disarticulation as recommended by the Animals (Scientific Procedures) Act 1986, UK (for a summary of the regulations see Drummond, 2009) and all the experiments conformed to the local animal welfare committee guidelines. The EDL and soleus muscles from both hindlimbs were then isolated and small muscle fibre bundles, containing approximately 10–15 fibres

(mean cross-sectional diameter  $350 \pm 17 \mu\text{m}$ ,  $n = 68$ ), were dissected and care was taken to ensure that all the fibres in a bundle were intact and electrically excitable.

During an experiment, the muscle fibre bundles were mounted horizontally between two stainless steel hooks in specially designed muscle chambers. Two types of muscle chambers, one with a total volume of 5 ml and the other 25 ml, were used for the determination of amino acid uptake and cellular-signal transduction events, respectively. The fibre bundles were continuously perfused with the standard mammalian Ringer solution alone or the standard Ringer solution plus the various compounds/inhibitors listed in Table 1. The composition of the Ringer solution was (in mM): 109 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 4 CaCl<sub>2</sub>, 24 NaHCO<sub>3</sub>, 1 NaHPO<sub>4</sub>, 10 sodium pyruvate plus 200 mg l<sup>-1</sup> bovine calf serum; and its pH was maintained at 7.42 by constantly bubbling it with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

#### Determination of the effects of dihydrotestosterone (DHT) and testosterone (T) on the uptake of isoleucine and $\alpha$ -(methylamino)isobutyric acid

To determine the uptake of the amino acids, eight fast-twitch and eight slow-twitch muscle fibre bundles were divided into two groups. Half were treated with the standard Ringer solution containing 6.3  $\mu\text{l}/100 \text{ ml}$  (107.9  $\mu\text{M}$ ) ethanol (the vehicle used to dissolve both DHT and T) plus either 2 mM [<sup>14</sup>C]isoleucine (L-[U-<sup>14</sup>C]Ile; radioactivity level 3.46  $\mu\text{Ci ml}^{-1}$ ) or 68.3  $\mu\text{M}$   $\alpha$ -[<sup>14</sup>C]methylaminoisobutyric acid ( $\alpha$ -[1-<sup>14</sup>C]-MeAIB; 2-(methylamino)-2-methylpropionic acid; radioactivity level 0.8  $\mu\text{Ci ml}^{-1}$ ) (both purchased from PerkinElmer, Beaconsfield, UK) for 1 h. These preparations acted as controls. The other half were treated with the Ringer solution containing 630 pg l<sup>-1</sup> 4,5 $\alpha$ -dihydrotestosterone (2.17 nM; DHT; 5 $\alpha$ -androstane-17 $\beta$ -ol-3-one) or testosterone propionate (1.83 nM; T; 17 $\beta$ -hydroxy-4-androsten-3-one 17-propionate) (both from Sigma-Aldrich, Gillingham, UK) plus the radio-labelled amino acids for the same duration (1 h). These fibre bundles acted as the experimental/treatment group. Radiolabelled Ile was used in these studies because, unlike leucine, it does not stimulate protein synthesis in adult mammalian skeletal muscle (Anthony *et al.* 2000).

At the end of the experiments described above, the Ringer solution was aspirated and the fibre bundles were washed twice in ice-cold phosphate-buffered saline (PBS). The excess buffer was blotted out and the bundles were then snap frozen in liquid nitrogen and pulverised, and cytosolic proteins were extracted using the non-ionic cell lysis buffer NP40 as described in Mutungi (2008). The

amount of proteins in each lysate was then determined using the quick Bradford assay (Bradford, 1976). The rest of the lysate was mixed with an equal amount of Optima Gold XR liquid scintillant (PerkinElmer) and the level of radioactivity in the mixture was determined using a liquid scintillation counter (Tri-Carb 2250 CA, Canberra-Packard) 1 h and 24 h after the end of each experiment. Also, each experiment was repeated at least twice. As T had no effect on amino acid uptake in either fibre type, the rest of the experiments were performed using DHT.

To investigate the cellular-signal transduction events underlying the effects of DHT on amino acid uptake, the experiments described above were repeated using eight fast- and eight slow- twitch muscle fibres bundles. Briefly, half of the fibre bundles were treated with the standard Ringer solution containing 107.9  $\mu\text{M}$  ethanol plus the radio-labelled amino acids for 1 h. The other half were pre-incubated for 20 min in the standard Ringer solution containing one of the inhibitors listed in Table 1. They were then treated, for 1 h, with the Ringer solution containing DHT plus either 2 mM L-[U-<sup>14</sup>C]Ile or 68.3  $\mu\text{M}$   $\alpha$ -[1-<sup>14</sup>C]-MeAIB plus the inhibitor whose effects were being investigated. Most of the inhibitors used and their concentrations are similar to those used previously in Hamdi & Mutungi (2010). The uptake of the amino acids in the presence and absence of the inhibitors as well as DHT were then determined as described above.

In adult mammals, muscle hypertrophy and hence protein synthesis is regulated by the Akt/mTOR pathway (Glass, 2003). Therefore, in another experiment we determined whether the Akt/mTOR pathway affected the basal uptake of amino acids or the DHT-induced increase in amino acid transport into the muscle fibre bundles. Briefly, 16 muscle fibre bundles (8 fast- and 8 slow-twitch) were divided into four groups each consisting of two fast- and two slow-twitch muscle fibre bundles. Each group was then treated for at least 20 min as follows: Group 1 (control) was treated with the standard Ringer solution plus 107.9  $\mu\text{M}$  ethanol; Group 2 was treated with the Ringer solution plus DHT alone; Group 3 was treated with Ringer solution containing 10 nM deguelin (a specific (PI3K)/protein kinase B (Akt) inhibitor) (Chun *et al.* 2003) or 100 ng ml<sup>-1</sup> rapamycin (an mTOR specific inhibitor) (Shepherd *et al.* 1995) plus 1 mM ethanol; and Group 4 was treated with the Ringer solution containing DHT plus the above concentrations of deguelin and rapamycin. At the end of this period, the solutions were replaced with the same solutions but this time containing 2 mM L-[U-<sup>14</sup>C]Ile. The fibre bundles were then incubated in these solutions for 1 h after which the uptake of L-[U-<sup>14</sup>C]Ile was determined as described above.

**Table 1. The proteins/receptors whose effect was being investigated and the inhibitors used**

| Protein/process   | Inhibitors   |               |                          | Source              |
|-------------------|--|---------------|--------------------------|---------------------|
|                   | Scientific name  | Common name   | Concentration            |                     |
| Transcription     | Actinomycin IV   | Actinomycin D | 1 $\mu\text{g ml}^{-1}$  | Sigma-Aldrich       |
|                   | 3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide   | Cycloheximide | 10 $\mu\text{g ml}^{-1}$ | Sigma-Aldrich       |
| Translation       | 2-Aminobicyclo[2,2,1]heptane-2-carboxylic acid   | BCH           | 100 $\mu\text{M}$        | Sigma-Aldrich       |
| Androgen receptor | 6-Chloro-1 $\beta$ ,2 $\beta$ -dihydro-17-hydroxy-3'H-cyclopropa(1,2)-pregna-1,4,6-triene-3,20-dione acetate | Cyproterone   | 1 $\mu\text{M}$          | Sigma-Aldrich       |
|                   | 2-Methyl-N-(4-nitro-3-[trifluoromethyl]phenyl)propanamide  | Flutamide     | 3 $\mu\text{M}$          | Sigma-Aldrich       |
| SNAT2             | 2-N-Dimethylalanine-2-(methylamino)-2-methylpropionic acid   | MeAIB         | 10 $\mu\text{M}$         | Sigma-Aldrich       |
| EGFR              | N-[3-Chlorophenyl]-6,7,-dimethoxy-4-quinazoline  | AG 1478       | 100 nM                   | Sigma-Aldrich       |
| MEK               | 2'-Amino-3'-methoxyflavone   | PD98059       | 20 $\mu\text{M}$         | Alexis Biochemicals |
| Akt               | (7a5,13a5)-13,13a-Dihydro-9,10-dimethoxy-3,3-dimethyl-3H-bis[1]benzopyrano[3,4-b:6',5'-e]pyran-7(7aH)-one    | Deguelin      | 10 nM                    | Sigma-Aldrich       |
| mTOR              | 23,27-Epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine   | Rapamycin     | 100 ng ml <sup>-1</sup>  | Sigma-Aldrich       |

### Determination of amino acid incorporation into proteins

In another experiment, a modification of the trichloroacetic acid (TCA) precipitation method was used (Jiang *et al.* 2003). Briefly, six fast-twitch and six slow-twitch fibre bundles were divided into two equal groups. Half (controls) were treated with the standard Ringer solution containing 107.9  $\mu\text{M}$  ethanol plus 2 mM L-[U-<sup>14</sup>C]Ile for 1 h. These preparations acted as controls. The other half (experimental) were treated with the Ringer solution containing 630 pg ml<sup>-1</sup> DHT plus the radio-labelled amino acid for the same duration (1 h). At the end of the experiment, proteins were extracted as described above. The lysates were then mixed with equal amounts of 10% ice-cold TCA and left standing on ice for 2–3 h. The lysate–TCA mixture was then centrifuged at 15,000 g for 20 min and the supernatant was discarded. The pellet was washed twice in ice-cold acetone and centrifuged at 15,000 g for 10 min, and the supernatant was discarded. Finally, the pellet was dried, pulverised and re-dissolved in 50–100  $\mu\text{l}$  urea buffer depending on the size of the pellet. The buffer contained 7 M urea, 2 M thiourea and 20 mM trisbase, pH 8.5. The radioactivity of the mixture was then determined as described above.

### Determination of the amino acid transporter mediating the effects of DHT on amino acid transport in mammalian skeletal muscle fibre bundles

Both MeAIB and Ile are neutral amino acids and are transported across the cell membrane through system A and system L amino acid transporters, respectively (Christensen, 1990; Palacin *et al.* 1998). Although each of these systems has several isoforms (Palacin *et al.* 1998; Mackenzie & Erickson, 2004), only the sodium-coupled neutral amino acid transporter (SNAT) 2 (Utsunomiya-Tate *et al.* 1996; Yao *et al.* 2000; Hyde *et al.* 2001; Mackenzie & Erickson, 2004) and the sodium-independent L-type amino acid transporter (LAT) 2 (Pineda *et al.* 1999; Segawa *et al.* 1999; Sugawara *et al.* 2000) have consistently been identified in skeletal muscle. Furthermore, both these transporters can be inhibited by the synthetic amino acid analogues  $\alpha$ -(methylamino)isobutyric acid (MeAiB; 2-(methylamino)-2-methylpropionic acid) and 2-aminobicyclo-(2,2,1)heptane-2-carboxylic acid (BCH), respectively (Christensen, 1985). Therefore, in another experiment we used these analogues to investigate the amino acid transporters mediating the effects of DHT on amino acid uptake observed in the preceding experiments.

Briefly, the muscle fibre bundles were divided into four groups each containing four fast- and four slow-twitch fibre bundles. Each group was then treated for at least 20 min as follows: Group 1 (control) was treated with the standard Ringer solution plus 107.9  $\mu\text{M}$  ethanol; Group 2 was treated with the Ringer solution plus DHT alone; Group 3 was treated with the Ringer solution containing either BCH or MeAIB alone plus 107.9  $\mu\text{M}$  ethanol; and Group 4 was treated with the Ringer solution containing DHT plus either 10 mM BCH or 100  $\mu\text{M}$  MeAIB. At the end of this period, the solutions were then replaced with the same solutions but this time 2 mM L-[U- $^{14}\text{C}$ ] Ile was added. The bundles were then incubated in these solutions for 1 h after which the uptake of Ile by the fibre bundles was determined as described above.

BCH inhibits both LAT1 and LAT2. Therefore, in another experiment we determined whether the DHT-induced uptake of Ile was through LAT2. Briefly, another set of fibre bundles (6 fast- and 6 slow-twitch) was treated for 20 min with Ringer solution containing 1:500 dilution of a rabbit polyclonal antibody against LAT2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). They were then incubated for 1 h in the Ringer solution containing 2 mM L-[U- $^{14}\text{C}$ ]Ile plus either the antibody alone or the antibody plus DHT. The uptake of Ile by the fibres was then determined as described above.

In another experiment, we examined whether DHT treatment affects the expression of LAT2 and SNAT2 in the fibre bundles. To do this, we treated another set of fibre bundles with the standard Ringer solution plus the vehicle (107.9  $\mu\text{M}$  ethanol) or the standard Ringer solution plus DHT. Cytosolic and crude membrane proteins were then extracted and immunoblotted for the expression of SNAT2 and LAT2 (Santa Cruz Biotechnology) as described below.

### Determination of the cellular signal transduction pathway(s) mediating the effects of DHT on amino acid uptake

To investigate the cellular signal transduction events mediating the effects of DHT on amino acid uptake, another set of fibre bundles was treated with the standard Ringer solution plus the vehicle or the standard Ringer solution plus DHT for 1 h. In some of the experiments, the fibre bundles were pre-incubated with the standard Ringer solution plus the EGFR inhibitor tyrphostin AG1478 or the MEK inhibitor PD98059 for 20 min. They were then treated with the Ringer solution containing the inhibitor whose effects were being investigated plus DHT for 1 h. At the end of the experiments, proteins were extracted from the muscle fibre bundles and immunoblotted as described below.

### Immunoblotting

At the end of the experiments described above, the fibre bundles were snap frozen in liquid nitrogen and pulverized. Cytosolic and crude membrane proteins were extracted using NP40 lysis buffer. To extract cytosolic/total cell proteins, the lysates were centrifuged for 10 min at 15,000 g. The supernatant was then collected and stored at  $-80^{\circ}\text{C}$  until required. To extract crude membrane proteins, the pellets from the cytosolic extractions were washed twice in ice cold PBS and blotted with filter paper to remove excess PBS. They were then snap frozen in liquid nitrogen and pulverized, and proteins were extracted as described above. Equal amounts (10  $\mu\text{g}$ ) of the proteins were then immunoblotted as described in Mutungi (2008). Briefly, the proteins were resolved in either 10% or 5% SDS-polyacrylamide gels, depending on the molecular weight of the proteins being probed. They were then transferred onto PVDF membranes. The membranes were blocked for non-specific antibody labelling with 5% milk for 30 min. They were then incubated overnight at  $4^{\circ}\text{C}$  with primary antibodies against pEGFR, pJNK, pp38 and pATF2 (from Cell Signaling Technology, Inc., Danvers, MA, USA); pEK1/2, myc, pcJUN, MyoD, SNAT2 and LAT2 (from Santa Cruz Biotechnology); and pRSK1/2, pMSK1/2 and pEF2 (Abcam, Cambridge, UK). The following day they were washed and incubated for 2 h with species specific secondary antibodies conjugated with horseradish peroxidase. Finally, they were visualised using SuperSignal WestPico (Perbio Science UK Ltd, Cramlington, UK) chemiluminescence substrate and exposure to film.

The following day, the membranes were stripped as described in Hamdi & Mutungi (2010). They were then re-probed with either a pan-actin antibody (ab3280) from Abcam or an antibody against the total (unphosphorylated) protein whose phosphorylation was being investigated. These blots were used as loading controls. In some experiments, the fibre bundles were also fibre typed using monoclonal antibodies against fast and slow MyHCs (Sigma-Aldrich).

### Analysis and data handling

The radioactivity of each lysate was counted and expressed as the disintegration per minute. It was then normalized to the protein content of the lysate and expressed as becquerel per microgram of protein ( $\text{Bq } \mu\text{g}^{-1}$ ). The radioactivity was then normalized to that recorded under control conditions and all the data are presented as the mean percentage change in uptake  $\pm$  SD.

All the Western blots were run in triplicate and each experiment was repeated at least three times. To determine the statistical significance of the changes, the intensity of the bands, from each experiment, was analysed using

ImageJ (Scion/NIH) and was normalized to that of the loading control (either actin or the total protein).

Statistical analysis of the data was performed using SigmaPlot 11.2 (Systat Software Inc., San Jose, CA, USA). The data obtained from the two fibre types under control and from the various experimental conditions were then compared using a three-way ANOVA and  $P < 0.05$  was considered statistically significant.

## Results

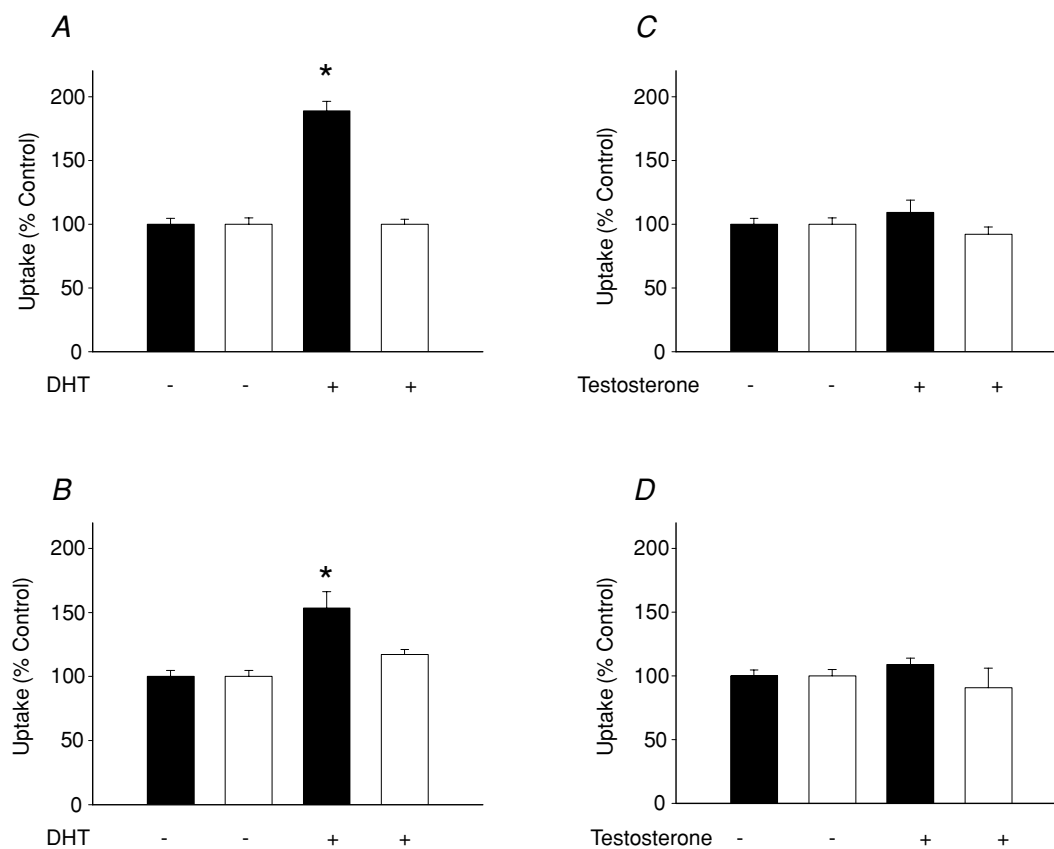
### Acute effects of DHT and T on amino acid uptake in fast- and slow-twitch skeletal muscle fibre bundles

Treatment of the fibre bundles with physiological concentrations ( $630 \text{ pg ml}^{-1}$ ) of DHT significantly ( $P = 0.001$ ) increased the uptake of L-[U- $^{14}\text{C}$ ]Ile and  $\alpha$ -[1- $^{14}\text{C}$ ]MeAIB in the fast-twitch fibre bundles without significantly ( $P = 0.72$ ) affecting that of the slow-twitch ones. For example, DHT led to an  $88.7 \pm 14\%$  ( $n = 4$  fibre bundles) increase in the uptake of L-[U- $^{14}\text{C}$ ]Ile in

the fast-twitch fibre bundles but only to a  $5.9 \pm 4.5\%$  ( $n = 4$  fibre bundle) increase in the slow-twitch ones (Fig. 1A). The corresponding values for  $\alpha$ -[1- $^{14}\text{C}$ ]MeAIB were  $53.9 \pm 12.8\%$  ( $n = 4$  fibres) and  $7.0 \pm 4.0\%$  in the fast- and slow-twitch fibre bundles, respectively (Fig. 1B). In contrast, T did not significantly ( $P = 0.47$  and  $0.59$  for Ile and MeAIB, respectively) affect the uptake of L-[U- $^{14}\text{C}$ ]Ile and  $\alpha$ -[1- $^{14}\text{C}$ ]MeAIB in either fibre type (Fig. 1C and D). For example, it led to a  $3.2 \pm 4.9\%$  ( $n = 4$  fibres) increase and to a  $4.4 \pm 5\%$  ( $n = 4$  fibres) decrease in the uptake of L-[U- $^{14}\text{C}$ ]Ile in the fast- and slow-twitch muscle fibres, respectively (Fig. 1C). The corresponding values for  $\alpha$ -[1- $^{14}\text{C}$ ]MeAIB were  $6.0 \pm 5\%$  ( $n = 4$  fibres) increase and  $6.5 \pm 5\%$  ( $n = 4$  fibres) decrease in the fast- and slow-twitch fibres, respectively (Fig. 1D).

### Amino acid transporter(s) mediating the effects of DHT on amino acid transport in isolated intact mammalian skeletal muscle fibre bundles

Figure 2 shows the effects of pre-incubating another set of fast- and slow-twitch muscle fibre bundles with



**Figure 1. DHT but not T increases amino acid uptake in fast-twitch skeletal muscle fibre bundles**

Bar graphs showing the effects of treating fast- (filled bars) and slow- (open bars) twitch fibre bundles with physiological levels of DHT (A and B) and T (C and D) on the uptake of L-[U- $^{14}\text{C}$ ]Ile (A and C) and  $\alpha$ -[1- $^{14}\text{C}$ ]MeAIB (B and D). Note that treating the fibre bundles with DHT, but not T, significantly ( $P = 0.001$ ) increases the uptake of both L-[U- $^{14}\text{C}$ ]Ile and  $\alpha$ -[1- $^{14}\text{C}$ ]MeAIB in the fast-twitch fibre bundles. In contrast, DHT had little effect on the uptake of both amino acids in the slow-twitch bundles. \* $P < 0.05$  when compared to control.

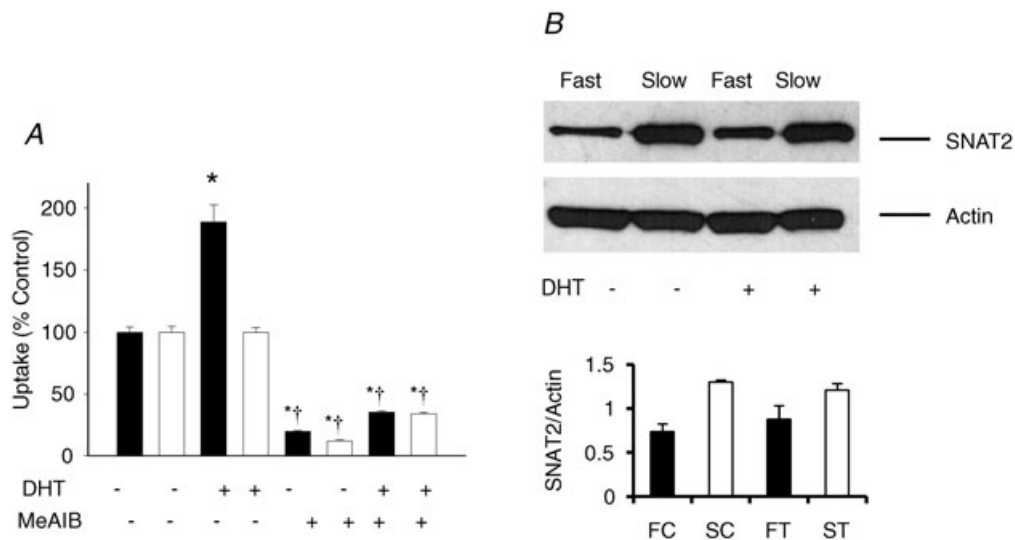
100  $\mu\text{M}$  MeAIB (a system A inhibitor) on the basal and DHT-induced uptake of L-[U- $^{14}\text{C}$ ]Ile. As the results show, treatment of the muscle fibre bundles with DHT significantly ( $P < 0.001$ ) increased the uptake of L-[U- $^{14}\text{C}$ ]Ile in the fast-twitch fibre bundles only. In contrast, pre-incubating the fibre bundles with MeAIB significantly ( $P < 0.05$ ) decreased the basal uptake of L-[U- $^{14}\text{C}$ ]Ile in both fibre types (Fig. 2A). Also, it completely abolished the DHT-induced increase in Ile uptake in both fibre types (Fig. 2A), suggesting that a system A amino acid transporter was probably involved. However, as the results displayed in Fig. 2B show, treatment of the muscle fibres with DHT did not significantly ( $P > 0.05$ ) affect the expression of SNAT2 (the commonest system A amino acid transporter identified in skeletal muscle) in both fibre types.

In another experiment, we investigated the effects of pre-treating the muscle fibre bundles with the L-type amino acid transporter inhibitor, BCH. Treatment of the muscle bundles with BCH alone significantly ( $P < 0.05$ ) decreased the basal transport of L-[U- $^{14}\text{C}$ ]Ile in both fibre types. Also, it completely abolished the DHT-induced increase in L-[U- $^{14}\text{C}$ ]Ile uptake (Fig. 3A), suggesting that the increase was via an L-type amino acid transporter. However, from these experiments it was uncertain whether the transporter was LAT1 or LAT2. Therefore, in another experiment we examined the effects of DHT on the expression of LAT2. In the untreated fibre bundles, the

slow-twitch muscle fibre bundles always expressed higher concentrations of the transporter than the fast-twitch ones. Moreover, treating the fibre bundles with DHT significantly ( $P = 0.01$ ) increased the expression of LAT2 in the fast-twitch fibre bundles without significantly ( $P = 0.13$ ) affecting its expression in the slow-twitch ones. LAT2 was extracted mainly in the membrane fraction of the fibres, whereas, SNAT2 was extracted mainly in the cytosolic fraction. Again these results suggest that the effects of DHT were probably mediated through LAT2. However, they were not conclusive. Therefore, in another experiment, we used an antibody against LAT2 as an inhibitor of the transporter. As the results displayed in Fig. 3C show, treatment of the fibre bundles with the antibody alone significantly ( $P < 0.05$ ) reduced the basal transport of L-[U- $^{14}\text{C}$ ]Ile in both fibre types. Also, it completely abolished the DHT induced uptake of L-[U- $^{14}\text{C}$ ]Ile (Fig. 3C) in the fast-twitch fibre bundles.

### Receptor(s) mediating the effects of DHT on amino acid transport in isolated intact mammalian skeletal muscle fibre bundles

DHT and T have both genomic and non-genomic actions (Heinlein & Chang, 2002; Hamdi & Mutungi, 2010). The genomic actions are mediated through

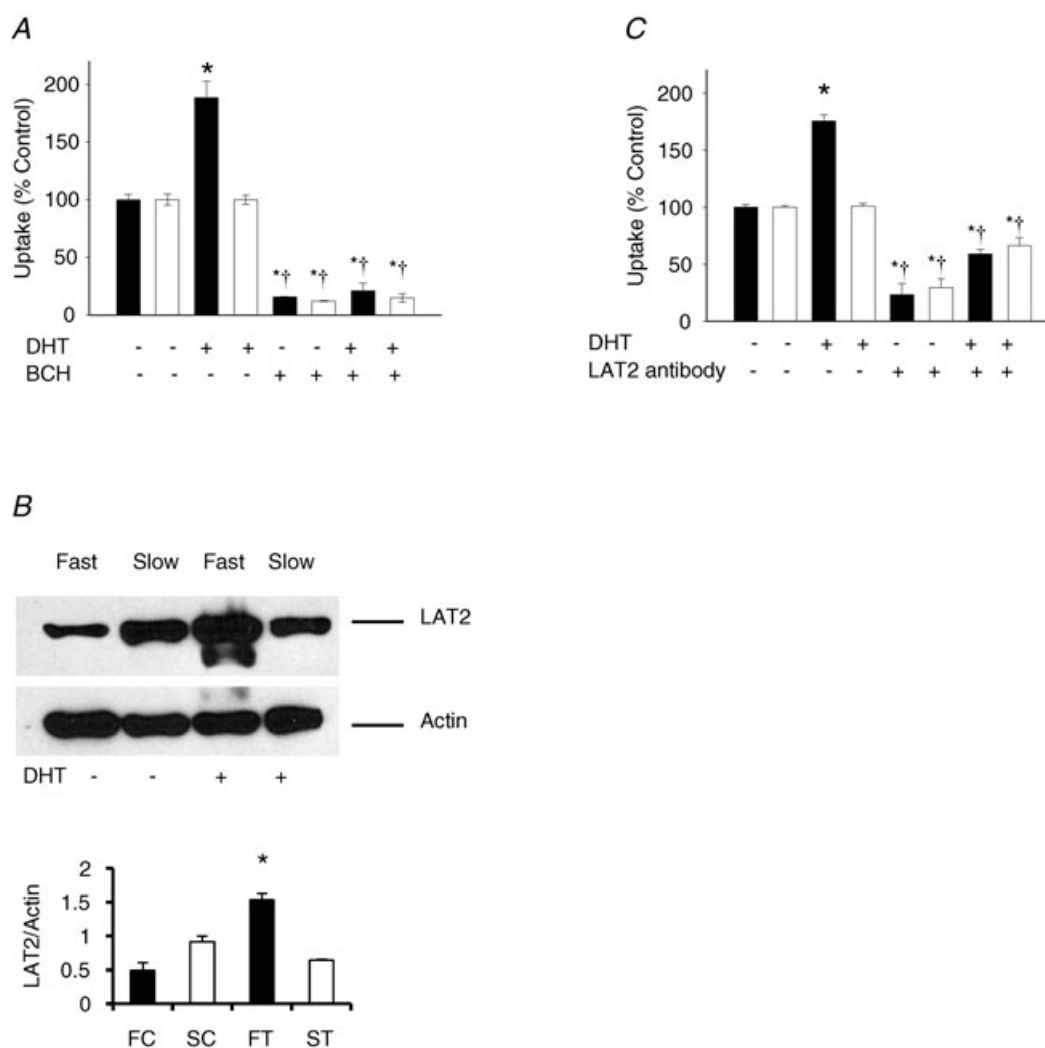


**Figure 2. The effects of DHT on amino acid uptake in mouse skeletal muscle fibre bundles are partly mediated through a system A amino acid transporter**

A, bar graphs showing the effects of treating fast-twitch (filled bars) and slow-twitch (open bars) muscle fibre bundles with DHT and MeAIB, as shown, on the uptake of L-[U- $^{14}\text{C}$ ]Ile. B, a representative Western blot and summary data showing the effects of DHT on the expression of SNAT2 in cytosolic proteins extracted from fast- (F, filled bars) and slow- (S, open bars) twitch muscle fibre bundles. Note that treating the muscle fibre bundles with MeAIB significantly ( $P < 0.05$ ) reduces the basal as well as the DHT-induced increase in Ile uptake in both fibre types. Furthermore the antibody labels a single band with a molecular mass of  $\sim 60$  kDa and DHT treatment has no effect on the expression of SNAT2. FT and ST are the fast- and slow-twitch preparations treated with DHT, respectively. \* $P < 0.05$  compared to control; † $P < 0.05$  compared to DHT treatment.

the classical androgen receptors (Fuller, 1991), whereas the non-genomic ones are mediated through surface membrane receptors such as the epidermal growth factor receptor (EGFR; Hamdi & Mutungi, 2010) and an unidentified G-protein coupled androgen receptor (GPCR; Estrada *et al.* 2003). Therefore, in another experiment we determined whether the effects of DHT on amino acid transport reported above were mediated through the androgen receptor. As the results displayed in Fig. 4 show, treatment of the fibre bundles with cyproterone (Fig. 4A; a steroidal inhibitor of the AR) alone significantly ( $P < 0.05$ ) increased the basal uptake

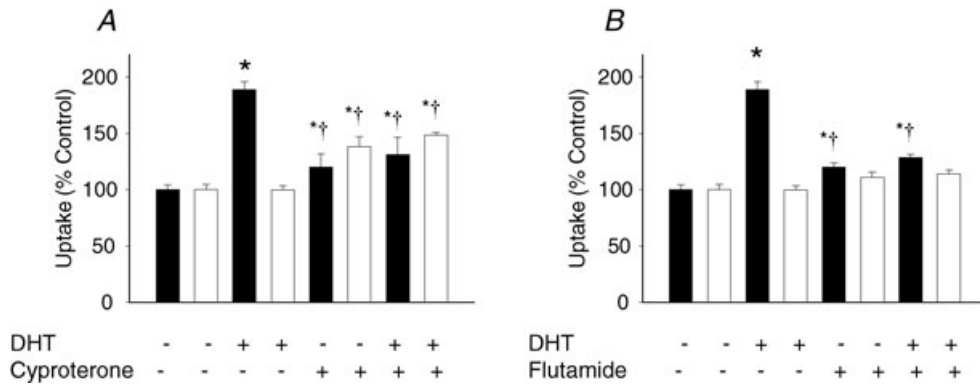
of L-[U-<sup>14</sup>C]Ile in both fibre types. On the other hand, it significantly ( $P < 0.05$ ) reversed the effects of DHT on amino acid uptake in both fibre types. Thus, pre-treating the fibre bundles with cyproterone significantly ( $P = 0.001$ ) reduced the DHT-induced increase in amino acid uptake in the fast-twitch fibre bundles, whereas in the slow-twitch ones it led to a further ( $12.4 \pm 4\%$ ) increase in the uptake of L-[U-<sup>14</sup>C]Ile. In contrast, treatment of the fibre bundles with flutamide (Fig. 3B; a non-steroidal AR inhibitor) alone significantly ( $P = 0.007$ ) increased the basal uptake of L-[U-<sup>14</sup>C]Ile in the fast-twitch fibre bundles only, whereas pre-treating



**Figure 3. The effects of DHT on amino acid uptake in mouse skeletal muscle fibre bundles are mediated through LAT2**

A, bar graphs showing the effects of treating fast- (filled bars) and slow- (open bars) twitch muscle fibre bundles with DHT and BCH, as shown, on the uptake of L-[U-<sup>14</sup>C]Ile. B, a typical Western blot and summary data showing the effects of DHT on the expression of LAT2 in membrane proteins extracted from fast- (F, filled bars) and slow- (S, open bars) twitch fibre bundles. Note that treating the muscle fibre bundles with BCH significantly ( $P < 0.05$ ) reduces the basal as well as the DHT-induced increase in Ile uptake in both fibre types. Additionally, the antibody labels a single band with a molecular mass of ~60 kDa and that DHT treatment increases the expression of LAT2 in the fast-twitch fibres only. FT and ST are the fast- and slow-twitch preparations treated with DHT, respectively. \* $P < 0.05$  compared to control; † $P < 0.05$  compared to DHT treatment.





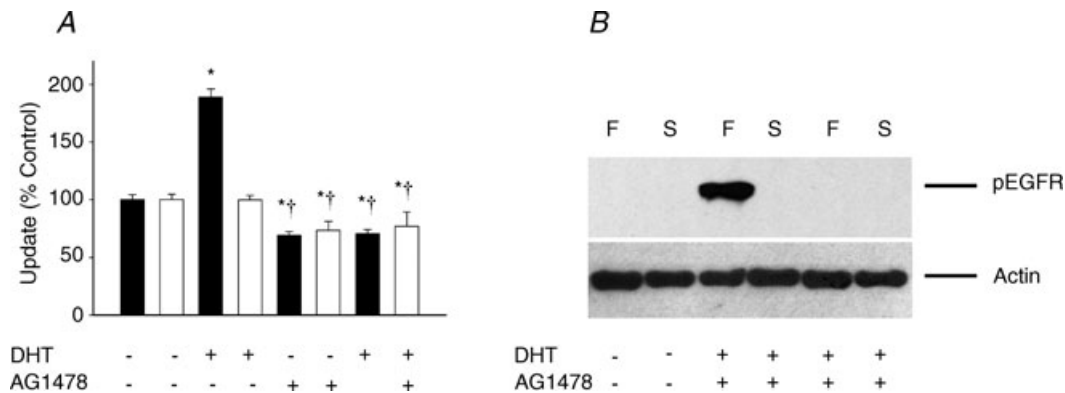
**Figure 4. The effects of DHT on amino acid uptake in mouse skeletal muscle fibre bundles are partly mediated through the androgen receptor**  
 Bar graphs showing the effects of treating fast- (filled bars) and slow- (clear bars) twitch skeletal muscle fibre bundles with the standard Ringer solution or the Ringer solution plus physiological levels of DHT alone or DHT plus the androgen receptor inhibitors cyproterone (A) and flutamide (B). Note that treatment of the fibre bundles with cyproterone alone significantly ( $P = 0.001$  and  $0.005$  in the fast- and slow-twitch fibres, respectively) increases the basal uptake of L-[U- $^{14}$ C]Ile in both fibre types. In contrast, flutamide on its own significantly ( $P = 0.028$ ) increases amino acid uptake in the fast-twitch fibre bundles only. Additionally, pre-treating the fibre bundles with both compounds significantly ( $P < 0.05$ ) reduces the DHT-induced increase in Ile uptake. However, these effects were greater in the fibre bundles pre-treated with flutamide than in those pre-treated with cyproterone. \* $P < 0.05$  compared to control; † $P < 0.05$  compared to DHT treatment.

the fibre bundles with it completely reversed the DHT-induced amino acid uptake seen in the fast-twitch fibre bundles.

Previously, we have shown that the acute/non-genomic effects of DHT on force production in isolated intact mammalian skeletal muscle fibre bundles are mediated through the epidermal growth factor receptor (EGFR) (Hamdi & Mutungi, 2010). Therefore, in another experiment we investigated whether this receptor (EGFR) mediated the acute/non-genomic effects of DHT on amino

acid uptake in these fibre bundles. As the results displayed in Fig. 5A show, treatment of the fibre bundles with the EGFR inhibitor AG1478 alone significantly ( $P < 0.05$ ) decreased the basal uptake of L-[U- $^{14}$ C]Ile in both fibre types. Also, it completely abolished the DHT-induced increase in amino acid uptake in the fast-twitch muscle fibre bundles (Fig. 5A).

Upon stimulation, the EGFR dimerises and then undergoes autophosphorylation (Mendelsohn & Baselga, 2000). Therefore, in another experiment we examined the effects



**Figure 5. The effects of DHT on amino acid uptake in mouse skeletal muscle fibre bundles are mediated through the epidermal growth factor receptor (EGFR)**  
 A, bar graphs showing the effects of treating fast- (filled bars) and slow- (clear bars) twitch muscle fibre bundles with either DHT or tyrosinase AG1478 as shown in the figure. Note that treating the fibre bundles with AG1478 significantly ( $P < 0.05$ ) reduces the basal uptake of L-[U- $^{14}$ C]Ile in both fibre types. Also, it completely abolishes the DHT-induced increase in amino acid uptake in the fast-twitch muscle fibre bundles. B, a Western blot showing the effects of treating the muscle fibre bundles with DHT alone or DHT plus AG1478 on the phosphorylation of the EGFR in membrane proteins. Note that the antibody labels a single band with a molecular mass of ~170 kDa and that treating the bundles with DHT increases the phosphorylation of EGFR and this effect is completely reversed by AG1478. \* $P < 0.05$  compared to control; † $P < 0.05$  compared to DHT treatment.

of DHT on the phosphorylation of the EGFR. As the results displayed in Fig. 4B show, treatment of the fibre bundles with DHT led to a marked increase in the phosphorylation of the EGFR in the fast-twitch fibre bundles. Furthermore, this DHT-induced increase in the phosphorylation of the EGFR was completely abolished by pre-treating the fibre bundles with Ringer solution containing the EGFR inhibitor tyrphostin AG1478.

### The cellular signal transduction events mediating the effects of DHT on amino acid transport in isolated intact mammalian skeletal muscle fibre bundles

To determine the cellular-signal transduction events mediating the acute/non-genomic actions of DHT on amino acid uptake in these muscles, another set of fibre bundles was treated with DHT alone or DHT plus the mitogen-activated protein kinase kinase (MEK) inhibitor, PD98059. The uptake of L-[U-<sup>14</sup>C]Ile as well as the phosphorylation of the extracellular-signal regulated kinases (ERK)1/2, the 90 kDa ribosomal S6kinases (RSK)1/2 and the mitogen and stress-activated protein kinases (MSK)1/2 were then determined. Treatment of the muscle fibre bundles with DHT increased the uptake of L-[U-<sup>14</sup>C]Ile in the fast-twitch fibre bundles (Fig. 6A). On the other hand, treating them with PD98059 alone significantly decreased the basal uptake of L-[U-<sup>14</sup>C]Ile in both fibre types. Also, PD98059 completely abolished the DHT-induced increase in amino acid uptake in the fast-twitch fibre bundles (Fig. 6A).

When the phosphorylation of ERK1/2 was examined, we found that the untreated fibre bundles expressed mostly phosphorylated ERK 2 (p42). Furthermore, its expression was always greater in the fast than in the slow-twitch fibre bundles. Moreover, phosphorylated ERK 1 (p44) was observed mainly in the fibre bundles treated with DHT. In contrast, the untreated fast- and slow-twitch fibres expressed similar concentrations of pRSK1/2 and treatment of the fibre bundles with DHT significantly ( $P = 0.03$ ) increased its phosphorylation in both fibre types (Fig. 6B). In Fig. 6C, the effects of pre-treating the fibre bundles with PD98059 on the phosphorylation of ERK1/2 and RSK1/2 are shown. As the results show, pre-treating the fibre bundles with PD98059 completely abolished the DHT-induced increase in the phosphorylation of both ERK1/2 and RSK1/2 in the two fibre types (Fig. 6C). Unlike, ERK and RSK, the untreated fibre bundles expressed mainly phosphorylated MSK and its phosphorylation was not significantly ( $P > 0.05$ ) affected by treatment with DHT except in the slow-twitch fibre bundles where it was slightly reduced (Fig. 6B).

The results reported in the previous section clearly showed that DHT was exerting its effects on amino acid uptake through activation of the ERK1/2 module of

the MAPK pathway. Its effects on the activation of the other modules have never been investigated. Therefore, in another experiment we determined the effects of DHT on the activation of c-Jun N-terminal kinase (JNK) and p38 (stress-activated protein kinase, SAPK). As the results displayed in Fig. 6D show, under control conditions the slow-twitch fibre bundles always expressed higher levels of the phosphorylated proteins than the fast-twitch ones. Additionally, treating the fibre bundles with DHT did not significantly ( $P = 0.08$ ) affect the phosphorylation of both kinases in either fibre type (Fig. 6D).

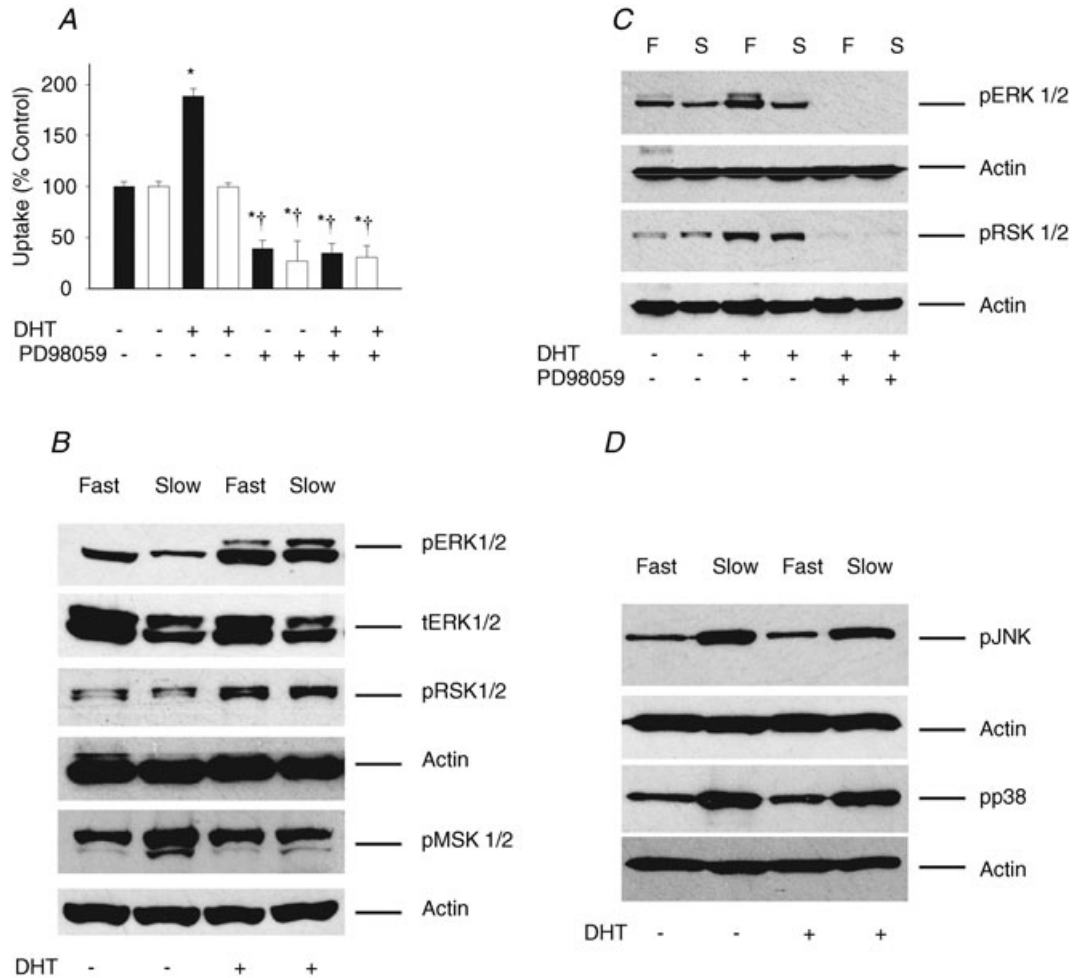
Another cell signalling pathway that has been implicated in the regulation of skeletal muscle mass (Glass, 2003) and hence amino acid uptake (Beugnet *et al.* 2003; Avruch *et al.* 2009) is the Akt (protein kinase B)/mammalian target of rapamycin (mTOR) pathway. Therefore, in another experiment we investigated the role of this pathway on the DHT-induced increase in amino-acid uptake. As the results in Fig. 7 show, treatment of the muscle fibre bundles with deguelin and rapamycin decreased the basal uptake of L-[U-<sup>14</sup>C]Ile in both muscle fibre types, suggesting that this pathway plays an essential role in the maintenance of basal amino acid transport in mammalian skeletal muscle fibres. Additionally, pre-treating the fibre bundles with rapamycin completely abolished the DHT-induced changes in amino acid uptake seen in the fast-twitch muscle fibres (Fig. 7B). In contrast, pre-treating the fibre bundles with deguelin blunted, without completely abolishing, the DHT-induced increase in L-[U-<sup>14</sup>C]Ile uptake in the fast-twitch muscle fibre bundles (Fig. 7A).

### DHT increases protein synthesis in isolated intact mammalian skeletal muscle fibre bundles

To determine whether the effects of DHT on amino acid uptake involved an increase in transcription or translation, another set of fibre bundles was treated with either the transcriptional inhibitor actinomycin D or translational inhibitor cycloheximide. The uptake of L-[U-<sup>14</sup>C]Ile was then examined in the presence or absence of DHT. As the results in Fig. 8 show, treatment of the fibre bundles with actinomycin D alone did not significantly ( $P = 0.47$  in the fast-twitch and 0.13 in the slow-twitch fibre bundles) affect the basal uptake of L-[U-<sup>14</sup>C]Ile in either fibre type. However, pre-treatment of the fibre bundles with it significantly ( $P = 0.001$ ) reduced the DHT-induced increase in L-[U-<sup>14</sup>C]Ile uptake without completely abolishing it (Fig. 8A). In contrast, treatment of the fibre bundles with cycloheximide significantly ( $P < 0.05$ ) reduced the basal uptake of L-[U-<sup>14</sup>C]Ile in both fibre types and pre-treating another set of fibre bundles with it completely abolished the DHT-induced increase in L-[U-<sup>14</sup>C]Ile observed in the fast-twitch fibre bundles (Fig. 9A).

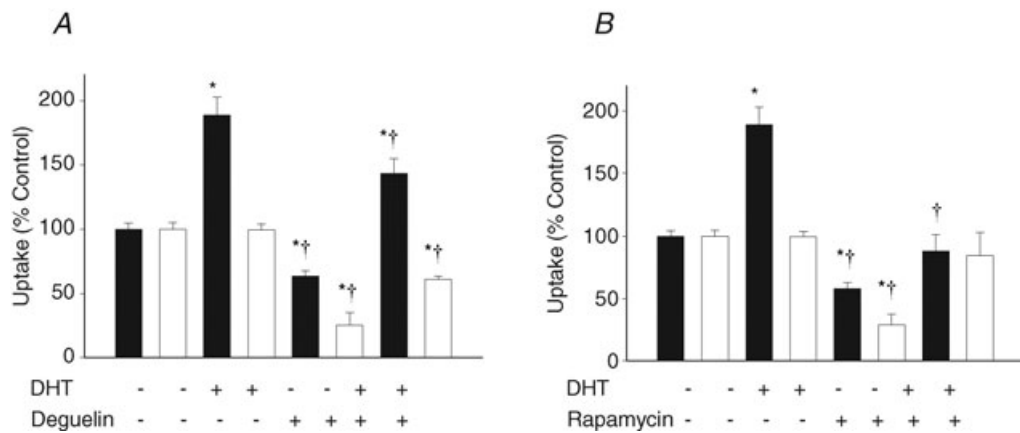
Upon activation, MAPKs can translocate into the nucleus where they regulate gene expression by phosphorylating many transcription factors including C-jun, c-myc and ATF (Krishna & Narag, 2008). Therefore, in another experiment, we examined the effects of DHT on some of these transcription factors as well as the translational regulator, eukaryotic elongation factor (eEF) 2. As the results displayed in Fig. 8B show, treating the muscle

bundle with DHT had little or no effect on the expression and activation of cmyc, MyoD and c-Jun. On the other hand, it increased the phosphorylation of ATF2 (Fig. 8B) and completely abolished that of eEF2 in both fibre types (Fig. 9B). Furthermore, treatment of the muscle fibre bundles with DHT significantly ( $P < 0.05$ ) increased the incorporation of Ile into proteins in the fast-twitch fibre bundles. In contrast, it led to a slight



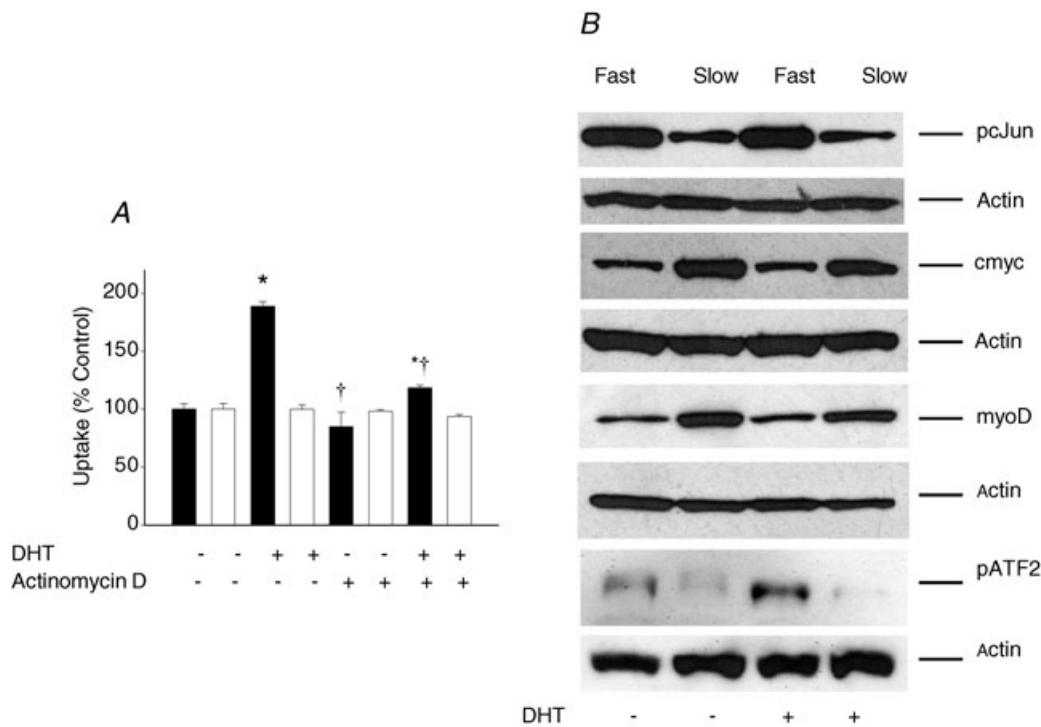
**Figure 6. The effects of DHT on amino acid uptake in mouse skeletal muscle fibre bundles are mediated through the ERK pathway**

A, bar graphs showing the effects of treating fast- (filled bars) and slow- (open bars) twitch muscle fibre bundles with either DHT or the MEK inhibitor PD98059, as shown in the figure, on the uptake of L-[U-<sup>14</sup>C]Ile. Note that treating the fibre bundles with PD98059 significantly reduces the basal uptake of Ile in both fibre types. Also, it completely abolishes the DHT-induced increase in Ile. B, Western blots showing the effects of treating muscle fibre bundles with DHT on the phosphorylation of ERK1/2, RSK1/2 and MSK1/2. Note that treating the muscle fibre bundles with DHT increases the phosphorylation of ERK1/2 and RSK1/2 but not that of MSK1/2. C, Western blots showing the effects of treating small muscle fibre bundles with DHT and PD98059 (as shown) on the cytosolic concentrations of phosphorylated ERK1/2 and RSK1/2. Note that the pERK antibody labels two bands with molecular masses of ~42 and 44 kDa, whereas that of pRSK labels two bands with molecular masses of ~78 and 80 kDa. Furthermore, treating the bundles with DHT increases the phosphorylation of ERK1/2 and RSK1/2 and that pre-treatment of the fibre bundles with PD98059 completely abolishes the effects of DHT. D, typical Western blots showing the effects of DHT on the cytosolic concentrations of phosphorylated JNK and p38. Note that the pJNK antibody labels a single band with a molecular mass of ~60 kDa, whereas that of pp38 labels a band with a molecular mass of ~42 kDa. Furthermore, DHT treatment has no effect on the phosphorylation of either kinase. \* $P < 0.05$  compared to control; † $P < 0.05$  compared to DHT treatment.



**Figure 7. The effects of DHT on amino acid uptake in mouse skeletal muscle fibre bundles are not mediated through the Akt**

A and B, bar graphs showing the effects of treating fast- (filled bars) and slow- (open bars) twitch muscle fibre bundles, with the compounds shown below each panel, on the uptake of L-[U-<sup>14</sup>C]Ile. Note that treating the fibre bundles with either deguelin or rapamycin alone significantly ( $P = 0.033$  for deguelin and  $0.025$  for rapamycin) reduces the basal uptake of L-[U-<sup>14</sup>C]Ile in both fibre types. Furthermore, rapamycin but not deguelin abolished the DHT-induced increase in Ile uptake. \* $P < 0.05$  compared to control; † $P < 0.05$  compared to DHT treatment.



**Figure 8. Actinomycin D blunts the effects of DHT on amino acid uptake in mouse skeletal muscle fibre bundles**

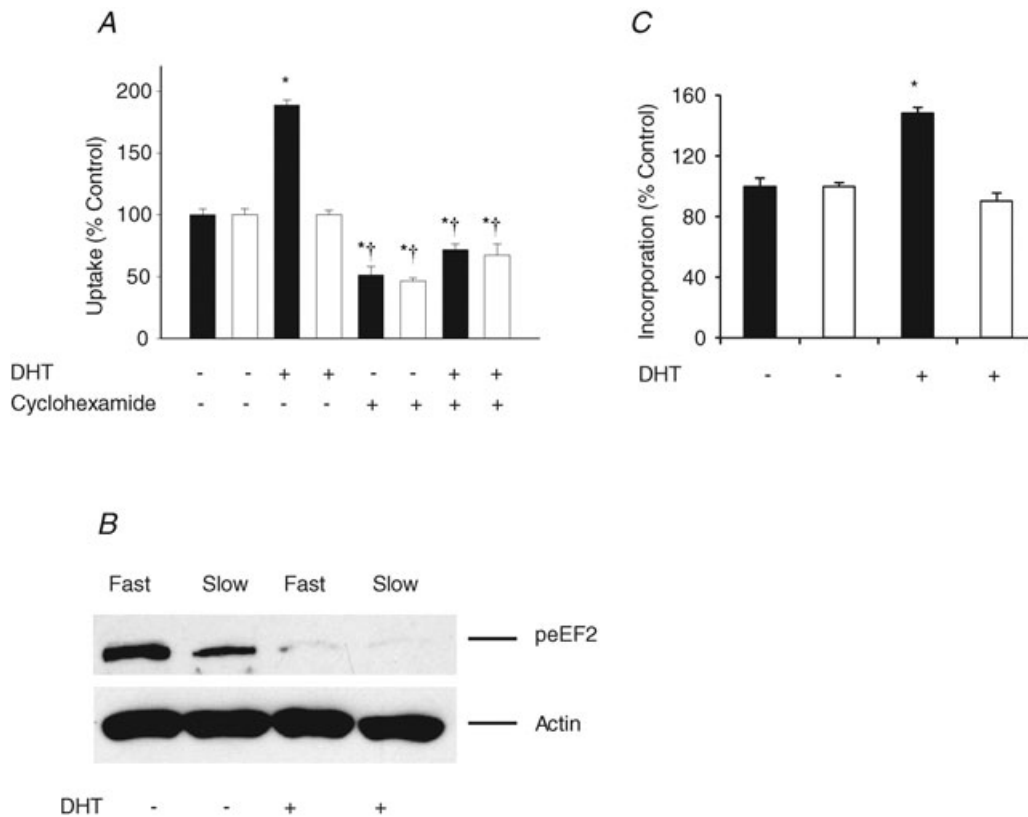
A, bar graphs showing the effects of treating fast- (filled bars) and slow- (open bars) twitch muscle fibre bundles with Ringer solution containing either DHT or actinomycin D on the uptake of L-[U-<sup>14</sup>C]Ile. Note that treating the muscle fibre bundles with actinomycin D alone had no effect on the basal uptake of Ile in both fibre types. Moreover, it blunts without completely abolishing the DHT-induced increase in Ile uptake. B, representative Western blots showing the effects of DHT on the expression of pc-Jun, myoD, cmc and pATF2. Note that DHT does not significantly ( $P > 0.05$ ) affect the phosphorylation/expression of any of the transcriptional factors except ATF2. \* $P < 0.05$  compared to control; † $P < 0.05$  compared to DHT treatment.

( $9.8 \pm 5.6\%$ ,  $n = 3$  fibres) decrease in Ile incorporation in the slow-twitch fibre bundles (Fig. 9C), suggesting that DHT treatment was also regulating protein synthesis in the fibre bundles.

### Discussion

A key finding in the present study was the observation that treating small skeletal muscle fibre bundles isolated from the EDL and soleus of adult female mice with physiological concentrations ( $630 \text{ pg ml}^{-1}$ ) of DHT, for 1 h, significantly ( $P = 0.001$ ) increased the uptake of both Ile and MeAiB in the fibre bundles isolated from the EDL but not in those isolated from the soleus. Although the acute administration of hormones such as insulin (Biolo *et al.* 1995), insulin-like growth factor 1 (IGF-1)

(Fryburg *et al.* 1995) and growth hormone (Fryburg *et al.* 1995) has been shown to increase protein synthesis and to promote amino acid uptake in human skeletal muscle, this is the first time that an increase in amino acid uptake in response to the acute administration of an anabolic-androgenic steroid in adult mammalian skeletal muscle has been demonstrated. Only two other studies have previously investigated the effects of T on amino acid uptake. Both studies used human subjects and were unable to demonstrate any changes in amino acid uptake in whole muscle groups (Bhasin *et al.* 1997; Ferrando *et al.* 1998). Although small skeletal muscle fibre bundles were used in the present study and T was applied directly to the fibre bundles, it is important to note that T had no effect on amino acid uptake in either fibre type. In contrast, treating the fibre bundles with DHT led to a marked increase in



**Figure 9. Actinomycin D blunts the effects of DHT on amino acid uptake in mouse skeletal muscle fibre bundles**

A, bar graphs showing the effects of treating fast- (filled bars) and slow- (open bars) twitch muscle fibre bundles with Ringer solution containing either DHT or cycloheximide on the uptake of L-[U-<sup>14</sup>C]Ile. Note that treating the muscle fibre bundles with cycloheximide significantly ( $P < 0.05$ ) reduces the basal uptake of Ile in both fibre types. Moreover, pre-treatment of the fibre bundles with it completely abolishes the DHT-induced increase in Ile uptake. B, representative Western blots showing the effects of DHT on the phosphorylation of eEF2. Note that the antibody labels a single band with a molecular mass of  $\sim 100$  kDa. Moreover, treatment of the muscle fibre bundles with DHT completely abolishes the phosphorylation of the protein in both fibre types. C, summary data showing the effects of treating muscle fibre bundles with DHT on Ile incorporation into the muscle fibre bundles. Note that treating the muscle fibre bundles with DHT significantly ( $P = 0.004$ ) increases the incorporation of Ile in the fast-twitch fibre bundles. In contrast it leads to a slight decrease ( $9.8 \pm 5.6\%$ ,  $n = 3$  fibres) in the incorporation of Ile in the slow-twitch fibre bundles. \* $P < 0.05$  compared to control; † $P < 0.05$  compared to DHT treatment.

the uptake of both Ile and MeAIB in the fast-twitch fibre bundles only (Fig. 1). Previously, we have also shown that T has no acute effects on force production in isolated intact mouse skeletal muscle fibre bundles, whereas DHT increased force production in the fast-twitch fibre bundles but decreased it in the slow-twitch ones (Hamdi & Mutungi, 2010). Taken together, these findings suggest that T may not have any acute/non-genomic effects in adult mammalian skeletal muscle fibres.

As mentioned in the introduction, in most tissues T is converted into DHT by the enzyme  $5\alpha$ -reductase. Therefore, it is likely that all the acute effects of T observed previously in cultured myocytes (Estrada *et al.* 2003) may have been exerted by DHT. Indeed, it has previously been suggested that the anabolic effects of T in human skeletal muscles may be indirect or secondary to the release of another hormone such as IGF-1 (Ferrando *et al.* 1998). Here we speculate that in addition to IGF-1, the effects of T in mammalian skeletal muscle may also be exerted through the release of DHT. We also suggest that DHT is the main anabolic-androgenic steroid in adult mammalian skeletal muscles.

Although all the experiments reported here were performed using muscle fibre bundles isolated from female mice, we suggest that these hormones have similar effects in muscle bundles isolated from male mice. Indeed, we have previously shown that DHT has similar effects on force production in male and female mouse skeletal muscle fibre bundles (Hamdi & Mutungi, 2010). The main reason we used female muscles in this study was to avoid previous exposure to these hormones as these may compromise their effects (see Exner *et al.* 1973*a,b*). As mentioned in the introduction, anabolic-androgenic steroids are major determinants of body composition and their presence or absence in plasma has huge effects on body structure and composition (Wilson *et al.* 1988; Regnier & Herrera, 1993; Bhasin *et al.* 2001). Therefore, we speculate that the presence of these hormones in males may be the main reason male muscles are larger and stronger than those of females. Indeed, in frogs the flexor carpi radialis muscle has been shown to be dimorphic and sensitive to testosterone (Regnier & Herrera, 1993). However, whether the differences between the muscles of males and females are due to these hormones only is uncertain and further investigation is needed.

Amino acids enter and leave cells through highly specialized proteins known as amino acid carriers or transporters. Moreover, each cell expresses numerous varieties of these transporters (Christensen, 1990). As the data displayed in Figs 2 and 3 show, in addition to other transporters, adult mouse fast- and slow-twitch skeletal muscle fibres express both SNAT2 (a system A amino acid transporter) and LAT2 (an amino acid transporter belonging to system L). Although mRNAs for these transporters have previously been identified in mammalian skeletal muscle

(Utsunomiya-Tate *et al.* 1996; Pineda *et al.* 1999; Segawa *et al.* 1999; Sugawara *et al.* 2000; Yao *et al.* 2000; Hyde *et al.* 2001), this is the first time the protein for LAT2 has been shown to be present in adult mammalian skeletal muscle. It is also the first time the expression of both transporters in different muscle fibre types has been described. As the data in Fig. 2 show, the untreated slow-twitch muscle fibre bundles express more ( $\sim 1.8$  times) transporter proteins than the corresponding fast-twitch fibres. However, whether this difference is physiologically important is uncertain and further studies are necessary.

It is now generally accepted that certain nutrients such as amino acids are essential for the maintenance of tissue structure, growth and function. Therefore, it has been suggested that their uptake and release by cells is highly regulated. Although many factors including pH (Sugawara *et al.* 2000; Yao *et al.* 2000), osmolarity (Horio *et al.* 1997), intracellular amino acid levels (Gazzola *et al.* 1972; Drummond *et al.* 2010), hormones (Edmondson & Lumeng, 1980; Barber *et al.* 1982), growth factors (Moule & McGivan, 1987) and mitogens (Franchi-Gazzola *et al.* 1999) have been shown to regulate the expression and activity of SNAT2 (so called adaptive response) in many cell types, far less is known about the factors that control the activity and function of L-type amino acid transporters. The results we report in this study (see Figs 2 and 3) show that although DHT increases the activity of SNAT2, it does not affect its expression. Instead, it increases the activity and expression of LAT2 (see Fig. 3). Furthermore, preloading the muscle fibre bundles with the classical system A and L-type amino acid inhibitors led to a marked decrease in the basal uptake of L-[U- $^{14}$ C]Ile in both fibre types and completely abolished the DHT-induced increase in Ile uptake. Similar effects were also observed when the fibre bundles were treated with Ringer solution containing an antibody against LAT2 (see Fig. 3C), suggesting that the effects of DHT on Ile are mediated through this transporter. From these observations we suggest that the two transporters (LAT2, SNAT2) are somehow linked. Thus, by modulating the expression and activity of LAT2, DHT also seems to indirectly regulate the activity of SNAT2. This type of coupling, commonly referred to as tertiary active transport, has been previously reported and is thought to exist in most cells (Hyde *et al.* 2007; Baird *et al.* 2009). Indeed, recent data published by Drummond *et al.* (2010) suggest that it may also exist between SNAT2 and LAT1 in human skeletal muscle, suggesting that this type of coupling may be widespread in skeletal muscle. However, further studies to confirm this are necessary.

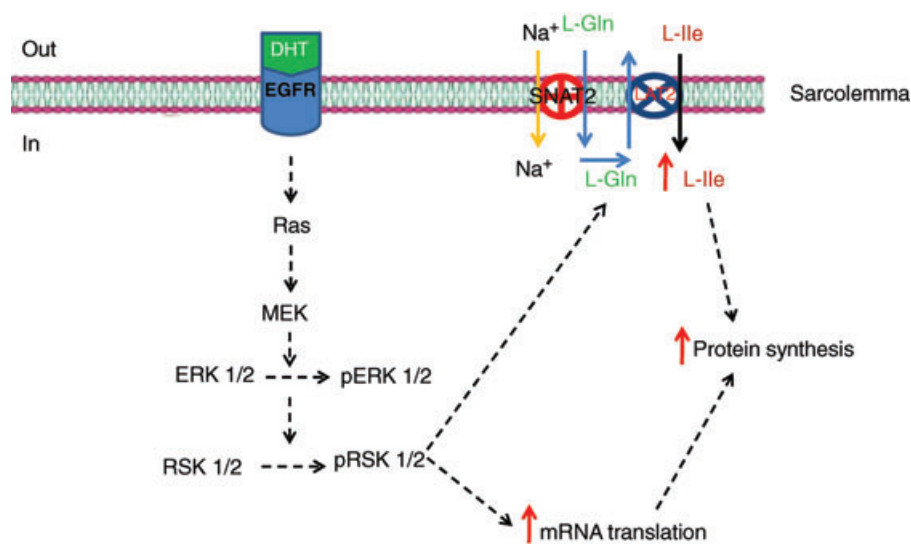
Previously, we have shown that the acute/non-genomic actions of DHT on force production in adult mouse skeletal muscle fibre bundles are mediated through the epidermal growth factor receptor (EGFR) and involve

activation of ERK1/2 (Hamdi & Mutungi, 2010). It is interesting to note that the results we report here suggest that the acute/non-genomic actions of DHT on amino acid uptake in adult mammalian skeletal muscle fibres are also mediated through the same receptor and pathway. However, as the data presented in Fig. 4 show, involvement of the classical androgen receptors in the DHT-mediated increase in amino acid uptake cannot be ruled out. Thus, pre-treating the fibre bundles with either cyproterone (Fig. 4A) or flutamide (Fig. 4B) significantly ( $P < 0.05$ ) blunted the effects of DHT on Ile uptake without completely abolishing them. Previously, it has been suggested that testosterone can activate the MAPK pathway via a G-protein coupled androgen receptor (Estrada *et al.* 2003). However, whether this mechanism(s), commonly referred to as transactivation, is the one that mediates the acute/non-genomic effects of DHT in adult mammalian skeletal muscle fibres is uncertain and further research to clarify this is necessary.

Upon activation, ERK1/2 can either remain in the cytosol or translocate into the nucleus where it plays a critical role in the regulation of both gene expression and DNA replication (Brunet *et al.* 1999). In the nucleus, ERK1/2 phosphorylates an array of targets, including many transcription factors and a family of RSK-related kinases, the mitogen- and stress-activated protein kinases

(MSKs) (Deak *et al.* 1998). Although a large number of the downstream targets of ERK1/2 were examined in the present study, DHT treatment increased the phosphorylation of RSK1/2 and ATF2 only. Additionally, DHT had no effect on the phosphorylation of the other MAPK modules. To us, these findings suggest that the acute/non-genomic actions of DHT in adult mammalian skeletal muscles are exerted mainly through the EGFR and involve activation of the ERK1/2 module of the MAPK pathway (for details see Fig. 10). Our hypothesis is that DHT, either directly or indirectly, activates the EGFR which in turn activates the MAPK pathway leading to an increase in amino acid transport into the fibres and protein synthesis by the fibres. Furthermore, DHT seems to target mainly fast-twitch fibres.

In adult animals, an increase in skeletal muscle mass (hypertrophy) occurs mainly as a result of an increase in the size rather than the number of muscle fibres and is generally thought to be regulated by the Akt/mTOR pathway (Glass, 2003). The Akt/mTOR pathway is activated by many stimuli including growth factors, hormones and nutrients. Moreover, its activation culminates in the block of apoptosis, induction of protein synthesis, gene transcription and cell proliferation (Dann *et al.* 2007). Therefore, we expected that DHT and T would activate this pathway. However, as the results displayed in Fig. 7 show, the AKT specific inhibitor deguelin blunted



**Figure 10. Cell signalling pathway mediating the effects of DHT on amino acid transport in mammalian skeletal muscle fibres**

A schematic diagram showing the cellular signalling pathway we suggest mediates the acute effects of DHT on amino acid in mammalian skeletal muscle fibre bundles. Our hypothesis is that DHT, through an unknown mechanism, activates the EGFR and this leads to the activation of RSK1/2 by ERK1/2. The activated RSK1/2 then increases the activity and expression of LAT2 (blue circle with a cross), which in turn increases the transport of Ile into the muscle fibre bundles. Additionally, RSK1/2 enhances the mRNA translation into proteins. Note that the transport of Ile is coupled to that of small neutral amino acids such as glutamine (Gln) that are transported into the cell by system A amino acid transporters such as SNAT2 (red circle with a cross). Thus, an increase in the activity of LAT2 indirectly increases the activity of SNAT2. The eventual effects of all these processes are to increase protein synthesis and hence skeletal muscle mass especially in fast-twitch muscle fibres.

the DHT-induced amino acid uptake in fast-twitch fibres without completely abolishing it, thereby suggesting that the acute/non-genomic actions of DHT are not mediated through Akt. Instead, the results we present suggest that DHT increases protein synthesis by regulating the translation of mRNA already present in the cells. Indeed, treatment of the muscle fibre bundles with DHT led to a marked decrease in the phosphorylation of eEF2 and a moderate (~50%) increase in protein synthesis in the fast skeletal muscle fibre bundles (see Fig. 9C). Furthermore, pre-treating the fibre bundles with the translational inhibitor cycloheximide completely abolished the DHT-induced increase in amino acid uptake. cycloheximide is a glutarimide antibiotic that binds the E-site on the 60S ribosomal subunit thereby inhibiting the binding of eEF2 on this site and tRNA translocation from the A-site to the P-site (Schneider-Petsch *et al.* 2010). Therefore, both of these observations provide further evidence in support of our hypothesis that DHT increases amino acid uptake by increasing mRNA translation. However, this cannot be the only mechanism involved because pre-treating the fibre bundles with the transcriptional inhibitor actinomycin D also abolished the DHT-induced increase in amino acid uptake. However, as DHT did not increase the activity of many of the transcription factors examined in this study, we hypothesize that it increases the transcription of genes that are already active.

It is also noteworthy that pre-treating the fibre bundles with the mTOR specific inhibitor rapamycin not only reduced the basal uptake of Ile, it also completely abolished the DHT-induced increase in the uptake of Ile in the fast-twitch fibre bundles. To us these findings suggest that some of the acute/non-genomic effects of DHT are probably mediated through mTOR. Indeed, a number of studies have suggested that mTOR is the link between amino acid availability and increased protein synthesis (Beugnet *et al.* 2003; Avruch *et al.* 2009). However, rapamycin can also induce autophagy (Ravikumar *et al.* 2004). Therefore, another possibility is that its effects are due to a build up of amino acids in the muscle fibres. However, the exact mechanism underlying the effects of rapamycin is uncertain and further studies are necessary.

In summary, the results we report in this study show that another physiological function of the acute/non-genomic actions of DHT in adult mammalian skeletal muscle fibres is to increase the uptake of essential amino acids. Although we cannot completely rule out androgen receptor involvement, the main findings suggest that the increase in amino acid uptake and protein synthesis are mediated through the EGFR and involve the activation of the ERK1/2 module of the MAPK pathway which in turn activates RSK1/2 leading to an increase in the expression of the L-type amino acid transporter LAT2.

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#### Author contributions

G.M. was involved in the conception and design of the experiments. He also contributed to the writing and revision of the manuscript. M.M.H. performed most of the experiments and most of the data analysis.

#### Acknowledgements

This work was supported by the University of East Anglia. M.M.H. is on a PhD studentship sponsored by the Malaysian Government.