THE PHYSIOLOGICAL ACTIONS AND CELLULAR SIGNALING PATHWAYS MEDIATING THE ACUTE NON-GENOMIC EFFECTS OF DHT IN ISOLATED INTACT MAMMALIAN SKELETAL MUSCLE FIBRE BUNDLES

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ABSTRACT

Anabolic-androgenic steroids (AASs; testosterone (T) and its many synthetic derivatives) have body-building properties. Therefore, they are widely prescribed for the treatment of muscle wasting conditions such as sarcopenia and cachexia. Although, the chronic effects of these compounds in skeletal muscle have been the subject of many previous studies and some of their chronic effects are well documented, little is known about their acute/non-genomic effects. Furthermore, in most tissues T is not the active hormone; instead it is converted to dihydrotestosterone (DHT). Despite this, the acute effects of DHT have never been investigated. Most of the previous studies were performed using animals or humans and some of the findings were highly variable. Therefore, to minimise this variability, small muscle fibre bundles isolated from the extensor digitorum longus and soleus of adult female CD1 mice were used to investigate the acute/non-genomic effects of DHT and T on amino acid uptake. Pharmacological interventions were then used to determine the cellular signalling pathway(s) and receptor(s) mediating these actions. My results, which form the bulk of this thesis, show that treatment of the muscle fibre bundles with DHT (but not T) led to increase in the uptake into proteins of the essential amino acid, isoleucine. These effects were mediated through the epidermal growth factor receptor (EGFR) and involved the activation of the extracellular-regulated kinases (ERK) 1/2 and the p90

ribosomal S6 kinases (RSK) 1/2. DHT treatment also increased the phosphorylation of the 20kDa regulatory myosin light chains and the expression of the L-type amino acid transporter (LAT) 2. From these results I suggest that DHT, and not T, is the more potent anabolic-androgenic steroid hormone in mammalian skeletal muscle fibres and that its derivatives may be better drugs for the management of sarcopenia and cachexia than those of T.

PUBLICATION AND ABSTRACTS

Paper

Hamdi, M. M. & Mutungi, G. (2010). Dihydrotestosterone activates the MAPK pathway and modulates maximum isometric force through the EGF receptor in isolated intact mouse skeletal muscle fibres. 588 (**3**) *The Journal of Physiology.* 511-525

Hamdi, M. M. & Mutungi, G. (2011). Dihydrotestosterone (DHT) stimulates amino acid uptake and the expression of LAT2 in mouse skeletal muscle fibres through an ERK1/2-dependent mechanism. May 23. *The Journal of Physiology.* [Epub ahead of print].

Abstracts

Hamdi, MM, Wileman, T, Mutungi, G. (2009). Dihydrotestosterone (DHT) modulates force production in isolated mouse skeletal muscles by regulating the phosphorylation of myosin light chains by ERK 1/2. **15** *Proceedings of The Physiological Society.* 111-112 (Presented to the Physiological Society at its annual meeting in Dublin, Ireland held on 7^{th}-10^{th} July 2009).

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Hamdi, M. M. & Mutungi, G. (2010). DHT increases isoleucine incorporation in isolated mouse fast skeletal muscle fibre bundles through an epidermal growth factor receptor (EGFR)-dependent mechanism. (Presented to the Physiological Society at its annual meeting in Manchester, UK, held on 30th June -2nd July 2010)

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LIST OF ABBREVIATIONS

5αRD	5 alpha-reductase
α-(1-^{14}C)-MeAIB	carbon-14 labeled MeAIB
AAS	anabolic androgenic steroids
AR	androgen receptor
BCH	2-Aminobicyclo (2,2,1)heptane-2-carboxylic acid
DHT	dihydrotestosterone
EDL	extensor digitorum longus
EGFR	epidermal growth factor receptor
ERK1/2	extracellular signal regulated kinase1/2
IGF-1R	insulin-like growth factor 1 receptor
IL-(U^{14}-C)	isoleucine carbon-14 labeled isoleucine
LAT	L-type amino acid transporter
MAPK	mitogen activated protein kinase
MeAIB	α-(Methylamino) isobutyric acid
MEK	MAPK/ERK1/2 kinase
MyHC	myosin heavy chain
PDGFR	platelet derived growth factor receptor
RMLCs	regulatory myosin light chains
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SNAT	sodium dependent neutral amino acid transporter
T	testosterone

AMINO ACID ABBREVIATIONS

A	Alanine (Ala)
R	Arginine (Arg)
N	Asparagine(Asn)
D	Aspartic acid (Asp)
C	Cysteine (Cys)
E	Glutamic acid (Glu)
Q	Glutamine (Gln)
G	Glycine (Gly)
H	Histidine (His)
I	Isoleucine (Ile)
L	Leucine (Leu)
K	Lysine (Lys)
M	Methionine (Met)
F	Phenylalanine (Phe)
P	Proline (Pro)
S	Serine (Ser)
T	Threonine (Thr)
W	Tryptophan (Trp)
Y	Tyrosine (Tyr)
V	Valine (Val)

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CHAPTER 1

INTRODUCTION

1.1 **Skeletal muscle structure and function**

In this study, I investigated the cellular signal transduction events underlying the acute (=rapid/non-genomic) actions of testosterone (T) and its metabolite dihydrotestosterone (DHT) in isolated intact mammalian skeletal muscle fibre bundles. Therefore, in this section of my thesis I will provide background information on the structure, organisation and function of mammalian skeletal muscles.

There are three types of muscles in the mammalian body namely; cardiac, skeletal and smooth muscle. Examined under polarising light, cardiac and skeletal muscles show highly regular repeats of dark and light bands or striations. Therefore, they are also referred to as striated muscles. On the other hand, smooth muscles show no striations when examined under a light microscope, hence the name smooth muscle (Randall *et al.* 1997).

Cardiac muscle is found in the heart, smooth muscle is located in the walls of all hollow visceral organs, whereas, skeletal muscle is closely associated with the skeletal system. As all the experiments reported here were performed using skeletal muscles, I will concentrate on the structure and function of this muscle type.

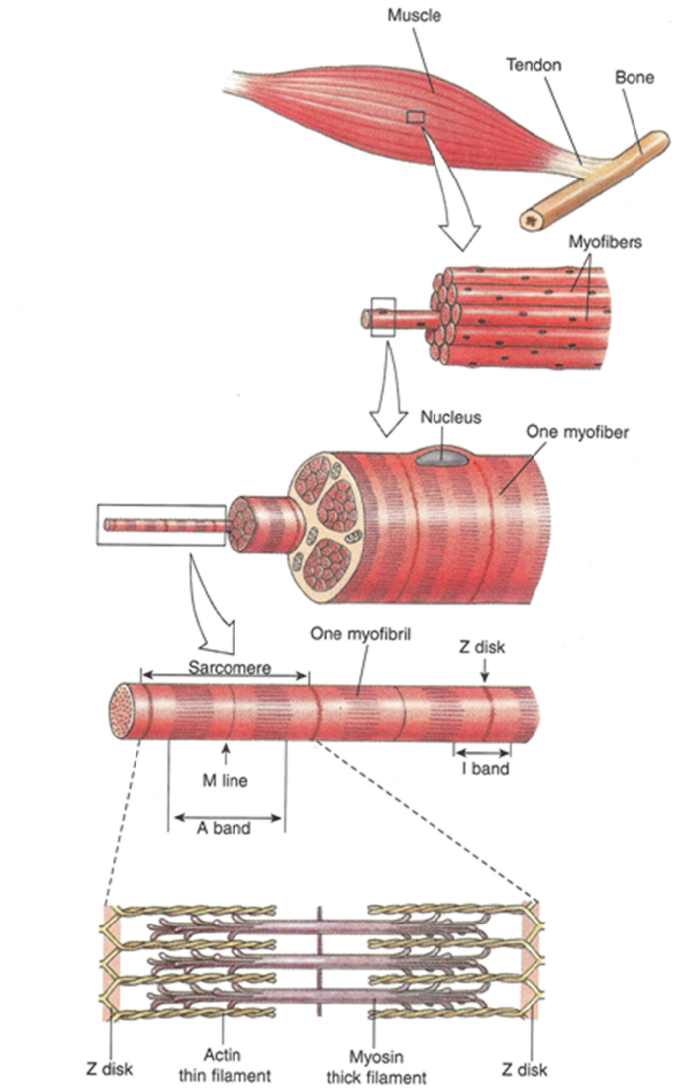

***Figure 1.1**: A schematic diagram showing the structural organisation of a skeletal muscle. Note that each muscle consists of long, cylindrical, multinucleated cells known as muscle fibres or myofibres that run from end to end. The myofibres are composed of myofibrils and other cellular components such as mitochondria. The myofibril in turn consist of three types of protein filaments namely; actin, myosin and titin filaments. Moreover, the muscle is tethered at either end by straps of tough connective tissues known as tendons or aponeurosis. Figure adapted from Randall et al. 1997.*

Skeletal muscle forms the bulk of body weight (~40 to 50% of body mass) (Rooyackers & Nair, 1997). Its main function is to provide the force required for postural maintenance and to move joints. Macroscopically, skeletal muscles can be divided into red and white muscle. Red muscle is rich in myoglobin (which gives it its red appearance) and oxidative enzymes. Its fibres express a slow form of the contractile muscle protein, myosin, which splits ATP slowly. Therefore, it is also referred to as slow-twitch muscle (McComas, 2005). It is used mainly for postural support and to power slow repetitive movements such as marathon running. In contrast, white muscle is devoid of myoglobin and is rich in glycolytic enzymes. It consists mainly of muscle fibres that express isoforms of myosin that split ATP rapidly. Therefore, it is also known as fast-twitch muscle (McComas, 2005). In the body, white muscle powers activities that require large amounts of energy for short periods of time such as sprint running (Pette & Staron, 2000).

Figure 1.1 shows the functional organisation of a typical skeletal muscle. As the diagram shows, each skeletal muscle is tethered at either end to bones by straps of tough connective tissues known as tendons or to other muscles/tissues via a sheath of connective tissue known as an aponeurosis. Depending on their location, the tendons are referred to as either the tendon of origin or insertion. Thus, the tendon proximal to the body is known as the tendon of origin;

whereas, the one distal to it is referred to as the tendon of insertion (Garrett, 1990).

As the diagram also shows, each muscle consists of long, cylindrical, multinucleated cells known as muscle fibres or myofibres. Except in immature or regenerating muscles where the nuclei are located in the centre of the fibre, in adult muscles the nuclei are arranged around the periphery of the fibre. The myofibres are arranged in groups of between 10 and 100 fibres, depending on their function, known as fascicles or fibre bundles. Each fascicle is surrounded by a thin connective tissue known as a perimysium and the muscle fibres in fascicle run parallel to each other from one tendon to the other. In adult mice, each muscle fibre is 30-60μm in diameter and 6-7mm long (Hegarty & Hooper, 1971).

Each muscle fibre consists of protein filaments grouped together in bundles known as myofibrils. In all muscles, the myofibrils are surrounded by cytoplasm (or sarcoplasm) and other cellular organelles such as mitochondria, myoglobin, and glycogen. Furthermore, the volume density of each of these components depends on the fibre type (Peachey, 1968). Each myofibril is approximately 1μm in diameter and in most muscles runs the whole length of the fibre (see figure 1.1). Moreover, 600-3000 of them can be packed into a single myofibre, which is equivalent to 60 to 70 % of its total volume (Luff & Goldspink, 1970).

As mention above, each myofibril consists of filaments or myofilaments that lie parallel to the long axis of the myofibril. In turn, the myofilaments consist of ordered repeats of thin and thick filaments. It is this ordered assembly of thin and thick filaments that gives skeletal and cardiac muscles their striated appearance when examined under a light microscope (Randall *et al.* 1997).

1.2 **Thin filament**

The thin filament consists of the contractile protein actin (Straub, 1942). The actin molecule exists in two forms, globular (G) and filamentous (F) actin (Pardee & Spudich, 1982; Pollard & Borisy, 2003). Actin is one of the most abundant proteins in eukaryotic cells and has a molecular weight of approximately 43kDa (Garells & Gibson, 1976). Additionally, its amino acid sequence is highly conserved and as a result actin is commonly used as a loading control in gel electrophoresis and Western blot analysis (Kabsch & Vandekerchovke, 1992). Therefore, in most of my studies, I used actin to check and correct for loading errors.

In higher vertebrates, the actin family consists of six isoforms namely; α-skeletal, α-cardiac, α-smooth muscle, γ-smooth muscle, γ-cytoplasmic non-muscle and β-cytoplasmic non-muscle actin (Garells & Gibson, 1976; Mounier & Sparrow, 1997). Furthermore, the expression of each of these isoforms in vertebrates has been shown to depend on the tissue, its function and developmental stage (Cox & Buckingham, 1992; Bertola *et al.* 2008). Thus, adult skeletal

muscles express mainly α-actin and β-actin is only expressed during early myogenesis (Cox *et al.* 1990).

Each thin filament consists of two strands of F-actin that are intertwined to form a double helix with a diameter of ~7nm (Gordon *et al.* 2000). Running along the grooves between the two strands is another protein known as tropomyosin. Tropomyosin is a rod shaped molecule that is composed of two intertwined polypeptides each approximately seven actin monomers long (Gordon *et al.* 2000). Each tropomyosin molecule is held in place by another protein known as troponin (Gordon *et al.* 2000). The troponin molecule consists of three subunits namely; troponin T (TnT), which tethers the tropomyosin to the actin molecule, troponin C (TnC) which is the Ca^{2+} binding subunit and finally troponin I (TnI) or the inhibitory subunit of troponin (Gordon *et al.* 2000). TnI partially covers the myosin binding sites on the actin molecule (Figure 1.2) and under resting conditions prevents myosin from binding to actin.

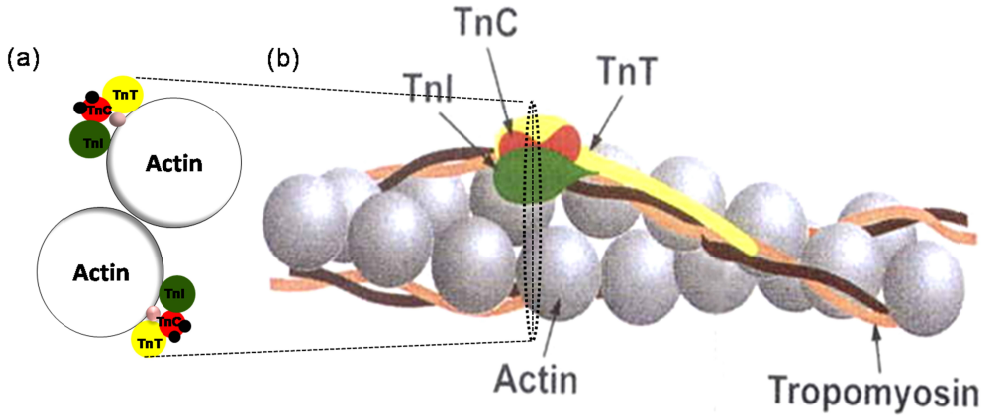

***Figure 1.2**: A schematic diagram showing the relationship between the various proteins that form a thin filament. In (a) a transverse cross-section of the filament is shown; whereas, in (b) a longitudinal section is displayed. Note that TnI (green globules) partially obscures the myosin binding sites on actin (grey/white spheres), TnT (yellow sphere/rod) tethers tropomyosin (black and brown strands) onto actin and TnC (red globules) binds calcium ions (black spheres in (a)). Figure from Gordon, et al. 2000.*

1.3 **Thick filament**

The thick filament consists mainly of the contractile protein myosin. However, it also contains several myosin binding proteins including C-protein, H-protein, myomesin and the giant muscle protein titin (Clark *et al.* 2003). Each myosin molecule consists of three distinct regions: a head or motor domain; a neck and a carboxylic terminal tail (Schiaffino & Reggiani, 1996). Members of the myosin

superfamily are defined by their conserved head domain sequences and based on these sequences can be grouped into 20 classes each with a different structure and function (Krendel & Mooseker, 2005). The myosin found in skeletal muscle belongs to class II or conventional myosin (Sellers, 2000). Therefore, in this section of my thesis I will concentrate on the structure of this class of myosins.

Conventional (also referred to as sarcomeric) myosin is composed of two heavy chains with molecular masses of ~200kDa and two pairs of light chains with molecular masses, ranging from 16-27kDa depending on the muscle type (see Fig 1.3). Myosin molecules form a parallel, two-chain coiled-coil structure over most of their length except in the NH_2-terminal region where they form two large, globular structures commonly referred to as myosin heads or subfragment 1(S1). One pair of light chains binds to each S1. The coiled-coil region of the myosin molecule, also known as the myosin rod, interacts with the rods of other myosin molecules *via* oppositely charged amino acids to form the myosin (thick) filaments. The molecules are also shifted along the filament by regular intervals of ~14.3nm. Because of this stagger the myosin heads project outwards from the thick filament and are helically arranged with repeats of ~14.3nm (Gordon *et al.* 2000).

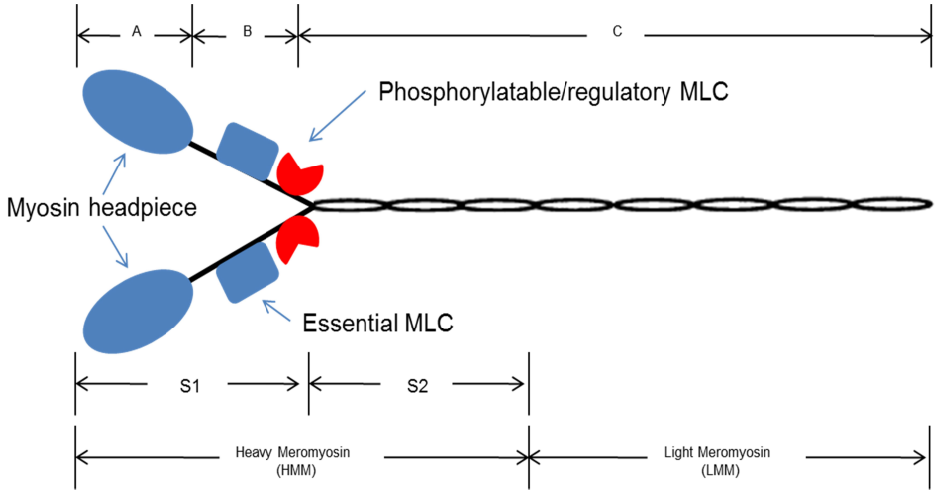

***Figure 1.3**: A schematic diagram showing the structure of conventional/class II myosin. Note that it consists of two molecules each composed of a head (A), neck (B) and tail (C) regions. Furthermore, the tails from the two molecules intertwine to form a α-helical coiled-coil. Limited proteolytic digestion of the molecule with trypsin yields two sub-fragments; heavy meromyosin (HMM) and light meromyosin (LMM). The HMM can be further split into two sub-fragments; S1 and S2. S1 consists of the myosin heads; whereas, S2 consists of the neck and its associated myosin light chains (MLC). Diagram adapted from Schiaffino & Reggiani (1996).*

In mammalian skeletal muscles both the myosin heavy chains (MyHCs) and MLCs exist in several isoforms that are differentially expressed in the various fibre types. Up to date six MyHC isoforms namely; MyHC-β/slow, MyHC-2A, MyHC-2X, MyHC-2B, MyHC-emb and MHC-neo have been identified in mammalian skeletal muscles (Schiaffino & Reggiani, 1996). MyHC-β/slow is found predominantly in slow-twitch/type 1 fibres; whereas, MyHC-2A, MyHC-2X and

MyHC-2B are found in fast-twitch/type 2 fibres. In contrast, MyHC-emb and MHC-neo are found mainly in embryonic and neonatal fibres, respectively. Because of their unique distribution in the various fibre types, MyHCs are commonly used in the identification/classification of skeletal muscle fibre types.

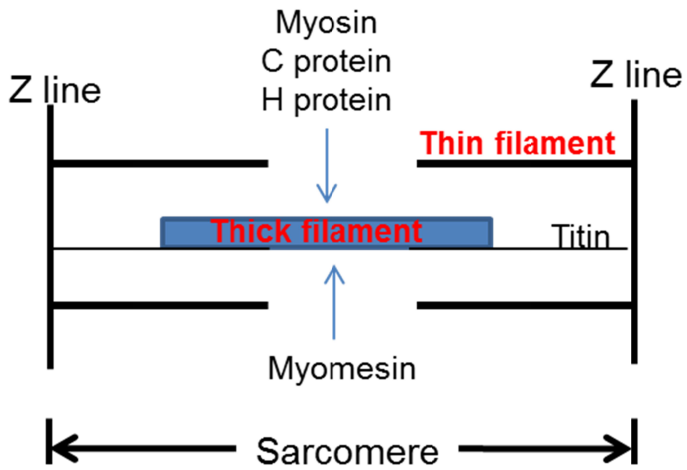

***Figure 1.4**: A schematic diagram of the sarcomere showing the location of the thin and thick filaments as well as the proteins associate with the thick filament. Diagram adapted from Schiaffino & Reggiani (1996).*

1.4 Muscle fibre types

Adult mammalian skeletal muscles perform a wide variety of functions ranging from those requiring prolonged/slow repetitive contractions for example the maintenance of posture to those requiring bursts of rapid movements such as sprint running. As a result they are composed of a mixture of fibre types that differ in their

metabolic capabilities, contractile properties, ultra-structural characteristics and fatigue resistance.

On the basis of these characteristics, adult mammalian skeletal muscle fibres have been classified as either fast-twitch (types 2A, 2B and 2X) or slow-twitch (type 1) (see summary in Table 1.1; Schiaffino & Reggiani, 1996). Both type 2B and 2X fibres express isoforms of the contractile protein, myosin (MyHCII/D and MyHCII, respectively) that hydrolyse ATP rapidly. In order to generate ATP quickly, they utilise anaerobic metabolism and hence they fatigue fairly rapidly (Pette, 1985). Therefore, they are adapted for movements such as sprint running that require short bursts of rapid contractions (Table 1.1; Pette, 1985). In contrast, type 1 fibres express a slow contracting isoform of myosin (MyHCI) that hydrolyses ATP slowly (Pette, 1985). These fibres are recruited when slow repetitive or prolonged postural movements are necessary (Pette & Staron, 2000). To power their slow contractions, type I fibres utilise predominantly aerobic metabolism and therefore, they are relatively fatigue resistant. Located in between these two groups are type 2A fibres. These fibres express a fast isoform of myosin (MyHCIIA) which utilises both oxidative and glycolytic metabolism (Delp & Duan 1996). Therefore, these fibres are relatively fatigue resistant and are recruited during movements that require high speeds sustained for long periods of time such as middle distance running (see Table 1.1; Sjostrom *et al.* 1987).

***Table 1.1**: A summary of some of the characteristics of the various muscle fibre types.*

Fibre type name	Histochemistry characteristics			Immunohistochemical characteristics	Metabolic characteristics	Use
	mATPase acid pre-incubation (pH 4.0)*	mATPase alkali pre-incubation (pH 10.0)*	SDH stain**	Myosin isoform***	Oxidative capacity	
Type I (Slow Oxidative)	Stains dark	Stains light	Stains dark	MyHC I	High	Postural maintenance, marathon running
Type 2A (Fast Oxidative Glycolytic)	Stains light	Stains dark	Stains light	MyHC IIA	Intermediate	Middle distance running
Type 2B	Stains light	Stains dark	Stains light	MyHC II/D	Low	Sprint running
Type2X (Fast Glycolytic)	Stains light	Stains dark	Stains light	MyHC IIX	Low	Sprint running

*Acid pre-incubation (pH 4.6) inhibits the myosin ATPase activity in fast (Type 2A, 2X, and 2B) fibre types, but not in the slow (Type I) type. Alkali pre-incubation (pH 10.2) inhibits myosin ATPase in slow fibre types only. In this stain, phosphate reacts with Ca^{2+} to form calcium phosphate which precipitates. The Ca^{2+} is then replaced with cobalt which reacts with hydrogen sulphide to form colbalt sulfide (CoS_2) (dark brownish) which precipitates to give the fibres the dark colour. As fast-contracting muscle fibres hydrolyse ATP faster than slow-contracting ones, when given equivalent times, fast-contracting fibres appear dark histochemically, and slow-contracting fibre appear light. When this method is used in conjunction with pre-incubation steps to accentuate or reverse this pattern, the myosin ATPase assay can be used to distinguish between fast- and slow-contracting muscle fibres.

**Succinic dehydrogenase (SDH) is found in all aerobic tissues and normally catalyses the hydrogenation of succinate to fumarate in the citric acid cycle. Therefore, it is very specific for mitochondrial density/activity. In this stain, the rate of the reaction is monitored using a redox dye such as sodium tetrazolium. As the conversion of succinate to fumarate occurs, the dye is reduced into an insoluble deep blue compound.

***Probed using monoclonal antibodies against the various myosin heavy chains.

Although most mammalian skeletal muscles consist of a mixture of the fibre types mentioned above, each fibre type shows a regional distribution (Wang & Kernell, 2001). Additionally, some skeletal muscles such as the extensor digitorum longus (EDL) and soleus are composed of mainly a single fibre type (Ranatunga & Thomas, 1990). Because of their fibre type composition, these muscles are commonly used as experimental models of fast- and slow-twitch fibres, respectively (Mutungi & Ranatunga, 1996; Mutungi & Ranatunga, 2000; Hamdi & Mutungi, 2010). For this reason, all the fibre bundles used in this study were isolated from the EDL and soleus of adult female mice.

1.5 **Skeletal muscle plasticity**

Each of these fibre types is the product of a multi-gene programme that is tightly regulated in a fibre-type specific manner (Butler-Browne and Whalen, 1984; Weydert et al. 1987) and in adult muscles can be rapidly switched from one type to another depending on the activity of the motor neurones innervating them; and the physiological and biochemical demands imposed upon them (Talmadge, 2000). This fibre type switching is commonly referred to as muscle plasticity (Schiaffino *et al.* 2007). During this process, fibre types are remodelled in a sequential manner (Ausoni *et al.* 1990; Conjard *et al.* 1998). For example, endurance exercise (Green *et al.* 1984) and chronic electrical stimulation (Talmadge, 2000) leads to muscle fibres switching in the following order; 2X/2B $\rightarrow$ 2A

→ I fibres; whereas, inactivity leads to the fibres switching in the opposite direction i.e. I → 2A → 2X/2B (Pette & Starron, 2000). Although this is what is thought to occur under most experimental conditions, it is now generally accepted that under certain conditions such as hind limb unloading or prolonged bed rest, the fibres can skip the expression of one or several of the myosin isoforms (Andersen *et al.* 1996). For example, when the soleus muscle switches from slow to fast fibres in response to inactivity or spaceflight unloading, a high number of fibres have been found to co-expressing type I and 2X myosin without expressing the type 2A myosin, so called 'jump fibres' (Talmadge, 2000). In addition to fibres that express a single myosin isoform, a considerable numbers of fibres have been shown to contain two or occasionally more myosin isoforms for example type I+2A, 2A+2X or 2X+2B (commonly termed' hybrid fibres') (Talmadge, 2000). The co-existence of different myosin isoforms in a single fibre is characteristics of muscles undergoing rapid changes and in these muscles the population of hybrid fibres has been shown to increase. For example, the number of hybrid fibres has been shown to increase in human muscle following strength training (Staron & Hikida, 1992), endurance training (Klitgaard *et al.*1990) and in chronically electro-stimulated vastus lateralis muscle of spinal-cord injured individual (Andersen *et al.* 1996).

Although, many factors including the patterns of innervation (Pette & Vrbova, 1985), altered weight bearing (Loughna *et al.* 1990), physical activity (Booth & Thomason, 1991) and various hormones (Izumo *et al.* 1986) have been shown to affect the fibre type composition and hence the gene programmes of adult mammalian skeletal muscles, the cellular signalling pathways and the transcription factors that regulate these changes are still not fully understood (Spangenburg & Booth, 2003).

Another major determinant of muscle fibre type composition is ageing. One consequence of the ageing process is the progressive loss of skeletal muscle mass (Grimby & Saltin, 1983; Bruce *et al.* 1989; Brooks & Faulkner, 1994; Frontera *et al.* 2000). Previously, most of the decline in force with age was attributed to the loss in skeletal muscle mass (Grimby & Saltin, 1983; Bruce *et al.* 1989; Frontera *et al.* 2000). However, recent evidence suggests that changes in the contractile properties of the muscle fibres *per se* and a reduction in the number and cross-sectional area of muscle fibres, especially type 2B/2X fibres (Larsson & Moss, 1983; Larsson *et al.* 1991, Lexell, 1995; Brown & Hasser, 1996; Kadhiresan *et al.* 1996) play a far greater role in the ageing process than previously thought (Li & Larsson, 1996; Larsson *et al.* 1997; Degens *et al.* 1998; Hook et al. 1999; D'Antona et al. 2006). Thus, ageing leads to a decrease in both specific muscle force (specific Po) (Larsson *et al.* 1997) and maximum velocity of unloaded shortening (V_o) in type 1 and type 2A

fibres but not in type 2B/2X fibres (Li & Larsson, 1996; Larsson *et al.* 1997; Degens *et al.* 1998).

1.6 **Synthesis, structure and metabolism of T and DHT**

As mentioned above, in this study I investigated the acute/rapid actions of T and its metabolite dihydrotestosterone (DHT). Therefore, in the next few pages I will describe the synthesis, metabolism and chronic effects of T. T, the principal sex hormone in males, is a 19 carbon (C_{19}) molecule that is synthesised from cholesterol in the Leydig cells of the male testes. It has 3 aromatic- and 1 five-carbon rings (see Figure 1.5; labelled A, B, C and D) with a hydroxyl (OH-) group at the C_{17} position. The synthesis of T occurs in the mitochondria and the endoplasmic reticulum of the Leydig cells. T, like all other steroid hormones, is synthesised from cholesterol and uses the same pathway but different enzymes. Thus, in Leydig cells the 11- and 21-hydroxylases found in the adrenal cortex are missing and instead these cells contain 17α-hydroxylase which hydroxylates pregnenolone at the C_{17} to produce 17α-hydroxypregnenolone (see figure 1.5). 17α-hydroxypregnenolone is then hydroxylated into androstenedione. The androstenedione is then subjected to side chain cleavage and conversion by 17β-hydroxysteroid dehydrogenase into T.

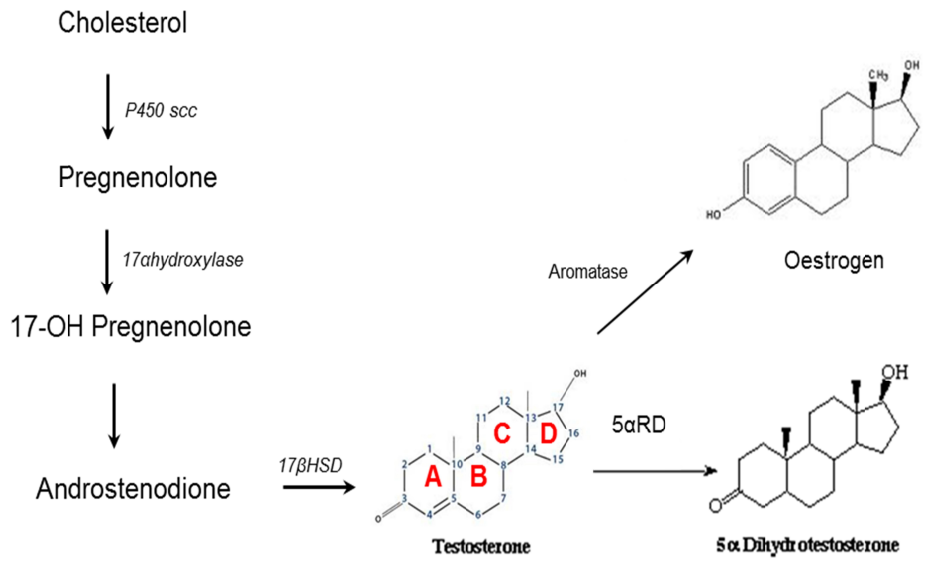

***Figure 1.5**: A schematic diagram showing the biosynthesis of T in Leydig cells. As the diagram shows, the synthesis of T starts with cholesterol which is cleaved between C_{20} and C_{22} by side chain cleavage enzyme $P450_{SCC}$ to produce pregnenolone. The enzyme 17α-hydroxylase then hydroxylates pregnenolone at C_{17} to produce 17-OH Pregnenolone. Further hydroxylation of pregnenolone by hydroxylases leads to the formation of androstenodione; whereas, the hydroxylation of androstenodione by 17βHSD leads to the formation of T. In certain target tissues, T is then reduced by 5α-reductase (5αRD) into DHT or it is aromatised by aromatase into oestrogen. 17-OH pregnenolone: 17α-hydroxypregnenolone, $P450_{SCC}$: P450 side chain cleavage, 17βHSD:17β-hydroxysteroid dehydrogenase, 5αRD: 5αReductase. A, B and C are the three aromatic ring and D is the five-carbon ring.*

In healthy young adult men, the plasma concentration of T ranges from 300 to 1000ngdl^{-1}. Most of the T is bound to either albumin or

sex hormones binding globulin (SHBG), and only 2% is free [normal range free T: 1.5 to 20ngdl^{-1}] (Pardridge, 1986, Evans, 2004) and hence physiologically active. In certain target tissues such as the liver, brain, prostate and pubic skin, T is irreversibly converted to DHT by the enzyme 5α-reductase (5αRD) (see figure 1.5) (Bruchovsky & Wilson, 1968). In healthy young adult men, the plasma concentration of DHT [normal range 4 to 57.5ngdl^{-1}] is similar to that of free T. DHT is also considered to be the more potent hormone because it has a higher receptor binding affinity and a lower dissociation constant than T (Saartok *et al.* 1984). However, as skeletal muscle is thought to lack 5αRD (Thigpen, *et al.* 1993), it is uncertain whether the anabolic effects of anabolic androgenic steroids arise from T itself or from its more potent metabolite DHT.

In the body, T has a short active-life of 1hour (Singh *et al.* 2001). However, it can be chemically modified to increase its anabolic potency, slow its rate of metabolism and decrease its aromatisation into oestrogen (Wilson, 1990). For example, T can be alkylated at C_{17} into methyltestosterone making it resistant to hepatic degradation or it can be made more lipophilic by esterification at C_{17} to produce testosterone cypionate thus prolonging its half-life in circulation (Evans, 2004). These synthetic derivatives of T are commonly referred to as anabolic androgenic steroids (AAS) (Kuhn, 2002). Although in the market, AAS come with range of effective

doses and active life, none of them have convincingly demonstrated any effects that are different from those of native T (Evans, 2004).

1.6.1 **Clinical use of T**

Because of their anabolic properties, T and its many derivatives (commonly known as anabolic-androgenic steroids, AAS) has been prescribed for the treatment of cachexia (muscle wasting associated with chronic debilitating diseases such as AIDS, chronic obstructive pulmonary disease, cancer) and sarcopenia (muscle wasting due to old age) since the 1940s (Kuhn, 2002). However, in recent years the number of patients on T has increased dramatically. For example, in the USA alone one million patients were on T in 1991. However, by 2004 this number had risen to 3 million (Evans, 2004). Worldwide, the incidence of chronic debilitating diseases such as chronic obstructive pulmonary disease (COPD) and cancer is increasing at an alarming rate, and the proportion of the population over 65 years old continues to increase rapidly (Bonita, 1998; Justl & Hartigan, 1999; Lievre *et al.* 2007). This will further increase the number of patients on T unless ways of arresting/reducing the incidence of both cachexia and sarcopenia are found. In addition to their use in clinical medicine, T and its derivatives is commonly abused by athletes and body builders. It is estimated that 1 to 3 million athletes in the USA alone have used T (Silver 2001). Thus, each year, the sales of T in the sports industry have increased by between 20 and 30%, and are

now estimated to be worth over 100 million US dollars (Tokish 2004).

Although T is widely used, as well as abused, there are many uncertainties surrounding its benefits (see Fig. 1.6). As the figure shows, T administration can induce alterations in muscle gene expression, resulting in increased skeletal muscle mass. However, the only change that is certain an increase in skeletal muscle mass.

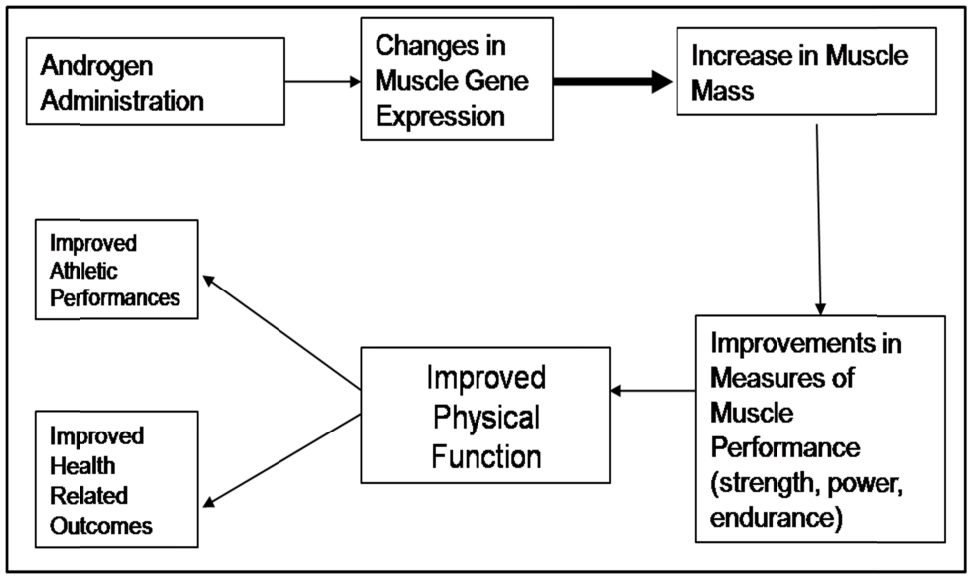

***Figure 1.6**: A schematic diagram showing the rationale for the abuse of T by athletes and their potential therapeutic use in the treatment of cachexia and sarcopenia. The narrow arrows indicate areas of uncertainty; whereas, the heavier ones indicate areas where more knowledge on the actions of T is available. Note that an increase in skeletal mass is the only certain outcome following the prolonged administration of T or its derivatives. Figure adapted from Bhasin et al. 2001.*

Although studies demonstrate an increase in mRNA synthesis following the chronic administration of T propionate to castrated animals (Xu *et al.* 2004); oxandrolone in healthy young men (Sheffield-Moore, *et al.* 1999) and T enanthate in elderly men (Urban *et al.*1995), no evidence showing that these compounds increase protein synthesis and decrease its breakdown exists. It is also uncertain whether they lead to an improvement in performance.

1.7 **Mechanism of action for DHT and T**

1.7.1 **Genomic mechanism**

Both DHT and T are lipid soluble and can freely cross the cell membrane. Once inside the cell, they bind to the classic androgen receptors (AR). The AR gene is located on the long arm of chromosome X at position 11-12 (Gelmann, 2002). It consists of 8 exons coding for the N-terminus DNA binding domain (DBD), nuclear localisation sequences (NLS), and C-terminal ligand/hormone binding domain (LBD) (for general structure see Figure 1.7) (Gelmann, 2002). The AR is a 110kDa receptor protein consisting of 919 amino acids forming a structure with 12 α-helices and 2 β-sheets (Rochette-Egly, 2003). It belongs to the large family of nuclear transcription factors and normally resides in the cytoplasm where it is bound to chaperone molecules such as heat shock protein 90 (HSP90) that protect it from enzymatic damage (Pratt & Toft, 2003). Studies have shown that AR can be found in all tissues

except in adrenal medulla and spleen (Takeda *et al.* 1990; Wilson & McPhaul, 1996).

***Figure 1.7**: A schematic diagram showing the structure of the classical androgen receptors (AR). Note that the receptor consists of a DNA-binding domain (DBD), a nuclear localisation domain (NLS) and a C-terminal ligand binding domain (LBD).*

Both T and DHT bind to the AR at the LBD leading to a conformational change in its tertiary structure. This leads to the release of the chaperones bound to it and exposes the NLS (Gelmann, 2002; Heinlein & Chang, 2002). The hormone-receptor complex then translocates into the nucleus, where the DBD binds to the hormone responsive elements (HRE) on the promoter regions of its target genes. Depending on the co-activators/co-repressors recruited, the hormone-receptor complex can then lead to either the activation or repression of these genes (see Fig. 1.8; Losel *et al.* 2003). This mechanism of steroid action is known as the classic or genomic pathway and because it involves gene transcription and mRNA translation, its effects usually take several hours to days to be manifested (Florini, 1970; Beato, 1989; Beato *et al.* 1996).

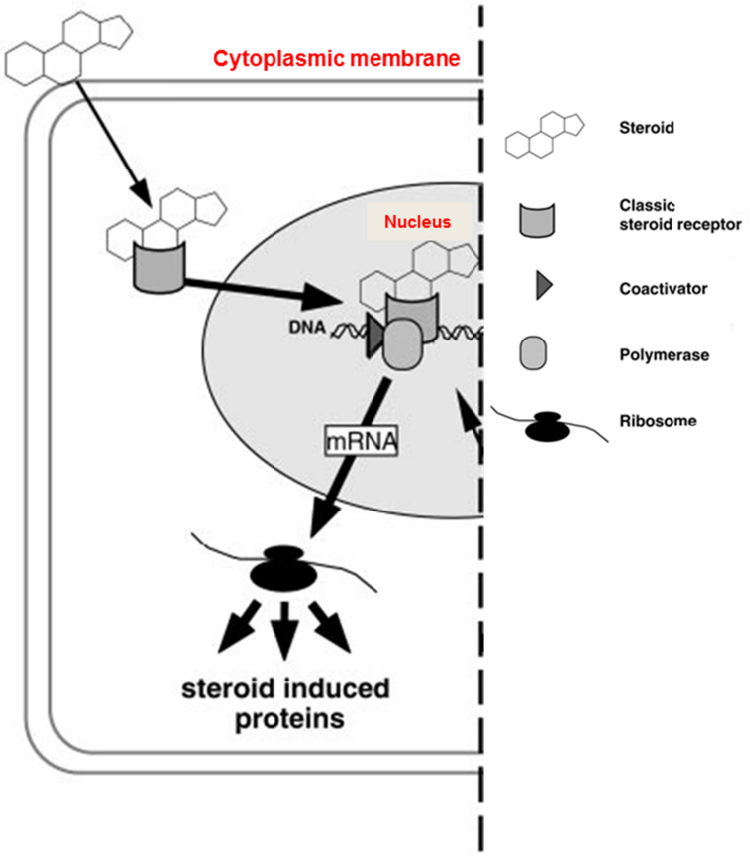

***Figure 1.8**: A schematic diagram showing the genomic mechanism of action of anabolic androgenic steroids. In this mechanism, the hormone crosses the cell membrane and once inside the cell, it binds the AR forming the hormone-AR complex. The complex then undergoes a conformational change leading to its translocation into the nucleus where it binds to the HRE on the promoter regions of its target genes leading to their activation or repression. Figure 1.8 adapted from Losel et al. (2003)*

1.7.2 **Non-genomic mechanism**

In addition to their genomic actions, it is now generally accepted that steroid hormones can also exert actions that are too rapid to be explained by the classic/genomic pathway (Falkenstein *et al.* 2000; Heinlein & Chang, 2002; Simoncini & Genazzani, 2003).

These actions occur within seconds to minutes after administration of the hormone, and are generally insensitive to inhibitors of the androgen receptor (Estrada *et al.* 2003); suggesting that they are regulated by cellular signalling pathways involving surface membrane receptors and second messengers (Heinlein & Chang, 2002). This mechanism of steroid action is commonly referred to as the non-genomic or non-classical mechanism. Because of the speed at which it occurs, it is also referred to as the rapid/acute action of AAS (see Fig. 1.9).

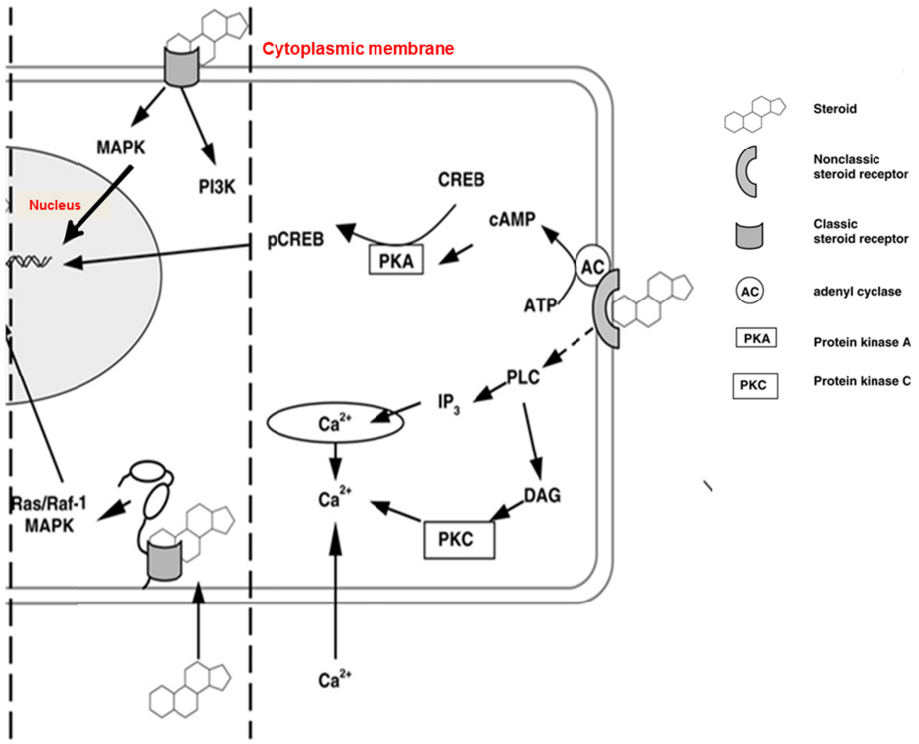

***Figure 1.9**: A schematic diagram showing the non-genomic mechanism of action of steroid hormones. In this mechanism, the hormone binds and activates a membrane receptor. Recruitment of the receptor activates second messengers such as cyclic AMP (cAMP) and intracellular calcium $[Ca^{2+}]_i$ leading to the activation of various cellular signalling pathways. Figure adapted from Losel et al. 2003.*

Although the non-genomic actions of other steroid hormones such as oestrogen, progesterone and aldosterone have received a lot of attention and their actions in many tissues are well characterised (for details see review by Losel *et al.* 2003), little is known about the non-genomic actions of DHT/T in adult mammalian skeletal muscles. So far, only two studies have investigated the acute effects of T in

skeletal muscles. However, both studies were performed on cultured muscle cells and used supra-physiological concentrations of T (Estrada *et al.* 2000; Estrada *et al*. 2003). Therefore, it is uncertain whether physiological levels of T or its metabolite DHT have any rapid/non-genomic actions in isolated intact mammalian skeletal muscle fibres. Furthermore, little is known about the identity of the receptor/s mediating these actions.

Although little is known about the receptor(s) mediating the non-genomic effects of steroid hormone (Falkenstein *et al.* 2000; Losel *et al.* 2003; Simoncini & Genazzani, 2003), there are reports suggesting that these effects may not be uniform and therefore, they may involve a variety of receptors. For example, some studies have implicated the classical AR (for general structure see figure 1.7) (Cinar *et al.* 2007); whereas others suggest that they involve a G protein receptor localised at the membrane (Estrada *et al.*2003). Therefore, in the following sections I will review some of the receptors that could mediate the acute/rapid actions of these hormones.

1.7.3 **G-protein coupled receptors (GPCRs)**

GPCRs are cytoplasmic membrane receptors with seven trans-membrane (heptahelical) domains that are closely associated to signal transducing heterotrimeric guanine nucleotide binding proteins (G proteins) (Dorsam & Gutkin, 2007). They comprise the largest group of cell surface receptors with more than 1000 members some

of which can be found in skeletal muscle tissue and skeletal muscle cells (for general structure see figure 1.10; Gutkind, 1998; Jean-Baptiste *et al.* 2005). GPCRs can be activated by a diverse array of external stimuli including growth factor, T and oestrogen (Gutkind, 1998; Hryb *et al.* 1985). GPCRs also play a role in normal cell growth (Gutkind, 1998) and based on their gene sequence homology and the size of the extracelluar loops, GPCR can be split into 4 classes including rhodopsin or class A, secretin and adhesion or class B, glutamate or class C and frizzled/olfactory (Bjarnadottir *et al.* 2007, Lagerstrom & Schioth, 2008).

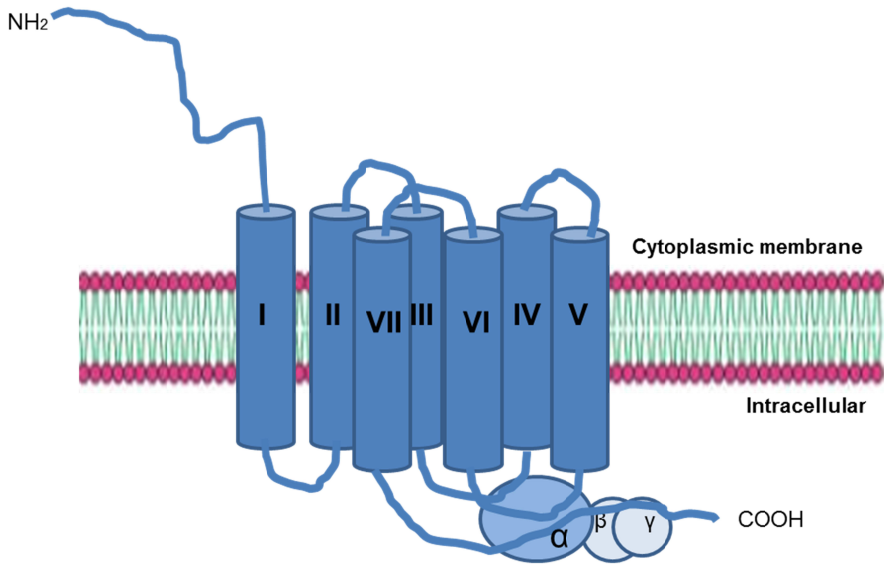

***Figure 1.10**: A schematic diagram of a GPCR showing the transmembrane domains and the G protein which consists of an α, β and γ sub-units*

GPCRs do not have tyrosine kinase activity; instead signal transmission is achieved through the separation of the α-, β and γ-

subunits of the G protein. The G proteins are composed of a 36-52KDa α-subunit, a 35-36KDa β-subunit and an 8-10KDa γ-subunit (Dorsam & Gutkind, 2007). The α-subunit particularly α_s, functions as a regulatory molecule for the integral membrane enzyme adenylyl cyclase (AC). AC hydrolyses ATP into cyclic adenosine monophosphate (cAMP) which induces cAMP dependant PKA signal transmission (Griner & Kazanietz, 2007). The β and γ-subunits form the βγ-dimer which is only separable under strong denaturing conditions (Bjarnadottir *et al.* 2007). The βγ-dimer activates the effector enzyme phospholipase C (PLC) which activates its downstream signalling cascade.

1.7.4 **Receptor tyrosine kinases**

Another group of receptors that can mediate the acute/non-genomic actions of AAS is the receptor tyrosine kinases. Many cellular activities including cell growth and proliferation are regulated by extracellular signals (Pawson, 2002). Most of these signals are transduced across the cell membrane through receptor tyrosine kinases (RTKs) such as insulin-like growth factor-1 receptor (IGF-1R) (Rennie *et al.* 2004), platelet derived growth factor receptor (PDGFR) (Albrecht & Tidball, 1997) and epidermal growth factor receptor (EGFR) (Zwick *et al.* 1999). In general, all RTKs are monomers in the cell membrane except insulin receptor (IR) and IGF-1R (Schlessinger, 2000). All RTKs consist of an extracellular N terminal ligand binding domain that is commonly glycosylated, a

transmembrane spanning domain and a cytoplasmic/cytosolic c-terminal domain (Hunter, 1998). The cytoplasmic domain contains a conserved protein tyrosine kinase core and additional regulatory sequences that can be phosphorylated by protein kinases that can transfer a phosphate from ATP to the hydroxyl groups of the tyrosine residues on these receptors (Hunter, 1998). This enables the receptors to recruit signalling molecules to the RTKs and activation of their downstream effectors (Hunter, 1998).

1.7.4.1 **Insulin growth factor-1receptor**

The chronic administration of nandrolone in female rats (Lewis *et al.* 2002); oxandrolone in healthy young men (Sheffield-Moore, *et al.* 1999; Bamman *et al.* 2001) and testosterone enanthate in healthy elderly men (Ferrando *et al.* 2002) is accompanied by an increase in the mRNA for IGF-1. Therefore, it has been suggested that some of the effects of T are exerted through the IGF-1R.

The IGF-1R consists of 2α and 2β subunits and has tyrosine kinase activity (see figure 1.11) (Alessi *et al.* 1996). A fully functional IGF-1R is a dimer that is activated by at least three ligands, IGF-1, IGF-2 and insulin (Alessi *et al.* 1996). IGF-2R, also known as cation independent mannose-6-phosphate receptor, is a monomeric receptor that consists of 15 extracellular domains, a transmembrane domain and Intracellular domain (MacDonald *et al.* 1988). However, the IGF-2R seems have no role in growth factor signalling (MacDonald *et al.* 1988). When activated, IGF-1R transmit a signal

to its two major substrates - insulin receptor substrate 1 (IRS1) - a cytoplasmic adaptor proteins with a phosphotyrosine binding domain, PTB leading to the Akt/mTOR - an important signalling pathway for protein synthesis, cell-proliferation and -survival (White & Kahn, 1994) and Shc leading to Ras/Raf/ERK signalling pathway (Baserga, 1995) an important pathway for cell growth and proliferation.

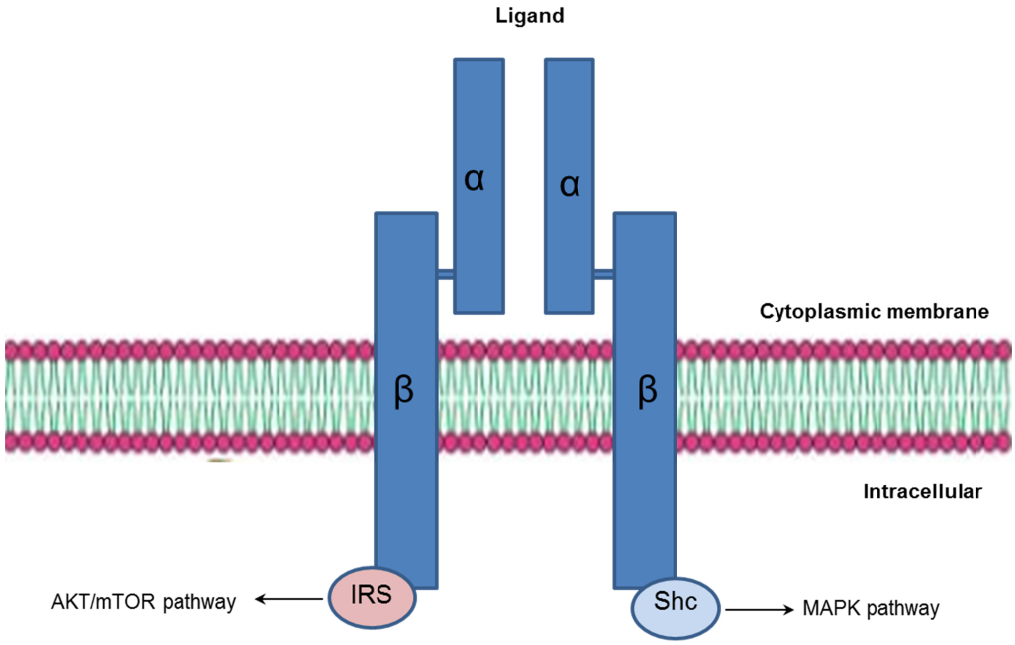

***Figure 1.11**: A schematic diagram of IGF-1R. Ligand binding to IGF-1R leads to the recruitment of IRS1 which activates the Akt/mTOR pathway; whereas, Shc activates MAPK pathway.*

1.7.4.2 **Platelet derived growth factor receptor**

Another receptor that can mediate the non-genomic actions of DHT and T is the PDGFR. The PDGFR was originally identified in fibroblasts, smooth muscle cells, skeletal muscle and glial cells (Raines & Ross, 1993; Albrecht & Tidball, 1997; Fiaschi *et al.* 2003). Each receptor consists of an extracellular region with five immunoglobulin like domains namely D1-D5, a single transmembrane domain, a juxtamembrane domain and an intracellular cytoplasmic tyrosine kinase domain (Williams, 1989). The kinase domain exists in two isoforms; α and β (Kelly *et al.* 1991). The α-isoform has a molecular weight of ~170kDa; whereas, that of the β isoform is 180 KDa (see figure 1.12). Thus, the receptor can contain two α-kinase domains, two β-kinase domains or a mixture of both. The α-kinase dimer binds PDGF-AA, PDGF-BB and PDGF-AB, the β-kinase binds PDGF-BB only; whereas, the heterodimer binds PDGF-AB and PDGF-BB (Williams, 1989). Autophosphorylation of tyrosine residues in the kinase domain serve as docking sites for various signalling molecules including src, PI3K and PLC which further activate downstream signalling pathways (Kypta *et al.* 1990)

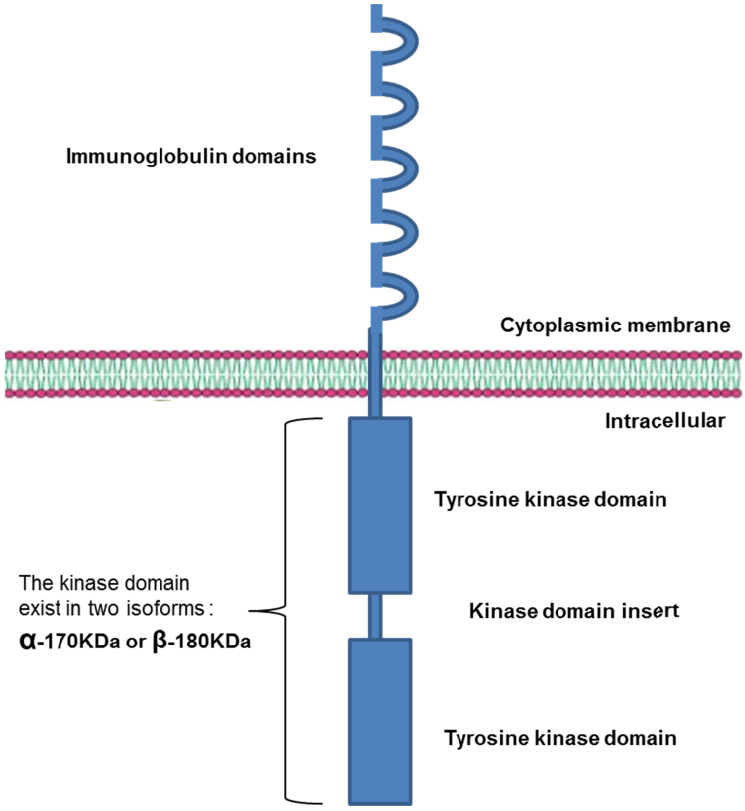

***Figure 1.12**: A schematic diagram showing the general layout of α- and β-PDGFR. Each consists of an extracellular immunoglobulin domain and tyrosine kinase domain separated by a kinase domain insert.*

1.7.4.3 Epidermal growth factor receptor

As mentioned in section 1.7.4 above, the epidermal growth factor receptor (EGFR) is another receptor tyrosine kinase found in many tissues including skeletal muscle (Thompson & Gill, 1985, Peng *et al.* 1997). It is a 170kDa transmembrane receptor that activates a wide range of biological responses including mitogenesis, differentiation and apoptosis (Wells, 1999). It belongs to a family of

four members EGFR (ErbB1/HER1), ErbB2 (neu/HER2), ErB3 (HER3) and ErbB4 (HER4) (Ulrich *et al.* 1984, Plowman *et al.* 1990).

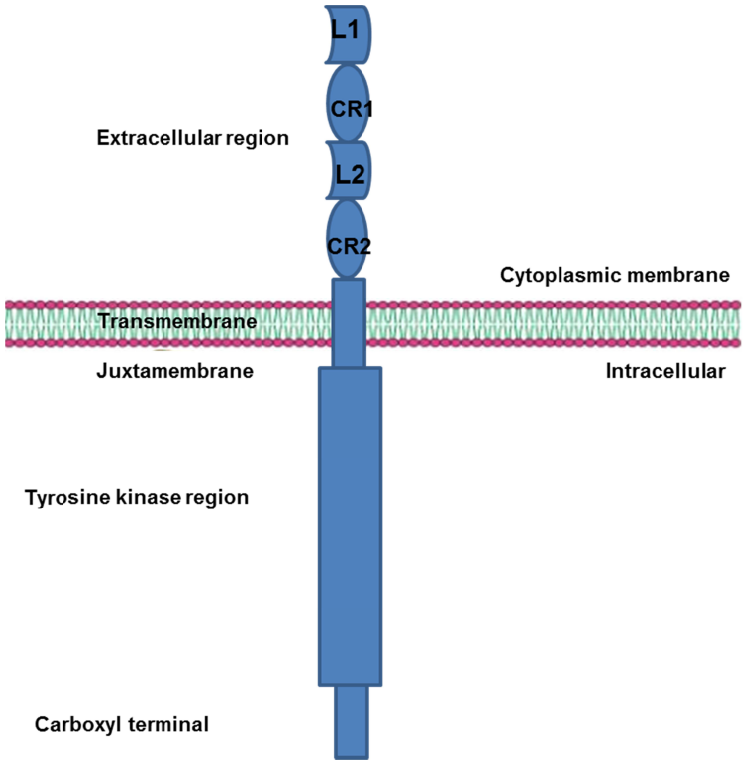

***Figure 1.13**: A schematic diagram showing the general structure of EGFR.*

The general layout of the protein consists of an extracellular ligand binding domain, a single α-helical transmembrane domain and a cytoplasmic domain containing tyrosine and serine phosphorylation sites with a regulatory carboxyl terminal segment (see Figure 1.13) (Ferguson, 2004). The extracellular C-terminal domain consists of four sub domains; two homologous large domains (L1 and L2) and two cysteine-rich domains (CR1 and CR2). These domains are also referred to as I, II,III and IV, respectively (Ferguson, 2004). The EGFR spans through the cell membrane with a single alpha helical

domain consisting of 23 amino acids. It has been suggested that this transmembrane domain regulates receptor dimerization (Schlessinger, 2002). The transmembrane domain is followed by the intracellular domain which has a juxtamembrane region, a tyrosine kinase region and a carboxyl terminal end region. Ligand binding to the receptor activates autophosphorylation of the intracellular region leading to recruitment of Ras/Raf and subsequently activation of the MAPK pathway (Jorissen *et al.* 2003).

1.8 **Amino acid transporters**

Although membrane receptors have an important role in the transduction of extracellular signals, the transport of solutes such as ions and nutrients (for example amino acids across cell membrane) is also important in all cells. Almost all transmembrane transport processes are conducted by integral membrane proteins, sometimes functioning in association with cytoplasmic receptors (Saier, 2000; Conde *et al.* 2010). Amino acids are the building blocks of proteins and come in all shapes and sizes, For example, they can be neutral with aliphatic side chains such as glycine, leucine and isoleucine or polar such as arginine and glutamic acid. However, they all require transporters to move from one side of the membrane to the other. Furthermore, since the intracellular amino acid concentrations in mammalian cells are generally higher than (or at least equal to) those in the extracellular fluid, amino acids need to be actively transported into the cell (Hyde *et al.* 2003).

Amino acids enter and leave cell through specialised proteins known as amino acid transporters (Christensen, 1990) which have been placed into distinct 'systems' depending on their substrate specificity, transport mechanism and regulatory properties as shown in table 1.2 and 1.3 (Hyde *et al.* 2003).

1.8.1 **Sodium dependent neutral amino acid transporters**

The sodium-coupled neutral amino acid transporters also known as sodium dependent neutral amino acid transporters (SNAT), are members of the solute like carrier 38 (SLC 38) family which consist of system A and system N amino acid transporters (see table 1.2) (Mackenzie & Erickson, 2004). Amino acid transporters in this group prefer small zwitterionic amino acids such as glycine (Mackenzie & Erickson, 2004) and are poor at transporting branched chain amino acids (Hyde *et al.* 2003). Of the two systems, those belonging to system N subtypes have a narrow range of substrates including glutamine, histidine and asparagine. On the other hand, transporters belonging to system A have a broader range of substrates including alanine, asparagine, cysteine, glutamine, methionine and serine (Hyde *et al.* 2003). Amino acid transporters in both systems are tolerant to lithium (Reimer *et al.* 2000) but are highly sensitive to pH (Baird *et al.* 2006). System A and N transporters depend on sodium gradient to pump amino acids into the cell through secondary active transport (Lingrel *et al.* 1994). Therefore, they tend to be coupled to the sodium/ potassium ATPase ($Na^{+/}K^{+}$ATPase) pump. Subtypes of

system A include SNAT1, SNAT2 and SNAT4; whereas, subtypes of system N include SNAT3 and SNAT 5 (Mackenzie & Erickson, 2004).

Transporters belonging to system A are all electrogenic and pH sensitive (Mackenzie & Erickson, 2004; Bevington *et al.* 2002) and can easily be distinguished from those belonging to system N because they are inhibited by the model substrate α-(methylamino)isobutyric acid (MeAIB) (Christensen, 1985). They are composed of eleven hydrophobic transmembrane alpha helices connected to each other by hydrophilic intra- and extracellular loops. Also, they have sites that can be glycosylated and phosphorylated by protein kinase C (PKC) (see Fig. 1.14; Yao *et al.* 2000). They have been identified in lung, skeletal muscle, spleen and intestine (Sugawara *et al.* 2000; Hatanaka *et al.* 2001) and their expression is up-regulated by many factors including hormones, growth factors and mitogens (Taylor, 2009).They are also subject to adaptive regulation mediated by intracellular and possibly extracellular amino acid levels (Franchi-Gazzola *et al.* 2004).

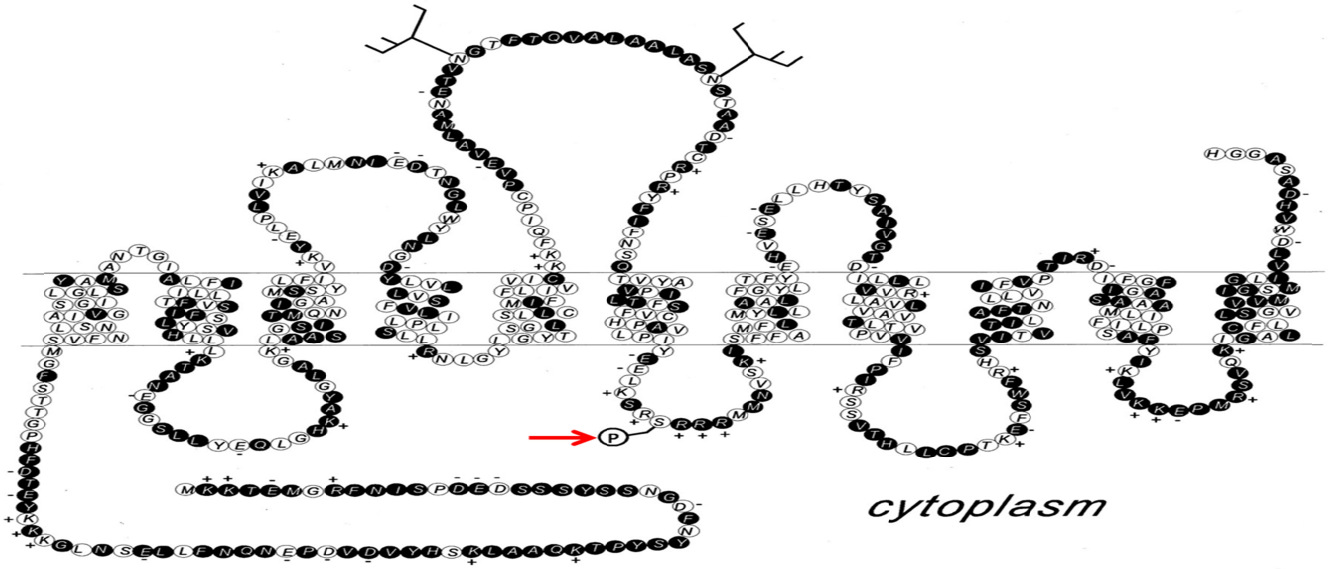

***Figure 1.14**: The primary amino acid sequence and proposed structure of SNAT2. SNAT2 is predicted to have eleven putative transmembrane domains with potential sites for N-linked glycosylation (three-pronged branches) and phosphorylation by protein kinase C (red arrow). Diagram adapted from Yao et al. (2000).*

***Table 1.2**: List of some of the amino acid transporter systems found in the cells of eukaryotes. Table adapted from Christensen, (1983); McGivan & Pastoranglada, (1994); Hyde et al. (2003)*

Neutral-amino-acid transporters: sodium-dependent				
System	Protein	Gene	Amino acid substrates (one- letter code)	Tissue distribution
A	SNAT1	SLC38A1	Me-AIB, G, A, S, C, Q, N,H, M, T, P, Y, V	Widespread including brain, heart, placenta
	SNAT2	SLC38A2	Me-AIB, G, P, A, S, C, Q N, H, M,	Muscle, kidney, intestine
	SNAT4	SLC38A4	G, P, A, S, C, N,M, H, K, R	Placenta
ASC	ASCT1	SLC1A4	A, S, C	Liver
	ASCT2	SLC1A5	A, S, C, T, Q,	Widespread
B°	ASCT2	SLC1A5	A, S, C, T, Q, F, W,Y	Epithelial cell
N	SNAT3	SLC38A3	Q, N, H	Liver
	SNAT5	SLC38A5	Q, N, H, S, G	Brain

Table 1.2 continued

Anionic-amino-acid transporters: sodium-dependent				
X^-_{AG}	EAAT1	SLC1A3	E, D	Widespread
	EAAT2	SLC1A2	E, D	Brain
	EAAT3	SLC1A1	E, D, C	Brain
	EAAT4	SLC1A6	E, D	Cerebellum
	EAAT5	SLC1A7	E, D	Retina
Cationic-amino-acid transporters: sodium-dependent				
$B^{o,+}$	ATB(o,+)	SLC6A14	K, R, A, S, C, T, N, Q, H, M, I, L, V, F,Y,W	Intestine
y+L*	y+ LAT1	SLC7A7	K, R, Q, H, M, L	Placenta
	y+LAT2	SLC7A6	K, R, Q, H, M, L,A,C	Erythrocytes

X^-_{AG} denotes systems that are substantially specific to Aspartate and Glutamate; ($^+$) plus, ($^-$) minus or (^{o}) and neutral sign indicate the need for appropriately charged amino acid substrates; (y+L*) heteromeric transporters formed upon association with glycoprotein CD98 (also known as 4F2hc) encoded by gene SLC3A2.

1.8.2 **Sodium independent amino acid transporters**

Another subset of neutral amino acid transporters are the L-type amino acid transporters. Transporters belonging to system L mediate the transport of large neutral amino acids with bulky branched and aromatic side chains (Palacin *et al.* 1998). They do not require a sodium gradient to transport amino acids (see table 1.3). Instead they function as amino acid exchangers (Hyde *et al.* 2003).

Members of this group are heterodimeric proteins that consist of a light chain subunit namely L-type amino acid transporter (LAT) 1 and 2; and a heavy chain subunit commonly referred to as 4F2hc (see Fig. 1.15. The heavy chain subunit is also known as CD98 and is approximately 80-kDa in size. It was first identified in activated lymphocytes and is highly glycosylated. On the other hand, the light chains are non-glycosylated and are approximately 40-kDa (Hemler & Strominger, 1982). The main function of 4F2hc is to support the translocation and insertion of the transporter complex into the cell membrane. Thus, without the heavy chain, the transporter resides mainly in the cytoplasm.

System L amino acid transporters are obligatory exchanger proteins with a 1:1 stoichiometry that exchange small neutral amino acids such as glutamine for large branched chain amino acids like leucine and isoleucine (so called tertiary amino acid transport) (Hyde *et al.* 2003). Therefore, for these transporters to function normally they

require an intracellular pool of these small neutral amino acids (Verrey, 2003). Therefore, they tend to be coupled to (for example) system A transporters which provide the amino acids they exchange.

One of the distinguishing features of L-type amino acid transporters is the observation that they are inhibited by the non-metabolisable amino acid analogues 2-aminoendobicycle (2,2,1)–heptane-2-carboxylic acid (BCH) and 3-aminoendobicyclo-(2,2,1)- heptane-2-carboxylic acid (BCH) (Palacin *et al.* 1998; Franchi-Gazzola *et al*. 2004). LAT1 has substrate affinity in the micromolar range and prefers to transport large neutral amino acids such as leucine, valine, phenylalanine, tryptophan methionine and histidine; and is found mainly in the brain, spleen, placenta and testis (Kanai *et al.* 1998). On the hand, LAT2 transports a broader range of substrates including all L-isomers of neutral amino acid and has been identified in may tissues including the small intestines, brain and skeletal muscle (Segawa *et al.* 1999).

Other members of system L include LAT3 and LAT4 L (Babu *et al.* 2003; Bodoy *et al.* 2005). These are relatively new members of the system that can be distinguished from LAT1 and LAT2 based on their structural features. For example LAT3 and LAT4 are not heteromeric transporter and hence do not require 4F2hc to elicit transport activity (Bodoy *et al.* 2005).

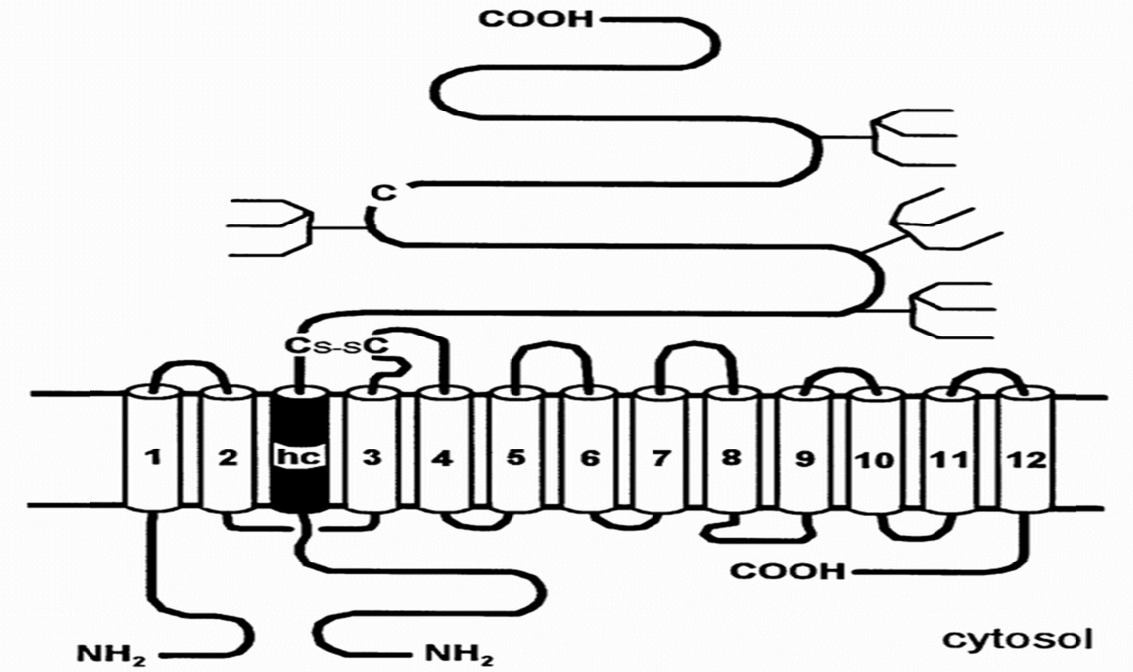

***Figure 1.15**: A schematic diagram showing the general structure of an L-type amino acid transporter. LATs consist of two sub-units a light (numbered cylinders) and a heavy chain (filled cylinder, hc). The light chain consists of 12 transmembrane domains (numbered 1–12). Diagram adapted from Mastroberardino et al. 1998.*

***Table 1.3**: List of some of the amino acid transporter systems found in the cells of eukaryotes. Table adapted from Christensen, (1985); Hyde et al. (2003); Verrey (2003)*

System	Protein	Gene	Amino acid substrates (one- letter code)	Tissue distribution
Neutral -amino -acid transporters: sodium -independent				
Asc	Asc1	SLC7A10	G, A, S, C, T	Brain
	Asc2	-	G, A, S, T	Erythrocytes, kidney
L	LAT1	SLC7A5	BCH, H, M, L, I, V, Y , W, Q	Placenta, adipocyte, smooth muscle
	LAT2	SLC7A8	BCH, A, S, C, T, N, Q, H, M, L, I, V, F, Y,W	Kidney, skeletal muscle, placenta
T	TAT1	SLC16A10	F, Y, W	Erythrocytes
Anionic -amino -acid transporters: sodium -independent				
X^{-c}	xCT	SLC7A11	ET	Brain
–	XAT2	–	D, E	Kidney

Table 1.3 continued

Cationic-amino-acid transporters: sodium-independent				
$b^{o,+**}$	b(o,+)AT	SLC7A9	K, R, A, S, C, T, N,Q, H, M, I, L, V, F, Y, W, Ci	Small intestine, blastocycts
	Cat-1	SLC7A1	R, K, H	Endothelial cells
y^+	Cat-2	SLC7A2	R, K, H	Liver
	Cat-3	SLC7A3	R, K	Thymus, uterus, brain
	Cat-4	SLC7A4	Unknown	Glioblastoma cells

X^{-c} where Cystine shares transport with Glutamate; System T is mainly found in human red blood cells; ($^+$) plus, ($^-$) minus or (^{o}) and neutral sign indicate the need for appropriately charged amino acid substrates; ($b^{o,+**}$) heteromeric transporter formed upon association with rBAT glycoprotein encoded by gene SLC3A1; y^+ consist of two subfamilies SLC7A1-4 (consist of 14 transmembrane domain) and SLC7A5-11 (not listed in this table) form heteromeric transporter with 4F2hc or rBAT (Verrey, 2003).

1.9 Protein synthesis

As stated in section 1.5, skeletal muscle is highly plastic and its fibres can rapidly switch from one type to another depending on the biochemical and physiological demands imposed upon them. This process, commonly known as skeletal muscle plasticity, involves the synthesis of new proteins and/or the remodelling existing ones, thereby, making the control of protein synthesis important in this process (Rooyackers & Nair, 1997). Protein synthesis is energetically a highly expensive process (Mamane *et al.* 2006). Therefore, it is tightly regulated. Protein synthesis is a two-step process and involves transcription and translation. Transcription is the initial step and involves the copying of the genetic information encoded by genes into messenger RNA (mRNA); whereas, translation involves decoding of the genetic information in the mRNA into proteins. Therefore in section 1.9.1 below, I will describe the transcription process and in the next section (1.9.2) I will briefly outline the translation process.

1.9.1 Transcription process

All the genetic information in a cell is encoded by genes located within the deoxyribonucleic acid (DNA). However, in this form the information is inaccessible and cannot be directly utilised by the protein synthesising machinery. Instead, the information has to be first transcribed into mRNA through a complex process known as transcription. Each DNA consists of two strands held together in a

double helix by weak hydrogen bonds. To prevent it from damage, the DNA strands are bound to acetylated histones (forming the nucleosomal complex) in a coiled transcriptional repressed orientation (Kornberg, 1974). Therefore, for transcription to occur, the histones are acetylated by histone acetylases (HATs). HATs add the acetyl residues removing the positive charge on the lysine residue of the histone protein. This allows the strands to unwind and increases the space between the nucleosome and the DNA wrapped around it allowing access for the recruitment of transcription factors to begin. The unwound DNA now is ready for transcription initiation.

At the 5'end, ~100 nucleotides upstream from the transcriptional start site, is the gene promoter and enhancer regions (see Figure 1.16). The promoter region contains AT rich sequences, known as the TATA box, which binds the TATA box binding protein (TBP). TBP, TBP associated factors (TAFs) together with RNA polymerase II complex forms the general transcription complex which then binds to the core promoter region.

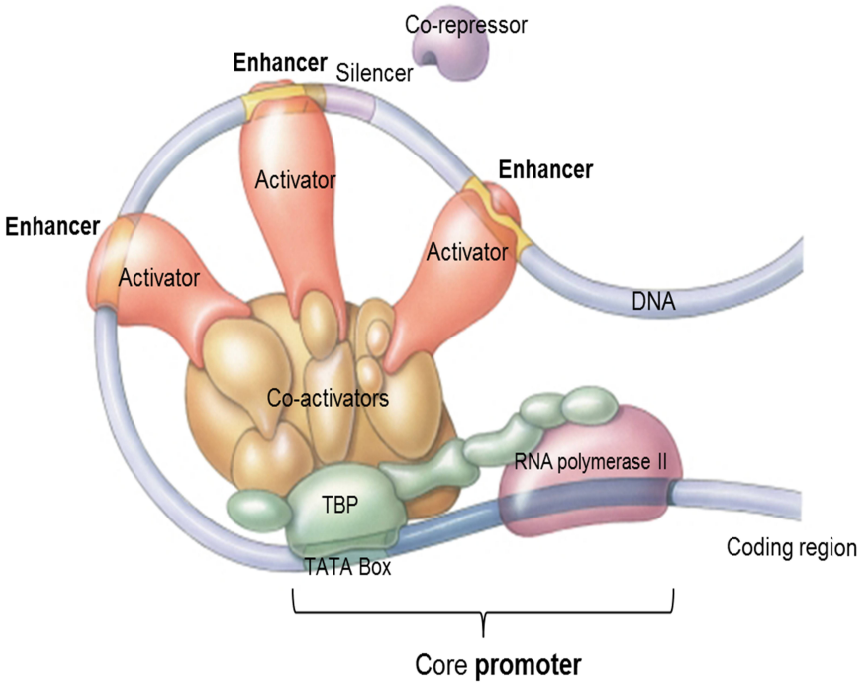

***Figure 1.16**: Assembly of the general transcription complex. The general transcription complex positions RNA polymerase II on the promoter region at the beginning of a gene. The general transcription complex consist of the basal factors which include TBP (green), co-activators collectively known as TAFs (brown) and activators (red) that bind the enhancer. The basal factors cannot by themselves increase or decrease the rate of transcription. However recruitment of co-regulators can either enhance (as co-activators) or reduce (as co-repressors) (purple) transcription. Diagram adapted from Johnson, (2001).*

Starting from the promoter region, during the transcription process, the transcription machinery moves along the DNA strand into the gene. As it encounters each DNA nucleotide, corresponding complementary RNA nucleotides are added to a chain of growing

RNA until it reaches the stop codon at the opposite end of the gene. At the stop site, it disengages from the DNA releasing the newly assembled RNA transcript. The transcribed RNA then undergoes post transcriptional processing, introns are excised and exons are spliced producing a mature messenger RNA (mRNA), which is the transcriptional unit conveying the encoded instruction that tells the cell to assemble amino acid to make a particular protein.

Although core promoters can accurately direct transcription initiation, *in vitro* intact eukaryotic transcription requires sequence specific regulatory transcription factors (also known as *trans* elements) (Pabo & Sauer, 1992). These interact with the DNA sequence *via* characteristic structural motifs that enter the DNA grooves. There are four types of general structural motifs including; helix-turn-helix (HTH), homeodomain, zinc finger and leucine zipper (Pabo & Sauer, 1992).

1.9.2 **Translation process**

After transcription, the mRNA must then be translated into proteins. This is a complex process and requires the ribosome, the mRNA and the aminoacylated tRNAs to come together in the correct configuration. For this to happen many proteins that bind each of these components are involved. Translation can be divided into three main steps, initiation, elongation and termination. During the initiation phase eukaryotic initiation factor (eIF)2 binds a complex consisting of the initiating methionyl-tRNA (MET-tRNA) and a

molecule of GTP and places this complex on the smaller (40S) ribosomal subunit. Simultaneously, eukaryotic initiation factors 4E (eIF4E) binds directly to mRNA through its 5' methylated guanosine cap structure (Clemens & Bommer, 1999). eIF4E is the rate limiting protein and is normally bound to translational repressor eukaryotic initiation factor binding protein (4EBP)1; and is only released when 4EBP1 is phosphorylated (Gingras *et al.* 1999). eIF4E has the ability to bind to the scaffolding protein eIF4G. This protein has binding site for many other initiation factors including the ribosome-associated protein eIF3 which enhances the binding the eIF2 complex to the 40S ribosomal subunit.

In order for initiation to start the correct site at the beginning of the mRNA open reading frame must be found. The 40S subunit.Met-tRNA, scans the mRNA and finds the initiating/start codon, usually AUG. Once the start codon is identified the GTP associated with eIF2 is hydrolysed to GDP and inorganic phosphate. The inorganic phosphate together with all the other initiation factors is released from the ribosome. The GDP bound eIF2 is also released and the GDP is exchanged for GTP in readiness for the start of another initiation process. The larger (60S) ribosomal subunit binds to the complex to form the complete 80S initiation complex.

During elongation, another aminoacyl-tRNA is added to the ribosome at the A-site by eukaryotic elongation factor (eEF) 1 and another amino acid is added to the growing polypeptide chain.

Following the formation of the peptide bond, the ribosome translocates from the A- to the P- site bringing the next codon into position and the whole process is repeated again until one of the three stop codons is reached (Sheikh & Fornace, 1999). The translocation from the A-site to the P-site is catalysed by unphosphorylated elongation factor (eEF2) and requires the hydrolysis of GTP to GDP (Clemens & Bommer, 1999).

This mode of protein synthesis is known as cap-dependent protein synthesis and is thought to be the main mechanism of protein synthesis in eukaryotic cells (Merrick, 2004). However, another mode of translation initiation occurs in eukaryotic cells subjected to stress known as cap-independent or internal ribosome entry site (IRES) mediated translation (see figure 1.17). This mechanism is faster and has been observed within 2 hours after transcription (Johannes *et al.*1999). In this process, the uncapped 5'mRNA-end interacts with the 40S ribosome subunit directly (Vagner *et al.* 2001).

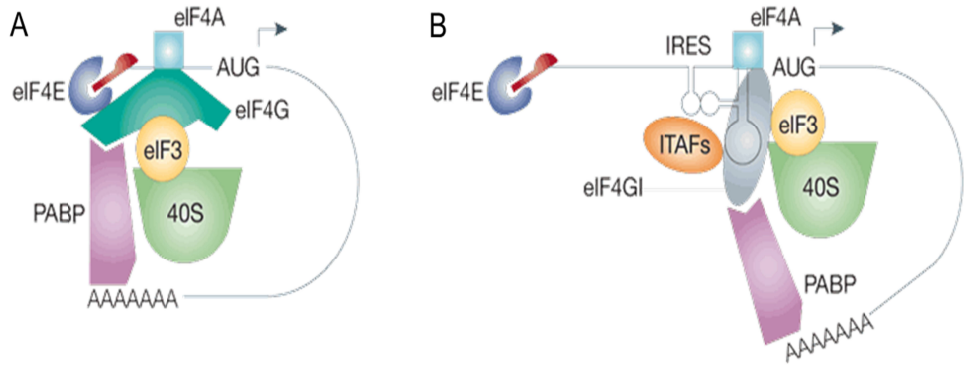

***Figure 1.17**: A schematic diagram showing cap-dependent and cap-independent protein synthesis. Under normal growth conditions, cap-dependent translation initiation occur (A); whereas, during periods of stress cap-independent initiation takes place (B). AUG is a start codon; eukaryotic initiation factors (eIFs) are: eIF4A, eIF4E, eIF3, eIF4GI; ribosomal subunit 40Svedberg (40S); poly A binding protein (PABP); IRES trans-acting factors (ITAFs); Internal ribosome-entry site (IRES). Diagram adapted from Holcik & Sonenberg, (2005).*

1.10 Regulation of gene expression

From the several thousand different genes, there are some of the gene products that are required by the cell under all condition such as housekeeping genes. An example is the genes encoding protein kinases. These genes are important for cell survival and are expressed all the time. On the other hand, there are gene products that are required only at certain times. Therefore, their expression varies according to demand a process known as gene expression regulation (see figure 1.18).

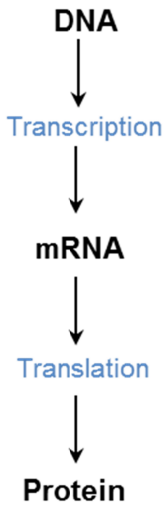

***Figure 1.18**: Schematic diagram showing the levels at which gene regulation can occur during protein synthesis. Gene regulation can either increase, decrease or abolish a particular gene product according to environmental demand.*

The regulation of gene expression can occur during transcription, a process known as transcriptional regulation. For example steroid hormones such as DHT or T, serves as effector molecules that can adjust the rate of transcription (Beato, 1989). As mentioned in section 1.7.1 above, binding of these hormones to the AR exposes its NLS which encourage its export into the nucleus. In the nucleus, the activated AR binds to a short DNA sequence located at the promoter regions of the target genes, known as androgen response elements (AREs). The AREs consists of 15-mer inverted palindromic sequences containing 3 unspecified nucleotide spacers (n) flanked by two half-sites: 5'-GGTACAnnnTGTTCT-3' (Beato, 1989). The ARE-bound AR dimer can either interact with the transcription

machinery to begin transcription or recruit co-regulators. The co-regulators then can either enhance (as co-activators) or reduce (as co-repressor) transcription (see figure 1.16) (Heemers & Tindall 2007).

Although It has been reported that acute T treatment can lead to the activation of co-activators such as cyclic AMP response element (CREB) in prostate (Unni *et al.* 2004) and sertoli cells (Cheng *et al.* 2007), no activation of transcription factors was observed following the acute treatment of cultured myotubes with T (Estrada *et al.* 2003). However, whether T treatment in adult skeletal muscles involves the activation of transcription factors is uncertain.

Gene regulation can also occur at the translational level. Compared to transcriptional regulation, translational regulation of existing mRNAs allows for more rapid changes in the cellular concentrations of the encoded proteins. Thus, it can be used for the general maintenance of cells (Sonenberg & Hinnebusch, 2009). However, as there is no data available it is uncertain whether the acute effects of anabolic-androgenic steroids involve the translational regulation of proteins.

1.11 General aims of the study

The primary aims of this study were to;

1. Develop an *ex vivo* technique suitable for investigating the acute/non-genomic effects of testosterone (T) and dihydrotestosterone (DHT) in adult mammalian skeletal muscle fibres
2. Use the small, isolated skeletal muscle fibre bundle technique developed to investigate;

a) The acute effects of DHT and T on amino acid uptake by fast- and slow-twitch mouse skeletal muscle fibre bundles.

b) The cellular signalling pathway(s) mediating the acute effects of T and DHT on amino acid uptake in mammalian skeletal muscle fibres.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 General materials

All the materials and methods specific to a particular experiment are described in the appropriate/relevant chapter. Therefore, in this chapter I will only describe the materials and methods that are common to several chapters.

2.1.1 Animals

All the experiments in this study were performed using small muscle fibre bundles isolated from the extensor digitorum longus (EDL) and the soleus muscles of adult CD1 mice. The mice belonged to an outbred stock obtained from Charles River (Charles River UK Ltd. Margate, Kent) and were maintained in the Disease Modelling Unit (DMU), University of East Anglia. They were kept in individual cages and received 12 hours light and 12 hours dark. They were fed standard rodent chow and fresh water *ad libitum.*

The CD1 mice originated from outbred stock from the Anticancereux laboratory in Switzerland. They were then imported to the Rockefeller Institute, United States in 1926. Later in 1948, the Hauschka Ha/ICR stock was initiated at the Institute for Cancer Research in Philadelphia from the Swiss mice. Then in 1959, they were designated as HaM/ICR or CD1 by Charles River Laboratory Incorporated (Rice & O'Brien, 1980). CD1 mice were used in this

study because they are relatively inexpensive (Aldinger *et al.* 2009), readily available in large numbers (Aldinger *et al.* 2009) and have short lifespans (Enthoven *et al.* 2008). Furthermore their genetic composition is relatively stable and retains the same amount of variation as that found in natural murine or human populations (Rice & O'Brien, 1980; Aldinger *et al.* 2009). They are also widely used in toxicological and cancer testing and as laboratory animals (Maugh, 1978; Chia *et al.* 2005; Aldinger *et al.* 2009). Thus, the CD1 outbred stock represents a general mouse population with minimal potential for genetic complications; thereby, making it a suitable mouse stock for this study (Rice & O'Brien, 1980; Enthoven *et al.* 2008; Aldinger *et al.* 2009).

In this study, a total of 95 CD1 mice, consisting of 7 males ~38g in weight and 88 females ~30g in weight, were used. The mice had a mean age (mean ± SEM) of 53.73 ±6.00 days (see table 2.1 for overall mice characteristics) and before they were released from the DMU, the mice were physically inspected for general health. Moreover, as shown in Fig. 2.1, their characteristics including weight, gender and mean age matched those of a normal healthy CD1 population (Rowe & Goldspink, 1969; Percy and Jonas, 1971). Thus, the inspection regime ensured that all the mice used in the study were healthy.

***Table 2.1**: The overall profile of the CD1 mice used in this study*

Animals used for	Male	Female	Mean ± SEM days/ (age range in days)
Investigating the effect of DHT/T on MAPK pathway in isolated intact skeletal muscle fibre bundles (Chapter 3)	3	17	48.61 ± 2.83 days (42 to 56)
Investigating the effect of DHT/T on Akt/mTOR pathway in isolated intact skeletal muscle fibre bundles (Chapter 4)	4	13	57.39 ± 4.49 days (49 to 63)
Investigating the physiological effects of DHT/T on amino acid uptake in isolated intact skeletal muscle fibre bundles (Chapter 5)	-	8	59.67 ± 1.33 days (57 to 62)
Investigating the pathway and the transporter mediating the amino acid uptake in isolated intact skeletal muscle fibre bundles (Chapter 5)	-	20	57.43 ± 2.01 days (54 to 61)
Investigating the receptor mediating the non-genomic effects of DHT in isolated intact skeletal muscle fibre bundles (Chapter 6)	-	30	52.13 ± 2.22 days (48 to 56)
Total numbers of CD1 mice used	7 (7.36%)	88 (92.64%)	53.73 ± 6.00 days (43 to 63)

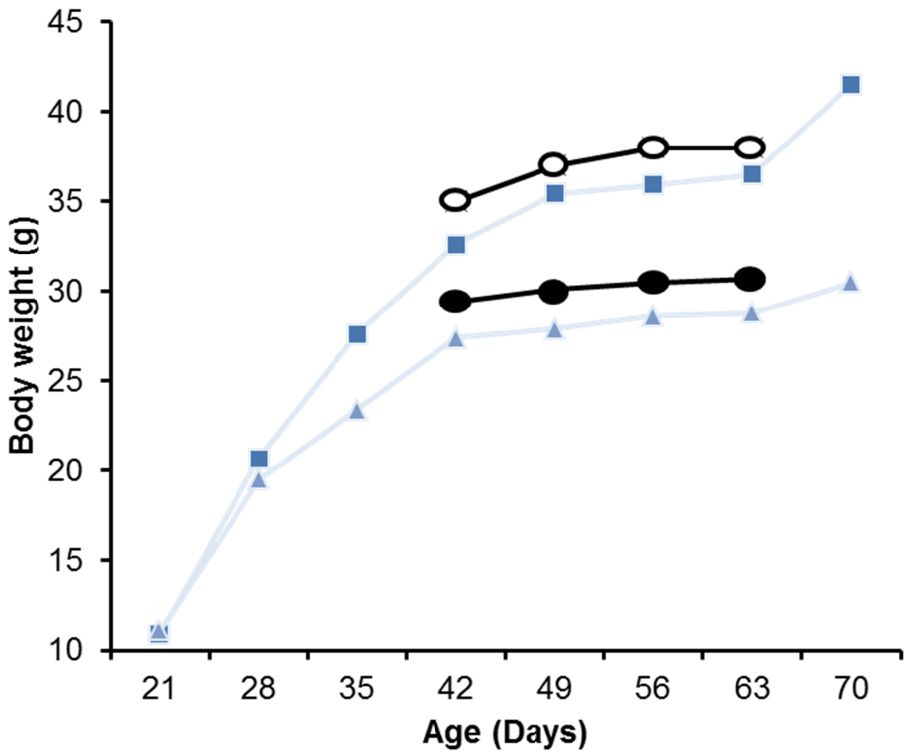

***Figure 2.1**: Growth charts of a typical mouse population. The graph shows bodyweights of a typical male (rectangles) and female (triangles) mouse population (blue lines); and that of the male (open circles) and female (close circles) mice used in this study (black lines). Note that the growth rate of the mice used in this study was well within the range expected and that their weights were within the plateau region of the general mouse population. Data adapted from Rowe & Goldspink (1969) and Percy & Jonas (1971).*

2.2 Isolation of the small muscle fibre bundles

The mice were killed by cervical disarticulation as recommended by the Animals (Scientific Procedures) Act 1986, UK (for details of the regulations see http: //www. legislation. gov. uk / ukpga / 1986 / 14 / contents) and all the experiments performed conformed to the UEA animal welfare committee guidelines. The *extensor digitorum longus* (EDL, a mainly fast-twitch muscle in adult mice) and the *soleus* (SOL, a predominantly slow-twitch muscle in adult mice) muscles from both hind limbs were isolated using a scalpel blade and fine forceps. They were then pinned at *in situ* length in a silicone elastomer (Sylguard®, Dow Corning Europe SA) coated petri dish filled with the standard Ringer's solution. The standard Ringer's solution contained (in mM); 109 NaCl, 5 KCl, 1 $MgCl_2$, 4 $CaCl_2$, 24 $NaHCO_3$, 1 $NaHPO_4$, 10 sodium pyruvate plus 200mgl^{-1} bovine calf serum; and its pH was maintained at approximately 7.4 by continuously bubbling it with 95% O_2 and 5% CO_2 (British Oxygen Company, Ltd Guilford). For details of the chemicals used to prepare the Ringer's solution and their source(s) see Table 2.2.

***Table 2.2**: A summary of the basic information such as scientific name, molecular weight and source of the chemicals used in the preparation of the standard Ringer's solution.*

Scientific Name	**Formula/Molecular weight/Formula Weight**	**Catalogue No.**	**Source**
Sodium chloride	NaCl/ FW:58.44	BDH4534-5KGP	BDH/VWR International Ltd, Lutterworth, Leicestershire
Potassium chloride	KCl/ FW:74.55	BDH4532-500GP	BDH/VWR International Ltd, Lutterworth, Leicestershire
Magnesium chloride	$MgCl_2$/ FW: 203.31	M/060053	Fisher Scientific UK Ltd. Loughborough, Leicestershire
Calcium chloride (1M solution)	$CaCl_2$/ MW: 110.98	21115-250ML	Sigma-Aldrich, Gillingham, Dorset

Sodium hydrogen carbonate	$NaHCO_3$/ FW: 84.01	S/4240/53	Fisher Scientific UK Ltd. Loughborough, Leicestershire
Sodium dihydrogen orthophosphate	$NaHPO_4$/ FW: 156.01	S/3760/53	Fisher Scientific UK Ltd. Loughborough, Leicestershire
Sodium pyruvate	$CH_3COCOONa$/ MW: 110.04	P5280	Sigma-Aldrich, Gillingham, Dorset
Bovine serum albumin	Protease free MW: 67000.00	05479-10G	Sigma-Aldrich, Gillingham, Dorset

To prepare the small fibre bundles, the muscles were transferred into a specially designed muscle chamber with the following internal measurements; 20mm width x 67mm length x 26mm depth (Figure 2.2). They were then mounted horizontally between two stainless steel hooks. The chamber was then filled with enough Ringer's solution to cover the muscles completely (~25mls).

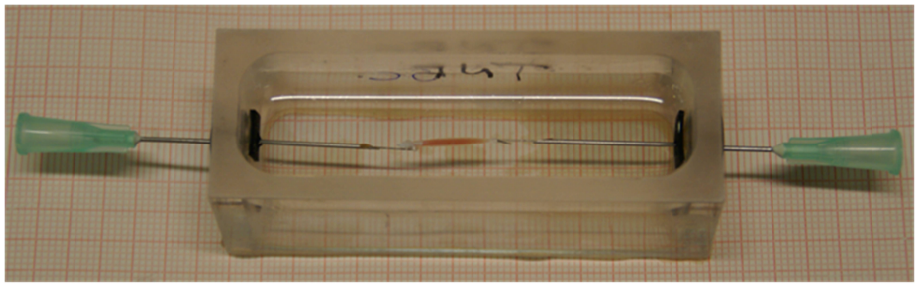

Figure 2.2*: A photograph of the muscle chamber used in the dissection of the small muscle fibre bundles with a slow-twitch fibre bundle mounted between the two stainless steel hooks.*

The hooks could be easily rotated thereby giving complete access to the muscles. Small muscle fibres bundles (~10-15 fibres, mean cross-sectional diameter 230.6 ± 17.7μm) were then dissected under dark-field illumination at X70 magnification using a dissecting microscope (BM 2, Meiji Techno, Japan). Care was taken to ensure that all the fibres in a bundle were intact and contracted uniformly when they were electrically stimulated. To elicit a contraction, the fibre bundles were electrically stimulated using two platinum electrodes placed on either side of the fibre bundle. For details of the instruments used in the preparation of the fibre bundles see Table 2.3.

***Table 2.3**: The basic information such as name, description, model and source of the instruments used in the preparation of the small muscle fibre bundles.*

Instrument	**Description**	**Model/ Catalogue No.**	**Source**
Long arm stereo dissection Microscope	Magnifications: X10, X20, X35, X70	BM 2	Meiji Techno Co. Ltd. Saitama, Japan.
Scalpel blade No. 15	Small curved cutting edge for short and precise incision.	0305	Swann-Morton Ltd. Sheffield, UK.
Scalpel blade handle No.3	Standard blade handle	No.3	Swann-Morton Ltd. Sheffield, UK.

2.3 **Myosin isoforms in the skeletal muscle fibre bundles used in this study**

Although there are many studies on fibre type composition in rat-extensor digitorum longus (Pullen *et al.* 1977; Dekleva & Sirca, 1978; Eddinger *et al.* 1985), guinea pigs-oesophageal muscularis externa (Gutmann *et al.* 1970;Whitmore, 1982) and rabbit-tibialis anterior (Salmons, 1992) muscle, no studies have investigated the fibre type composition of the mouse EDL and soleus muscle. Therefore, in this study I randomly performed Western blot analysis to determine the fibre type composition of the bundles I used. As the results displayed in Fig. 2.2 show, the fibre bundles isolated from the EDL expressed entirely fast myosin; whereas those from the soleus expressed the slow isoform of myosin.

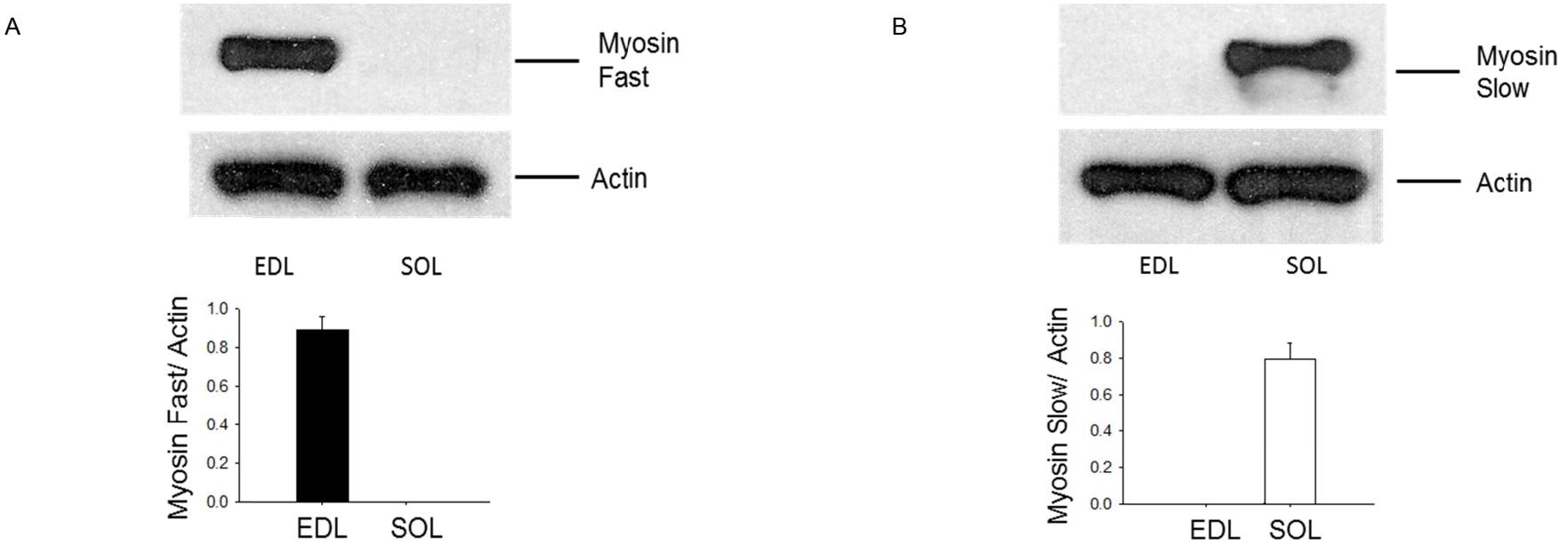

***Figure 2.3**: Representative Western blots showing the myosin isoform expressed by the fibre bundles isolated from the EDL and soleus muscles used in this study (A,B). The bundles were probed using mouse monoclonal antibodies purchased from Sigma-Aldrich, UK. Note that the antibodies labelled a single protein with a molecular weight of 200KDa in both the EDL (A; myosin fast, filled bar) and SOL (B; myosin slow, open bar) muscle fibre bundles. Furthermore, the bundles from the extensor digitorum longus (EDL) expressed only the fast isoform of myosin; whereas those from the soleus (SOL) expressed slow myosin only.*

2.4 **Treatment of the muscle fibre bundles with DHT and T**

All the experiments reported here were performed at room temperature (~20 °C) on small muscle fibre bundles isolated from the EDL and SOL of adult female CD1 mice. During an experiment, the fibre bundles were mounted horizontally between two stainless steel hooks in one of the various muscle chambers used in the study (Figure 2.2). The fibre bundles were divided into two groups; half were treated with the standard Ringer's solution plus 6.3µl per 100ml (1.07mM) of the vehicle (absolute ethanol) used to dissolve the DHT and T for 1hour. These fibre bundles acted as the control samples. The other half was treated for the same period of time (1hour) with Ringer's solution containing either 630pgml^{-1} (2.16nM) DHT or (1.82nM) T. These fibre bundles acted as the experimental/treated samples. For the details of the chemicals used in these experiments see Table 2.4.

***Table 2.4**: The basic information such as scientific name, molecular weight and source of the chemicals used in treatment of the fibre bundles with DHT and T.*

Scientific Name/Commercial name	**Formula/Molecular weight/ Density**	**Catalogue No.**	**Source**
Absolute ethanol	C_2H_5OH/ FW: 46.04 Density:0.789gml^{-1}	NC9602322	Fisher Scientific UK Ltd. Loughborough, Leicestershire
17β-Hydroxy-4-androsten-3-one 17-propionate/ Testosterone propionate	$C_{22}H_{32}O_3$/ MW: 344.49	T1875	Sigma-Aldrich, Gillingham, Dorset
17β-hydroxy-5α-androstan-3-one/ Androstanolone	$C_{19}H_{30}O_2$/ MW: 290.45	A8380	Sigma-Aldrich, Gillingham, Dorset

2.5 **Determination of the cellular signalling pathway(s) mediating the effects of DHT and T**

In some experiments, the fibre bundles were pre-incubated for 20minutes in Ringer's solution containing the inhibitors (described in later chapters). They were then treated with DHT or T plus the inhibitor whose effects were being investigated for a further 1hour (for summary of the protocol used see figure 2.4). During all the experiments, the fibre bundles were continuously subjected to twitch contractions and the Ringer's solution was continually aerated with medical gas (95% oxygen-5% carbon dioxide).

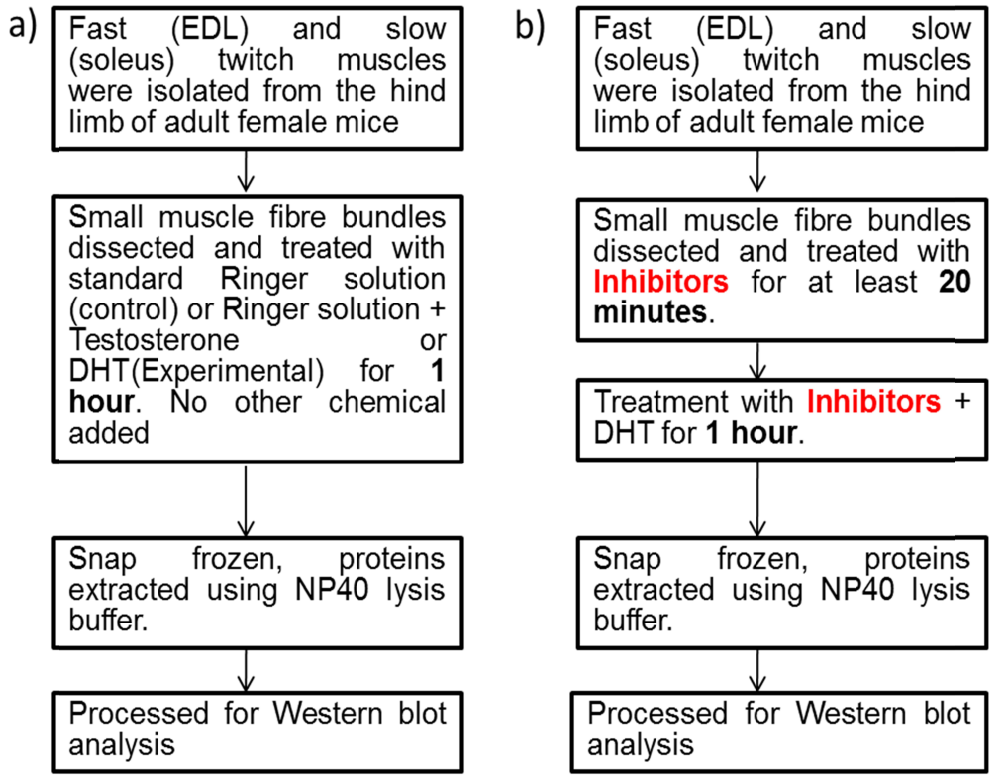

***Figure 2.4**: A flowchart summarising the protocol used in these experiments*

2.6 **Protein extraction and quantitation**

At the end of the experiments described above, the muscle fibre bundles were snap frozen in liquid nitrogen and pulverised using a mortar and pestle. The samples were then mixed with 100 to 200µl of the non-ionic lysis buffer NP40. The buffer contained (in mM); 150 NaCl, 50 Tris (pH 7.5), 1.5 $MgCl_2$, 10% Glycerol and 1% NP-40. 100µl of complete phosphatase inhibitor cocktail and one tablet of complete protease inhibitor cocktail were then added to 10ml of buffer just before use. For details of the chemicals used to prepare the NP40 buffer are shown in Table 2.5,

***Table 2.5**: Summary of the chemicals used in the preparation of the NP40 lysis buffer.*

Scientific Name/Commercial name	**Formula/Molecular weight/Formula Weight/ Description**	**Catalogue No.**	**Source**
Sodium chloride	NaCl/ FW:58.44	BDH4534-5KGP	BDH/VWR International Ltd, Lutterworth, Leicestershire
Tris(hydroxymethyl) aminomethane	$C_4H_{11}NO_3$/ MW: 121.14	B2005	Melford Laboratories Ltd. Chelsworth, Ipswich
Magnesium chloride	$MgCl_2$/ FW: 203.31	M/060053	Fisher Scientific UK Ltd. Loughborough, Leicestershire
Glycerol	$C_3H_8O_3$/ MW: 92.09	G5516	Sigma-Aldrich, Gillingham, Dorset
4-Nonylphenyl-polyethylene glycol /NP40	$C_{18}H_{30}O_3$/ MW: 294.429	74385-1L	Sigma-Aldrich, Gillingham, Dorset

(Ethylenedinitrilo)tetraacetic acid /EDTA	$C_{10}H_{16}N_2O_8$/ MW: 292.24	EDS-100G	Sigma-Aldrich, Gillingham, Dorset
Phosphatase inhibitor cocktail set II	A cocktail of phosphatase inhibitors for the inhibition of acid, alkaline and tyrosine phosphatases during protein extraction	524625	Calbiochem, Nottingham, UK
Protease inhibitor cocktail tablets	For complete inhibition of proteases during protein extraction	04693116001	Roche, Basel, Switzerland
Hydrochloric acid	HCl/ FW: 36.46	H/1000/PB17	Fisher Scientific UK Ltd. Loughborough, Leicestershire

The frozen samples were then transferred into 0.5ml eppendorf tubes. They were then vortexed and left on ice for 10minutes before they were centrifuged for 10minutes at 15000g. The supernatant, containing mainly cytosolic proteins, was collected and the pellet was kept for the isolation of membrane proteins.

To isolate membrane proteins, the pellet was washed 3 times using phosphate buffer saline (PBS). It was then snap frozen in liquid nitrogen, pulverised and membrane proteins extracted as described above. Briefly, the samples were mixed with 30-50 µl NP40buffer. The lysate was then vortexed and centrifuged at 15000g. The supernatant was collected and the pellet was discarded this time.

The concentration of proteins in each lysate was determined using the Quick Bradford Assay (Bradford 1976) and the details of the chemicals used are shown in Table 2.6. During an assay, various volumes (0,1,5,10 and 20µl) of a 1 $mgml^{-1}$ solution of bovine serum albumin (BSA) were mixed with double distilled water and the protein assay dye in a 1ml disposable cuvette. These samples were used to generate the standard curve. The concentration of proteins in each lysate was then assayed by mixing 5µl of the sample with double distilled water and the protein assay dye. The mixture was then thoroughly mixed and the optical density of each mixture was determined using a spectrophotometer.

Table 2.6: *The volume of 1µg/µl BSA standard; double distilled water; sample and 1X Quick Bradford dye used in the Quick Bradford protein assay.*

BSA standard (µl)	Double distilled water (ml)	1X Protein Assay dye (ml)
0*	0.8	0.2
1	0.8	0.2
5	0.795	0.2
10	0.79	0.2
20	0.78	0.2
Sample(s)		
5	0.795	0.2

*Blank used for zeroing the spectrophotometer

The spectrophotometer (Helios-γ, Fisher Scientific, UK) was set at a wavelength of 595nm (for details of the spectrophotometer and the dye see table 2.7). The blank sample was then used to zero the spectrophotometer reading. The absorbance or optical densities (OD) of the samples were then measured. The ODs of the BSA mixtures were then used to generate a standard curve. The amounts of protein (in µg/µl) in the lysates was then determined from the standard curve.

The assay is based on the direct binding of proteins to Coomassie brilliant blue G-250 dye (CBBG). The dye exists in three forms; cationic (red), neutral (green) and anionic (blue) (Compton & Jones 1985). Under acidic conditions the protonated dye is predominantly red. However; when it binds to arginine, tryptophan, tyrosine, histidine, and phenylalanine residues in proteins, its absorbance shifts from 495nm to 595nm (Reisner *et al.* 1975). In the Quick Bradford assay that I described above, it is this unprotonated blue protein-dye complex that is detected using the spectrophotometer.

***Table 2.7**: Details of the dye reagent and the spectrophotometer used in the Quick Bradford assay.*

Reagent and Instrument	**Description**	**Model/ Catalogue No.**	**Source**
Protein assay dye reagent	1L Coomasie Brilliant Blue dye G-250 reagent for Quick Bradford	500-0201	BioRad Laboratories Ltd, Hemel Hempstead, UK.
Spectrophotometer	UV-Visible wavelength range: 190-1100nM	Thermo spectronic Helios-γ	Fisher Scientific UK Ltd. Loughborough, Leicestershire

2.7 **Preparing the proteins for gel electrophoresis**

Antibodies recognise a modest portion of the antigen, known as an epitope which may still remain in its tertiary structure. To enable access of the antibody to the epitope the protein needs to be unfolded or denatured. To denature, 10 µg of the sample was mixed with 5µL of loading buffer containing denaturing and reducing agents. The loading buffer contained; 2g sodium dodecyl sulphate (SDS) which served as the denaturing detergent, 10µl/ml β-mercaptoethanol (added just before use) which served as the reducing agent, 8ml trisbase pH 6.8, 6ml glycerol, 1.2mg bromophenol blue. The mixture was then boiled for 2 minutes followed by a quick spin at 13000g. The samples were then loaded in individual wells and resolved using a discontinuous gel (Shapiro *et al.* 1967, Weber & Osborn 1969).

The SDS binds strongly to proteins at an approximate ratio of 1 dodecyl sulphate molecule per 2 amino acid residues and neutralises the charges on the proteins (Reynolds & Tanford, 1970). It also disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negative charge with equal charge densities per unit length (Reynolds & Tanford, 1970). Normally proteins form random-coil configuration created by disulfide bonds. Therefore addition of β-mercaptoethanol reduces the disulfide bonds within-and-between the proteins. Other chemicals such as, glycerol and bromophenol

blue have their own functions. For example, glycerol increases the density of the sample, thereby preventing uneven gel loading and sample overflow; whereas, bromophenol blue serves as an indicator of how far the sample has travelled (Laemmli, 1970). For the details of the chemicals used in the preparation of the loading buffer see table 2.8.

***Table 2.8**: Summary of the chemicals used for preparation of the loading buffer.*

Scientific Name	**Formula/Molecular weight**	**Catalogue No.**	**Source**
Tris(hydroxymethyl) aminomethane	$C_4H_{11}NO_3$/ MW: 121.14	B2005	Melford Laboratories Ltd. Chelsworth, Ipswich
Glycerol	$C_3H_8O_3$/ MW: 92.09	G5516	Sigma-Aldrich, Gillingham, Dorset
Sodium dodecyl sulphate	C_{12} H_{25} NaO_4S/ MW: 288.38	B2008	Melford Laboratories Ltd. Chelsworth, Ipswich
3',3'',5',5''-tertrabromophenol sulphonephthalein (Bromophenol blue)	$C_{19}H_{10}Br_4O_5S$/ MW: 669.96	B0126-25G	Sigma-Aldrich, Gillingham, Dorset
β-Mercaptoethanol	$HSCH_2CH_2OH$/ MW: 78.13	7154	Sigma-Aldrich, Gillingham, Dorset

2.8 **Gels**

Most of the gels used in this study were 10% SDS/polyacrylamide and consisted of a 4% acidic (pH 6.8) stacking gel at the top and a 10% alkali (pH8.8) separation gel at the bottom. The stacking gel consisted of 1ml polyacrylamide (40%), 6.4ml double distilled water, 2.5ml 1.5M Tris buffer (pH 6.8), 100µl of 10% SDS, 50µl of 10% ammonium persulphate and 10µl of TEMED (a cross-linker); whereas, the separation gel consisted of 5ml polyacrylamide (40%), 9.69ml double distilled water, 5.0ml of 1.5M Tris buffer (pH 8.8), 200µl of 10% SDS, 100µl of 10% ammonium persulphate and 10µl of TEMED. For details of the chemicals used in the preparation of both the separation and stacking gel see table 2.9.

***Table 2.9**: Summary of the chemicals used for preparing separation and stacking gel.*

Scientific Name	**Formula/Molecular weight/Formula Weight/Description**	**Catalogue No.**	**Source**
40% Acrylamide/Bis solution	Contains 99.9% acrylamide and bis-acrylamide at 9:1 ratio	161-0141	BioRad Laboratories Ltd, Hemel Hempstead, UK.
Tris(hydroxymethyl) aminomethane	$C_4H_{11}NO_3$/ MW: 121.14	B2005	Melford Laboratories Ltd. Chelsworth, Ipswich
Glycine	NH_2CH_2COOH/ MW: 75.0	G0709	Melford Laboratories Ltd. Chelsworth, Ipswich
Sodium dodecyl sulphate	$C_{12}\ H_{25}\ NaO_4S$/ MW: 288.38	B2008	Melford Laboratories Ltd. Chelsworth, Ipswich
Ammonium persulphate	$(NH_4)_2S_2O_8$/ MW: 228.20	A3678-25G	Sigma-Aldrich, Gillingham, Dorset
1,2-Bis(dimethylamino)ethane/ TEMED	$(CH_3)_2NCH_2CH_2N(CH_3)_2$/ MW: 116.20	T9281-25G	Sigma-Aldrich, Gillingham, Dorset

In some experiments, 6% gels were used because they provided better resolution especially for proteins larger than 100kDa. For the volume of each chemical and buffers required to prepare the separation and stacking gels see Table 2.10.

***Table 2.10**: The chemicals and buffers required for preparing the various gels*

	Separation			Stacking
Chemicals/buffers	6%**	12%*	10%	4%
Acrylamide (40%)	3.0ml	6.0ml	5.0ml	1.0ml
Distilled water	11.69ml	8.69ml	9.69ml	6.4ml
Tris HCl pH8.8	3.0ml	5.0ml	5.0ml	-
Tris HCl pH6.8	-	-	-	2.5ml
10% SDS	200.0µl	200.0µl	200.0µl	100.0µl
10% AMPS	100.0µl	100.0µl	100.0µl	50.0µl
TEMED	10.0µl	10.0µl	10.0µl	10.0µl
Total volume	20.0ml	20.0ml	20.0ml	10.0ml

*12% separation gel was used to obtain better separation for protein less than 50KDa. ** As for protein with a molecular weight of more than 100KDa, a 6% gel was used.

2.9 **Gel electrophoresis**

To separate the proteins, the gels were placed in a mini electrophoresis tank (BioRad, Hempstead, UK) filled up to the level recommended by the manufacturer with running buffer. The running buffer contained $(1L)^{-1}$; 25mM trisbase, 1.9M glycine, 1% SDS and its pH was set at 8.3 using concentrated HCL. Each well was then loaded with the sample plus loading buffer. To ensure that the molecular weight of the separated proteins could be easily determined, one of the wells was loaded with 8-10µl of a dual precision protein standard (Bio-Rad, Hempstead, UK). The samples were then resolved by applying a constant voltage of ~80V for 2-3 hours. For details of the apparatus used and the chemicals used for preparing the running buffer see table 2.11.

***Table 2.11**: Details of the apparatus used for gel electrophoresis and the chemicals used for preparing the running buffer.*

Items and chemicals	**Formula/Molecular weight/Formula Weight/ Description**	**Catalogue No.**	**Source**
Mini-Protean Tetra-Cell	Consist of 4-gel vertical electrophoresis system for 1 and 0.75 mm gel thickness. (includes casting stands, casting frames, 10-well combs, thick and thin glass plates)	165-8000	BioRad Laboratories Ltd, Hemel Hempstead, UK.
PowerPac HC	High current power supply (250V/0.3mA/300W)	164-5052	BioRad Laboratories Ltd, Hemel Hempstead, UK.
Tris(hydroxymethyl) aminomethane	$C_4H_{11}NO_3$/ MW: 121.14	B2005	Melford Laboratories Ltd. Chelsworth, Ipswich
Glycine	NH_2CH_2COOH/ MW: 75.0	G0709	Melford Laboratories Ltd. Chelsworth, Ipswich

Sodium dodecyl sulphate	$C_{12}H_{25}NaO_4S$/ MW: 288.38	B2008	Melford Laboratories Ltd. Chelsworth, Ipswich
Sodium hydroxide	NaOH/ MW: 40.00	80085-362	BDH/VWR International Ltd, Lutterworth, Leicestershire

2.10 Transfer of the proteins from the gel onto membranes

For the resolved proteins to be analysed by Western blotting, they need to be immobilised onto a pliable membranes (Towbin *et al.* 1979). Immobilisation of the proteins on a pliable membrane makes them accessible and easy to probe. Therefore, in this study, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using either the wet or semi dry technique.

2.10.1 Wet transfer

This technique is also known as the tank transfer technique and was used during the early part of my studies before the semi-dry technique was introduced into the laboratory. It was performed using a mini trans-blot cell assembly from Bio-Rad (Bio-Rad, Hempstead, UK). Briefly, the gel holder cassette was placed with the black side down on a flat surface. A thick fibre pad/sponge was placed on the black side of the cassette. Two sheets of pre-soaked thick filter paper were placed on the fibre pad. The equilibrated gel was then placed on the filter paper. The PVDF membrane soaked in methanol was placed on the gel and the sandwich was completed by adding two sheets of pre-soaked thick filter paper on the PVDF membrane. Small air bubbles trapped in the sandwich were removed by gently rolling a glass tube on the filter paper. Finally, a fibre pad was placed on the thick filter paper. The cassette was firmly closed and placed in the electrode module. A bio-ice cooling unit and the electrode module were placed in the transfer tank. The transfer tank was filled

with cooled (4°C) transfer buffer. For details of the membrane and chemicals used in the preparation of the transfer buffer see table 2.12. A magnetic flea was then added to maintain ion distribution and even buffer temperature. The lid was secured and a constant current of 200mAmps for 1 hour was applied. This technique requires the temperature of the whole system to be maintained at 4°C and thus was performed in a cold room (4°C).

***Table 2.12**: Characteristics of the membrane and summary of the chemicals used for preparing the transfer buffer.*

Items/ Scientific Name	**Formula/Molecular weight/Formula Weight/Description**	**Catalogue No.**	**Source**
Polyvinyl difluoride /PVDF transfer membrane	One roll of the transfer membrane measuring 26.5cm width x 3.75m length with pore size: 0.45µm	88518	Fisher Scientific UK Ltd. Loughborough, Leicestershire
Absolute methanol	CH_3OH/ FW: 32.04 The methanol is for soaking the transfer membrane	67-56-1	Fisher Scientific UK Ltd. Loughborough, Leicestershire
Tris(hydroxymethyl) aminomethane	$C_4H_{11}NO_3$/ MW: 121.14	B2005	Melford Laboratories Ltd. Chelsworth, Ipswich
Glycine	NH_2CH_2COOH/ MW: 75.0	G0709	Melford Laboratories Ltd. Chelsworth, Ipswich

Sodium dodecyl sulphate	C_{12} H_{25} NaO_4S/ MW: 288.38	B2008	Melford Laboratories Ltd. Chelsworth, Ipswich
Absolute ethanol	C_2H_5OH/ FW: 46.04	NC9602322	Fisher Scientific UK Ltd. Loughborough, Leicestershire

2.10.2 **Semi dry transfer (semi dry)**

This is the technique currently used in the laboratory. It is performed using a trans-blot SD assembly (Bio-Rad, Hempstead, UK), that consists of a flat platinum anode bed and does not require extra electrode assembly. This technique also uses a small quantity of transfer buffer. Firstly, a pre-soaked sheet of extra thick filter paper was placed onto the platinum anode bed. The methanol soaked PVDF membrane was then placed onto the extra thick filter paper. This was followed by the gel and the sandwich was completed by adding a sheet of pre-soaked thick filter paper on top of the gel. Small air bubbles trapped in the sandwich were removed by gently rolling a glass tube on the filter paper. Finally, the cathode was placed on top of the stack. A constant current of 200mA for 45 minutes was then applied. This technique was performed at room temperature. For details of the instruments used for protein transfer see table 2.13. Although transfer can be done in wet or semi-dry conditions there are advantages and disadvantages of each of these techniques (see table 2.14 for comparison of the two transfer systems). Both techniques use transfer buffer whose composition was as follows $(1L)^{-1}$ 0.2% ethanol, 390mM Glycine, 480mM Trisbase and 1%SDS.

***Table 2.13**: Details of the instruments used to transfer proteins from the gels to the membranes*

Instrument	Description	Catalogue No.	Source
PowerPac HC	High current power supply (250V/0.3mA/300W)	164-5052	BioRad Laboratories Ltd, Hemel Hempstead, UK.
Mini-Transblot Electrophoretic Transfer Cell	Consist of gel transfer cell, for two 10 x 7.5 cm gels, 2 gel holder cassettes, foam pads, electrodes, tank, cooling unit, lid with cables	170-3930	BioRad Laboratories Ltd, Hemel Hempstead, UK.
Trans-Blot SD Semi Dry Transfer Cell	Semi-dry electrophoretic transfer cell	170-3940	BioRad Laboratories Ltd, Hemel Hempstead, UK.

***Table 2.14**: Comparison of the two transfer systems*

	Wet transfer	Semi-dry transfer
Molecular weight range	Broad molecular weight range (>100kDa)	For medium size proteins (20-70KDa)
Transfer time	Extended transfer is possible (up to 24 hours) without buffer depletion. Under high-intensity conditions rapid transfer (15min-1hour) can be performed	Extended transfer is not possible due to buffer depletion
Temperature control	Specific temperature regulation (4-10°C), refrigerated buffer circulator and cooling unit permit native protein transfer	Temperature regulation by external cooling is not possible
Buffer capacity	Use 2 – 3 litres of transfer buffer	Use approximately 250ml per experiment; reduced cost of reagents and experiment time

2.11 **Blocking of the membranes**

The use of antibodies raised against specific proteins enables further analysis of the immobilised proteins (Renart *et al.* 1979, Burnette 1981). To increase specificity of antigen detection, the membrane needs to be blocked for non-specific binding of the primary and/or secondary antibodies to the membrane. Two commonly used blocking solutions are 0.5-5% non-fat dairy milk (NFDM) or 1-5% BSA. Although NFDM is cheaper it contains the phosphoprotein, casein, which causes high background. On the other hand, some antibodies give a stronger signal on membranes blocked with BSA as opposed to NFDM for unknown reasons.

For the blocking process each of the membranes was placed in a square petri dish with a single compartment approximately 100x100mm. The membrane was then blocked in 5% (w/v) dried milk powder dissolved in TBST for 1 hour. The TBST contained $(1L)^{-1}$; 250mM Trisbase, 1.4M NaCl, 30mM KCl, 0.5% Tween 20. The pH of the buffer was set at 7.4 using concentrated HCl. For details of the chemicals used to prepare the TBST buffer are given in table 2.15.

***Table 2.15**: Summary of the chemicals used in the preparation of TBST buffer.*

Scientific Name	**Formula/Molecular weight/Formula Weight/ Density**	**Catalogue No.**	**Source**
Tris(hydroxymethyl) aminomethane	$C_4H_{11}NO_3$/ MW: 121.14	B2005	Melford Laboratories Ltd. Chelsworth, Ipswich
Sodium chloride	NaCl/ FW:58.44	BDH4534-5KGP	BDH/VWR International Ltd, Lutterworth, Leicestershire
Potassium chloride	KCl/ FW:74.55	BDH4532-500GP	BDH/VWR International Ltd, Lutterworth, Leicestershire
Polyoxyethelene-20-sorbitan monolaurate/Tween 20®	$C_{58}H_{114}O_{26}$/ MW: 1228.00	BP337-100	Fisher Scientific UK Ltd. Loughborough, Leicestershire
Concentrated Hydrochloric acid	HCl/Density: 1.18 kgl^{-1}	BDH3028-2.5LG	BDH/VWR International Ltd, Lutterworth, Leicestershire

2.12 **Incubation with antibodies**

Once blocked, the PVDF membranes are now ready for incubation with the primary antibodies. The primary antibody was diluted to the required concentration using TBST. The appropriate dilution was determined by testing a range of dilutions (usually 1:100 - 1: 2000). The PVDF membranes were then incubated overnight at 4°C in 5% milk in TBST (w/v) containing the mono/polyclonal antibodies raised against the various proteins of interest (the antibody dilutions used are given in the appropriate chapters). The specificity of some of the antibodies was tested by incubating the membranes with blocking peptides.

The following day, the membranes were given 4 x 15 minute washes in TBST. The membranes were then incubated with species-specific secondary antibodies conjugated to horse-radish peroxidise for 1hour at room temperature. The details of the secondary antibodies and the dilutions used are provided in the relevant chapters.

The membranes were then given 4x15minute washes using TBST. They were then washed for 10 minutes in PBS. All washes were done on an orbital shaker (Bibby Scientific Ltd. Staffordshire) set at 90rpm.

2.13 **Development of the membranes**

Protein bands were developed by the enhanced chemiluminescent reaction between HRP-conjugated secondary antibodies on the

membrane and the enhanced chemiluminescent (ECL) developing solution. After the last wash with PBS, each of the membranes was treated with ECL developing solutions (SuperSignal®, Thermo Fisher, Loughborough, UK) as per the manufacturer's instructions, and incubated in the dark for 10 minutes. After the incubation period, the membranes were dabbed on tissue paper to remove any excess ECL liquid. A thin transparent double-layer plastic sheet was prepared and the membrane was placed in between the sheets. Any air bubbles trapped between the sheet and the membrane were removed by pressing the bubbles toward the edges of the plastic sheet. The assembly was then placed in an x-ray cassette to protect the membranes from light.

The optimum exposure time was then determined and the membranes were then exposed to film in the darkroom. The exposed x-ray film was then processed in the darkroom using a medical x-ray film processor (Konica Minolta, SRX101A, Hampshire, UK) for permanent documentation. Oversaturated films were discarded, and those which clearly showed the probed band of interest were selected for digitisation. For details of the ECL developing system see table 2.16.

***Table 2.16**: Summary of the apparatus and chemicals used in the exposure and development of the membranes.*

ECL developing system	**Description**	**Model/ Catalogue No.**	**Source**
SuperSignal West Pico Chemiluminescent Substrate	Consist of Luminol/Enhancer, 500mL stable peroxide buffer, 500mL	34078	BioRad Laboratories Ltd, Hemel Hempstead, UK.
X-Ray cassette	Kodak X-Omatic Regular, 18 x 24cm	525-7068	GB Medical Ltd. Andover, Hampshire, UK.
X-Ray Film processor	Konica Minolta SRX 101A	SRX101A	GB Medical Ltd. Andover, Hampshire, UK.

2.14 **Membrane stripping and re-probing for actin/total protein**

Stripping is the term used for describing the removal of the primary and secondary antibodies from the membrane. It is used when more than one protein is investigated on the blot or the same protein is being probed with a different antibody. For example, a membrane is probed using a phosphor-specific antibody. Then it is re-probed with an antibody against the total protein. There are three stripping techniques; soft stripping - which uses two subsequent washes in PBS each for 10minutes followed by two subsequent washes in TBST each for 20minutes; medium stripping - which uses a stripping buffer containing 0.1%SDS and harsh stripping - which employs a stripping buffer containing 10%SDS and β-mercaptoethanol. As stripping may remove some of the protein from the membrane and damage fragile proteins, it is always advisable to use soft stripping before proceeding to harsher ones.

For my study, the medium stripping technique was adopted. The membranes were stripped using a medium stripping buffer, which contained ($1L^{-1}$) 200mM Glycine, 0.1%(w/v) SDS, 1%(v/v) Tween 20. The pH of the buffer was set at 2.2 using concentrated HCl. For details of the chemicals used and their source(s) see table 2.17.

Briefly, the membrane was incubated in the stripping buffer for 20 minutes. This was followed by 2x10 minute washes in PBS. The PBS contained $(1L)^{-1}$; 1.4M NaCl, 155mM Na_2HPO_4, 47mM NaH_2PO_4, 27mM KCl and its pH was set at 7.2 using concentrated HCl. For details of the chemicals used in the preparation of the PBS and their sources see table 2.18.

***Table 2.17**: Summary of the characteristics and source of the chemicals used to prepare the stripping buffer.*

Scientific Name	**Formula/Molecular weight/Formula Weight**	**Catalogue No.**	**Source**
Glycine	NH_2CH_2COOH/ MW: 75.0	G0709	Melford Laboratories Ltd. Chelsworth, Ipswich
Sodium dodecyl sulphate	$C_{12}H_{25}NaO_4S$/ MW: 288.38	B2008	Melford Laboratories Ltd. Chelsworth, Ipswich
Polyoxyethelene-20-sorbitan monolaurate/Tween 20®	$C_{58}H_{114}O_{26}$/ MW: 1228.00	BP337-100	Fisher Scientific UK Ltd. Loughborough, Leicestershire
Hydrochloric acid	HCl/ FW: 36.46	H/1000/PB17	Fisher Scientific UK Ltd. Loughborough, Leicestershire

***Table 2.18**: Summary of the chemicals used to prepare the PBS buffer and their sources.*

Scientific Name	**Formula/Molecular weight/Formula Weight**	**Catalogue No.**	**Source**
Sodium chloride	NaCl/ FW:58.44	BDH4534-5KGP	BDH/VWR International Ltd, Lutterworth, Leicestershire
Disodium hydrogen phosphate/ Sodium phosphate dibasic	Na_2HPO_4/ FW: 141.96	42437-0025	Fisher Scientific UK Ltd. Loughborough, Leicestershire
Sodium dihydrogen phosphate/ Sodium phosphate monobasic	NaH_2PO_4/ FW: 119.98	20780-0025	Fisher Scientific UK Ltd. Loughborough, Leicestershire
Potassium chloride	KCl/ FW:74.55	BDH4532-500GP	BDH/VWR International Ltd, Lutterworth, Leicestershire
Hydrochloric acid	HCl/ FW: 36.46	H/1000/PB17	Fisher Scientific UK Ltd. Loughborough, Leicestershire

After stripping, the membrane was blocked in 5% (w/v) dried milk powder dissolved in TBST for 1 hour. It was then incubated for 1 hour at room temperature in 5% milk in TBST (w/v) containing mono/polyclonal antibodies raised against the protein of interest.

The membranes were then washed as described above, and incubated for 1-hour in HRP- conjugated species specific secondary antibodies diluted in 5% milk dissolved in TBST (w/v). The membranes were then washed with TBST as described in sections 2.10 and 2.11. They were then prepared for development and visualisation as described in 2.12.

2.15 Data handling

2.15.1 Western (-Immunoblotting)

After development, the films were digitised, at a resolution of 600dpi, using a scanner (HP PSC 1400, Hewlett Packard, Berkshire, UK) and the images were saved in a computer as black and white tiff files.

The intensity of each protein band was then analysed using Scion® image software from NIH. The intensity of the bands of interest were then normalised to those of the corresponding total protein or actin in the same sample and the data are presented as mean of the ratios ± S.D.

2.16 **Statistical analysis**

Statistical analyses was performed on the mean intensities of the probed bands from the western blot analysis. A paired t-test was used to compare the control and the DHT/T treated samples; whereas,a three-way ANOVA was used to compare the data from the two fibre types and to perform comparisons between the various treatment groups i.e. untreated (control, group 1), treated with DHT/T (group 2), treated with inhibitor only (group 3), and those treated with DHT plus the inhibitor (group 4). A Sidak-Holmes Post-Hoc test was then used to confirm the statistical significance of the data. All of the statistical tests were carried out using Sigma Plot for Windows version 11.0 (Systat Software Inc., San Jose California) and a p-value <0.05 was considered to be statistically significant.

CHAPTER 3

EFFECTS OF DHT AND T ON THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) PATHWAY

3.1 Introduction

As stated in section 1.7, anabolic androgenic steroids have both genomic/chronic and non-genomic/acute/rapid effects (Heinlein & Chang, 2002; Simoncini & Genazzani, 2003). Although the long term/genomic actions of AAS have been the subject of numerous previous studies and some of their physiological functions are well known, little is known about their acute/non-genomic actions especially in adult mammalian skeletal muscle fibres (Estrada *et al.* 2000; Estrada *et al.* 2003). Indeed, only one study has previously investigated the acute/non-genomic effects of testosterone (T) and nandrolone (a synthetic derivative of testosterone) in myotubes. Treatment of the myotubes with both hormones led to a rapid and transient increase in inositol triphosphate (IP_3) and intracellular Ca^{2+} as well as the phosphorylation of the extra-cellular signal regulated kinases (ERK)1/2 (Estrada *et al.* 2003).

ERK1/2 are members of the ubiquitous proline-directed protein-serine/threonine kinases commonly referred to as mitogen-activated protein kinases (MAPKs). These kinases are arranged in a series to form a cascade commonly referred to as the MAPK pathway. MAPK signal transduction pathways are the most wide-spread of all eukaryotic cellular regulation mechanisms. They are activated by a

wide variety of extracellular signals including hormones, inflammatory cytokines and environmental stresses such as irradiation, osmotic shock and ischemia (Krishna & Narang, 2008). To date, six distinct MAPK pathways have been identified in mammalian cells namely; extracellular signal-regulated kinases (ERK1/2), c-Jun NH_2-terminal kinases or stress activated protein kinases (JNK or SAPK), p38, ERK3/4, ERK5 and ERK6/7 (Paul *et al.* 1997; Johnson & Lapadat, 2002). However, the three that have been extensively studied and whose functions are well known are ERK1/2, p38 and JNK (Pearson *et al.* 2001; Krishna & Narang, 2008). Therefore, in this study I investigated the effects of DHT and T on these three modules only.

Although each MAPK pathway is unique and responds to specific stimuli, all six pathways share some common features. For example, they all consist of a set of three evolutionary conserved kinases that are sequentially activated namely, a MAPKK kinase (MAP3K), MAPK kinase (MAPKK) and MAPK (see Fig 3.1; Krishna & Narang, 2008).

The downstream targets of the MAPK or MAPK activated protein kinases (MKs) add extra specificity, diversity and amplification to each pathway (Cargnello & Roux, 2011). Kinases in this group include p90 ribosomal S6 kinases (RSK) 1-4, mitogen and stress-activated kinases (MSK) 1/2 and the MAPK-activated protein kinases (MK) 2/3. However, amongst these kinases only the RSK family has been shown to be exclusively activated by ERK1/2 (Roux *et al.* 2007). So far, no MKs have been assigned to JNK (Cohen, 1997; Cargnello & Roux, 2011) and p38 (Ronkina *et al.* 2008; Cargnello & Roux, 2011). Once activated, ERK1/2 have been shown to accumulate in both the cytoplasm as well as the nucleus where they activate numerous proteins including cytoskeletal proteins (Crean *et al.* 2002), transcription factors (Xing *et al.* 1996) and MKs (Dalby *et al.* 1998; Frodin & Gammeltoft, 1999).

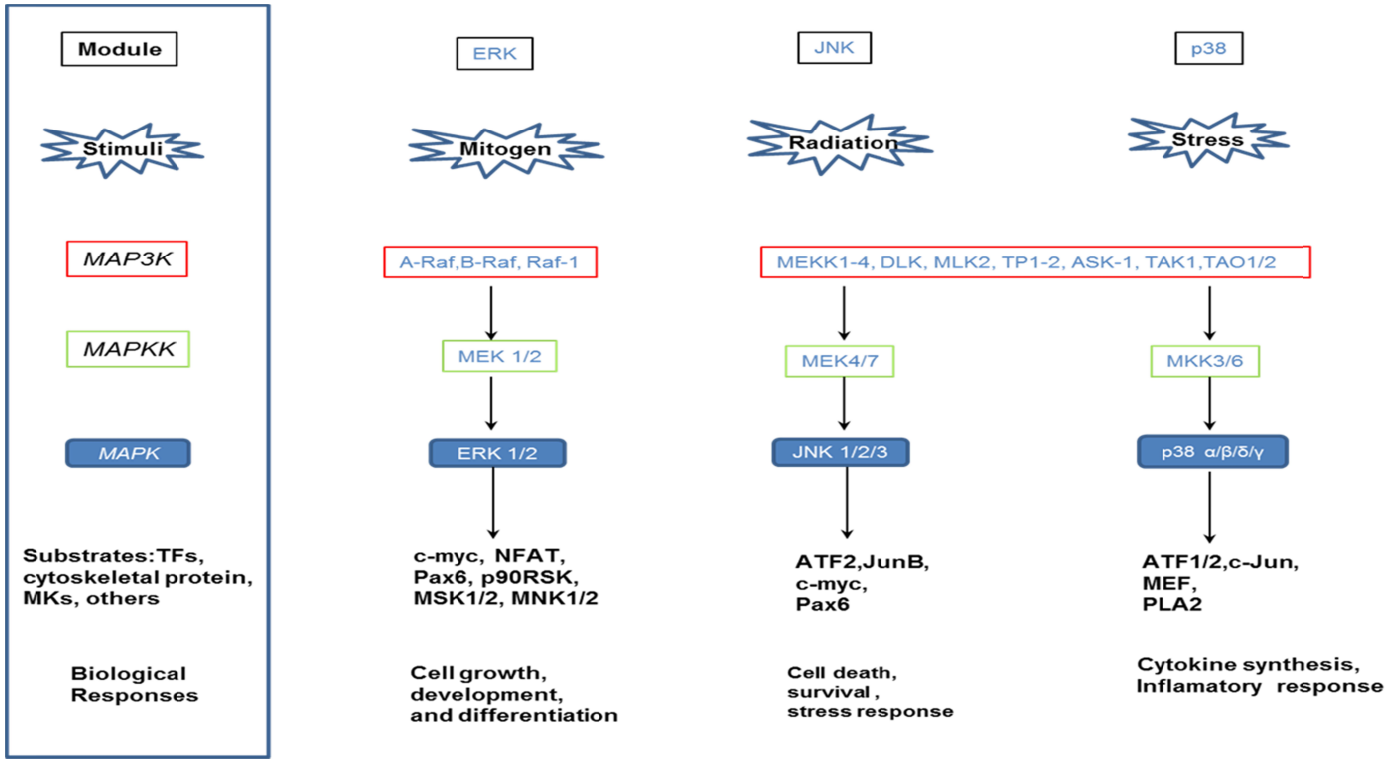

Figure 3.1*: A schematic diagram showing the arrangement and biological responses of the three well studied module of the MAPK pathway namely ERK, JNK and p38. Note that the three modules are activated by different extracellular stimuli and induce biological responses. Diagram adapted from Krishna & Narang (2008).*

As mentioned above, only one study has previously investigated the effects of T and nandrolone in skeletal muscle. However, the study was performed using cultured myotubes and supramaximal levels of T and nandrolone (Estrada *et al.* 2003). Therefore, it is uncertain whether physiological levels of T or its active metabolite dihydrotestosterone (DHT) have any acute/non-genomic actions in adult mammalian skeletal muscle fibres. Also, the study by Estrada *et al* investigated the effects of T and nandrolone on ERK1/2 only. Therefore, it is unknown whether T and DHT affect the other modules of the MAPK pathway.

3.2 **Aim of this Chapter**

Therefore, the primary aim of the experiments reported in this chapter were to investigate the effects of treating small skeletal muscle fibre bundles, isolated from the EDL (a fast-twitch muscle) and soleus (a slow twitch-muscle) of adult CD1 mice, with physiological concentrations of DHT and T on the activation of ERK1/2, JNK, p38 and their downstream substrates.

3.3 **Materials and method**

3.3.1 **Treatment of the fibre bundles with DHT and T**

The general materials and methods used in this study were described in detail in chapter 2 and will only be briefly mentioned in this chapter. All the experiments were performed at room temperature (~20°C) using small muscle fibre bundles isolated from either the EDL (a fast-twitch muscle) or soleus (a slow-twitch

muscle) of female adult CD1 mice prepared as described in section 2.2. The fibre bundles were then mounted horizontally between two stainless steel hooks in purpose built muscle chambers (internal measurement approximately 20mm width x 67mm length x 26mm depth) filled with the standard Ringer's solution. They were then divided into two equal groups. Half were treated with the standard Ringer's solution plus the vehicle (1.07mM absolute ethanol) used to dissolve DHT and T for 1hour. Proteins extracted from these fibres acted as controls. The other half was treated for the same period of time (1hour) with the Ringer's solution containing 630pgml^{-1} of DHT (2.16nM DHT) or T (1.82nM T). Proteins extracted from these fibre bundles acted as the experimental/treated samples. During the experiments, the Ringer's solution was continuously bubbled with medical gas (95%O_2, 5%CO_2). Additionally, every 10 minutes, the Ringer's solution was completely replaced and the fibre bundles were electrically stimulated to elicit a series of twitch contractions.

To investigate the cellular signalling pathway(s) mediating the effects of DHT and T, some of the fibre bundles were pre-incubated for 30minutes in Ringer's solution containing 20μM of the MEK inhibitor, PD98059 (Alexis Biochemicals). They were then treated for 1hour with Ringer's solution containing the inhibitor alone or the inhibitor plus 630pgml^{-1} DHT or T.

3.3.2 **Immunoblotting**

At the end of the experiments described above, proteins were extracted and separated using gel electrophoresis as described in chapter 2 sections 2.6 and 2.9. They were then transferred on to PVDF membranes and blocked as described in sections 2.10 and 2.11. The membranes were then incubated overnight with the antibodies listed in table 3.1.The following day, they were incubated with species-specific secondary antibodies conjugated to horse-radish peroxidase for 1 hour at room temperature. Later, they were developed using ECL substrate and exposure of the membranes to film. On the third day, the membranes were stripped and re-probed for total ERK 1/2(Santa Cruz Biotechnology, USA) or actin (Abcam, Cambridge England). These blots acted as loading controls.

3.3.3 **Data handling and analysis**

All the Western blots were run in triplicate and each experiment was repeated at least three times. The Western blots were digitised as described in section 2.14.1 and the intensity of each protein band was analysed using Scion® image software from NIH. The intensity of the bands of interest were then normalised to those of the corresponding total protein or actin in the same sample and the data are presented as the Mean ± S.D. Statistical analysis of the data performed using SigmaPlot® 11.2 (Systat Software Inc., London, UK). The data obtained from the two fibre types under control and the various experimental conditions were then compared using a three way ANOVA and a $p<0.05$ was considered statistically significant.

***Table 3.1**: List of the antigens and the antibodies used in these experiments.*

ANTIBODIES/PEPTIDES			SOURCE
Antigens	Description	Dilution	
ERK 1/2 phosphorylated at Threonine 202 and Serine 204	Rabbit polyclonal antibody	1:1000	SantaCruz Biotechnology Inc. (USA).
Total ERK 1 [C-16]	Rabbit polyclonal antibody	1:1000	SantaCruz Biotechnology Inc. (USA).
JNK phosphorylated at Threonine 183 and Threonine 185	Mouse monoclonal antibody	1:500	Cell signaling technology Inc. (Beverly, USA)
MSK 1&2 phosphorylated at Serine 360	Rabbit monoclonal antibody	1:500	Abcam (Cambridge, England)
p38 phosphorylated at Threonine 180 and Tyrosine 182	Rabbit polyclonal antibody	1:500	Cell signaling technology Inc. (Beverly, USA)

p90RSK phosphorylated at Serine 380	Rabbit monoclonal antibody	1:500	Abcam (Cambridge, England)
Myosin Light Chain phosphorylated at Serine 20	Rabbit polyclonal antibody	1:1000	Abcam (Cambridge, England)
Total myosin light chain	Rabbit polyclonal antibody	1:1000	Abcam (Cambridge, England)
Actin	Mouse monoclonal antibody	1:1000	Abcam (Cambridge, England)
Goat anti-rabbit	IgG-HRP	1:1000	SantaCruz Biotechnology Inc. (USA).
Goat anti mouse	IgG-HRP	1:1000	SantaCruz Biotechnology Inc. (USA).

3.4 **Result**

3.4.1 **Effects of DHT and T on the activation of ERK1/2**

The results in figure 3.2 (A,B) show the basic observation in this study. As the results show, the untreated fibre bundles expressed mostly phosphorylated pERK 2 (p42). Furthermore, its expression was always greater in the fast- than in the slow-twitch fibre bundles.

Moreover, treatment of fibre bundles with Ringer's solution containing physiological levels of DHT for 1hour increased the phosphorylation of ERK 1 (p44) in both fibre types (see Fig. 3.2A). On the other hand, treating them with in Ringer's solution containing physiological levels of T for 1 hour, increased the phosphorylation of ERK1 (p44) in slow-twitch fibre type only (Fig. 3.2B).

In a previous study, T was shown to have no effects on the force production in isolated intact mammalian skeletal muscle fibre bundles (Hamdi & Mutungi, 2010) and because in this study it only increased the phosphorylation of ERK1/2 in the slow-twitch fibre bundles, its effects were not investigated any further.

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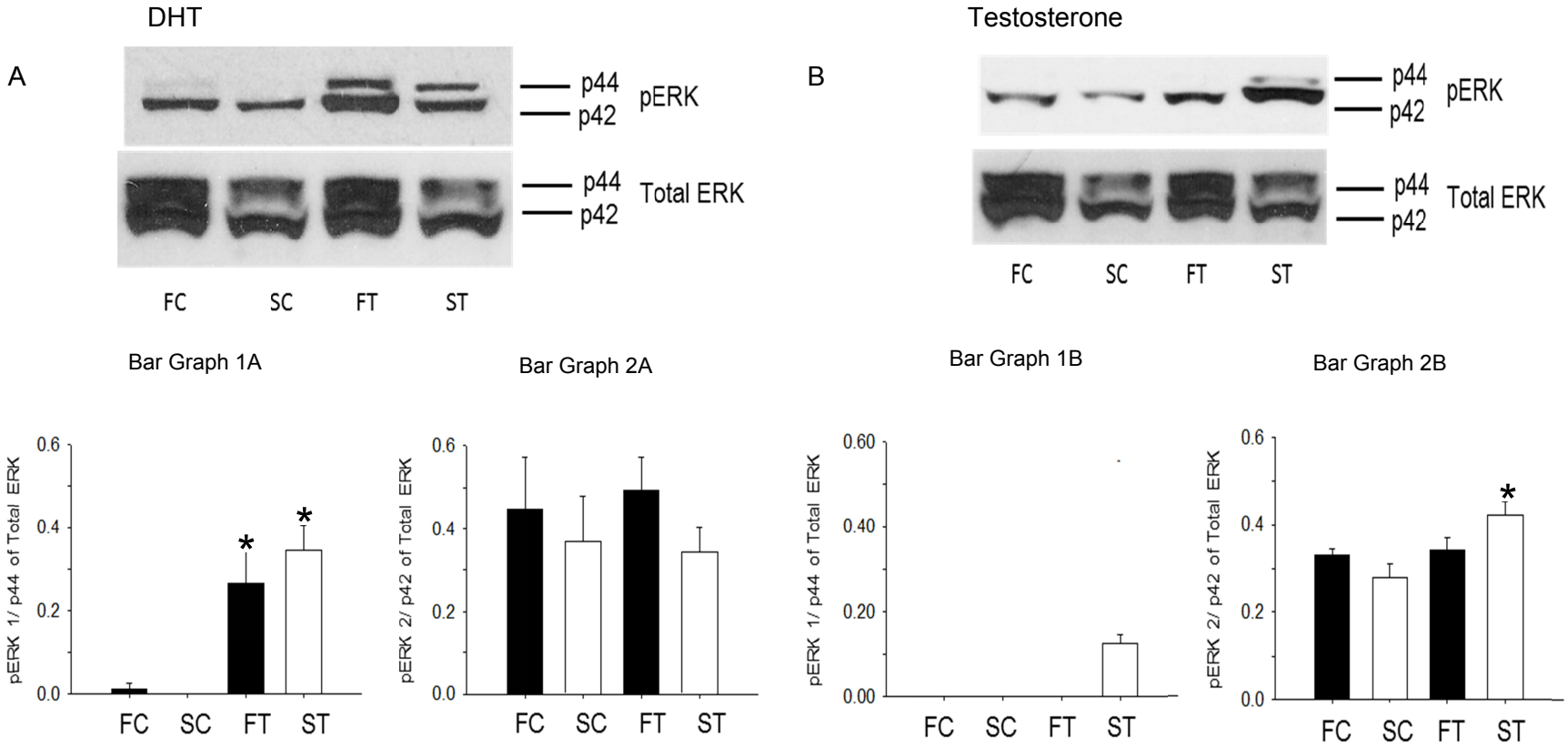

Figure 3.2 *: DHT increases the phosphorylation of ERK 1/2 in both fibre types* .

Representative Western blots showing the concentration of phosphorylated ERK1/2 (p44/p42 respectively) and total/ non-phosphorylated ERK1/2 (p44/p42) in female fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles treated with the standard Ringer's solution (A,B,FC,SC) or the standard Ringer's solution plus 2.16nM DHT (A,FT,ST) or 1.82nM T (B,FT,ST). Note that treatment of the fibre bundles with DHT led to a significant increase ($p=0.04$; $p<0.05$) in the phosphorylation of ERK 1(p44) in both fibre types (A,FT,ST, Bar Graph 1A); whereas, treatment with T increased the phosphorylation of ERK 1(p44) in slow-twitch fibre bundles only (B,ST, Bar Graph 1B). Also note that the concentration of the non-phosphorylated (total) p44/p42 in fast-twitch (A,B,FC,FT) fibre bundles was always higher than that in the slow-twitch fibre bundles (A,B,SC,ST) ($p=0.043$;$p<0.05$).

3.4.2 **Effects of DHT and T on activation of JNK and p38**

As mentioned in section 3.1, the MAPK pathway has three parallel modules ERK, JNK and p38. Therefore, in another experiment, I examined the effects of treating small muscle fibre bundles with physiological levels of DHT on the phosphorylation of JNK and p38. The results from these experiments are shown in Fig. 3.3. As the results show, the expression of phosphorylated JNK and p38 in untreated fibre bundles was always 2-3 times higher in the slow-twitch than in the fast-twitch fibre bundles. Furthermore, treatment of the muscle fibre bundles with DHT had no effect on the phosphorylation of both JNK and p38 in either fibre type.

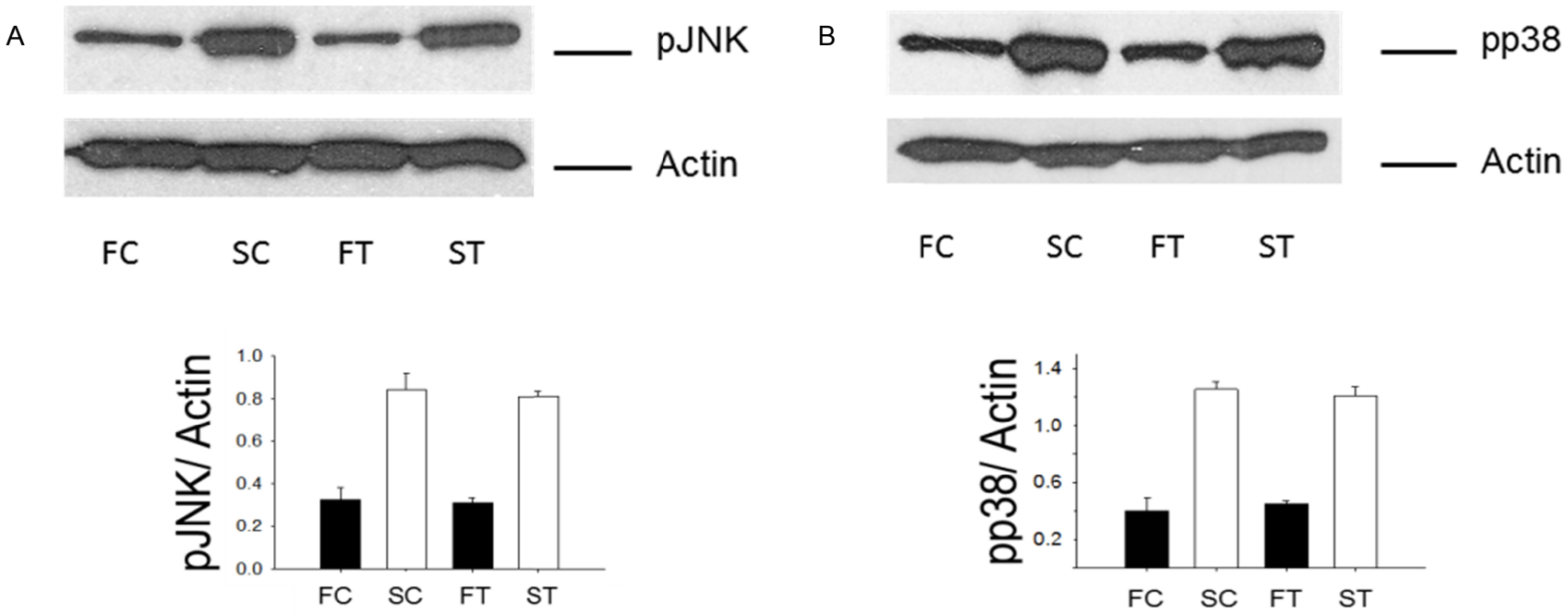

***Figure 3.3**: DHT had no effect on the phosphorylation of JNK and p38 in both fibre types .*

Representative Western blots showing the effects of DHT on the phosphorylation of JNK (A, pJNK) and p38 (B, pp38). Note that both antibodies identified a single band with a molecular weight of ~54KDa in the case of pJNK and ~43KDa in the case of pp38. Moreover, the untreated slow-twitch muscle fibre bundles expressed higher concentrations of the phosphorylated proteins than the fast-twitch ones. FC=control fast-twitch; SC=control slow-twitch; FT=DHT treated fast-twitch; ST=DHT treated slow-twitch

3.4.3 **Comparison of female DHT-treated muscles with untreated male muscles**

T and DHT are male sex steroids and their concentrations are higher in male than in female animals. Therefore, in another experiment I investigated whether the DHT- and T-induced changes in the phosphorylation of ERK1/2 were similar to those found naturally in male animals. The results from this experiment are shown in Fig. 3.4 and as they show, treatment of the muscle fibre bundles with DHT and T were similar to those reported in section 3.4. Thus, DHT increased the phosphorylation of ERK1/2 in both fibre types; whereas, T increased its phosphorylation in the slow-twitch fibre bundles only. Moreover, treatment of the muscle fibre bundles with these hormones did not replicated the levels of phosphorylation of ERK1/2 found in the untreated male muscles. Instead, the phosphorylation of ERK1/2 in the untreated male muscles was very similar to that found in the corresponding untreated female muscle.

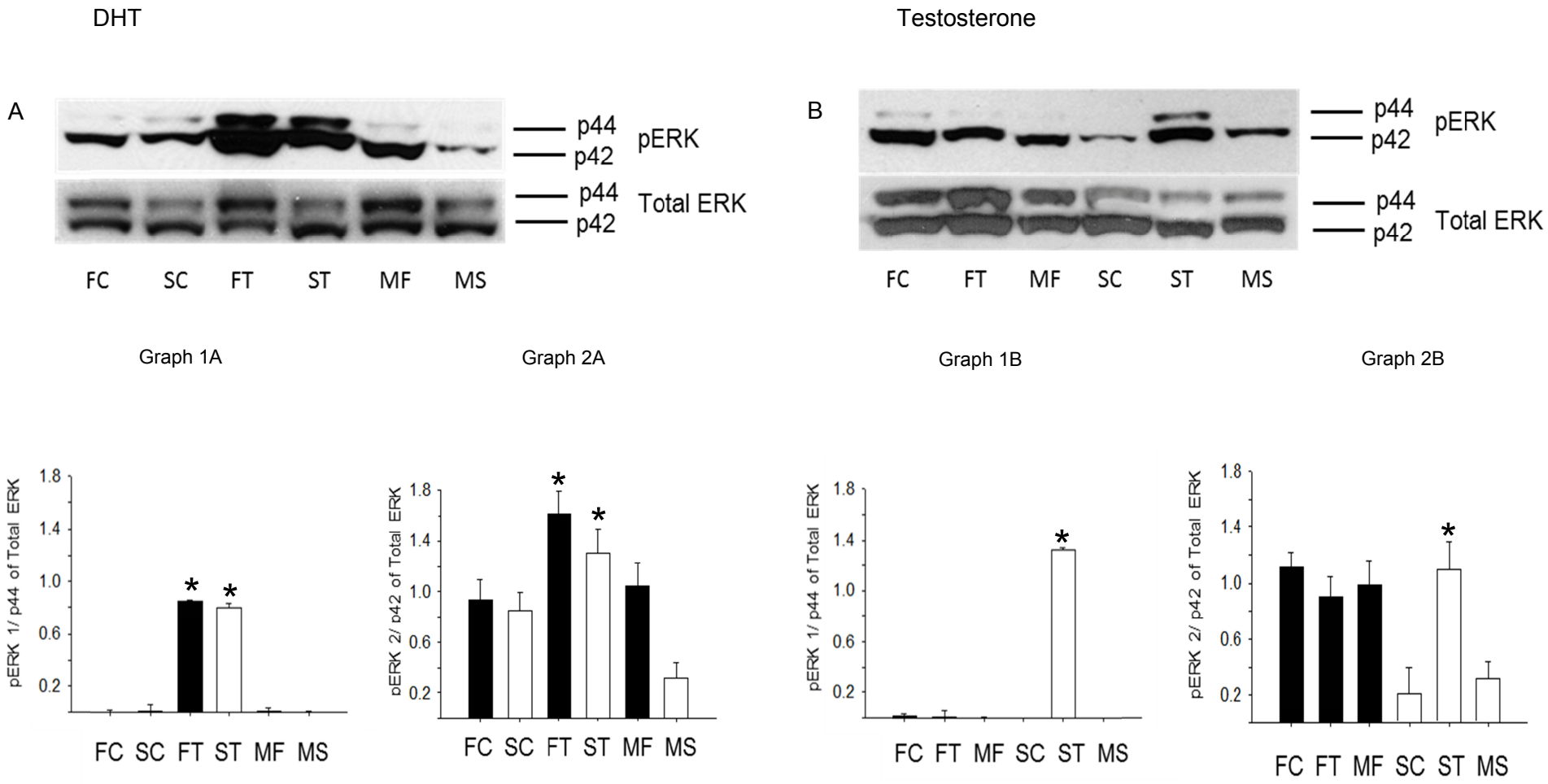

***Figure 3.4**: Untreated female and male muscle fibre bundles express similar concentration of phosphorylated ERK1/2.*

Representative Western blots showing the effects of treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) muscle fibre bundles isolated from adult female (FC,SC,FT,ST) and male (MF,MS) with the standard Ringer's solution (FC,SC,MF,MS) or the standard Ringer's solution plus 2.16nM DHT (A, FT,ST) or 1.82nM T (B,FT,ST). Note that treatment of the fibre bundles with DHT led to a significant increase ($p=0.04$; $p<0.05$) in the phosphorylation of ERK 1(p44) in both fibre types (A,FT,ST, Bar Graph 1A); whereas, treatment with T increased its phosphorylation in the slow-twitch fibres only (B,ST, Bar Graph 1B). Also note that the concentrations of the phosphorylated proteins in the untreated with DHT or T female and male muscle fibre bundles are similar.

3.4.4 **The effects of DHT on the activation of the MAPK pathway are abolished by the MEK inhibitor PD98059**

All MAPK pathways are regulated mainly at the level of MAPK (Garrington & Johnson, 1999; Johnson & Lapadat, 2002). At the level of MAPK, each protein kinase is activated by their respective upstream dual specificity enzyme, MEK, resulting in the phosphorylation of threonine and tyrosine residues in the area known as the activation loop of each substrate (Pearson *et al.* 2001; Shaul & Seger 2007). Both ERK1/2 are phosphorylated by their upstream kinases, MEK1/2, at their dual phosphorylation motifs, Thr-Glu-Tyr (TEY) (Payne *et al.* 1991). Therefore, in another experiment I investigated the effects of pre-treating the fibre bundles with the MEK specific inhibitor, PD98059 for 20minutes, followed by a 1hour incubation in Ringer's solution containing DHT and PD98059. The results from this experiment are displayed in Fig.3.5 and as they show, pre-treatement of the fibre bundles with PD98059 completely abolished the effects of DHT on the phosphorylation of ERK1/2.

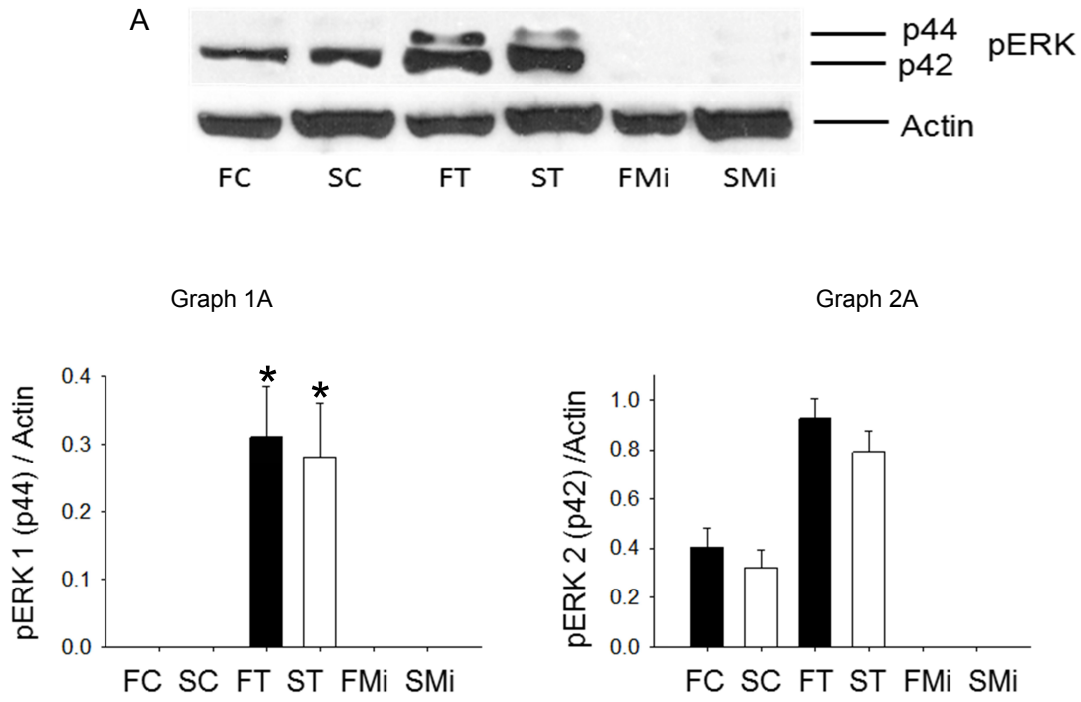

Figure 3.5: *Pre-treatment of the muscle fibre bundles with the MEK inhibitor PD98059 abolishes the DHT-induced increases in the phosphorylation of ERK1/2 in both fibre types*

*Representative Western blots showing the effects of pre-treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) fibre bundles with 20μM PD98059 (FMi,SMi) on the phosphorylation of ERK1/2. Note that treatment of the fibre bundles with DHT led to a significant increase (p=0.01; *p<0.05) in the phosphorylation of ERK 1(p44) in both fibre types. Furthermore, pre-treatment of the fibre bundles with PD98059 completely abolished the DHT-induced increase in the phosphorylation of ERK1/2 in both fibre types.*

3.4.5 **Effects of DHT on signal transduction downstream of the ERK1/2**

p90 ribosomal S6 kinases (RSK) 1/2 as well as mitogen- and stress-activated protein kinases (MSK1 and MSK2) have been identified as downstream substrates of ERK1/2 (Blenis, 1993; Deak *et al.* 1998). Therefore, in another experiment, I determined whether the acute effects of DHT in adult mammalian skeletal muscle fibre bundles were transduced through RSK1/2 or MSK1/2. To do this, another set of fibre bundles was treated with the standard Ringer's solution or the standard Ringer's solution plus DHT. The phosphorylation of RSK1/2 and MSK1/2 was then determined in the control and treated fibre bundles. As the results shown in Fig.3.6 show, the untreated fast- and slow-twitch fibre bundles expressed very low concentrations of phosphorylated RSK and its expression was similar in the two fibre types. Moreover, treating the fibre bundles with DHT increased its expression in both fibre types. In contrast, the untreated slow-twitch fibre bundles expressed higher levels of

phosphorylated MSK1/2 than the fast-twitch fibre bundles. Additionally, their treatment with DHT did not significantly affect its expression in either fibre type.

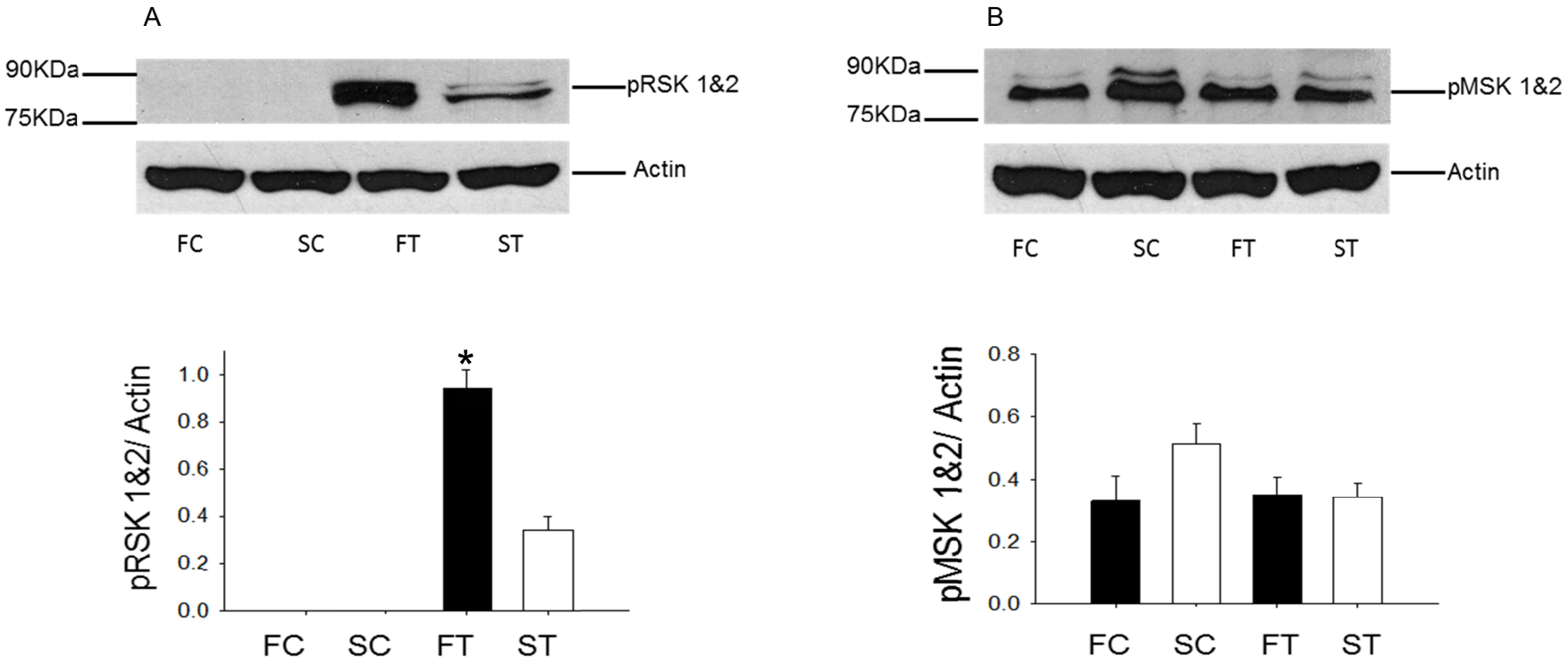

***Figure 3.6**: The acute effects of DHT in adult mammalian skeletal muscle fibres are mediated through RSK1/2*

*Representative Western blots showing the effects of treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) muscle fibre bundles with physiological concentrations of DHT on the phosphorylation of RSK1/2 (A) and MSK1/2 (B). Note that treatment of the fibre bundles with DHT significantly increases (p=0.02; *p<0.05) the phosphorylation of RSK 1/2 in both fibre types. In contrast, DHT had no effect on the phosphorylation of MSK1/2 in either fibre types.*

To investigate whether the effects of DHT on the phosphorylation of RSK1/2 were mediated through MEK1/2/ERK1/2 pathway, in some experiments the fibre bundles were pre-treated with the MEK specific inhibitor PD98059. As the results in Fig.3.7 show, pre-treating the fibre bundles with the inhibitor completely abolished the effects of DHT on the phosphorylation of RSK1/2.

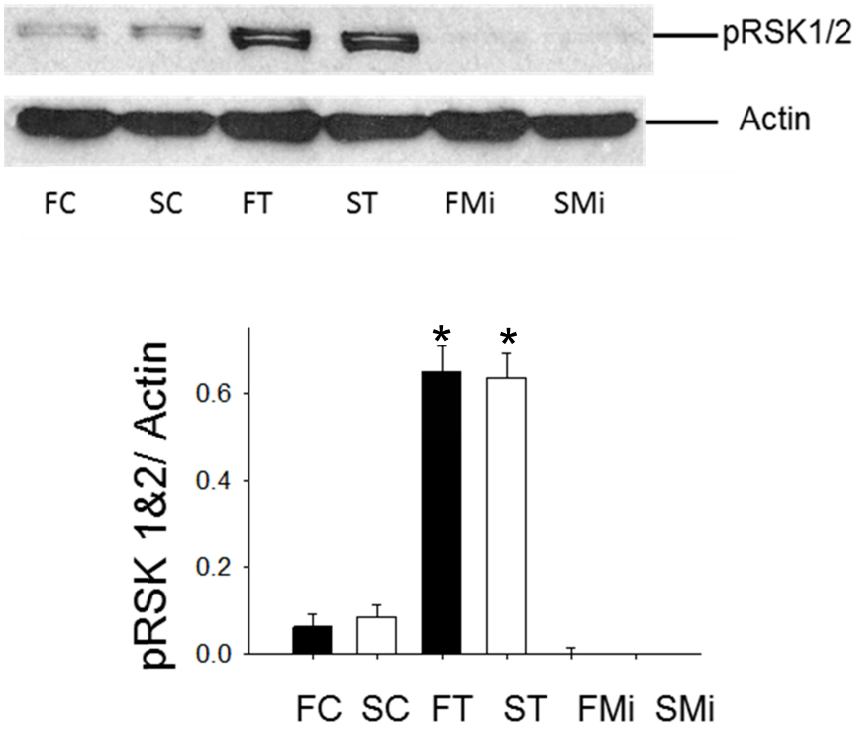

***Figure 3.7**: DHT increases the phosphorylation of RSK1/2 through MEK in both fibre types*

Representative Western blots showing the effects of pre-treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) muscle fibre bundles with 20μM PD98059 (FMi,SMi) on the phosphorylation of RSK1/2. Note that treatment of the fibre bundles with DHT led to a significant increase ($p=0.01$; $*p<0.05$) in the phosphorylation of RSK1/2 in both fibre types. Furthermore, pre-treatment of the fibre bundles with PD98059 completely abolished the DHT-induced increase in phosphorylation of RSK1/2 in both fibre types.

3.4.6 **Effects of DHT and T on the phosphorylation of the 20KDa RMLCs**

It has previously been shown that treating small muscle fibres bundles with DHT, but not with T, increases force production in fast-twitch fibre bundles and decreases it in slow-twitch fibre bundles (see Hamdi & Mutungi, 2010). However, the mechanism(s) underlying these observations is still poorly understood. Therefore, in another experiment I examined whether DHT exerted these effects by regulating the phosphorylation of the 20kDa regulatory myosin light chains (RMLCs).

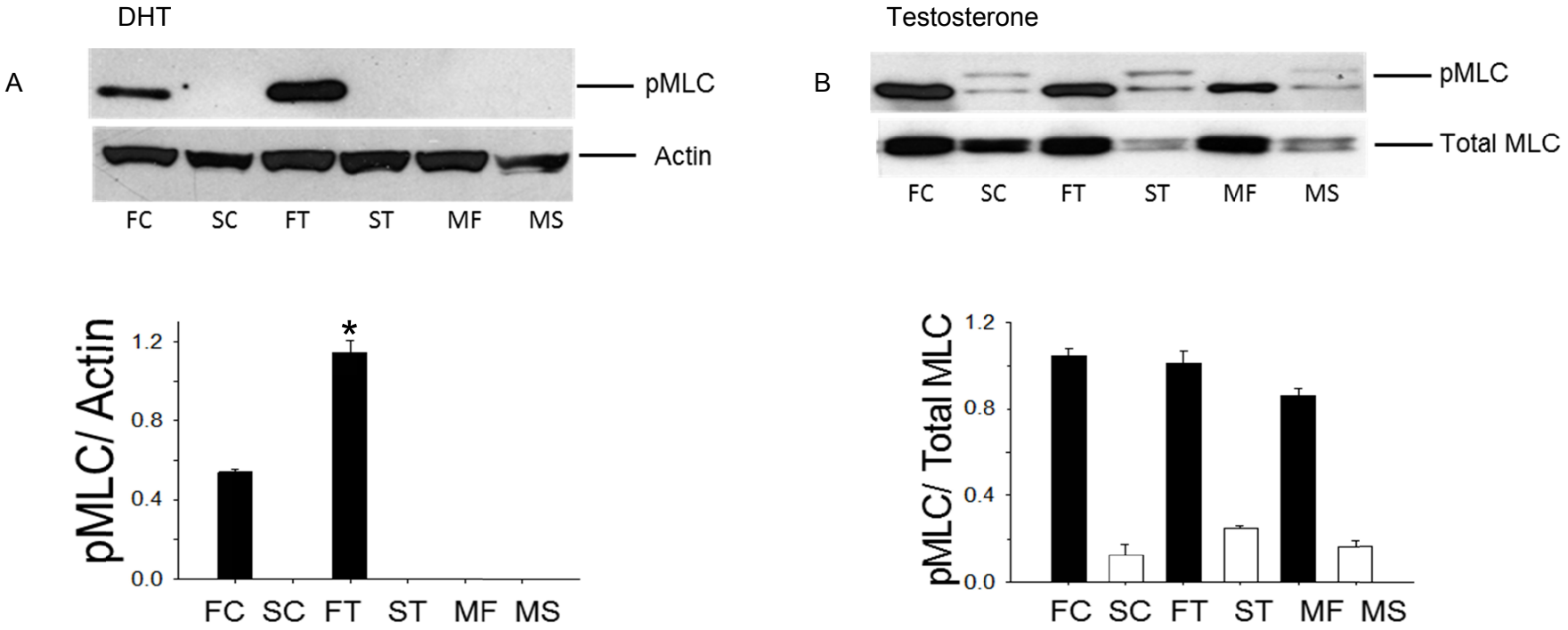

***Figure 3.8**: DHT increases the phosphorylation of the 20kDa RMLCs in fast-twitch fibre bundles*

*Representative Western blots showing the effects of treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) muscle fibre bundles with physiological concentrations of DHT (A) and T (B) on the phosphorylation of the 20kDa RMLCs. Note that treatment of the fibre bundles with DHT significantly increases (0.018;*p<0.05) the phosphorylation of RMLC in the fast-twitch fibre bundles only. In contrast, T has no effects on the phosphorylation of RMLC in either fibre type.*

As the results displayed in Fig.3.8 show, treating the muscle fibre bundles with DHT led to a 20-40% increase in the phosphorylation of the RMLCs in fast twitch fibre bundles only (Fig. 3.8A). In contrast, T led to a decrease in the phosphorylation of RMLCs in both fibre types (Fig. 3.8B).

Previously, a number of studies have shown that the motility of non-muscle cells results from an ERK1/2-dependent increase in the phosphorylation of the 20KDa RMLCs (Klemke *et al.* 1997; Iwabu *et al.* 2004). Therefore, to examine whether the DHT-induced increase in the phosphorylation of the 20kDa RMLCs was mediated through the same pathway, some of the fibre bundles were pre-treated with PD98059. They were then treated with DHT plus the inhibitor. As the results in Fig. 3.9 show, pre-treatment of the fibre bundles with the inhibitor completely abolished the effects of DHT on the phosphorylation of the 20kDa RMLCs.

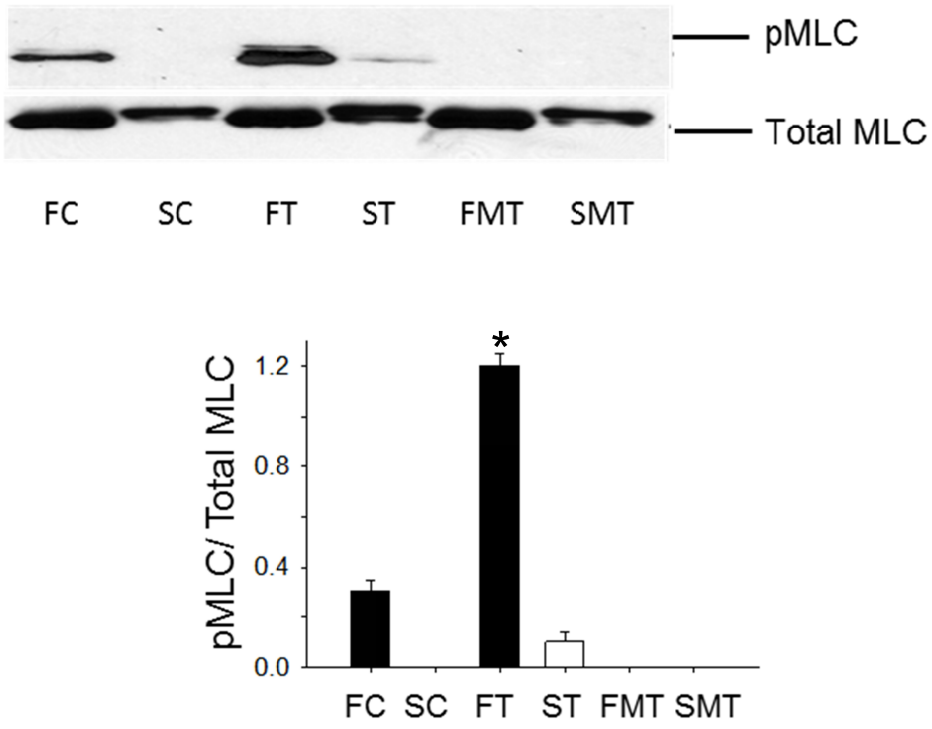

***Figure 3.9**: Inhibiting MEK abolishes the effects of DHT in isolated intact mammalian skeletal muscle fibres.*

Representative Western blots and summary data showing the effects of treating fast (F) and slow (S) twitch skeletal muscle fibre bundles isolated from female mice with the standard Ringer's solution (FC, SC), DHT alone (FT, ST) or DHT plus PD98059 (FMT, SMT) on the phosphorylation of regulatory myosin light chains (pMLC). Note that treating the muscle fibre bundles with DHT significantly ($p=0.01$;$p<0.05$) increases the phosphorylation of the regulatory myosin light chains in fast fibres. And that pre-treating the fibre bundles with PD89059 abolishes these effects.*

3.5 Discussion

A key finding in this study was the observation that treating fast- and slow twitch muscle fibre bundles isolated from adult female mice with physiological concentrations of DHT for 1hr increases the phosphorylation of ERK1 (p44) in both fibre types. Although similar results have previously been reported (see the study by Estrada et al. 2003), this the first time that DHT has been shown to have acute effects in mammalian skeletal muscle fibres. It is also the first time that any anabolic-androgenic steroid has been shown to have acute effects in adult mammalian skeletal muscle fibres. The study by Estrada and colleagues used cultured myocytes and the cells were treated with either T or nandrolone. In the present study, treatment of the muscle fibre bundles with T increased the phosphorylation of ERK1 in the slow-twitch fibre bundles only. Why T treatment has different effects in cultured myocytes and adult mammalian skeletal muscle fibres is uncertain. However, this might arise from the differences in concentration of T used in the two studies. The study by Estrada *et al.* (2003) used supraphysiological concentrations of T; whereas, physiological concentrations were used in the present study.

It noteworthy that although DHT treatment increased the phosphorylation of ERK1, it had no effect on the activation of the other MAPK modules, Thus, DHT treatment had no significant effect on phosphorylation of JNK (Fig. 3.3A) and p38 (Fig. 3.3B) in both fibre types. This finding is similar to those of other studies (Shi *et al.* 2007; Shi *et al.* 2008) and probably suggests that p38 and JNK might not be involved in skeletal muscle plasticity.

Another key finding in this study was all the observation that all fibre bundles expressed similar concentrations of phosphorylated/active ERK2 (p42) irrespective of whether they were treated or not and their fibre type composition. Moreover, the untreated fibre bundles expressed little or no phosphorylated ERK1 (p44) and only the DHT-treated muscle fibre bundles expressed this isoform in large quantities. This observation suggests that the two isoforms may have different roles in mammalian skeletal muscle fibres. My hypothesis is that ERK2 is essential for the survival of both the fast- and slow-twitch muscle fibres; whereas, ERK1 is involved in the regulation of muscle plasticity and is what is regulated by external stimuli. This hypothesis is supported by the findings of Saba-El-Leil and co-workers who showed that mice lacking ERK2 died inutero (Saba-El-Leil *et al*. 2003). On the other hand, mice deficient in ERK1 were viable and of normal size (Pages *et al.* 1999). Taken together these findings suggest that ERK2 maybe essential for the development and maintenance of all cells in the body; whereas, ERK1 is what is transduced and regulated by environmental factors

including mitogens and growth factors. Indeed, in ERK1 knockout mice, thymocyte maturation and differentiation was severely reduced suggesting that ERK1 may be essential for cell differentiation (Pages et al. 1999).

As mentioned above, treatment of the muscle fibre bundles with physiological concentrations of DHT increased the phosphorylation/activation of ERK1 in both fibre types; whereas, treatment with T increased its phosphorylation in the slow-twitch fibre bundles only (see Fig. 3.2). There are two interpretations of these findings; (1) DHT is the more potent anabolic-androgenic steroid in adult mammalian skeletal muscles. Indeed, DHT has been shown to have higher receptor binding kinetics than T (Bruchovsky & Wilson, 1968). (2) DHT is the only anabolic-androgenic steroid that has acute effects in adult mammalian skeletal muscle fibres. Therefore, for T to exert its effects it has to be converted in to DHT first. This process requires time and therefore, can account for the increase in the phosphorylated ERK1 in slow-twitch fibres. However, whether slow-twitch fibres have higher concentrations of 5α-reductase (the enzyme that converts T into DHT) that fast-twitch fibres is uncertain and further studies are needed.

Another important result from this study is the observation that treatment of the muscle fibre bundles with DHT increases the phosphorylation of RSK1/2 in both fibre types and that of the 20kDa myosin light chains in the fast-twitch fibre bundles only. On the other hand, DHT had no effect on the phosphorylation of MSK1/2. RSK 1/2 is one of the downstream effector molecules of ERK1/2; the other being MSK1/2 (Blenis, 1993). RSK has two kinase domains, a linker region, C- and N-terminal tails and the Arg-X-X-Ser consensus N terminal tail sequence. It is activated upon sequential phosphorylation by ERK (Zhao *et al.* 1996; Frodin & Gemmeltoft, 1999) and its activation is believed to promote cell growth and survival (Roux *et al.* 2007) and therefore, its activation may be important in skeletal muscle fibre hypertrophy. Indeed, one of the certain outcomes of long-term administration of anabolic-androgenic to both humans and rodents is an increase in skeletal muscle mass.

As mentioned above, in addition to its effects on RSK1/2, DHT treatment also increases the phosphorylation of the 20kDa regulatory myosin light chains (RMLCs). In non-muscle cells, the phosphorylation these light chains is essential for cell motility to occur (Iwabu *et al.* 2004). Additionally, mutation of the phosphorylation sites on these myosin light chains impairs muscle function in *Drosophila melanogaster;* suggesting that phosphorylation of these chains might be important in force production (Tohtong *et al.* 1995). Indeed, recent studies from this laboratory (Hamdi & Mutungi, 2010) and other previous studies (see

review by Sweeny *et al.* 1993) suggest that phosphorylation of these light chains may be important in the regulation of force production in adult mammalian skeletal muscle fibres.

Another important finding from this study was the observation that all the above effects of DHT could be abolished by pre-treating the muscle fibres with the MEK inhibitor PD98059; suggesting that they were all mediated via the MAPK pathway.

In summary, the results I present here show that one of the physiological functions of the acute effects of DHT in adult mammalian skeletal muscle fibres is to regulate the phosphorylation of the 20kDa RMLCs. These effects are mediated through the MAPK pathway and involve an increase in the phosphorylation ERK1 and RSK1/2 (see Fig. 3.10). From these results I suggest that one of the physiological actions of DHT is to modulate force production in adult mammalian skeletal muscles by regulating the phosphorylation of the 20kDa RMLCs by myosin light chain kinase.

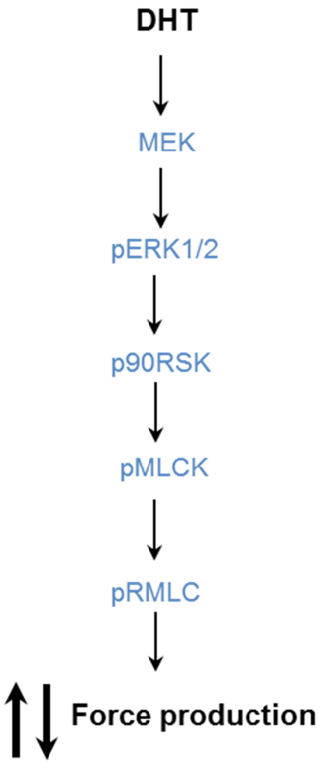

***Figure 3.10**: A schematic diagram summarising the non-genomic effects of DHT in isolated intact adult skeletal muscle fibre bundles. Treatment of the fibre bundles with physiological concentrations of DHT but not T activates the ERK pathway leading to the phosphorylation the 20kDa RMLCs which in turn regulates force production.* Note that force can either increase or decrease depending on the fibre type.

CHAPTER 4

EFFECTS OF DHT AND T ON THE PHOSPHORYLATION OF AKT

4.1 Introduction

As stated in 1.6.1, the prolonged administration of testosterone (T) or any of its many synthetic analogues increases skeletal muscle mass and decreases fat mass in both humans (Brodsky *et al.*1996; Bhasin *et al.* 2001; Ferrando *et al.* 2002) and rodents (Exner *et al.* 1973). Although this has been a constant finding in most previous studies (Brodsky *et al.*1996; Bhasin *et al.* 2001 Ferrando *et al.* 2002), little is known about the cellular signalling pathway(s) involved.

After the post-natal period, skeletal muscle mass increases mainly as a result of an increase in the size rather than the number of skeletal muscle fibres (Sandri, 2008). Furthermore, in most tissues including skeletal muscle, cell size is generally believed to be regulated by the protein kinase B (also known as Akt)/mammalian target of rapamycin (mTOR) pathway (Glass, 2003).

Protein kinase B (PKB) (or Akt) is a serine threonine kinase that occurs in three different isoforms, namely Akt-1 (PKB-α), Akt-2 (PKB-β) and Akt-3 (PKB-γ), that are encoded by different genes (Coffer *et al.* 1998). However, their protein sequences are closely related and show an 80% homology. All three isoforms have a pleckstrin homology, PH, domain that binds phospholipids and at least one phosphorylation site (Coffer *et al.* 1998). Akt-1 is

phosphorylated at Threonine 308 and Serine 473; Akt-2 is phosphorylated at Threonine 309 and Serine 474, while Akt-3 is phosphorylated at Threonine 302 and Serine 472 (Kandel & Hay, 1999). Akt-1 is found in many tissues including skeletal muscle (Coffer *et al.* 1998; Cleasby *et al.* 2007), Akt-2 is highly abundant in embryonic brown fat (Altomare *et al.* 1998) and Akt-3 is expressed mainly in breast and prostate tissue (Nakatani et al 1999). Since they have a high homology all three isoforms are capable of phosphorylating the same substrates (Kandel & Hay, 1999).

As stated in chapter 1 (section 1.7.4.1), the binding of growth factors to the tyrosine kinase receptor IGF-1R recruits insulin receptor substrate (IRS). PI3 Kinase is activated by binding of its p85 regulatory subunit to IRS, which in turn increases the activity of its p110 catalytic domain. Upon activation, PI3 Kinase phosphorylates phosphoinositides at the 3-position of the inositol ring generating phosphatidyl (3) phosphate (PIP); phosphatidyl (3,4) diphosphate (PIP_2) and phosphatidyl (3,4,5) triphosphate (PIP_3). PIP_3 binds the PH domain of Akt and this brings it into close proximity with the PIP_3-dependant protein kinase 1 (PDK1) at the plasma membrane. PDK1 then phosphorylates Akt at Threonine 308 and PDK2 at Serine 473 for full activation (Belham *et al.* 1999). The PI3 Kinase signal is then attenuated by the phosphatase, phosphate and tensin homolog on chromosome 10 (PTEN) which dephosphorylate PI3 Kinase. Once phosphorylated, Akt then translocates from the cytosol into the nucleus where it phosphorylates its downstream signalling

molecules such as mTOR leading to the regulation of protein synthesis and hence cell size (Richardson *et al.* 2004).

Therefore, an increase in skeletal muscle mass, as occurs following the chronic administration of T, must be accompanied by an increase in skeletal muscle fibre size and/or protein synthesis or a decrease in protein degradation; and for this to occur I hypothesised that T must lead to the activation of the Akt/mTOR pathway. So far, no studies have ever investigated the acute/non-genomic effects of DHT and T on the activation of Akt.

4.2. **Aim of this chapter**

Therefore, the primary aim of this study was to investigate the acute/non-genomic effects of treating small skeletal muscle fibre bundles with physiological levels of DHT and T on the phosphorylation of Akt.

4.3 **Materials and methods**

4.3.1 **Treatment of the fibre bundles with DHT and T**

The general materials and methods used in study are similar to those described in detail in Chapter 2. Therefore, they will only be briefly mentioned here. All the experiments reported here were performed at room temperature (~20°C) using small muscle fibre bundles isolated from either the EDL (a fast-twitch muscle) or soleus (a slow-twitch muscle) of female adult CD1 mice prepared as described in section 2.2. The fibre bundles were mounted

horizontally between two stainless steel hooks in purpose built muscle chambers (internal measurement 20mm width x 67mm length x 26mm depth) filled with the standard Ringer's solution. They were then divided into two equal groups. Half were treated for 1hour with the standard Ringer's solution plus 1.07mM of the vehicle (absolute ethanol) used to dissolve DHT and T. Proteins extracted from these fibres acted as controls. The other half was treated for the same period of time (1hour) with the Ringer's solution containing 630pgml^{-1} of DHT (2.16nM DHT) or T (1.82nM T). Proteins extracted from these fibre bundles acted as the experimental/treated samples. During the experiments, the Ringer's solution was continuously bubbled with medical gas (95%O_2, 5%CO_2). Additionally, every 10 minutes, the Ringer's solution was completely replaced and the fibre bundles were electrically stimulated to elicit a series of twitch contractions.

To determine whether the effects of Akt were time dependent, some of the fibre bundles were treated with DHT for 3hours (3hrs). In one experiment, the fibre bundles were stimulated every 10 minutes with a short burst of twitch contractions (intermittent); whereas, in another they were subjected to twitch contractions every 2 minutes (continuous).

To determine whether the Akt pathway was intact and functional, another set of fibre bundles was treated for 1hr with Ringer's solution containing 300ηM insulin (Sigma-Aldrich, UK).

4.3.2 **Immunoblotting**

At the end of the experiments described above, proteins were extracted and separated as described in chapter 2 sections 2.6 and 2.9. They were then transferred on to PVDF membranes and blocked as described in sections 2.10 and 2.11. The membranes were then incubated overnight with the antibodies listed in table 4.1 and the following day they were incubated with species-specific secondary antibodies conjugated to horse-radish peroxidase for 1hour at room temperature. They were then developed using ECL substrate and exposure of the membranes to film. On the third day, the membranes were stripped and re-probed for either total Akt (Santa Cruz Biotechnology, USA) or actin (Abcam, Cambridge England). These blots acted as loading controls.

***Table 4.1**: A list of the antibodies used to probe the various proteins described in this chapter*

ANTIBODIES/PEPTIDES			SOURCE
Antigens	Description	Dilution	
Akt phosphorylated at Threonine 308	Rabbit polyclonal antibody	1:1000	Cell signaling technology Inc. (Beverly, USA)
Akt phosphorylated at Serine 473	Rabbit polyclonal antibody	1:1000	Cell signaling technology Inc. (Beverly, USA)
Total Akt 1/2/3	Rabbit polyclonal antibody	1:1000	SantaCruz Biotechnology Inc. (USA).
Goat anti-rabbit	IgG-HRP	1:1000	SantaCruz Biotechnology Inc. (USA).
Goat anti mouse	IgG-HRP	1:1000	SantaCruz Biotechnology Inc. (USA).

4.3.3 **Data handling and analysis**

All the Western blots were run in triplicate and each experiment was repeated at least three times. The Western blots were digitised as described in section 2.15.1 and the intensity of each protein band was analysed using Scion® image software from NIH. The intensity of the bands of interest were then normalised to those of the corresponding total protein or actin in the same sample and are presented as mean ± S.D. Statistical analysis of the data performed using SigmaPlot® 11.2 (Systat Software Inc., London, UK). The data obtained from the two fibre types under control and the various experimental conditions were then compared using a three way ANOVA and a $p<0.05$ was considered statistically significant.

4.4 Result

4.4.1 Effects of DHT and T on the phosphorylation of Akt

The basic observations in this study are displayed in Fig. 4.1. As the results show, incubating the fast- and slow-twitch muscle fibre bundles in Ringer's solution containing physiological levels of DHT or T for 1hour completely abolished the phosphorylation of Akt at Thr308 (Fig.4.1). Similar observations were made when the effects of DHT and T on the pAkt-Ser473 were examined (Fig. 4.2). Thus, phosphorylated Akt was observed only in the untreated fibre bundles and was higher in the slow-twitch fibre bundles than in the fast-twitch ones.

Although the Western blots shown were obtained using an antibody from Cell Signalling Technology, similar results were obtained when the experiments were repeated using antibodies from Santa Cruz Biotechnology and Epitomics.

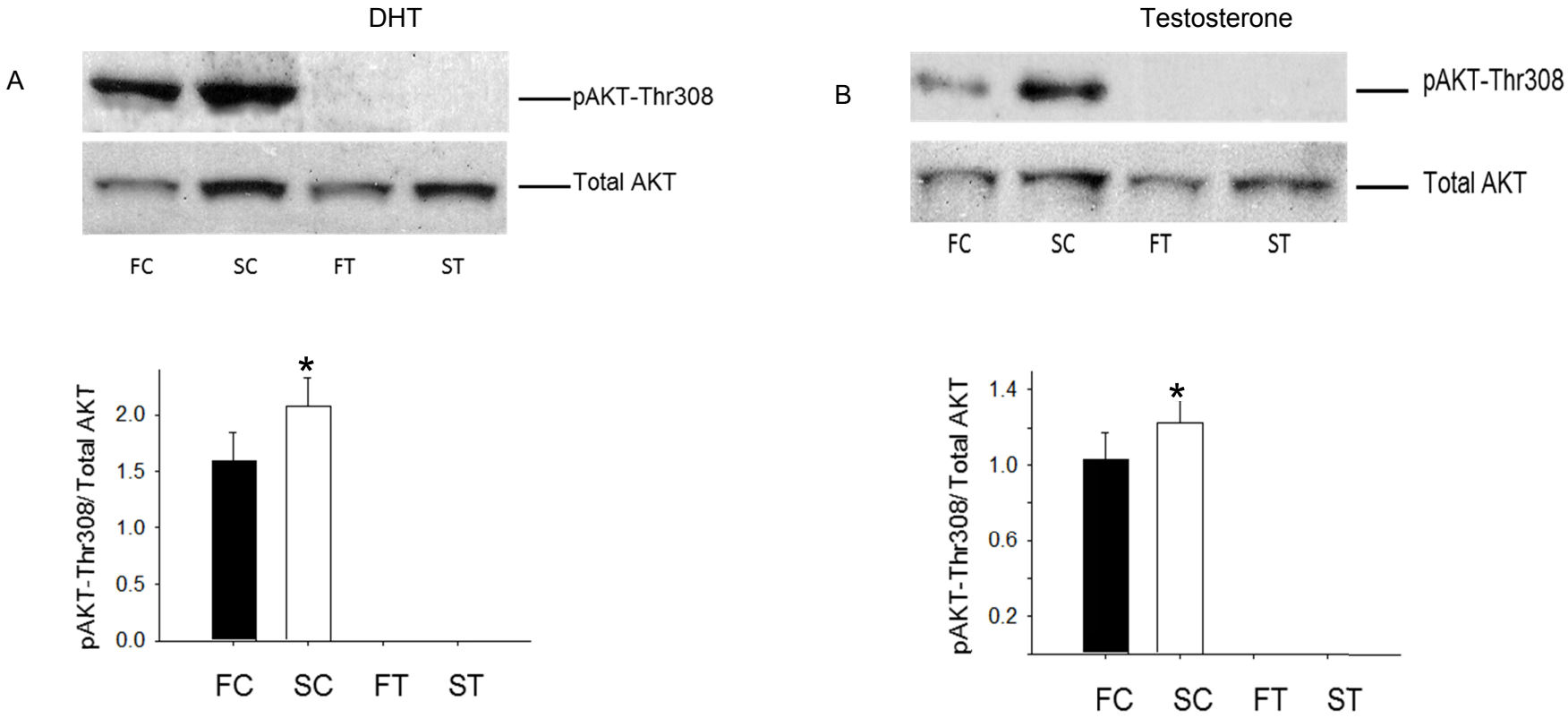

***Figure 4.1**: DHT and T abolish the phosphorylation of Akt at Threonine 308* .

Representative Western blots showing the effects of treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) muscle fibre bundles with DHT (A, FT, ST) and T (B, FT, ST) on the phosphorylation of Akt at Threonine 308 (pAKT-Thr308) probed using rabbit polyclonal antibody. Note that the antibody identified only one protein band at ~60KDa. Furthermore, treatment of the fibre bundles with DHT and T abolished the phosphorylation of Akt in both fibre types. The bar graphs show the intensity of the phosphorylated Akt bands normalised to that of total Akt in the same samples.

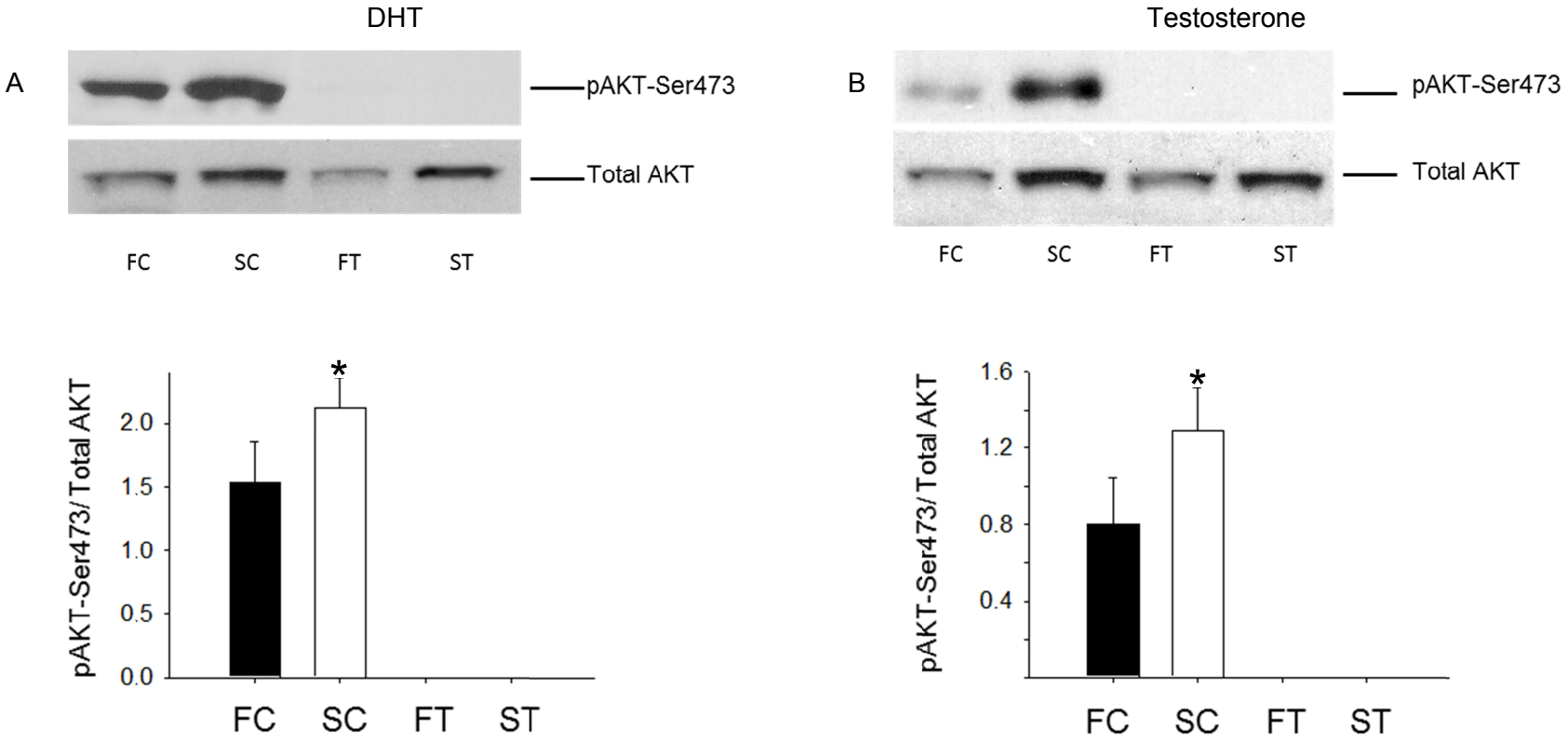

***Figure 4.2**: DHT and T abolish the phosphorylation of Akt at Serine 473*

Representative Western blots showing the effects of treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) muscle fibre bundles with DHT (A, FT, ST) and T (B, FT, ST) on the phosphorylation of Akt at serine 473 (pAKT-Ser473) probed using a rabbit polyclonal antibody. Note that the antibody identified only a single protein band at ~60KDa. Furthermore, treatment of the fibre bundles with DHT and T abolished the phosphorylation of Akt in both fibre types. The bar graphs show the intensity of the bands normalised to that of total Akt in the same samples.

4.4.2 **Comparison of Akt phosphorylation in DHT and T treated female muscle fibre bundles with untreated male muscle fibre bundles**.

As mentioned in Chapter 3, T and DHT are male sex steroids and their concentrations are higher in males than in females. Therefore, in another experiment, I examined whether the phosphorylation of Akt induced by DHT and T and that found naturally in male mice were similar.

As the results displayed in Fig.4.3 show, the concentration of phosphorylated Akt was always higher in the untreated male muscle fibre bundles than in the female ones. Moreover, treating the female mice muscle fibre bundles with DHT had the opposite effects on the phosphorylation of Akt to those I expected i.e. it reduced rather than increased its phosphorylation (similar observations were made when the phosphorylation of Akt at Ser473 was examined, result not shown).

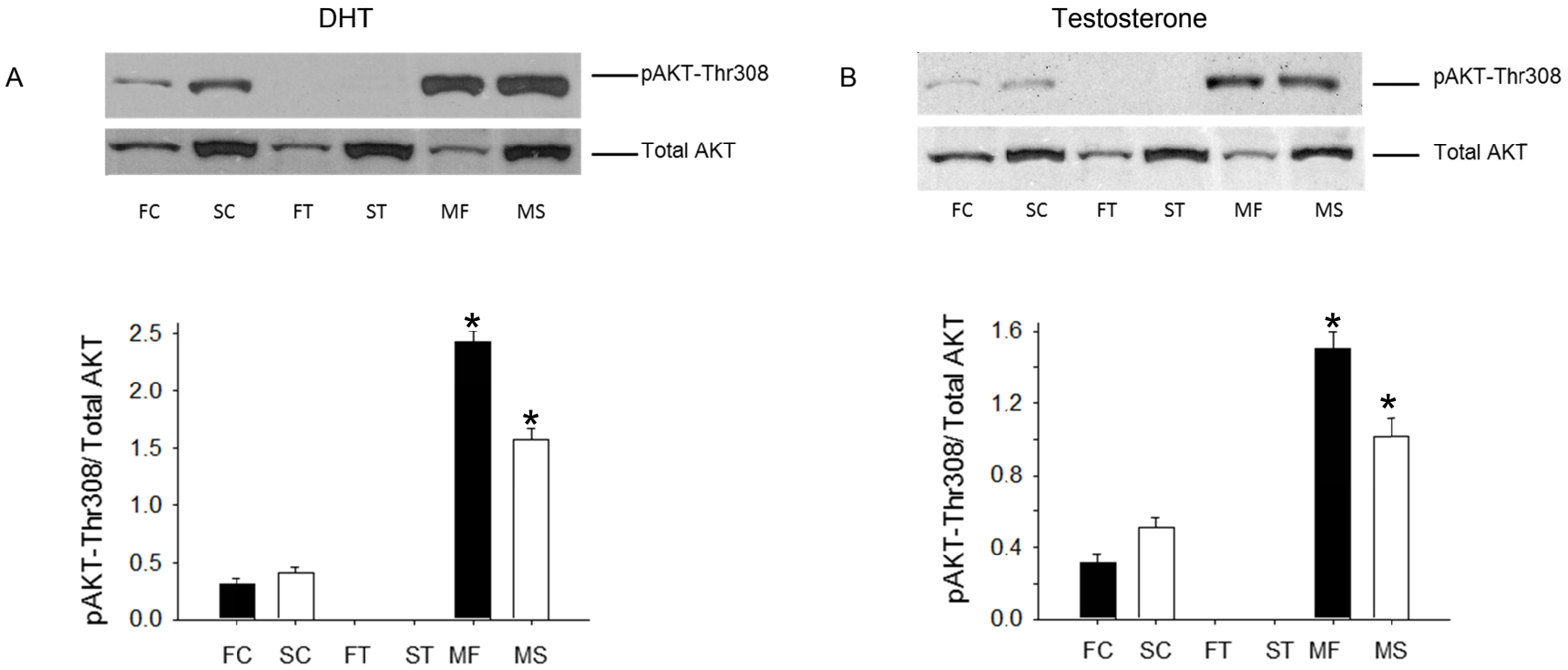

***Figure 4.3**: Male and female muscles express different levels of Akt phosphorylated at Threonine 308*

Representative Western blots showing the effects of treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) muscle fibre bundles with the standard Ringer's solution (FC, SC, MF, MS), DHT (A, FT, ST) and T (B, FT, ST) on the phosphorylation of Akt at threonine 308 (pAKT-Thr308) probed using a rabbit polyclonal antibody. Note that the muscles fibre bundles isolated from male mice (MF, MS) always expressed higher concentrations of pAkt308 than the corresponding female fibre bundles. Furthermore, treatment of the fibre bundles with DHT and T abolished the phosphorylation of Akt in both fibre types. The bar graphs show the intensity of the phosphor Akt bands normalised to those of total Akt in the same samples.

4.4.3 **Effects of Insulin on the phosphorylation of Akt in mouse skeletal muscle fibre bundles**

As DHT and T treatment abolished the phosphorylation of Akt at both threonine 308 and serine 473, I decided to investigate whether this pathway was intact and fully functional. To do this, I treated another set of fibre bundles with 300nM insulin. Insulin-like growth factor (IGF)1 is a potent stimulus of the PI3K/Akt pathway (Alessi *et al.* 1996) and can be mimicked by insulin (Alessi *et al.* 1996). As the results displayed in Fig.4.4 show, treatment of the muscle fibre bundles with insulin increased the phosphorylation of Akt in both fibre types. Furthermore, the insulin-treated female muscles and the untreated male muscles this time expressed similar concentrations of phosphorylated Akt.

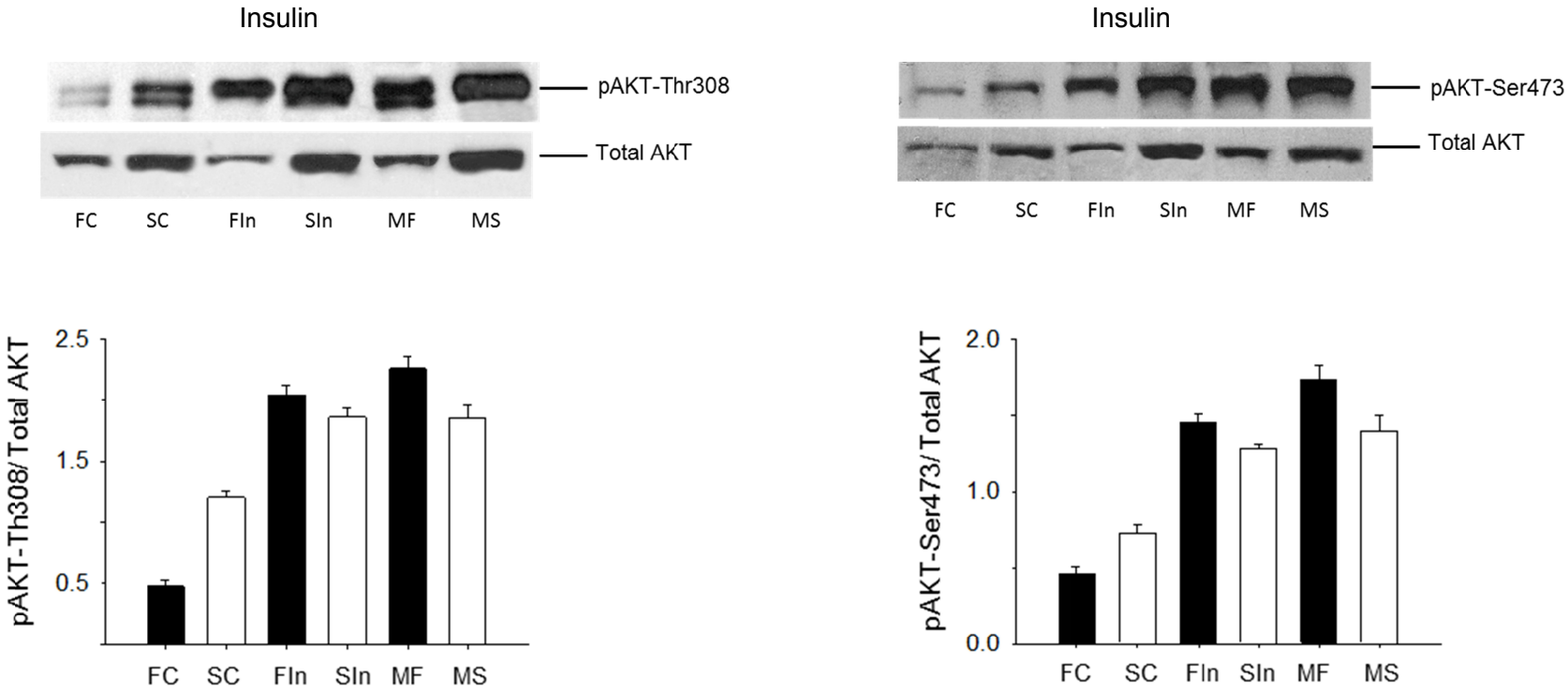

***Figure 4.4**: Insulin increases the phosphorylation of Akt at Thr308 and Ser473 in skeletal muscle fibre bundles isolated from female mice.*

Representative Western blots showing the effects of treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) muscle fibre bundles with the standard Ringer's solution (FC, SC, MF, MS) or the Ringer's solution plus 300ηM insulin on the phosphorylation of Akt at threonine 308 (Thr-308) and serine 473 (pAKT-Ser473). Note that treatment of the fibre bundles with insulin increases the phosphorylation of Akt at Thr308 and Ser473 in both fibre types. Furthermore, the male muscles fibre bundles (MF, MS) and the insulin treated (FIn, SIn) muscle fibre bundle, express similar concentrations of Akt phosphorylated at both sites. The bar graphs show the intensity of the pAkt bands normalised to those of total Akt in the same samples.

4.4.4 **Effects of treating skeletal muscle fibre bundles with DHT for 3hrs on the phosphorylation of Akt**

As shown above, muscle fibres isolated from untreated male mice always expressed higher concentrations of pAkt than the corresponding untreated fibres from female mice. To me this suggested that the effects of DHT on the phosphorylation of Akt may be time dependent. Therefore, in another experiment I tested this hypothesis. As the results displayed in figures 4.5 and 4.6 show, perfusing the fibre with DHT for three hours increased the phosphorylation of Akt at both Thr308 and Ser473 irrespective of whether the fibres were electrically stimulated continuously (ST-Con) or intermittently (FT-Inter).

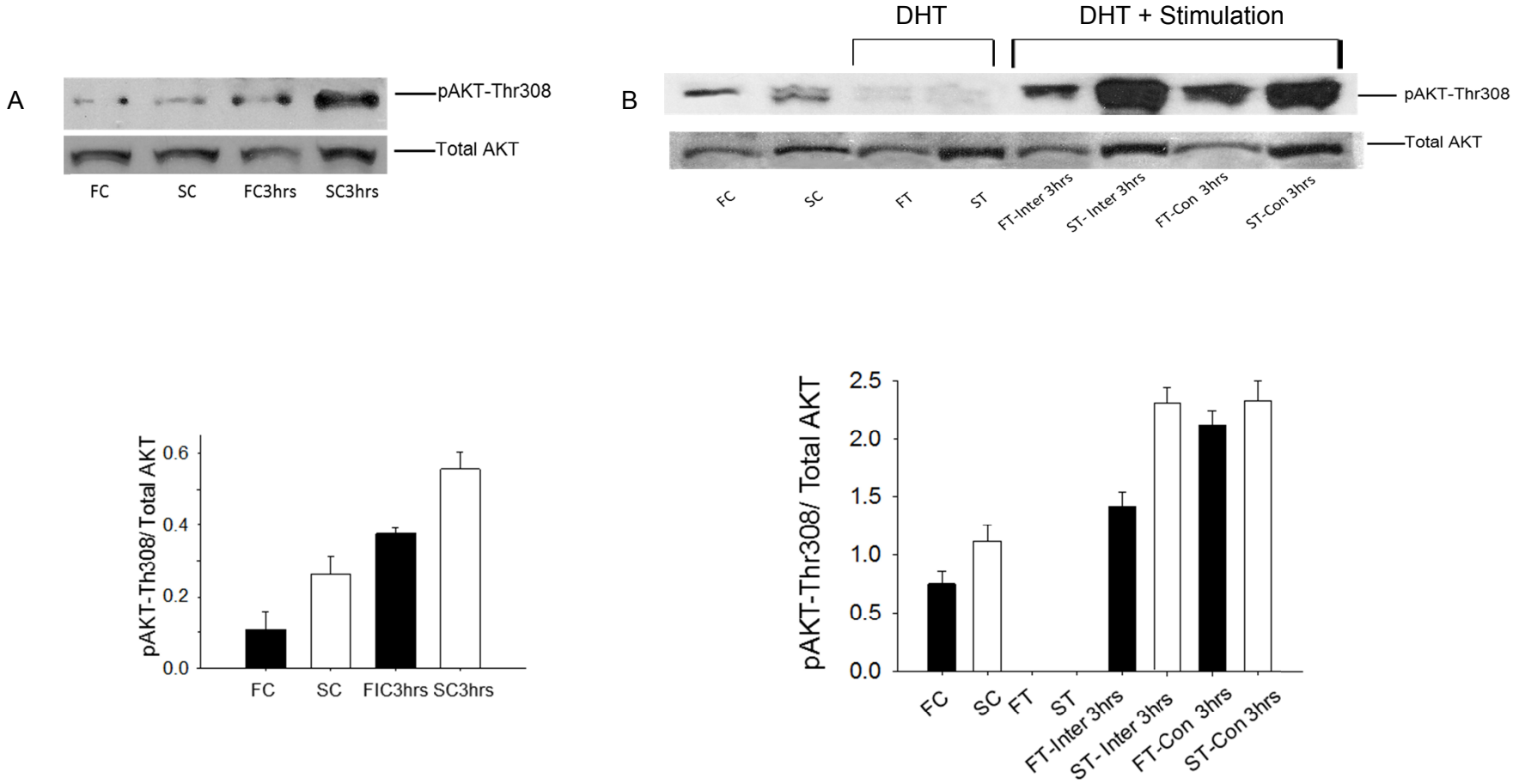

***Figure 4.5**: Treatment of the muscle fibre bundles with DHT for 3hrs increases the phosphorylation of Akt at Thr308 in both fibre types*

Representative Western blots showing the effects of treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) muscle fibre bundles with the standard Ringer's solution (B, FC, SC) or the standard Ringer's solution plus 630pgml^{-1} DHT for 3hrs on the phosphorylation of Akt at threonine 308 (Thr-308). Note that treatment of the fibre bundles with DHT for 3hours increases the phosphorylation of Akt at Thr308 in both fibre types irrespective of whether the fibre bundles were subjected to intermittent (B, FT-inter 3hrs, ST-inter 3hrs) or continuous (B, FT-Con 3hrs, ST-Con 3hrs) twitch stimulation. Also note that (A, FC, SC) and (A, FC3hrs, SC3hrs) are untreated control in standard Ringer's solution for 1hour and 3hours respectively.

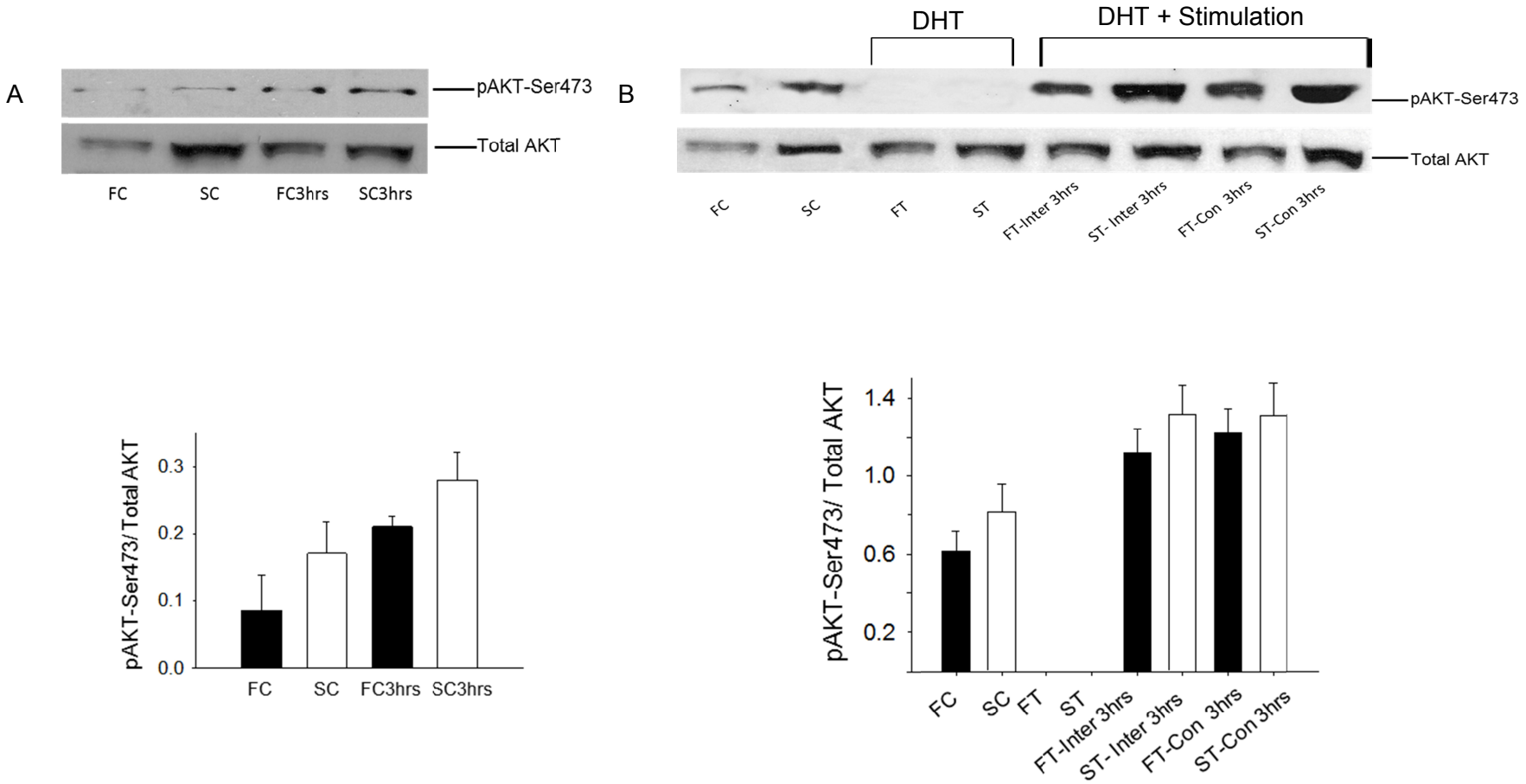

***Figure 4.6**: Treatment of the muscle fibre bundles with DHT for 3hrs increases the phosphorylation of Akt at Ser473 in both fibre types*

Representative Western blots showing the effects of treating fast-twitch (F, filled bars) and slow-twitch (S, clear bars) muscle fibre bundles with the standard Ringer's solution (B, FC, SC) or the standard Ringer's solution plus 630pgml^{-1} DHT for 3hrs on the phosphorylation of Akt at Serine 473 (Ser-473). Note that treatment of the fibre bundles with DHT for 3hours increases the phosphorylation of Akt at Ser473 in both fibre types irrespective of whether the fibre bundles were subjected to intermittent (B, FT-inter 3hrs, ST-inter 3hrs) or continuous (B, FT-Con 3hrs, ST-Con 3hrs) twitch stimulation. Also note that (A, FC, SC) and (A, FC3hrs, SC3hrs) are untreated control in standard Ringer's solution for 1hour and 3hours respectively.

4.5 **Discussion**

As stated in section 4.1 above, the prolonged administration of anabolic-androgenic steroids always increases skeletal muscle mass (Exner *et al.* 1973; Bhasin *et al.* 2001; Ferrando *et al.* 2002). Therefore, when I started performing these experiments I expected DHT and T to increase the phosphorylation of Akt at both Thr308 and Ser473. However, as results presented in Figs. 4.1 and 4.2 show, treatment of the muscle fibre bundles with Ringer's solution containing physiological levels of DHT and T, for 1hour, completely abolished the phosphorylation in both fibre types (Fig. 4.1). Contrary to my expectations, these findings suggest that one of the immediate/early effects of DHT and T treatment in mammalian skeletal muscle fibres is to switch off the Akt/mTOR pathway and to

switch on the MAPK pathway. Although this is the first time the acute effects of DHT and T on the activation of this pathway in adult mammalian skeletal muscle fibres have been investigated, treating castrated mice with T also switches off the genes involved in Akt pathway (Haren *et al.* 2011), As suggested in the previous chapter, one of the effects of the MAPK pathway in these fibres is to initiate the conversion of slow-twitch fibres into fast-twitch fibres. Therefore, switching off the Akt pathway allows the changes initiated by activation of the MAPK to occur first. However, why and how DHT and T switch off this pathway is unknown and further studies are needed.

Initially, when I made the above observations I thought that there may be a problem with the antibodies or the fibres. However, as the results displayed in Fig. 4.4 show, treatment of the fibre bundles with insulin led to a marked increase in the phosphorylation of Akt. Similar observations were also made when the fibre bundles were treated with DHT for 3hrs (see Figs. 4.5 and 4.6); suggesting that the pathway was intact and the fibre bundles were working properly. Therefore, from these findings it seems as if the effects of DHT on the Akt pathway are time dependent. However, further studies are needed to confirm this.

Another key finding in this study was the observation that the untreated male muscle always expressed higher concentrations of active Akt than the corresponding female muscle fibre bundles. Here, I postulate that the higher concentrations of active Akt in the

male muscles than in the female ones may be the reasons why males have bigger muscles than females. However, this is the first time that effects of gender on the phosphorylation of Akt have been investigated and more research is needed to ascertain this.

In summary, the results I presented here show that the acute/non-genomic effects of DHT on the phosphorylated of Akt are time dependent. Thus, DHT initially switches of the Akt pathway, when the MAPK pathway is active, and switches it on later. However further studies to confirm this and to investigate the mechanism(s) underlying this effect are necessary.

CHAPTER 5

THE NON-GENOMIC EFFECTS OF DHT ON THE TRANSPORT OF AMINO ACIDS IN ISOLATED INTACT FAST- AND SLOW TWITCH MAMMALIAN SKELETAL MUSCLE FIBRE BUNDLES

5.1 Introduction

In the body testosterone (T) is rapidly and irreversibly reduced into dihydrotestosterone (DHT) by 5α-reductase (Bruchovsky & Wilson, 1968). Although the free unbound concentrations of the two hormones are similar, it is generally believed that DHT is the more potent hormone because it has higher receptor binding kinetics than T (Bruchovsky & Wilson, 1968). However as the expression of 5α-reductase (5αRD) in adult mammalian skeletal muscle is controversial it has been suggested that DHT may not have physiological functions in this tissue (Thigpen *et al.* 1993). Nevertheless, in a recent study, we showed that DHT, but not T, can modulate force production in isolated intact mouse skeletal muscle fibres (Hamdi & Mutungi, 2010). Furthermore, as the data reported in Chapter 3 of this thesis show these effects were mediated through the MAPK pathway and involving the activation of ERK1. From these studies, it is clear that DHT has acute/non-genomic actions in adult mammalian skeletal muscle fibres (Hamdi & Mutungi, 2010). However, whether it has any other acute/non-genomic physiological functions in skeletal muscle is unknown.

In adult animals/humans, T and its derivatives commonly referred to as anabolic-androgenic steroids (AASs) are major determinants of body composition (Wilson, 1988; Bhasin et al. 2001). For example, treating adult female laboratory animals (Exner *et al.*1973), hypogonadal men (Brodsky *et al.* 1996), elderly individuals with serum low T level (Ferrando *et al.* 2002) and men suffering from AIDS (Bhasin *et al.* 2000) with AASs for several weeks has been shown to increase lean body mass. Although the mechanisms underlying the changes in lean body mass induced by AAS treatment are still poorly understood, it is generally thought that these compounds increase protein synthesis (Kochakian 1949; Ferrando *et al.* 2002) while at the same time decreasing its breakdown (Ferrando *et al.* 2002).

Skeletal muscle is the largest pool of essential amino acids in the body and these amino acids form up to 35% of its total muscle proteins (Harper *et al.* 1984). Therefore, for skeletal muscle to increase in mass, 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 the effects of AAS administration on amino acid uptake by skeletal muscles were unable to demonstrate any changes in amino acid uptake following the administration of T to human subjects (Bhasin *et al.* 1997; Ferrando *et al.*1998). Thereby leaving the source of the new amino acids unresolved.

Amino acids enter and leave cells through specialized transport systems 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 its physiological functions and the location of the transporters; suggesting that the expression and activity of these transporters may be carefully regulated (McGivan & Pastoranglada, 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 (Bevington *et al.* 2002; Franchi-Gazzola *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 muscles.

5.2 **Aims of this chapter**

Therefore, the primary aims of this study were to investigate the acute/non-genomic effects of DHT and T on;

(1) Amino acid uptake,

(2) The expression of the amino acid transporters SNAT2 and LAT2,

(3) The cellular signalling pathway(s) mediating these actions,

in fast- and slow-twitch muscle fibre bundles isolated from adult female mice.

5.3 **Materials and methods**

The details of the general material and methods used in this chapter are given in chapter 2 and will only be briefly mentioned in this chapter. All the experiments were performed at room temperature (~20°C) on small muscle fibre bundles isolated from the EDL and soleus muscles of adult female CD1 mice. The fibre bundles were mounted horizontally between two stainless steel hooks in a muscle chamber specially designed for these experiments. The chamber was made of perspex and had a glass bottom. Unlike the muscle chambers used in all the other experiments it was much smaller and had internal measurements of approximately 20mm width x 50mm length x 6mm depth and a total volume of ~5ml (Figure 5.1).

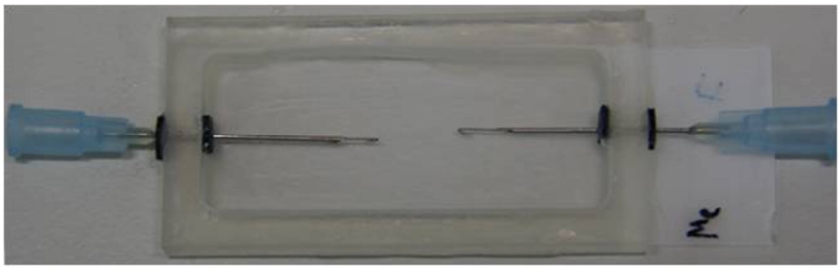

Figure 5.1*: A photograph showing one of the muscle chambers used in the amino acid uptake experiments*

5.3.1 **Determination of the effects of DHT and T on the uptake of isoleucine (Ile) and α-(Methylamino)isobutyric acid (MeAIB)**

To determine the uptake of the amino acids, 8 fast-twitch and 8 slow-twitch muscle fibre bundles, were divided into two groups. Half were treated with the standard Ringer's solution containing 6.3µl/100ml (1.07mM) ethanol (the vehicle used to dissolve both DHT and T) plus either 2mM C^{14} isoleucine (L-(U-^{14} C) Ile; radioactivity level 3.46µCiml^{-1}) (Doi *et al.* 2007) or 68.3µM C^{14} α-methylaminoisobutyric acid (α-(1-^{14}C) MeAIB; radioactivity level 0.8µCiml^{-1}) (Kashiwagi *et al.* 2009) (both purchased from PerkinElmer, Buckinghamshire, UK) for 1hour.

These preparations acted as controls. The other half were treated with the Ringer's solution containing 630pgml^{-1} DHT or testosterone propionate (T) (both from Sigma, Gillingham, Dorset, UK) plus the radio-labelled amino acids for the same duration (1hour). These fibre bundles acted as the experimental/treatment group. Radio-labelled 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 this experiment the fibre bundles were processed for liquid scintillation counting as described in section 5.3.4

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

To investigate the amino acid transporters mediating the effects of DHT on amino acid uptake observed in the preceding experiments, the following experiment was performed. The muscle fibre bundles were divided into four groups each containing 10 fast- and 10 slow-twitch fibre bundles. Each group was then treated for at least 20 minutes as follows; Group 1 (control) was treated with the standard Ringer's solution plus 1.07mM ethanol, Group 2 was treated with the Ringer's solution plus DHT alone, Group 3 was treated with the Ringer's solution containing either BCH or MeAIB alone plus 1.07mM ethanol; whereas, Group 4 was treated with the Ringer's solution containing DHT plus either 10mM BCH or 100μM MeAIB. At the end of this period, the solutions were then replaced with the same solutions but this time 2mM L-(U-^{14} C) Ile was added. The bundles were then incubated in these solutions for 1hour after which the uptake of Ile by the fibre bundles was determined as described in section 5.3.4

To determine the effects of DHT on the expression of LAT2 and SNAT2, another set of fibre bundles was treated with the standard Ringer's solution plus the vehicle (1.07mM ethanol) or the standard Ringer's solution plus DHT. Cytosolic and crude membrane proteins were then extracted and immunoblotted for the expression of SNAT2 and LAT2 as described below.

5.3.3 **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 described above, 10 fast- and 10 slow- twitch muscle fibres bundles were treated with the standard Ringer's solution plus the vehicle or the standard Ringer's solution plus DHT for 1hour. In some of the experiments, the fibre bundles were pre-incubated with the standard Ringer's solution plus PD98059 (a MEK specific inhibitor), deguelin (a PI3K/Akt inhibitor), rapamycin (an mTOR specific inhibitor), actinomycin D (a transcription inhibitor) or cyclohexamide (a translational inhibitor) for 20minutes. They were then treated with the Ringer's solution containing the inhibitor whose effects were being investigated plus DHT for 1hour (see table 5.1 for list of inhibitor). At the end of the experiments, the muscle fibre bundles were immunoblotted as described in section 5.3.6.

***Table 5.1**: A table showing details of the inhibitors used in the experiments described in this chapter*

PROTEIN/PROCESS	INHIBITORS			
	Scientific name	Common name	Concentration	Source
LAT2	2-Aminobicyclo[2,2,1]heptane-2-carboxylic acid	BCH	100μM	Sigma-Aldrich
SNAT2	2,N-Dimethylalanine,2 (Methylamino)-2-methylpropionic acid	MeAIB	10μM	Sigma-Aldrich
Transcription	Actinomycin IV	Actinomycin D	1μgml^{-1}	Sigma-Aldrich
Translation	3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide	Cyclohexamide	10μgml-1	Sigma-Aldrich
MAP kinase kinase (MEK)	2´-Amino-3´-methoxyflavone	PD98059	20μM	Alexis Biochemicals
AKT	(7aS,13aS)-13,13a-Dihydro-9,10-dimethoxy-3,3-dimethyl-3H-bis[1]benzopyrano[3,4-b:6′,5′-e]pyran-7(7aH)-one	Deguelin	100nM	Sigma-Aldrich
mTOR	23,27-Epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine	Rapamycin	100ngml -1	Sigma-Aldrich

5.3.4 **Liquid scintillation counting**

At the end of the experiments described above, the Ringer's solution was aspirated and the fibre bundles were washed twice in ice cold phosphate buffer saline (PBS). The excess buffer was blotted out and the bundles were then snap frozen in liquid nitrogen, pulverised and cytosolic proteins were extracted using the non-ionic cell lysis buffer NP40 as described in section 2.4. 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, Buckinghamshire, UK), in a capped plastic vial measuring 15mm in diameter x 55mm in height. The level of radioactivity in the mixture was then determined using a liquid scintillation counter (Tri-Carb 2250 CA, Canberra-Packard) 1hour and 24hours after the end of each experiment. Also, each experiment was repeated at least twice.

Carbon-14 is a long half-life, low energy (0.156MeV) β emitter that can be used to label amino acids including isoleucine. Using a radio scintillation counter, the radioactivity in the protein lysates can then be counted. Liquid scintillation counting is a common technique for measuring radiation emitted from β nuclide of the C-14 in biological samples suspended in a liquid scintillant cocktail (LSC) (Reid *et al.* 2000; Reid *et al.* 2001). As stated earlier, the lysate was mixed with LSC because the cocktail contains ethoxylated alkyphenol which serves as a surfactant enabling the aqueous lysate, in contact with the aromatic solvent, to form a stable micro emulsion (Stolzenberg *et*

*al.*1982). Sixty to 99% of the LSC is an aromatic solvent such as diisopropylnaphthalene (DIN) and serves as a source of high density π (pi) electron for efficient energy transfer (Birks, 1964). Phosphor or scintillants such as 2,5,-diphenyloxazole (PPO) and 1,4-bis(2-methylstyryl) benzene (bis-MSB), that constitute 0.3-1% of the LSC cocktail converts the captured energy into emitted light (Dianu *et al.* 2007). In theory, during the liquid scintillation counting, β emission from the muscle lysate transfers energy to the solvent molecules which in turn transfer their energy to the scintillant. The excited scintillant molecules dissipate the energy by emitting light. In this way, each β emission results in a pulse of light detected by the liquid scintillation counter (Birks, 1964).

Quenching is the loss of count due to the characteristics of the sample or LSC cocktail (Horrocks, 1974). In my study, Optima Gold XR® was used because it has a good quench resistance characteristic (Reid *et al.* 2001; Verrezen *et al.* 2008). Sometimes energy transfer such as heat and room light or sunlight can excite aromatic solvent molecules in the LSC, which causes unwanted background reading which in turn interfere with actual signal (Bruno & Christian, 1961). Therefore in my study the lysate was kept for 24 hours in the dark to minimise non-specific emission.

Two liquid scintillation counting machines a Tri-Carb 2250 CA (Packard Instrument Co, Meridien, USA) and a Canberra-Packard or Wallac 1409 DS (Perkin Elmer, Waltham, USA) were used in this study. Most of the experiments were performed using the Tri-Carb 2250 except when this was being used by others when the Wallac 1409 was used. Therefore, the performance of both machines was assessed by measuring radioactivity from four different samples FC (Fast control); FTesto (Fast testosterone); FCyp only (Fast Cyproterone only) and FCyp+DHT (Fast Cyproterone plus DHT).

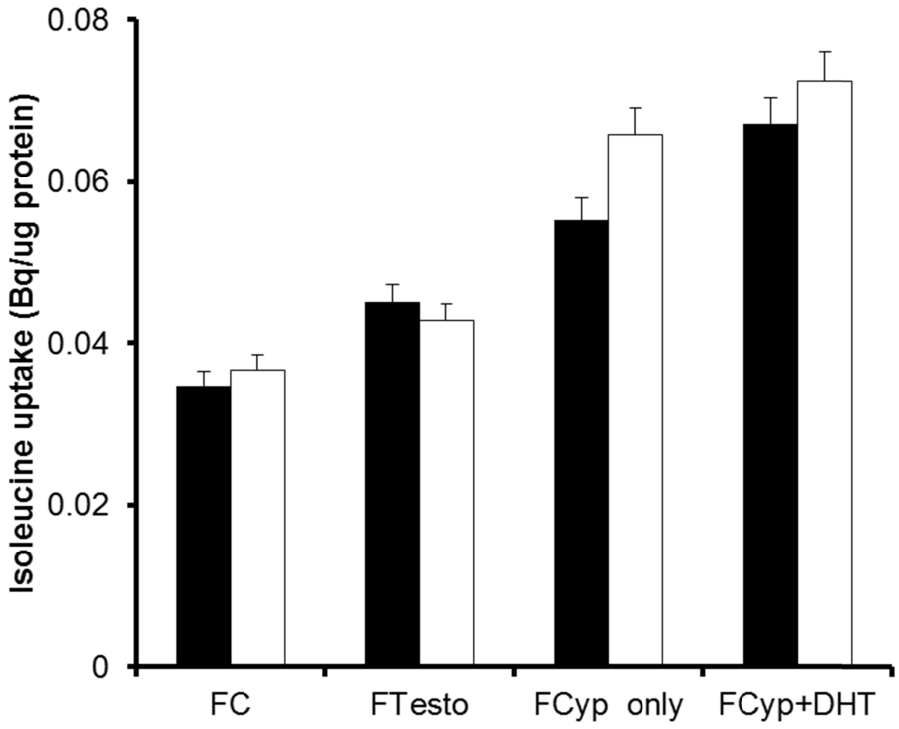

Figure 5.2: *There is no difference in the performance of the Wallac and Tri-Carb scintillation counters*

Bar graphs showing the radioactivity (Bq/µg protein) of four different samples FC (Fast control); FTesto (Fast Testosterone); FCyp only (Fast Cyproterone only) and FCyp+DHT (Fast Cyproterone plus DHT)) measured using the Wallac 1409 (filled bars) and the Tri-Carb 2250 (clear bars). Note that the measurements from the two machines were not statistically different (p>0.05;p=0.35) .

5.3.5 **Calibration**

In this study I used two radiolabeled amino acids; isoleucine and MeAIB that have never been used in the laboratory before. Therefore, my first test was to ensure that the concentrations I am using were within the linear range of the liquid scintillation machines. To do this I used 0, 2, 4, 6, 8, 16, 32, 40 and 50µl of -L-(U^{14}-C) isoleucine and α-(1-^{14}C)-MeAIB in 50mls of the standard Ringer's solution. The radioactivity of the samples was then measured using liquid scintillation counting and plotted as shown in Fig.5.3.

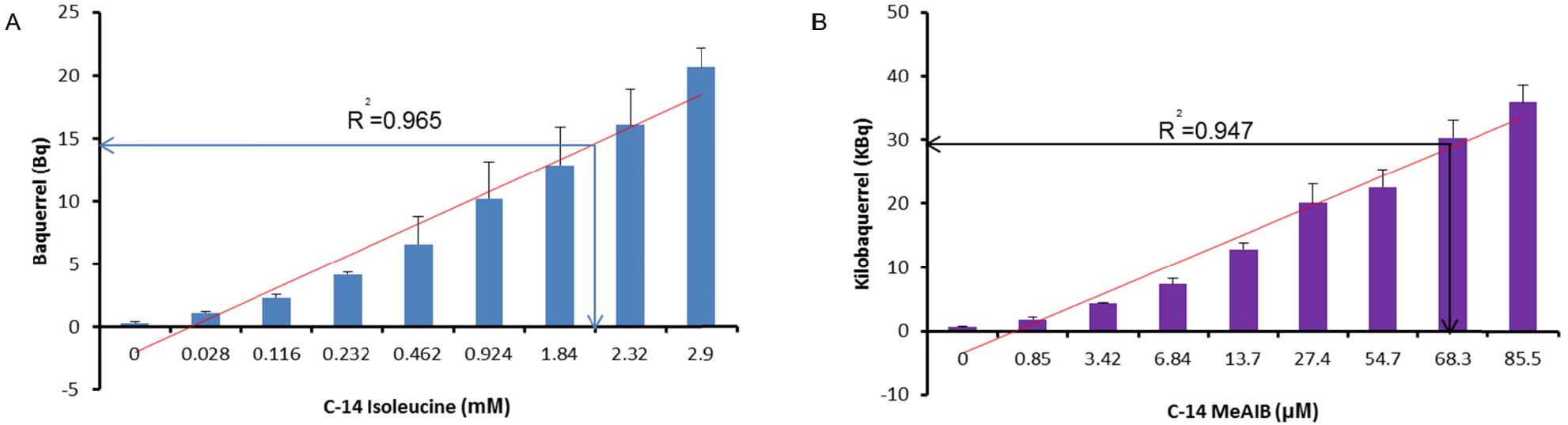

Figure 5.3: *Calibration of the liquid scintillation counter*

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Calibration curves showing the radioactivity (Bq) of pre-determined concentrations of L-(U^{14}-C) isoleucine (A) and MeAIB α-(1-^{14} C) measured using the liquid scintillation counter. Note that the radioactivity of the samples is almost linear. For example the R^2 for isoleucine was 0.965; whereas that of MeAIB was 0.947 for α-(1-^{14} C)-MeAIB (B), Note that the volume range for isoleucine are 0.39µl and 19.47µl and those of MeAIB are 6.80µl and 25.02µl respectively. Therefore, the liquid scintillation counting system is calibrated and the concentration of amino acids used in the study (2.07 – 8µl per 3ml) are within the detectable range of the machine

As in the results plotted in Fig. 5.3 show, the radioactivity of the samples was within the linear range of the machine. For example, R^2 for L-(U^{14}-C) isoleucine was 0.965; whereas, that for MeAIB was 0.947.

***Table 5.2**: List of the proteins and the antibodies used in this chapter.*

ANTIBODIES/PEPTIDES			SOURCE
Antigens	Description	Dilution	
SNAT2	Mouse monoclonal antibody	1:500	SantaCruz Biotechnology Inc. (USA).
LAT2	Rabbit polyclonal antibody	1:500	SantaCruz Biotechnology Inc. (USA).
CREB phosphorylated at Serine 133	Rabbit monoclonal antibody	1:500	Abcam (Cambridge, England)
Total cmyc	Mouse monoclonal antibody	1:500	SantaCruz Biotechnology Inc. (USA).
ATF2 phosphorylated at Threonine 71	Rabbit polyclonal antibody	1:500	Cell signaling technology Inc. (Beverly, USA)
eEF2 phosphorylated at Threonine 56 and Threonine 58	Rabbit monoclonal antibody	1:500	Abcam (Cambridge, England)
Actin	Mouse monoclonal antibody	1:1000	Abcam (Cambridge, England)

Goat anti-rabbit	IgG-HRP	1:1000	SantaCruz Biotechnology Inc. (USA).
Goat anti mouse	IgG-HRP	1:1000	SantaCruz Biotechnology Inc. (USA).

5.3.6 **Immunoblotting**

At the end of the experiments described above, the fibre bundles were snap frozen in liquid nitrogen, pulverized and cytosolic as well as crude membrane proteins were extracted using NP40 lysis buffer. To extract cytosolic proteins, the lysates were centrifuged for 10min at 15000rpm. The supernatant was then collected and stored at -80°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 the excess buffer. They were then snap frozen in liquid nitrogen, pulverized and proteins were extracted as described above. Equal amounts (10µg) 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 minutes. They were then incubated overnight at 4°C with primary antibodies against; L-type amino acid transporter 2 (LAT2), sodium neutral amino acid transporter 2 (SNAT2), phosphorylated CREB, cmyc, phosphorylated ATF2 and phosphorylated eEF2 (for details see Table 5.2). The following day they were washed and incubated for 1hour with species specific secondary antibodies conjugated with horse-radish peroxidase. Finally, they were visualised using SuperSignal WestPico (Perbio Science UK Ltd,

Cramlington, Northumberland) 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 or an antibody against the total (unphosphorylated) protein whose phosphorylation was being investigated. These blots were used as loading controls.

5.3.7 **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 (Bq/µg). The radioactivity was then normalized to that recorded under control conditions and all the data are presented as the mean percentage change in uptake ±S.D.

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 analyzed using Scion Image® from 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., London, UK). The data obtained from the two fibre types under control and from the various experimental conditions were then compared using a 3-way ANOVA and $p<0.05$ was considered statistically significant.

5.4 **Results**

5.4.1 **Effects of DHT and T on Isoleucine Uptake in adult mammalian skeletal muscle fibre bundles**

The basic observations from this study are shown Fig. 5.4. As the results show, incubating the fast- and slow-twitch muscle fibre bundles in Ringer's solution containing physiological levels of DHT for 1 hour significantly ($p<0.05$) increased the uptake of isoleucine (Ile)in the fast-twitch fibre bundles only. In contrast, treating the fibre bundles with similar concentrations of T did not significantly ($p>0.05$) increase the uptake of (Ile) in either fibre type. For example, treating the muscle fibre bundles with DHT led to an 88.83 ± 14.12% and to a 15.89 ± 4.55% increase in the uptake of Ile in the fast- and slow-twitch fibre bundles, respectively. On the other hand, treatment of the fibre bundles with T led to a 9.19 ± 9.67% increase in the uptake of Ile in the fast-twitch fibre bundles but to a 8.04 ± 5.67 % decrease in the uptake of Ile in the slow-twitch fibres.

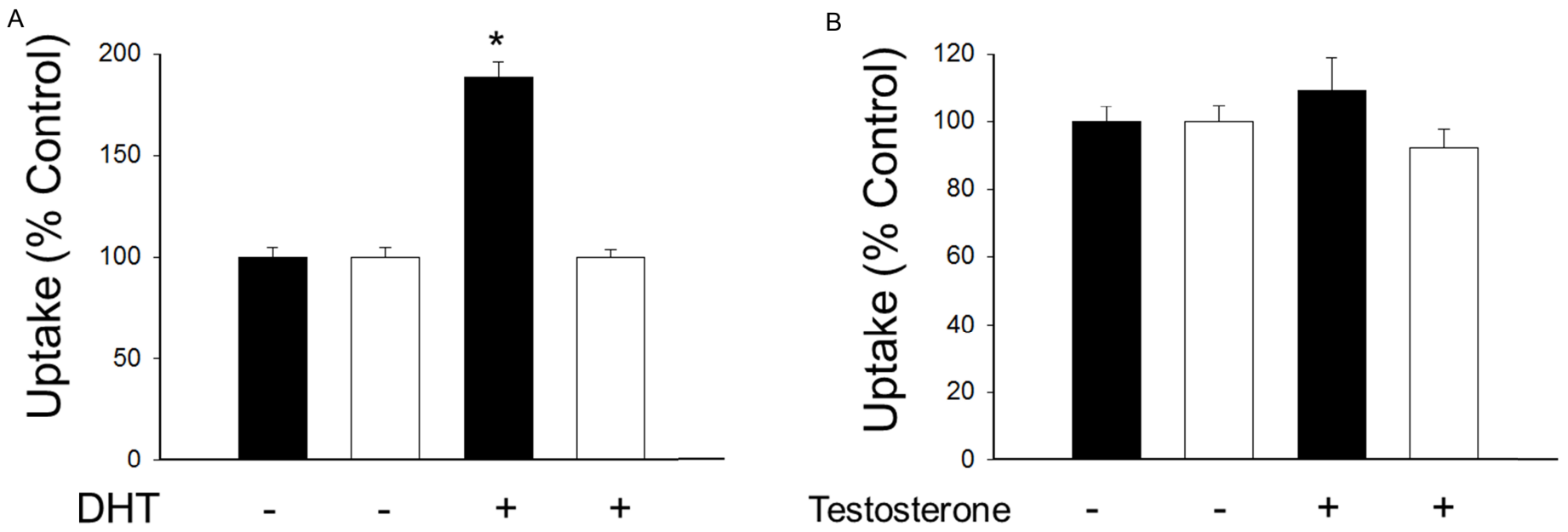


Figure 5.4: *DHT but not T increases isoleucine uptake in fast-twitch skeletal muscle fibre bundles*

Bar graphs showing the effects of treating fast- (filled bars) and slow- (clear bars) twitch fibre bundles with physiological levels of DHT (A) and T (B) on the uptake of L-(U-^{14} C) Ile. Note that treating the fibre bundles with DHT, but not T, significantly (p=0.001) increases the uptake of L-(U-^{14} C) Ile in the fast-twitch fibre bundles. In contrast, DHT had little or no effect on the uptake of L-(U-^{14} C) Ile in the slow-twitch bundles. In contrast, T leads to a slight decrease in the uptake of L-(U-^{14} C) Ile in these fibre bundles. p<0.05 when compared to control.*

5.4.2 **Effects of DHT and T on α-(Methylamino)isobutyric acid uptake in adult mammalian skeletal muscle fibre bundles**

The results in figure 5.5 show, the effects of treating the muscle fibre bundles with DHT (A) and T (B) on the uptake of MeAIB. As the results show, treating the fibre bundles with DHT significantly ($p<0.05$) increased the uptake of MeAIB in the fast-twich fibre bundles only. In contrast, treating the fibre bundles with T did not significantly ($p>0.05$ increase the uptake of MeAIB in either fibre type. Thus, treating the fibre bundles with DHT led to a 36.89 ± 7.86% increase in the uptake of α-(1-^{14} C)-MeAIB in the fast-twitch fibre bundles and to a 17.09 ± 4.05% increase in its uptake in the slow-twitch fibre bundles (Fig. 5.5A). In contrast, treating the fibre bundles with T led to a 8.98 ± 4.79 % increase in α-(1-^{14} C)-MeAIB uptake in fast twitch fibre bundles and to a 9.48 ± 5.65 % decrease in α-(1-^{14} C)-MeAIB uptake in slow-twitch fibre bundles (Fig. 5.5B).

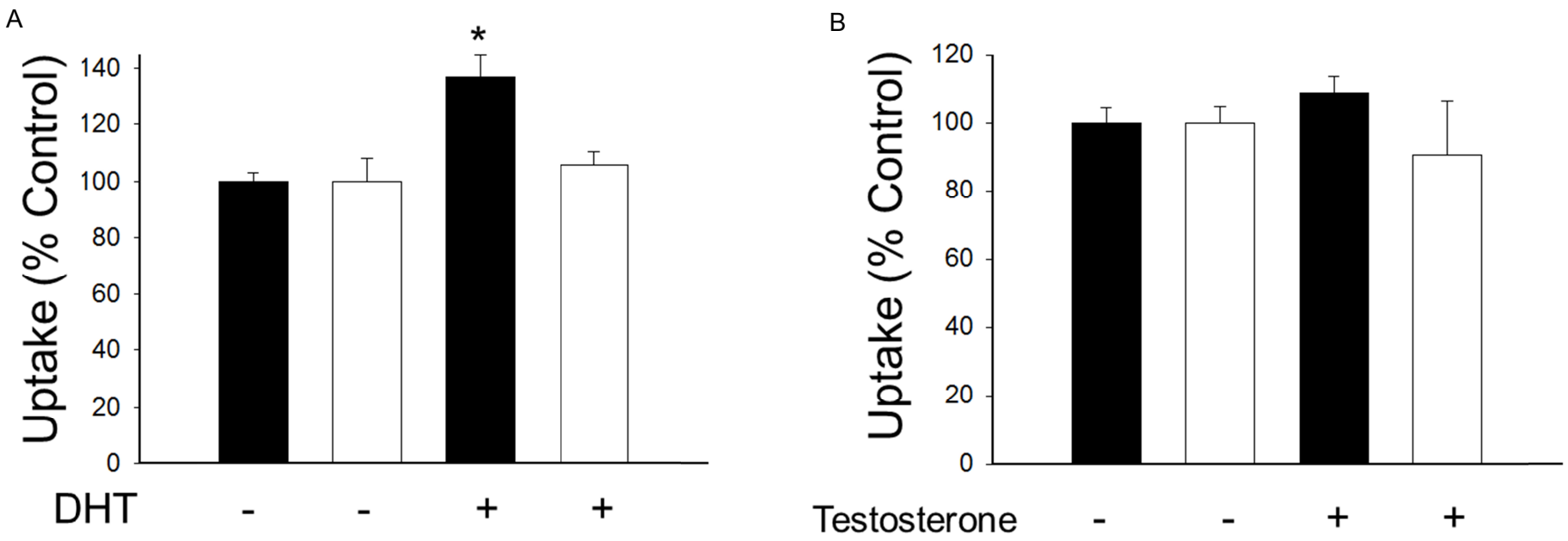


***Figure 5.5**: DHT but not T increases α-(Methylamino)isobutyric acid uptake in fast-twitch skeletal muscle fibre bundles*

*Bar graphs showing the effects of treating fast- (filled bars) and slow- (clear bars) twitch fibre bundles with physiological levels of DHT (A) and T (B) on the uptake of α-(1-^{14} C)-MeAIB. Note that treating the fibre bundles with DHT, but not T, significantly (p=0.001) increases the uptake of α-(1-^{14} C)-MeAIB in the fast-twitch fibre bundles. In contrast, DHT had little or no effect on the uptake of α-(1-^{14} C)-MeAIB in the slow-twitch bundles. In contrast, T led to a slight decrease in the uptake of α-(1-^{14} C)-MeAIB in these fibre bundles. * p<0.05 when compared to control.*

5.4.3 **The transporter mediating amino acid uptake in adult mammalian skeletal muscle fibre bundles**

In another experiment, I decided to investigate the amino transporter(s) mediating the effects of DHT reported in the previous sections. To do this, I pre-treated another set of fiber bundles with the L-type amino acid transporter inhibitor BCH and the system A inhibitor MeAIB for 20minutes. The Ringer's solution was then changed to one containing radiolabelled isoleucine, DHT and the inhibitor whose effects was being investigated for 1hour. The uptake of the radiolabelled isoleucine was then determined. In some experiments, proteins were extracted and probed for the expression of LAT2 and SNAT2 proteins.

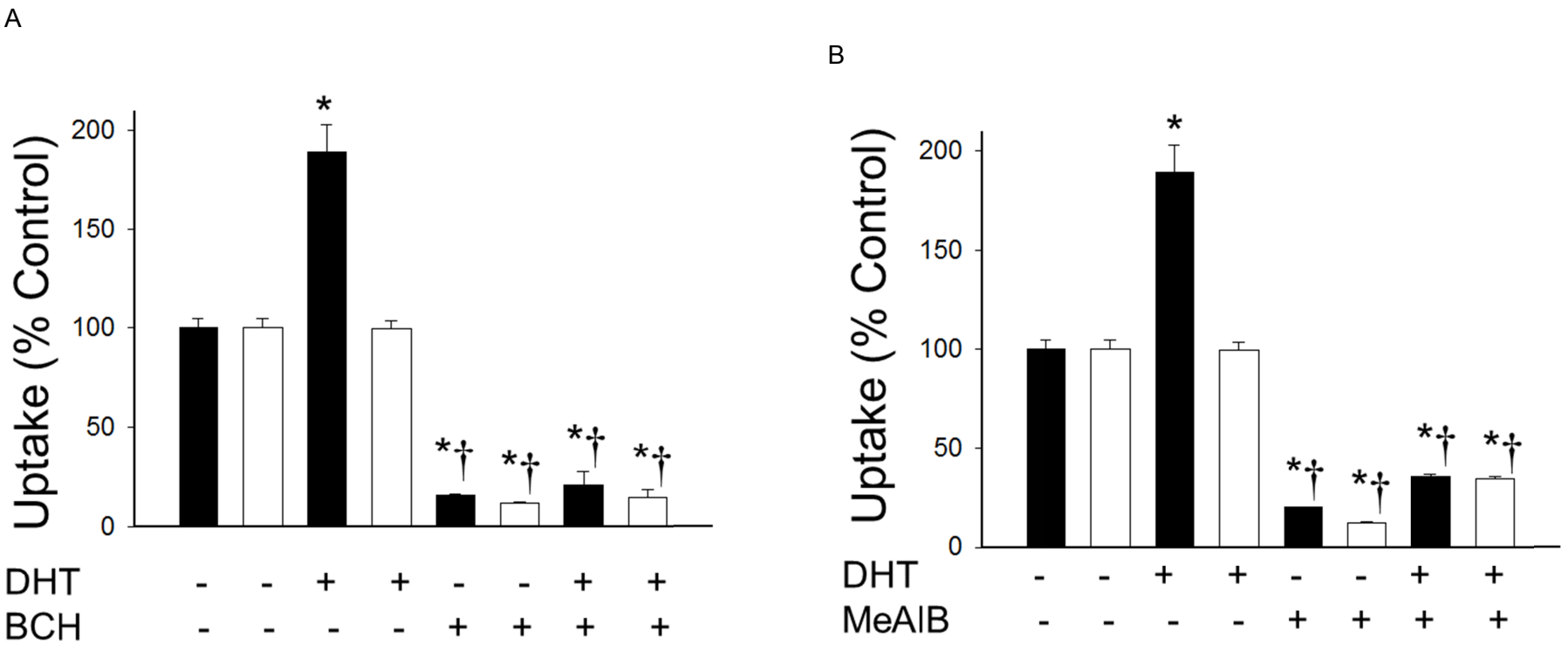


***Figure 5.6**: BCH and MeAIB inhibit the effects of DHT on amino acid uptake by the muscle fibre bundles.*

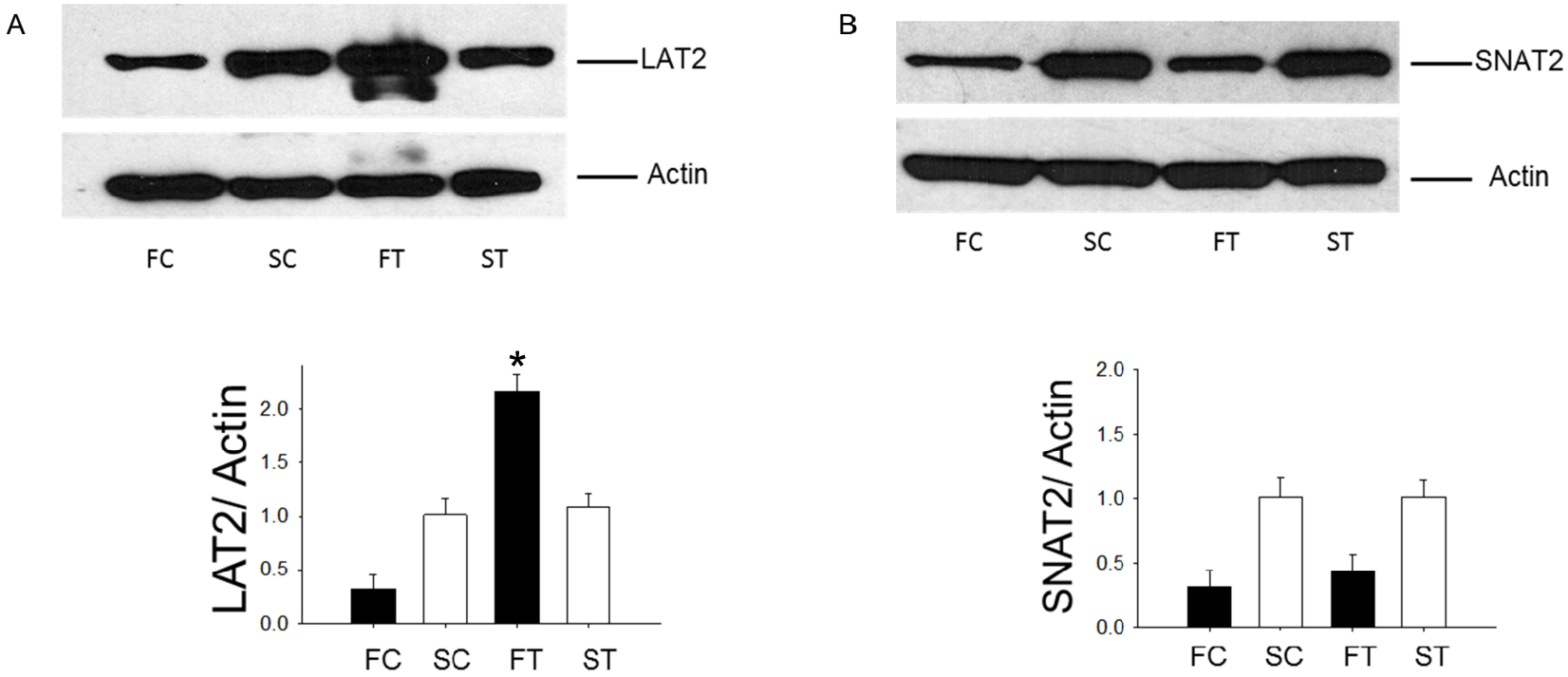


Figure 5.7: *The effects of DHT on amino acid uptake in skeletal muscle fibre bundles are mediated through LAT2 but require SNAT2 activity*

In figure 5.6; bar graphs showing the effects of treating fast-twitch (filled bars) and slow-twitch (clear bars) muscle fibre bundles with DHT plus BCH (A) or DHT plus MeAIB (B) on the uptake of L-(U-^{14} C) Ile. Note that treating the muscle fibre bundles with both BCH and MeAIB significantly ($p<0.05$) reduces the basal as well as the DHT-induced increase in Ile uptake. Furthermore, DHT increases the expression of LAT2 but not that of SNAT2. FT and ST are the fast- and slow-twitch samples treated with DHT, respectively.

In figure 5.7; (A) A typical Western blot and bar graphs showing the effects of DHT on the expression of LAT2 (A) in membrane proteins and SNAT2 (B) in cytosolic proteins extracted from fast- (F, filled bars) and slow- (S, clear bars) twitch muscle fibre bundles. Note that DHT increases the expression of LAT2 but not that of SNAT2. FT and ST are the fast- and slow-twitch samples treated with DHT, respectively. $p<0.05$ compared to control. † $p<0.05$ compared to DHT treatment.*

As the results displayed in Figure 5.6 show, treatment of the muscle fibre bundles with DHT significantly ($p<0.01$) increased the uptake of L-(U-^{14} C) Ile in the fast-twitch fibre bundles only. In contrast, pre-incubating the fibre bundles with BCH and MeAIB significantly ($p<0.05$) decreased the basal uptake of L-(U-^{14} C) Ile in both fibre types. Pre-treatment with BCH and MeAIB also completely abolished the DHT-induced increase in Ile uptake (Fig. 5.6A and Fig. 5.6B).

The effects of DHT on the expression of LAT2 and SNAT2 are shown in Fig.5.7. As the results show, under control conditions the slow twitch fibre bundles always expressed higher concentration of the two transporters than the fast twitch ones. Whether this difference is physiologically important is uncertain as the uptake of Ile and MeAIB under control conditions in the two fibre types only showed a small difference. For example the uptake of Ile was 0.042±0.0013Bqμg^{-1} muscle protein in fast twitch fibres (n=4) and the uptake of Ile was 0.039±0.007Bqμg^{-1} muscle protein in slow twitch fibres (n=4). On the other hand, treatment of the muscle fibre bundles with DHT significantly increased the expression of LAT2 in the fast-twitch fibre bundles only (Figure 5.7A) without significantly ($p=0.13$) affecting the expression of SNAT2 in ether fibre type (Figure 5.7B).

5.4.4 **The signaling pathway mediating the non-genomic effects of DHT on isoleucine uptake in adult mammalian skeletal muscle fibre bundles**

To determine the cellular-signal transduction pathway(s) mediating the acute effects of DHT on amino acid uptake, another set of fiber bundles was treated with DHT alone or DHT plus the mitogen-activated protein kinase kinase (MEK) inhibitor, PD98059, the PI3K/Akt inhibitor deguelin or the mTOR inhibitor rapamycin. The uptake of L-(U-^{14}C) Ile was then determined.

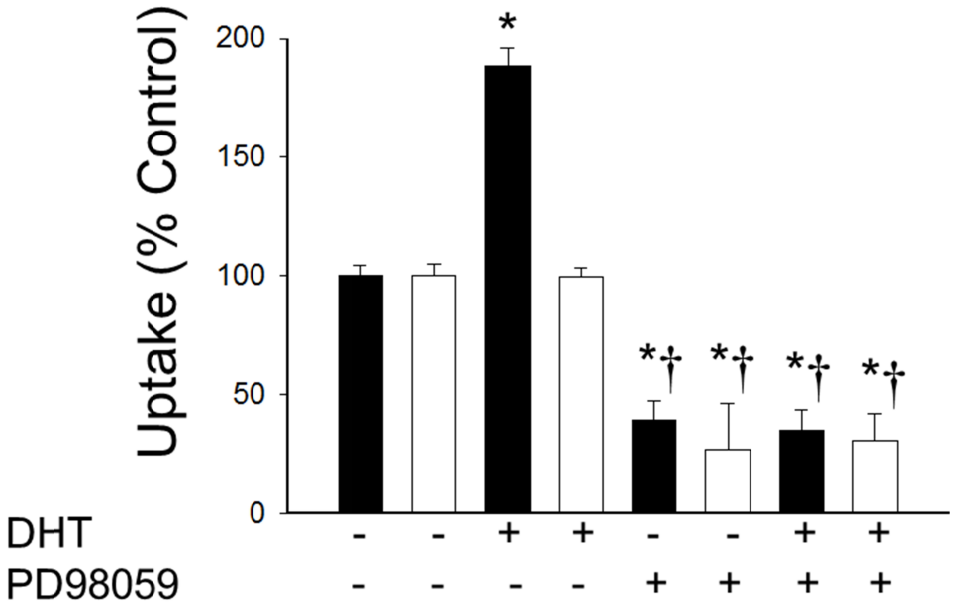


***Figure 5.8**: The effects of DHT on amino acid uptake in isolated intact muscle fibre bundles are mediated through the MAPK pathway.*

Bar graphs showing the effects of DHT on L-(U-^{14} C) Ile uptake in fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles treated with the standard Ringer's solution, the standard Ringer's solution plus DHT, the standard Ringer's solution plus MEK inhibitor PD98059 or the standard Ringer's solution plus DHT and PD98059. Note that treating the fibre bundles with DHT alone significantly increases the uptake of L-(U-^{14} C) Ile in the fast-twitch fibre bundles only. That treatment of the fibre bundles with PD98059 alone significantly (p=0.001) reduces the basal amino acid uptake of L-(U-^{14} C) Ile in both fibre types. Additionally, it completely abolished the DHT-induced uptake of L-(U-^{14} C) Ile in the fast-twitch fibre bundles. p<0.05 compared to control. † p<0.05 compared to DHT treatment.*

As the result in Figure 5.8 show treatment of the muscle fibre bundles with the MEK inhibitor, PD98059 significantly decreased the basal uptake of isoleucine. Also PD98059 completely abolished the DHT-induced increase in amino acid uptake in the fast-twitch fibre bundles.

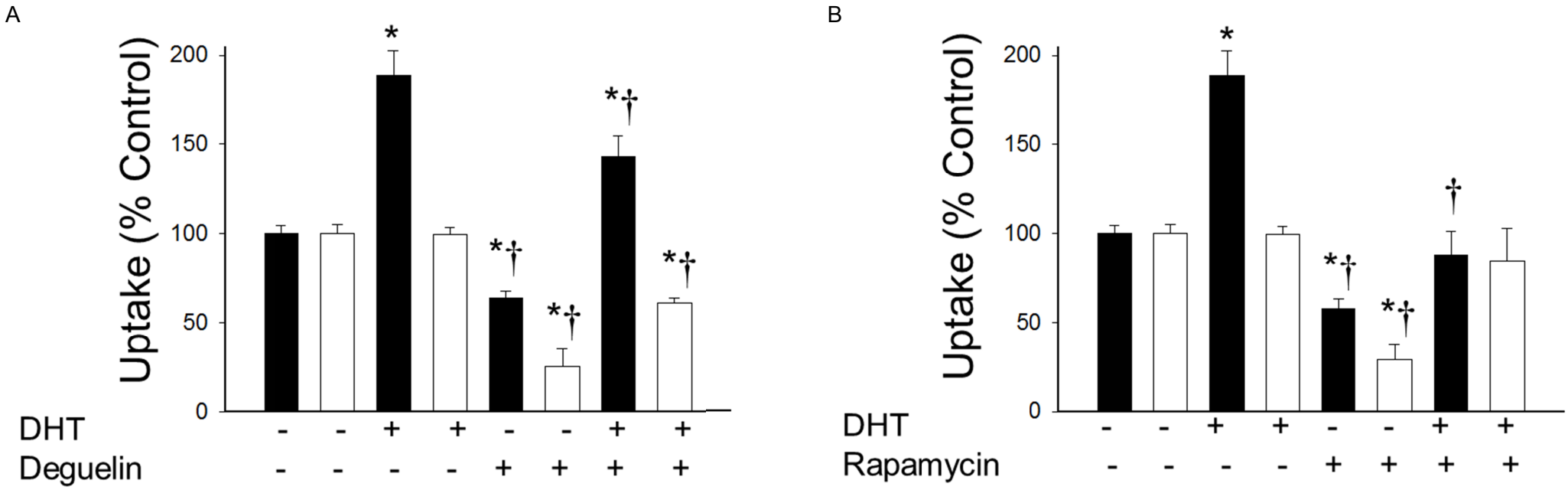


***Figure 5.9**: Rapamycin blunts the effect of DHT on amino acid uptake in mouse skeletal muscle fiber bundles*

Bar graph showing the effects of DHT on L-(U-^{14} C) Ile uptake in fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles treated with the standard Ringer's solution, the standard Ringer's solution plus DHT, the standard Ringer's solution plus the Akt specific inhibitor deguelin (A) or the mTOR inhibitor rapamycin (B) or the standard Ringer's solution plus DHT and deguelin or rapamycin. Note that treating the fibre bundles with either deguelin alone (A) or rapamycin alone (B) significantly decreases the basal uptake of L-(U-^{14} C) Ile in both fibre types. Additionally, pre-treating the fibre bundles with rapamycin but not deguelin completely abolishes the DHT-induced increase in L-(U-^{14} C) Ile in the fast-twitch fibre bundles. In contrast, deguelin blunts without abolishing the DHT-induced L-(U-^{14} C) Ile uptake.

The effects of treating the fibre bundles with either deguelin or rapamycin are shown in Fig 5.9. As the results show, treatment of the muscle fibre bundles with either deguelin alone or rapamycin alone decreased the basal uptake of isoleucine in both fibre types, suggesting that the Akt/mTOR pathway plays an essential role in the maintenance of basal amino acid transport in mammalian skeletal muscle fibres. Furthermore, pre-treating the fibre bundles with deguelin blunts without abolishing the effects of DHT on isoleucine uptake in the fast-twitch fibre bundles (Figure 5.9 A). In contrast, pre-treatment of the fibre bundles with rapamycin completely abolished the effects of DHT isoleucine uptake in the fast-twitch fibre bundles (Figure 5.9 B).

5.4.5 **The effects of actinomycin D on DHT-induced increase in isoleucine uptake in adult mammalian skeletal muscle fibre bundles**

To investigate whether the effects of DHT on isoleucine uptake involved transcription, another set of fiber bundles was treated with the transcriptional inhibitor actinomycin D. The uptake of L-(U-^{14} C) Ile was then examined in the presence or absence of DHT.

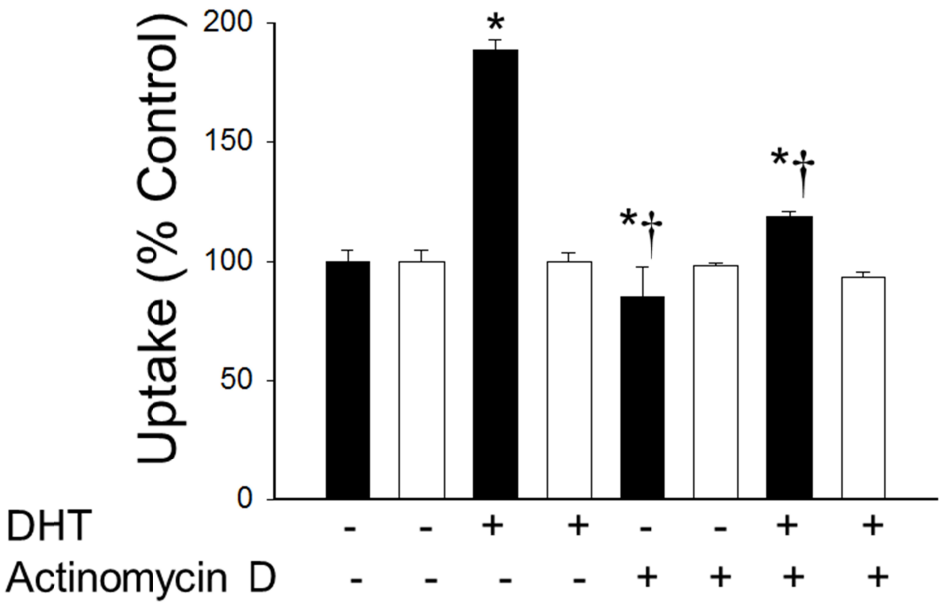


***Figure 5.10**: Actinomycin D blunts the effects of DHT on isoleucine uptake in mammalian skeletal muscle fibre bundles.*

Bar graph showing the effects of DHT on L-(U-^{14} C) Ile uptake in fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles treated with the standard Ringer's solution, the standard Ringer's solution plus DHT, the standard Ringer's solution plus actinomycin D or the standard Ringer's solution plus DHT and actinomycin D. Note that treating the fibre bundles actinomycin D alone does not significantly affect the basal uptake of L-(U-^{14} C) Ile in both fibre types. Additionally, pre-treating the fibre bundles with actinomycin D completely abolishes the DHT-induced increase in L-(U-^{14} C) Ile in the fast-twitch fibre bundles. p<0.05 compared to control. † p<0.05 compared to DHT treatment.*

The results from this experiment are shown in Fig. 5.10. As the results 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-^{14} C) Ile in either fibre type. However, pre-treatment of the fibre bundles with actinomycin D significantly (p=0.001) reduced the DHT-induced increase in L-(U-^{14} C) Ile uptake without completely abolishing it (Figure 5.10).

As stated in 3.1, once activated RSK1/2 translocate into the nucleus where it activates a large numbers of transcription factors including CREB, cmyc and ATF2 (Krishna & Narang, 2008). Furthermore, as the results displayed in Fig. 5.10, suggested that the effects of DHT involved transcription, in another experiment, I examined the effects of DHT on the activation of some of these transcription factors.

The results from this experiment are displayed in Fig. 5.11 and as they show, treatment of the muscle fibre bundles with DHT had little or no effect on the expression and activation of cmyc (Fig. 5.11B) in both fibre types. On the other hand, it increased the phosphorylation of ATF2 (Fig. 5.11C) and completely abolished phosphorylation of CREB in fast fibre type only (Fig. 5.11A).

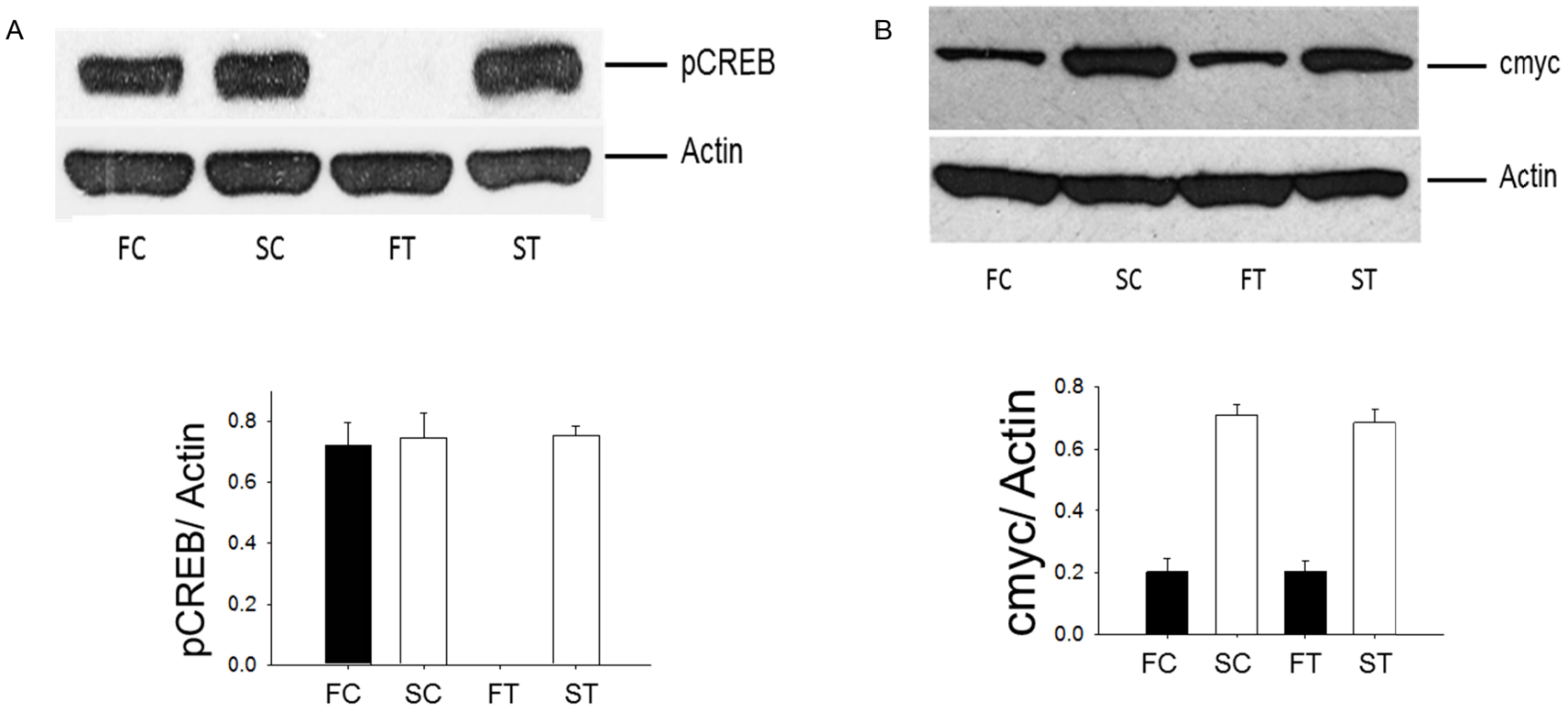
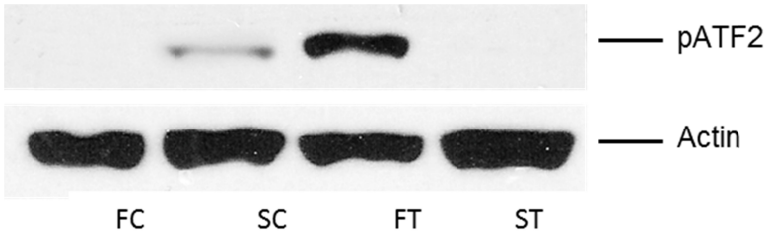


***Figure 5.11**: The effects of DHT on the expression of c-myc and the phosphorylation of CREB*

Figure 5.11 continued

C



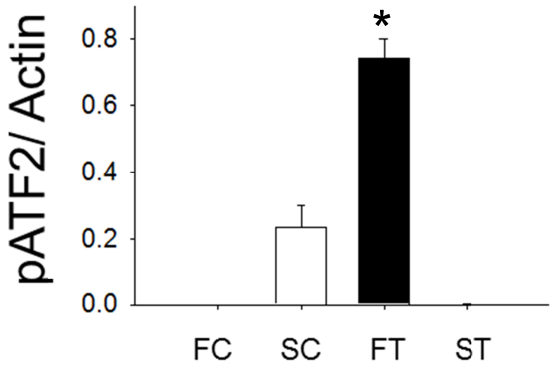


***Figure 5.11**: The effects of DHT on the phosphorylation of ATF2*

Representative Western blots and summary bar graphs showing the effects of DHT on the expression c-myc and the phosphorylation of CREB and ATF2 in fast-(F, filled bars) and slow- (S, clear bars) twitch muscle fibre bundles treated with the standard Ringer's solution (FC, SC) or the standard Ringer's solution plus DHT (FT, ST). Note that treating the muscle fibre bundles with DHT had no effect on the expression of c-myc in either fibre type. Instead, it increases the phosphorylation of ATF2 and completely abolishes that of CREB in the fast-twitch muscle fibre bundles only. p<0.05 when compared to control.*

As the results displayed in Figure 5.11 show, treating the muscle fibre bundles with DHT had little or no effect on the expression and activation of cmyc (Fig. 5.11 B) in both fibre types. On the other hand, it increased the phosphorylation of ATF2 (Fig. 5.11 C) and completely abolished phosphorylation of CREB in fast fibre type only (Fig. 5.11 A). . .

5.4.6 **The effects of cyclohexamide on DHT-induced increase in isoleucine uptake in adult mammalian skeletal muscle fibre bundles**

To investigate whether the effect of DHT-induced increase in isoleucine uptake involved the modulation of translation, another set of fibre bundles was treated with translational inhibitor cyclohexamide. The uptake of L-(U-^{14} C) Ile was then examined in the presence or absence of DHT.

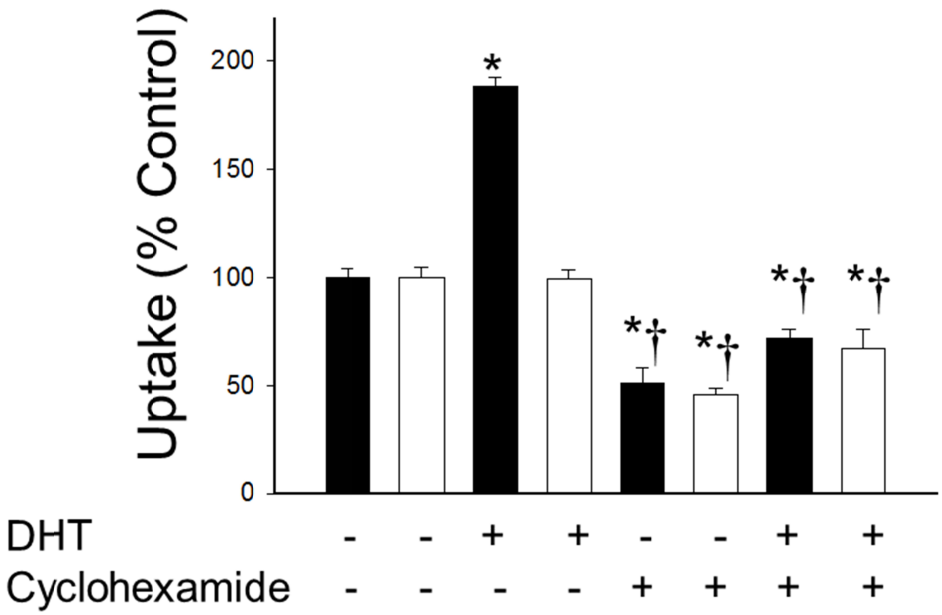


***Figure 5.12**: Cyclohexamide completely abolishes the effects of DHT on isoleucine uptake in mammalian skeletal muscle fibre bundles Bar graph showing the effects of DHT on L-(U-^{14} C) Ile uptake in fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles treated with the standard Ringer's solution, the standard Ringer's solution plus DHT, the standard Ringer's solution plus cyclohexamide or the standard Ringer's solution plus DHT and cyclohexamide. Note that treating the fibre bundles with cyclohexamide alone significantly ($p<0.05$) reduces the basal uptake of L-(U-^{14} C) Ile in both fibre types. Additionally, pre-treating the fibre*

bundles with cyclohexamide completely abolishes the DHT-induced increase in L-(U-^{14} C) Ile in the fast-twitch fibre bundles. p<0.05 compared to control. † p<0.05 compared to DHT treatment.*

As the result displayed in Figure 5.12 show, treatment of the fibre bundles with cyclohexamide alone significantly (p<0.05) reduced the basal uptake of L-(U-^{14} C) Ile in both fibre types. Moreover,pre-treating the fibre bundles with cyclohexamide completely abolished the DHT-induced increase in L-(U-^{14} C) Ile observed in the fast-twitch fibre bundles (Fig. 5.12).

5.4.7 **The effects of DHT on translational eukaryotic elongation factor (eEF) 2 in adult mammalian skeletal muscle fibre bundles**

Phosphorylation of eEF2 inhibits elongation and translation. To confirm the effects of cyclohexamide reported in the previous experiment, another set of fibre bundles was treated with DHT. The phosphorylation of eEF2 was then examined. As the results displayed in Fig. 5.13 show, the untreated fast-twitch fibre bundles expressed significantly (p<0.05) higher concentrations of phosphorylated eEF2 than the slow-twitch ones. However, treatment of the fibre bundles with DHT abolishes the phosphorylation of eEF2 in both fibre types.

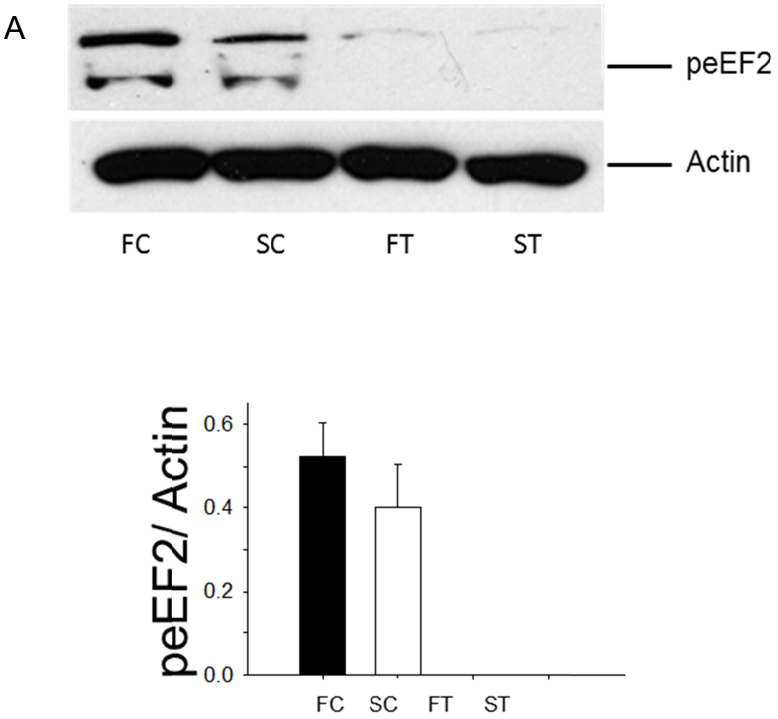


***Figure 5.13**: DHT abolish phosphorylation of eEF2 in both fibre types*

Representative Western blots and summary bar graphs showing the effects of DHT on the phosphorylation of eEF2 in fast-(F, filled bars) and slow- (S, clear bars) twitch muscle fibre bundles treated with the standard Ringer's solution (FC, SC) or the standard Ringer's solution plus DHT (FT, ST). Note that treating the muscle fibre bundles with DHT completely abolishes the phosphorylation of eEF2 in both the fast-and slow-twitch fibre bundles.

5.5 **Discussion**

A key finding in the present study was the observation that treating small skeletal muscle fibre bundles isolated from the EDL (a fast-twitch muscle) and soleus (a slow-twitch muscle) of adult female mice with physiological concentrations (630pgml^{-1}) of DHT, for 1hour, 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 (Figs. 5.4 and 5.5).

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 the fast- or slow twitch fibre bundles (Figures 5.4B and 5.5B). In contrast, treating the fibre bundles with DHT led to a marked increase in the uptake of both Ile and MeAIB in the fast-twitch fibre

bundles only (Fig.5.4A and 5.5A). Previous data from the laboratory has also shown that T has no acute effect 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 section 1.6, in most tissues T is converted into DHT by the enzyme 5α-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 I speculate that in addition to IGF-1, the effects of T in mammalian skeletal muscle may also be exerted by DHT. I also suggest that DHT is the main anabolic-androgenic steroid in adult mammalian skeletal muscles.

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 Fig. 5.7 shows, in addition to other transporters, adult mouse fast- and slow-twitch skeletal muscle fibres express both SNAT2 (a system A amino acid

transporter) (Fig. 5.7 B) and LAT2 (an amino acid transporter belonging to system L) (Fig. 5.7 A). Although mRNAs for these transporters have previously been identified in mammalian skeletal muscle (Utsunomiya-Tate *et al.* 1996; Segawa *et al.* 1999; Pineda *et al.* 1999; Yao *et al.* 2000; Hyde *et al.* 2001; Sugawara *et al.* 2000), 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 displayed in Fig.5.7 show the untreated slow-twitch muscle fibre bundles express more (~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 (Bevington *et al.* 2002; Yao *et al.* 2000; Sugawara *et al.* 2000), osmolarity (Horio *et al.* 1997), intracellular amino acid levels (Gazzola *et al.* 1972; Drummond et al. 2010), hormones (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

reported in this study 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. 5.7 A). 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 (Figure 5.6 A and B). From these observations, I suggest that although the effects of DHT on amino acid uptake are mediated through LAT2, SNAT2 is somehow involved. 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 (see Figure 5.14) (Hyde *et al.* 2007; Baird *et al.* 2009). Indeed, recent data published by Drummond and his co-workers suggest that it may also exist between SNAT2 and LAT1 in human skeletal muscle (Drummond *et al.* 2010); suggesting that this type of coupling may be widespread in skeletal muscle. However, further studies to confirm this are necessary.

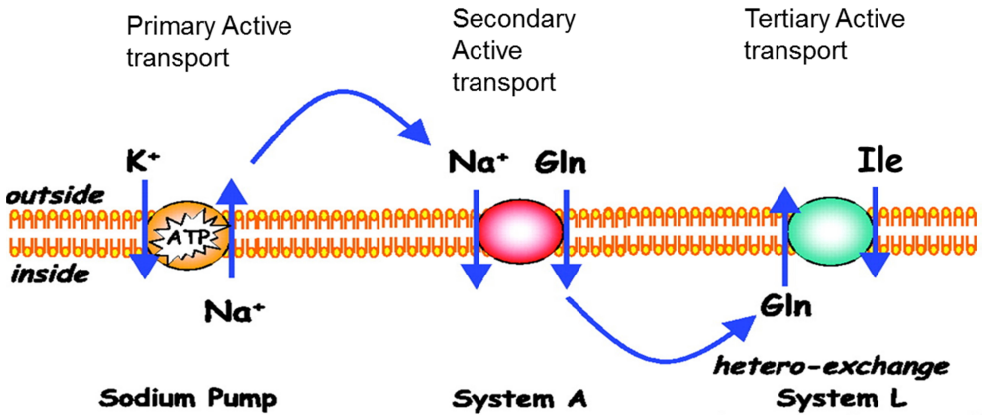


***Figure 5.14**: A schematic diagram showing the proposed organisation of amino acid transporters generally. The primary active transport systems provide the sodium ions used by the secondary active transporters to move small neutral amino acids such as glutamine into the cell. These amino acids are then exchanged by the tertiary active transporters such as system L for amino acids such as isoleucine from the extracellular amino acid pool. Diagram adapted from Hundal & Taylor (2009).*

In chapter 3 I showed that the non-genomic effects of DHT involve the phosphorylation of ERK1/2. 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 ERK phosphorylates an array of targets, including many transcription factors (Deak *et al.* 1998). In the present study, DHT treatment increased the phosphorylation of ATF2 (Fig. 5.11) and significantly decreased that of CREB. The findings further suggest that the non-genomic actions

of DHT in adult mammalian skeletal muscles are exerted mainly through the ERK1/2 module of the MAPK pathway (Fig. 5.8).

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 many growth factors, hormones and nutrients and its activation culminates in the block of apoptosis, induction of protein synthesis, gene transcription and cell proliferation (Dann *et al.* 2007). Therefore, my expectation was that DHT and T would activate this pathway. However, as the results displayed in Fig.5.9A show, the Akt specific inhibitor deguelin blunted the DHT-induced amino acid uptake in fast-twitch fibres without completely abolishing it. Thereby, suggesting that the non-genomic actions of DHT are not mediated through Akt. Instead, the results present in this chapter 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 (see Fig. 5.13). Furthermore, pre-treating the fibre bundles with the translational inhibitor cyclohexamide completely abolished the DHT-induced increase in amino acid uptake (Fig.5.12).

Cyclohexamide 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-

Poetsch *et al.* 2010). Therefore, both of these observations provide further evidence in support of my hypothesis that DHT increases amino acid uptake by increasing protein 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 (Fig. 5.10). However, as DHT did not increase the activity of many of the transcription factors examined, I hypothesis 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 (Fig. 5.9B). These findings suggest that some of the 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 (Avruch *et al.* 2009; Beugnet *et al.* 2003). 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 reported in this study show that another physiological function of the non-genomic actions of DHT in adult mammalian skeletal muscle fibres is to increase the uptake of essential amino acids mediated through the L-type amino acid transporter LAT2.

CHAPTER 6

RECEPTOR MEDIATING THE RAPID/NON-GENOMIC EFFECTS OF DHT IN ISOLATED INTACT FAST- AND SLOW-TWITCH FIBRE BUNDLES

6.1 Introduction

As the results reported in Chapters 3, 4 and 5 show, DHT like all other steroid hormones has acute/non-genomic effects in adult mammalian skeletal muscle fibres. Thus, treating small skeletal muscle fibre bundles isolated from the EDL (a fast-twitch) and soleus (slow-twitch) of adult female mice with physiological concentrations of DHT significantly increases amino acid uptake in the fast-twitch fibres (see the results reported in Chapter 5). This effect was mediated through the MAPK pathway (see the results reported in Chapter 3 and involved an increase in the expression of the L-type amino acid transporter LAT2 (see the results reported in Chapter 5). These effects occurred within 1hour; suggesting that they were mediated via a cell surface receptor rather than through the classical androgen receptors. However, very little is known about the receptor involved.

Only one other study has previously investigated the acute/non-genomic effects of T in skeletal muscle cells (Estrada *et al.* 2003). From their results, the authors suggested that the effects of the hormones used (testosterone (T) and nandrolone) were mediated via a G-protein coupled receptor (GPCR) on the cell membrane.

However, they never identified the GPCR involved. Therefore, it is uncertain whether the acute/non-genomic effects of anabolic androgen steroids are mediated through a GPCR as previously suggested (Estrada *et al.* 2003) or another cell surface receptor.

6.2 **Aim of this chapter**

Therefore, the primary aim of this study was to investigate the receptor(s) mediating the acute/non-genomic effects of DHT in isolated intact mouse fast- and slow- twitch skeletal muscle fibre bundles.

6.3 **Materials and methods**

The materials and methods used in this study are similar to those described in previous chapters and will only be briefly outlined here. Like in all the experiments reported in previous Chapters, the experiments reported here were performed at room temperature (~20°C) using small muscle fibre bundles (mean cross-sectional diameter 350±17µm, n=60) isolated from the EDL and soleus of adult female CD1 mice aged 52.1 ± 2.2 days (n=30; range 48 - 56 days) killed as described elsewhere in this thesis.

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 25ml and the other 5ml, were used in this study. The 25ml chamber was used in the experiments investigating the receptor mediating the acute/non-genomic effects of DHT in the muscle fibre

bundles; whereas, the chambers with a total volume of 5mls were used in the amino acid uptake experiments. The fibre bundles were continuously perfused with the standard mammalian Ringer's solution alone or the standard Ringer's solution plus the various compounds/inhibitors listed in Table 6.1. The composition of the Ringer's solution was as detailed in section 2.2 and its pH was maintained at 7.42 by constantly bubbling it with 95% O_2 and 5% CO_2.

6.3.1 **Determination of the receptor mediating the non-genomic effects of DHT in isolated mouse skeletal muscle fibre bundles**

To investigate the receptor(s) mediating the acute effects of DHT on amino acid uptake 32 muscle fibre bundles (16 fast-twitch and 16 slow-twitch) were divided into four equal groups. Each group was then treated for at least 20 minutes as follows; Groups 1 (control), 2 and 4 were treated with the standard Ringer's solution; whereas, Group 3 was treated with the Ringer's solution plus one of the inhibitors listed in Table 6.1. At the end of this period, they were then treated for 1hour as follows; Group 1 was treated with the standard Ringer's solution plus 107.9μM ethanol (the vehicle used to dissolve DHT), Group 2 was treated with the standard Ringer's solution plus 630ρgml-1 (2.16μM) DHT, Group 3 was treated with the Ringer's solution plus the inhibitor whose effects were being investigated and Group 4 was treated with the Ringer's solution containing DHT plus the inhibitors whose effects being investigated. At the end of the experiments, the muscle fibre bundles were processed for Western

blotting as described in previous chapters. In another study, the above experiments were repeated in the presence of 2mM L-(U-^{14}C) Ile. At the end of this experiment the fibre bundles were processed for liquid scintillation counting.

6.3.2 **Immunoblotting**

At the end of all the experiments described above, proteins were extracted and separated as described in section 2.24. They were then immunoblotted overnight with antibodies raised against the C-terminus- and N-terminus- of the AR, pERK1/2-Thr202/Ser204, the 20kDa pMLC, pEGFR-Tyr1173 and peEF2 (for details of the antibodies and concentrations used see table 6.2). The following day, the membranes were incubated with species-specific secondary antibodies conjugated to horse-radish peroxidise for 1 hour at room temperature. They were later developed using ECL substrate and exposure of the membranes to film. On the third day, the membranes were stripped and re-probed for with total ERK, total MLC, or actin for loading control and normalisation.

6.3.3 **Liquid scintillation counting**

The proteins from the second study were processed for liquid scintillation counting as described in section 5.3.4. Briefly, 5µl of each lysate was used to determine the protein concentration in the sample. The rest of the lysate was mixed with an equal amount of Optima Gold XR liquid scintillant (PerkinElmer, Buckinghamshire, UK) and the level of radioactivity in the mixture was determined using a liquid scintillation counter (Tri-Carb 2250 CA, Canberra-Packard) 1hour and 24hours after the end of each experiment.

***Table 6.1**: The concentration and source of the inhibitors used in this chapter*

PROTEIN	INHIBITORS			
	Scientific name	Common name	Concentration	Source
Androgen receptor (AR)	6-Chloro-1β,2β-dihydro-17-hydroxy-3′H-cyclopropa(1,2)-pregna-1,4,6-triene-3,20-dione acetate	Cyproterone acetate	1μM	Sigma-Aldrich
	2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide	Flutamide	3μM	Sigma-Aldrich
Src	4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4,d]pyrimidine	PP_2	10μM	Alexis Biochemicals, Nottingham, UK.
Insulin-like growth factor 1 receptor (IGF-1R)	α-Cyano-(3-methoxy-4-hydroxy-5-iodocinnamoyl)-(3′,4′-dihydroxyphenyl)ketone	Tyrphostin AG 538	100μM	Sigma-Aldrich

Platelet derived growth factor receptor (PDGFR)	4-(6,7-Dimethoxy-4-quinazolinyl)-N-(4-phenoxyphenyl)-1-piperazinecarboxamide	PDGFR Tyrosine kinase Inhibitor III	200nM	Calbiochem
Epidermal growth factor receptor (EGFR)	(N-[3-Chlorophenyl]-6,7,-dimethoxy-4-quinazolamine)	Tyrphostin AG 1478	100nM	Sigma-Aldrich

***Table 6.2**: List of the antigens of interest probed and the antibodies used to probe them in this chapter*

ANTIBODIES/PEPTIDES			SOURCE
Antigens	Description	Dilution	
Androgen receptor (C-terminal domain)	Rabbit polyclonal antibody	1:1000	SantaCruz Biotechnology Inc. (USA)
Androgen receptor (N-terminal domain)	Rabbit monoclonal antibody	1:1000	Abcam (Cambridge, England)
ERK 1/2 phosphorylated at Thr202 and Ser204	Rabbit polyclonal antibody	1:1000	SantaCruz Biotechnology Inc. (USA)
Myosin Light Chain phosphorylated at Serine 20	Rabbit polyclonal antibody	1:1000	Abcam (Cambridge, England)
eEF2 phosphorylated at Thr56 and Thr 58	Rabbit monoclonal antibody	1:500	Abcam (Cambridge, England)

EGFR phosphorylated at Tyrosine 1173	Rabbit polyclonal antibody	1:500	Cell signaling technology Inc. (Beverly, USA)
Total ERK 1 [C-16]	Rabbit polyclonal antibody	1:1000	SantaCruz Biotechnology Inc. (USA)
Total myosin light chain	Rabbit polyclonal antibody	1:1000	Abcam (Cambridge, England)
Actin	Mouse monoclonal antibody	1:1000	Abcam (Cambridge, England)
Goat anti-rabbit	IgG-HRP	1:1000	SantaCruz Biotechnology Inc. (USA)
Goat anti mouse	IgG-HRP	1:1000	SantaCruz Biotechnology Inc. (USA)

6.3.4 **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 (Bq/µg). The radioactivity was then normalized to that recorded under control conditions and all the data are presented as the mean percentage change in uptake ±S.D.

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 analyzed using Scion Image® from 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., London, UK). The data obtained from the two fibre types under control and from the various experimental conditions were then compared using a 3-way ANOVA and $p<0.05$ was considered statistically significant.

6.4 Results

6.4.1 Receptor mediating the acute effects of DHT in isolated intact mammalian skeletal muscle fibre bundles

6.4.1.1 Androgen receptor involvement

A few studies have previously shown that that the acute/non-genomic effects of DHT in the prostate cancer cell line, LnCaP (Peterziel *et al.* 1999) and sertoli cells (Cheng *et al.* 2007) are mediated through classical androgen receptor. Therefore, in this study I investigated whether the acute/non-genomic effects of DHT in adult mammalian skeletal muscle fibre bundles are mediated through the AR. To determine this, I pre-treated some muscle fibre bundles with 1μM cyproterone (a steroidal AR inhibitor) and 3μM flutamide (a non-steroidal inhibitor of the AR).

I then examined the effects of DHT on the phosphorylation of ERK1/2 and the 20kDa RMLCs as well as the uptake of isoleucine by the muscle fibres. The results from this study are displayed in Figs. 6.1, 6.2 and 6.3 and as they show, both inhibitors did not affect the effects of DHT on the phosphorylation of ERK1/2 and the 20kDa RMLCs. On the other hand, they both blunted the effects of DHT-induced increase in Ile uptake by the fast-twitch fibre bundles. Moreover, the effects were greater when the fibre bundles were pre-treated with flutamide than when they were pre-treated with cyproterone. Indeed, treating the muscle fibre bundles with cyproterone alone increased the uptake of Ile in both fibre bundles.

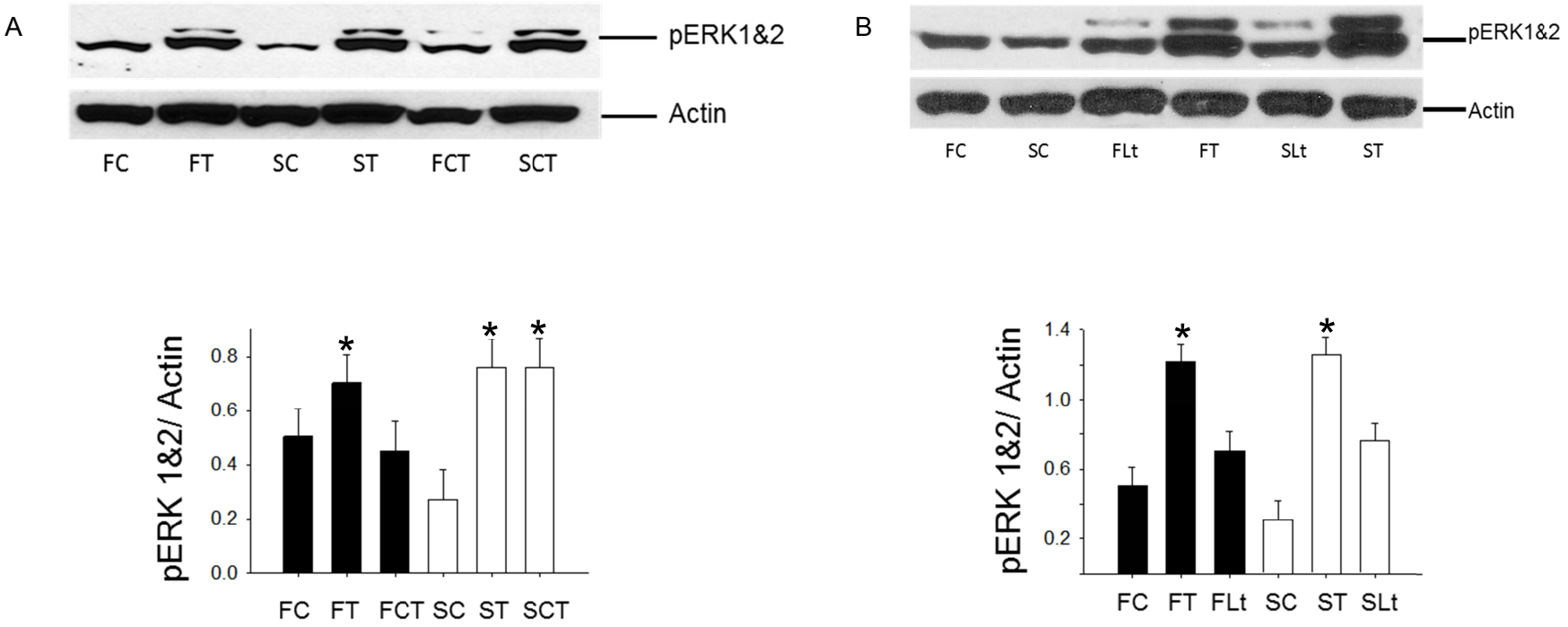


Figure 6.1: *Inhibiting the AR does not abolish the effects of DHT on the phosphorylation of ERK 1/2 in both fibre types*

Representative Western blots and summary bar graphs showing the effects of treating female fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles with the standard Ringer's solution (A,B,FC,SC), DHT alone (A,B,FT,ST), and the standard Ringer's solution containing both DHT and cyproterone (A,FCT,SCT) or flutamide (B,FLt,SLt) on the phosphorylation of ERK1/2. Note that treating the muscle fibre bundles with DHT significantly ($p=0.015$; $*p<0.05$) increases the phosphorylation of ERK1/2 in both fibre types and that pre-treating the fibre bundles with cyproterone (A) or flutamide (B) does not reverse these effects.

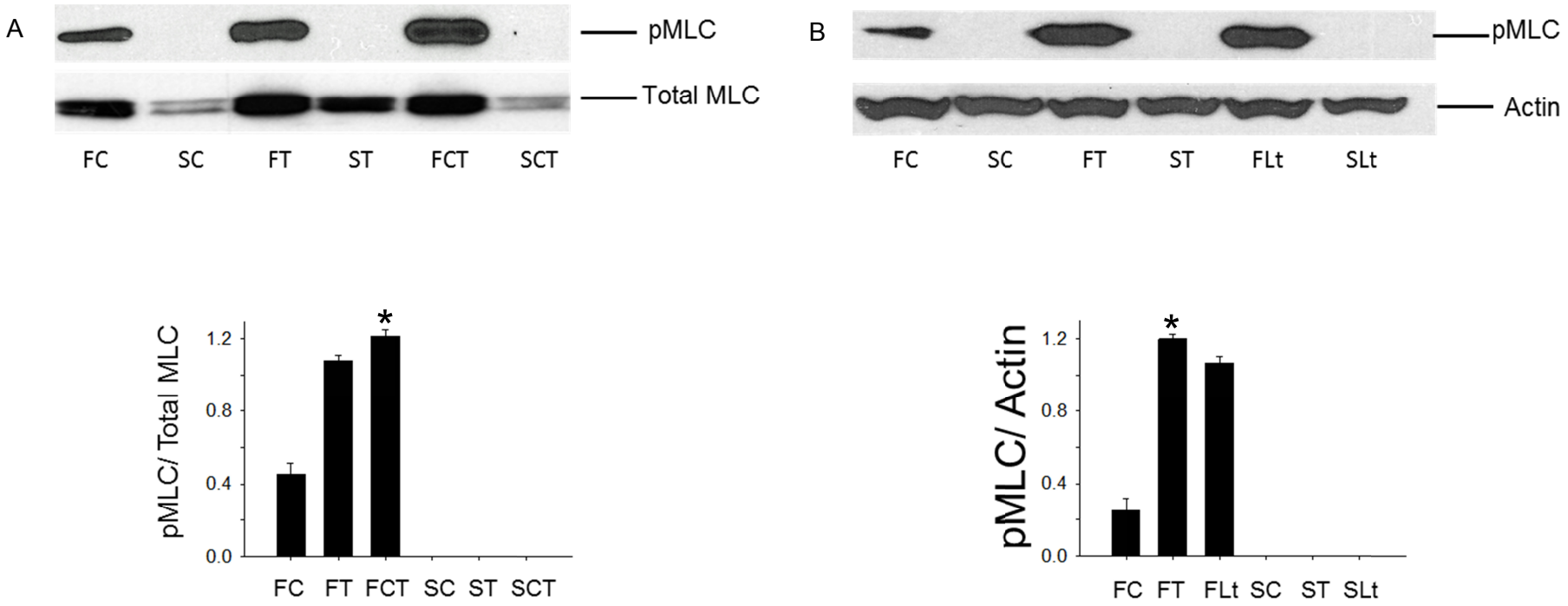


***Figure 6.2**: Inhibiting the AR does not abolish the effects of DHT on the phosphorylation of regulatory MLC in both fibre types*

Representative Western blots and summary bar graphs showing the effects of treating female fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles with the standard Ringer's solution (A,B,FC,SC), DHT alone (A,B,FT,ST), and the standard Ringer's solution containing both DHT and cyproterone (A,B,FCT,SCT) or flutamide (B,FLt,SLt) the on the phosphorylation of the 20kDa regulatory myosin light chain (pMLC). Note that treating the muscle fibre bundles with DHT significantly ($p=0.015$; $*p<0.05$) increases the phosphorylation of RMLCs in both fibre types and that treating fibre bundles with cyproterone (A) or flutamide (B) does not reverse these effects.

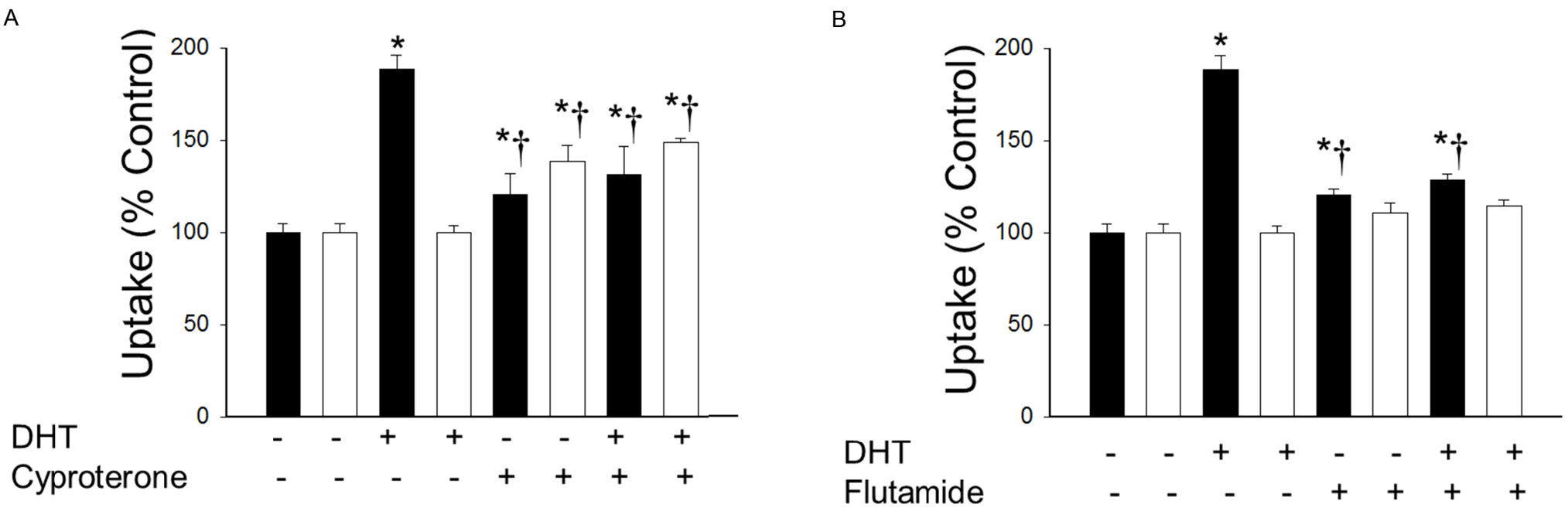


***Figure 6.3**: The effects of DHT on amino acid uptake in mouse skeletal muscle fibre bundles are partly mediated through the androgen receptor*

Bar graphs showing the effects of treating fast- (filled bars) and slow- (clear bars) twitch skeletal muscle fibre bundles with the standard Ringer's solution or the Ringer's 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 0.007, 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.*

6.4.1.2 **Androgen receptor density**

Previously, it has been suggested that the diverse effects of chronic steroid hormones treatment observed in animal experiments arise from differences in their androgen receptor (AR) densities (Eisenberg & Gordon 1950; Metcalf & Gross, 1960; Egginton, 1987; Salmons, 1992; Heinlein & Chang 2002). Furthermore, the AR density in mammalian skeletal muscles has never been directly investigated. I also wanted to rule out the involvement of AR density in the fast-/slow fibre differences seen in uptake of Ile.

Therefore, in another experiment I examined the AR densities in the fast- and slow-twitch skeletal muscle fibre bundles. Additionally, I compared fibre bundles isolated from female animals with those from male animals. As the results displayed in Fig. 6.4 show, the fibre bundles expressed relatively similar concentrations of the receptor irrespective of their phenotype or gender of the animal they were from.

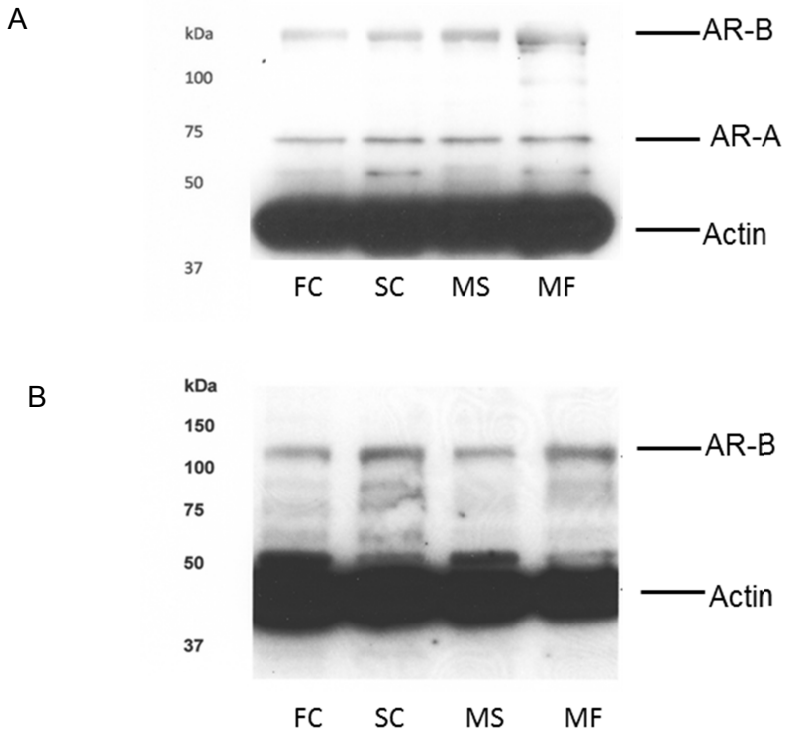


***Figure 6.4**: Fast twitch and slow twitch muscles express similar concentrations of the androgen receptor*

Representative Western blots showing the androgen receptor expression in female fast (FC), female slow (SC), male fast (MF) and male slow (MS) twitch skeletal muscles immunoblotted with a polyclonal antibody against the C terminus of the human AR (A) or a monoclonal antibody against the N terminus of the human AR (B).

Note that the polyclonal antibody identified two proteins, one with a molecular weight of ~110kDa (AR-B) and the other with a molecular weight of ~67kDa (AR-A); whereas, the monoclonal antibody identified only a single band with a molecular weight of ~110kDa. Note that the fast- and slow-twitch muscles from the same sex animal express similar concentrations of the AR and no difference was observed when the concentration of the receptor in the male and female muscles were compared.

6.4.2 **Src involvement in mediating the non-genomic effects of DHT in adult mammalian skeletal muscle fibre bundles**

It has been suggested that in the non-genomic mechanism, DHT recruits signaling pathways that are often associated with cell membrane receptors such as GPCR (Heinlein & Chang, 2000; Falkenstein *et al.* 2000; Simoncini & Genazzani, 2003; Estrada *et al.* 2003). One of the pathways implicated in these studies is the src/MAPK pathway (Kousteni *et al.* 2001; Cheng *et al.* 2007). Therefore, to test the involvement of src in the acute effects of DHT in adult mammalian skeletal muscle fibre bundles, I pre-treated some muscle fibres with src inhibitor PP_2. I then examined the effects of DHT on the phosphorylated ERK1/2 in the fibre bundles.

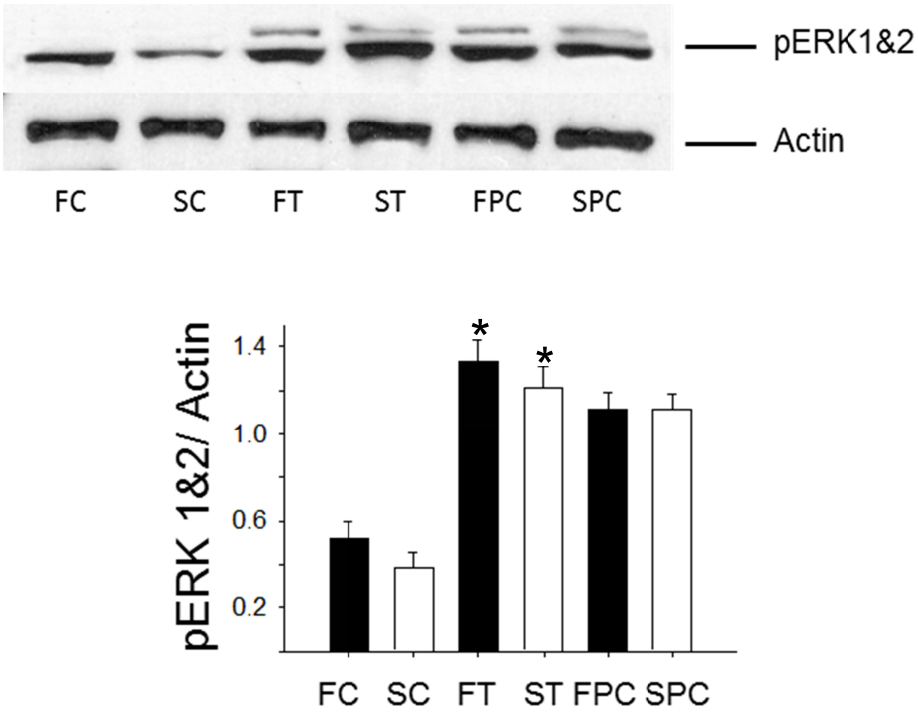


***Figure 6.5**: Inhibiting src does not abolish the effects of DHT on the phosphorylation of ERK 1/2 in both fibre types*

Representative Western blots and summary bar graphs showing the concentration of phosphorylated ERK1/2 (pERK1&2) probed using rabbit polyclonal antibody and actin in female fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles treated with the standard Ringer's solution (FC,SC), DHT alone (FT,ST), DHT plus 10μM PP_2 inhibitor (FPC, SPC). Note that when compared, treatment with DHT led to a significant increase ($p=0.01$; $*p<0.05$) in phosphorylation of ERK 1/2 in both fast- and slow-twitch fibres from female (FT,ST). Pre-treatment of the fibre bundles with PP_2 does not reverse this effect.

As the results displayed in figure 6.5 shows pre-treatment of the fibre bundles with PP_2 (a potent and specific inhibitor of src) had no effect on the DHT induced phosphorylation of ERK1/2 in both fibre types. This suggests that the non-genomic effect of DHT is not mediated through the src.

6.4.3 Receptor tyrosine kinase involvement

6.4.3.1 IGF-1R, PDGFR and EGFR Involvement

As stated in section 1.7.4, the non-genomic actions of DHT and T can be mediated through surface membrane receptor such as Receptor tyrosine kinases (RTKs) which includes insulin-like growth factor-1 receptor (IGF-1R) (Rennie *et al.* 2004), platelet derived growth factor receptor (PDGFR) (Albrecht & Tidball, 1997) and epidermal growth factor receptor (EGFR) (Zwick *et al.* 1999). Therefore, to determine whether the acute effects of DHT in adult mammalian skeletal muscle fibre bundles were exerted through these receptors, another set of fibre bundles was pre-treated for 20minutes with the IGF-1R inhibitor tyrphostin AG538, the PDGFR inhibitor PDGFR tyrosine kinase Inhibitor III or the EGFR specific inhibitor Tyrphostin AG1478. The effects of DHT on the phosphorylation of ERK1/2 and the 20kDa RMLCs was then determined.

As the results displayed in Fig.6.6 show, treating the fibre bundles with tyrphostin AG538 (Fig 6.6A) and PDGFR Tyrosine kinase Inhibitor III (Fig.6.6B) had no effect on effects of DHT on the phosphorylation of ERK1/2 in both fibre types. Similarly, they also did not affect the effects of DHT on the phosphorylation of the 20kDa RMLCs (Fig.6.7).

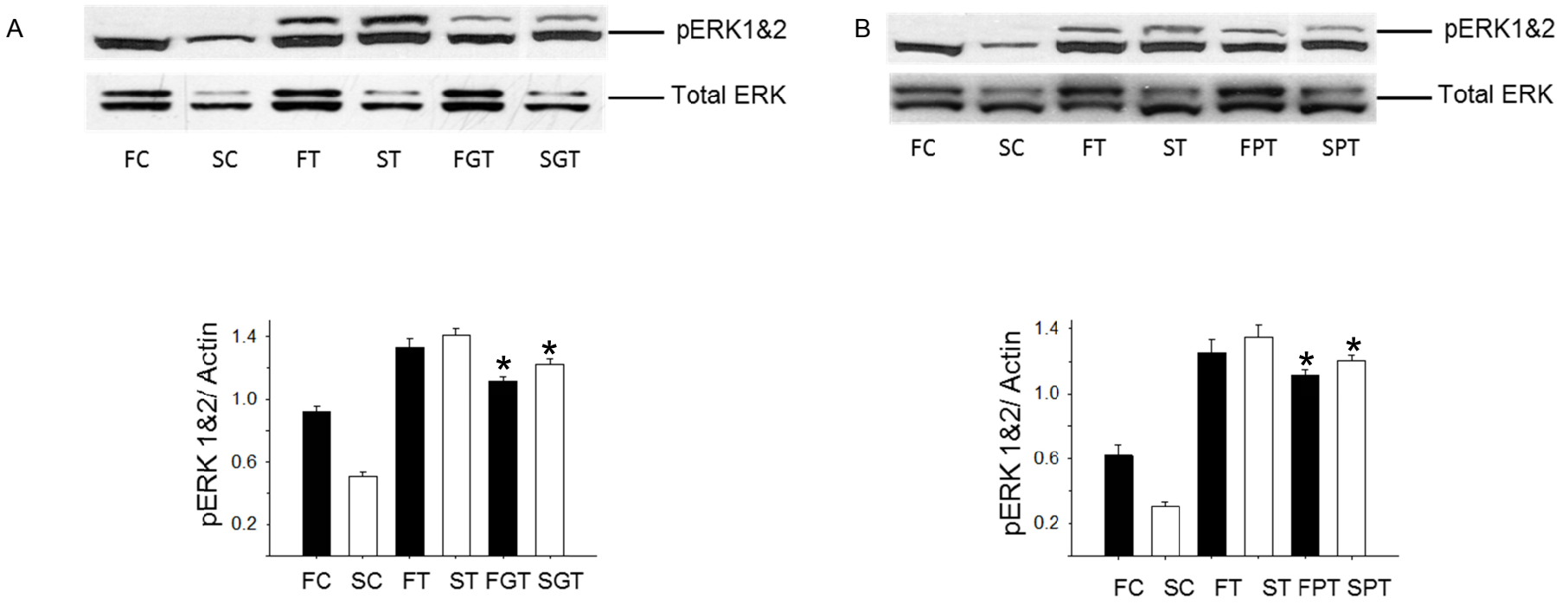


***Figure 6.6**: Inhibiting the IGF-1R and PDGFR does not abolish the effects of DHT on the phosphorylation of ERK 1/2 in both fibre types*

Representative Western blots and summary bar graphs showing the effects of treating female fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles with the standard Ringer's solution (A,B,FC,SC), DHT alone (A,B,FT,ST), and the standard Ringer's solution containing both DHT and tyrphostin AG538 (A,B,FGT,SGT) (A) or Tyrosine Kinase Inhibitor III (FPT,SPT) (B) on the phosphorylation of ERK1/2. Note that treating the muscle fibre bundles with DHT significantly ($p=0.015$; $*p<0.05$) increases the phosphorylation of ERK1/2 in the fast- and slow- twitch muscle fibre bundles. And that adding the inhibitors does not abolish these effects.

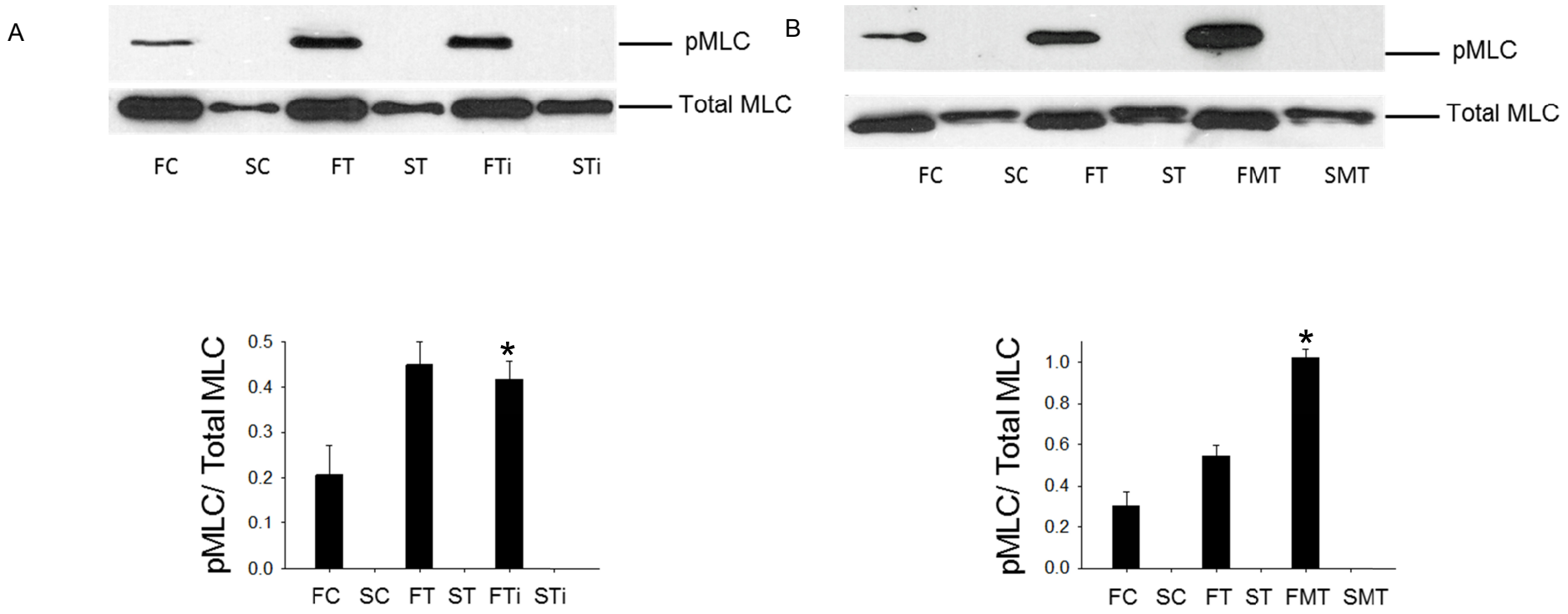


Figure 6.7: *Inhibiting the IGF-1R and PDGFR does not abolish the effects of DHT on the phosphorylation of MLC in both fibre types*

*Representative Western blots and summary bar graphs showing the effects of treating female fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles with the standard Ringer's solution (A,B,FC,SC), DHT alone (A,B,FT,ST), and the standard Ringer's solution containing both DHT and Tyrphostin AG538 (A,B,FGT,SGT) (A) or the Tyrosine Kinase Inhibitor III (FPT,SPT) (B) on the phosphorylation of the 20kDa regulatory myosin light chain (pMLC). Note that treating the muscle fibre bundles with DHT significantly (p=0.01; *p<0.05) increases the phosphorylation of MLC in both fibre types. And that adding the inhibitors does not abolish these effects.*

A number of studies have suggested that the ERK dependent phosphorylation of myosin light chain and hence motility of non-muscle cell is mediated through EGFR (Klemke *et al.* 1997; Iwabu *et al.* 2004). Therefore, to test whether the acute effects of DHT in adult mammalian skeletal muscle fibre bundles were mediated through the EGFR, I pre-treated another set of fibre with the EGFR specific inhibitor, Tyrphostin AG1478. The effects of DHT on the phosphorylation of ERK1/2 and the 20kDa RMLC was then determined.

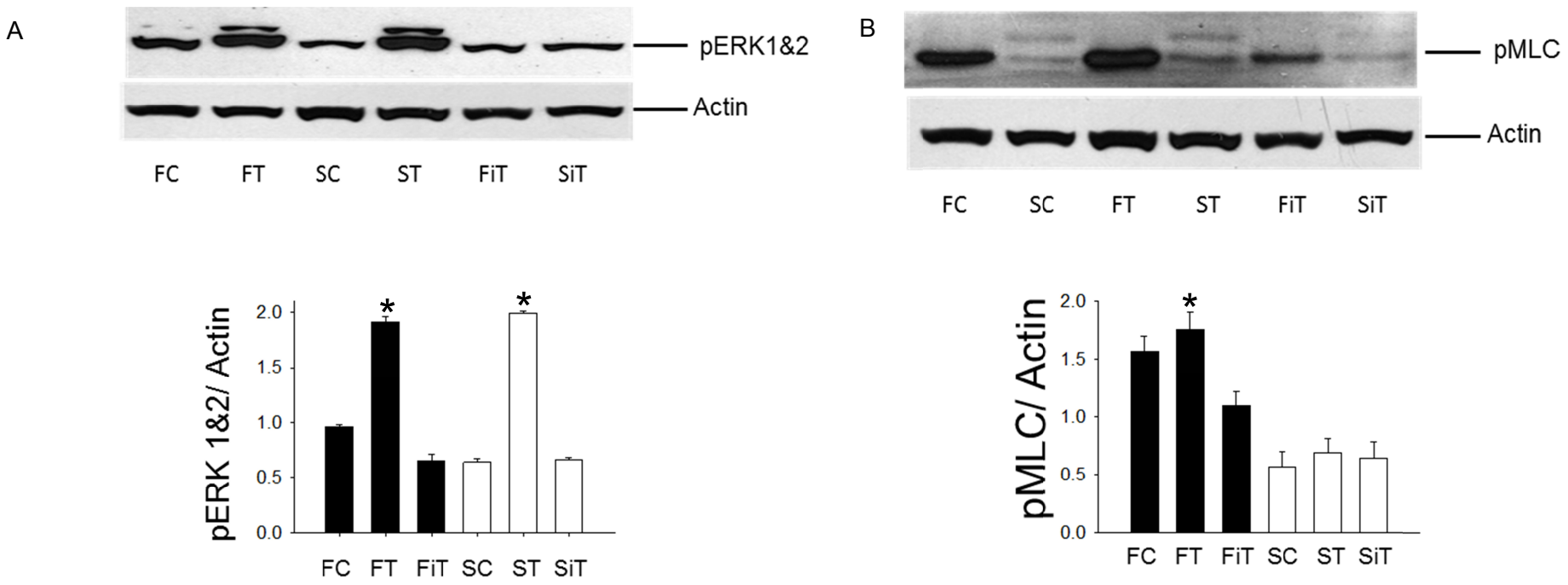


Figure 6.8: *Inhibiting the EGFR abolishes the effect of DHT on the phosphorylation of ERK 1/2 and MLC in both fibre types*

*Representative Western blots and summary bar graphs showing the effects of treating female fast-(filled bars) and slow-(clear bars) twitch skeletal muscle fibre bundles treated with the standard Ringer's solution (A,B,FC,SC), DHT alone (A,B,FT,ST), and the standard Ringer's solution containing both DHT and Tyrphostin AG1478 (A,B,FiT,SiT) on the on the phosphorylation of ERK1/2 (A) and phosphorylation of the 20kDa regulatory myosin light chain (pMLC) (B). Note that treating the muscle fibre bundles with DHT significantly increased (p=0.01; *p<0.05) the phosphorylation of ERK1/2 in fast-and slow- twitch fibres and that this was abolished when they were pre-treated with Tyrphostin AG1478.*

As the result in Figure 6.8 A,B show, pre-treatment of the muscle fibre bundles with Tyrphostin AG1478 completely abolished the effects of DHT on the phosphorylation of ERK1 (p44) and the 20kDa RMLCs in both fibre types; thereby suggesting that the non-genomic effect of DHT in adult mammalian skeletal muscle fibre bundles were mediated through EGFR.

In another experiment, I investigated whether this receptor (EGFR) also mediates the acute/non-genomic effects of DHT on amino acid uptake in these fibre bundles. To do this, I treated another set of fibre bundles with the Ringer's solution containing DHT only or the Ringer's solution containing DHT plus EGFR inhibitor, Tyrphostin AG1478. The uptake of Ile and the concentration of phosphorylated eEF2 were then determined.

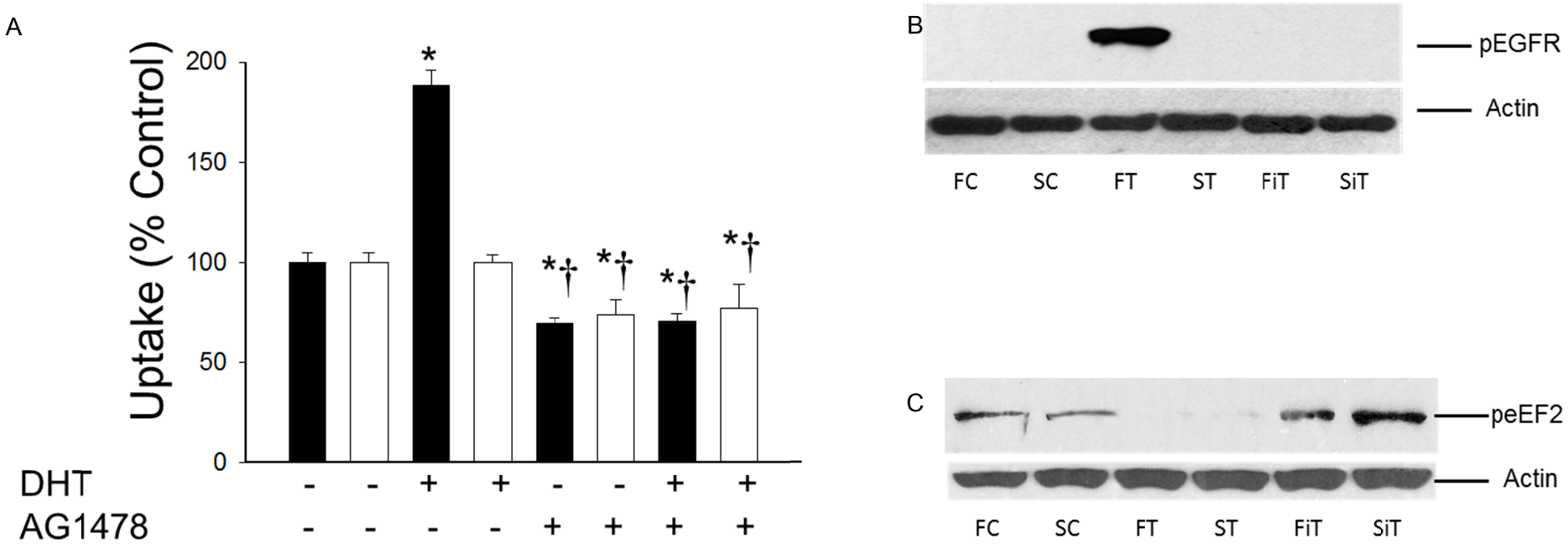


Figure 6.9: *The effects of DHT on amino acid uptake in skeletal muscle fibre bundles are mediated through the EGFR*

(A) Bar graphs showing the effects of treating fast- (filled bars) and slow- (clear bars) twitch skeletal muscle fibre bundles with the standard Ringer's solution or the standard Ringer's solution plus physiological levels of DHT alone or DHT plus tyrphostin AG1478 (A). Note that treatment of the fibre bundles with tyrphostin AG1478 significantly (p=0.004) 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) Representative Western blot showing the effects of treating the muscle fibre bundles with DHT alone (B, FT,ST) or DHT plus AG1478 (B,FiT,SiT) on the phosphorylation of the EGFR in crude membrane proteins. Note that treating the bundles with DHT increases the phosphorylation of EGFR and this effect is completely reversed by AG1478. (C) Representative Western blot showing the effects of treating the muscle fibre bundles with DHT alone (C, FT,ST) or DHT plus AG1478 (C,FiT,SiT) on the phosphorylation of eEF2 in cytosolic proteins. Note that treating the bundles with DHT abolishes the phosphorylation of eEF2 and this effect is completely reversed by AG1478. p<0.05 compared to control. † p<0.05 compared to DHT treatment. F=fast-twitch; S=slow-twitch.*

As the results displayed in Fig. 6.9A 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 and adding DHT did not recover the basal amino acid uptake. Also, it completely abolished the DHT-induced increase in amino acid uptake in the fast-twitch muscle fibre bundles (Fig.6.9B). Furthermore, treatment of the fibre bundles with DHT led to a marked increase in the phosphorylation of the EGFR in the fast-twitch fibre bundles (Fig. 6.9 B). This marked increase in the phosphorylation was also completely abolished when the fibre bundles were pre-incubated in Ringer's solution containing the EGFR inhibitor AG1478 (Fig. 6.9B). Treatment of the fibre bundles with DHT abolished the phosphorylation of eEF2 in both fibre types and the pre-treatment of the fibre bundles with AG1478 completely reversed the reduction.

6.5 Discussion

One of the key findings in this study was the observation that pre-treating small muscle fibre bundles isolated from the EDL and soleus of adult female mice with inhibitors of androgen receptors had no effect on DHT induced phosphorylation of ERK 1 (Fig. 6.1) and RMLCs (Fig. 6.2). Although a number of studies have recently suggested that the AR mediates the non-genomic effects of DHT in the AR positive prostate cancer cell line LNCaP (Migliaccio *et al.* 2000) and osteocytic cell line MLO-Y4 (Kousteni *et al*. 2001), the results I report here suggest that the AR is not the receptor mediating the non-genomic effects of DHT in adult mammalian skeletal muscle fibre bundles. However, as the results displayed in Fig.6.3 show, this cannot be completely ruled out.

In the LNCaP and MLO-Y4 cell lines, it is thought that the DHT-AR complex interacts with Src forming a DHT-AR-Src complex which in turn recruits and activates the adaptor protein Shc (Kousteni *et al*. 2001). Once activated, Shc rapidly forms Shc/Grb2/SOS complexes which further activate Ras or Ras-like GTPase and subsequently stimulate Raf-MAPKKK and activation of the MAPK pathway (Migliaccio *et al.* 2000, Song *et al.* 2002). However, as the results displayed in Fig. 6.5 show, pre-treating the fibre bundles with the cell permeable src specific inhibitor PP2 has no effect of the DHT-induced changes in the phosphorylation of ERK1/2; suggesting that src does not mediate the acute effects of DHT in mammalian skeletal muscle fibres either.

The results I present here, also suggest that IGF-1R and PDGFR do not mediate the acute/non-genomic effects of DHT in adult mammalian skeletal muscle fibre bundles. Thus, pre-treating the muscle fibre bundles with inhibitors of both receptors did not abolish the DHT-induced increase in the phosphorylation of ERK (Fig. 6.6A,B) and RMLCs (Fig. 6.7A,B).

In plasma the majority of steroid hormones are bound to either steroid hormone binding globulin (SHBG) or albumin. Therefore, a number of studies have suggested that steroid hormones exert their non-genomic effects through the binding of a cell surface SHBG receptor. Although a SHBGR has been identified in the prostate, testis, breast and liver (Hryb *et al.* 1985; Krupenko *et al.* 1994; Fortunati, 1999), it is uncertain whether this receptor is expressed in skeletal muscle. Although the SHGBR has not been cloned, it is thought to be either a G-PCR or coupled to one (Nakhla *et al.* 1999; Rosner *et al.* 1999). Previously, it has been suggested that the non-genomic effects of T in cultured myocytes are mediated via an AR coupled to a GPCR (Estrada *et al.* 2003). However, as the GPCR binding T in that study was not identified, it is uncertain whether the SHBGR was involved and further studies to confirm this are needed

In the present study cyproterone increase the uptake of Ile in both the fast- and slow-twitch skeletal muscle fibre bundles. Cyproterone is a steroidal AR inhibitor that is structurally similar to DHT. Therefore, it might be competing and binds to any of the receptors linked to a G protein such as sex hormone binding globulin receptor

(SHBGR) (Nakhla *et al.* 1999) leading to the transactivation of EGFR (Prenzel *et al.* 1999) and activation of the MAPK/ERK pathway (see Fig. 6.10). However, it is uncertain whether this is the mechanism underlying the effects of cyproterone on amino acid uptake in these fibre bundles and further studies to confirm this are needed.

Instead, the results I present here suggest that the acute/non-genomic effects of DHT in adult mammalian skeletal muscle are mediated through the EGFR. Thus, pre-treating the muscle fibre bundles with the cell permeable and EGFR specific inhibitor AG1478 completely abolished the effects of DHT on the phosphorylation of ERK1/2 and the 20kDa RMLCs. It also completely reversed the effects of DHT on amino acid uptake in the fibre bundles. Although in non-muscle cells the EGFR has been shown to regulate cell motility by modulating the phosphorylation of RMLCs by MLCK (Klemke *et al.* 1997), this is the first time that the EGFR has been implicated in the control of the non-genomic effects of anabolic-androgenic steroids. DHT can stimulate the EGFR in a number ways (see Fig. 6.10). It can also activate a metalloproteinase leading to the release of EGF (Prenzel *et al.* 1999). However, the exact mechanism involved is uncertain and further studies to confirm this are needed.

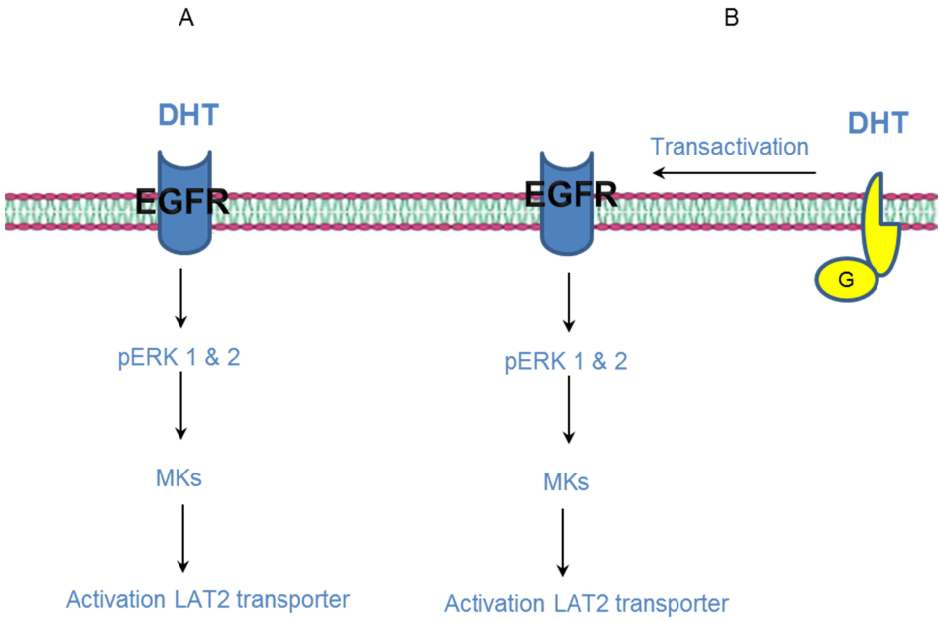


***Figure 6.10**: Possible mechanism underlying the activation of the EGFR by DHT. A schematic diagram showing two ways in which DHT can activate the EGFR. In (A) DHT directly binds and activates the EGFR; whereas, in (B) it binds to a GPCR which in turn transactivates the EGFR leading to the activation of the MAPK/ERK and an increase in LAT2 expression.*

In summary, the results reported in this chapter show that the physiological function of the non-genomic actions of DHT in adult mammalian skeletal muscle fibres is mediated through EGFR. However the involvement of AR cannot completely be ruled out.

CHAPTER 7

GENERAL DISCUSSION

7.1 The acute effects of DHT on the MAPK pathway

In chapter 3, I showed that all the fibre bundles expressed similar levels of phosphorylated/active ERK2 (p42) irrespective of their fibre type and treatment. Moreover, the untreated fibre bundles expressed little or no phosphorylated ERK1 (p44) and only the DHT-treated muscle fibre bundles expressed this isoform in large quantities. In contrast, T treatment increased the phosphorylation of ERK1 in the slow-twitch fibre type only. To me these findings reflect the relative roles played by the different isoforms of ERK1/2. My hypothesis is that ERK2 is essential for the survival of both the fast- and slow-twitch muscle fibres; whereas, ERK1 is involved in the regulation of the fast-fibre phenotype.

This hypothesis is supported by the findings of Saba-El-Leil and co-workers who showed that mice lacking ERK2 died *in utero*; suggesting that ERK2 may be essential for the maintenance of normal cell function and survival (Saba-El-Leil *et al*. 2003). On the other hand, mice deficient in ERK1 were viable and of normal size. However, thymocyte maturation was severely reduced in these mice suggesting that ERK1 may be essential for cell differentiation (Pages *et al.* 1999). Taken together these findings suggest that ERK2 may be essential for the development and maintenance of all cells in the

body; whereas, ERK1 is what is transduced and regulated by environmental factors including mitogens and growth factors.

Another key finding from the results reported in Chapter 3 is the observation that DHT but not T increases the phosphorylation/activation of RSK1/2 and the 20kDa myosin light chains. RSK 1/2 is one of the downstream effector molecules of ERK1/2, the other being MSK1/2 (Blenis, 1993). RSK has two kinase domains, a linker region, C- and N-terminal tails and the Arg-X-X-Ser consensus N terminal tail sequence. It is activated by the sequential phosphorylation by ERK (Frodin & Gemmeltoft, 1999) and its activation is believed to promote cell growth and survival (Itoh *et al.* 2005). On the other hand, the phosphorylation of the 20kDa regulatory myosin light chain by myosin light chain kinase has been implicated in the modulation of force production (Sweeny *et al.* 1993; Hamdi & Mutungi, 2010). From these observations, I propose that DHT acts though RSK1/2 and the phosphorylation of the 20kDa RMLCs to modulate force production in skeletal muscle fibres. Indeed, DHT has been shown to increase force production in fast-twitch muscle fibres and to decrease it in slow-twitch fibres (Hamdi & Mutungi, 2010). However, the molecular mechanism(s) underlying these effects is uncertain and further studies are necessary.

7.2 **The acute effects of DHT on the Akt/mTOR pathway**

The results presented in chapter 4 showed that treating fast- and slow- twitch skeletal muscle fibre bundles with Ringer's solution containing physiological concentrations of DHT or testosterone for 1hour abolishes the phosphorylation of Akt at both Thr308/Ser473 in both fibre types (Figs. 4.4, 4.5, 4.7 and 4.8). However, when the fibre bundles were treated with DHT for a longer period (3hours) the reverse effect was observed i.e. DHT led to an increase in the phosphorylation of Akt at both sites. From these observations, I suggest that the acute effects of DHT are to inhibit the Akt/mTOR pathway. This pathway is important in the regulation of skeletal muscle hypertrophy (Glass, 2003); whereas, the MAPK pathway has been implicated in fibre type switching (Shi *et al.* 2008). From these results, it seems as if DHT is activating ERK1/2 and Akt sequentially. Indeed, these two pathways have been shown to have different roles and to be activated sequentially in differentiating C2C12 cells. To me this is important as it allows the ERK pathway to initiate fibre type switching and any other effects it has in skeletal muscle before Akt is switched on. However, whether this is a common phenomenon is uncertain and further studies are needed.

7.3 **The effects of DHT and T on amino acid uptake**

In chapter 5 I investigated the physiological role of the acute/ non-genomic effects of DHT and T. As the results reported in that chapter show, treatment of the muscle fibre bundles with DHT increases amino acid uptake in the fast-twitch bundles without significantly affecting that of the slow-twitch ones. In contrast, treatment of the fibre bundles with T had no effect on amino acid uptake in either fibre type (Figs. 5.4 and 5.5). Although the acute administration of other 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. Although in the present study T was applied directly to the fibre bundles it still had no effect on amino acid uptake in the muscle fibre bundles suggesting that T may not have any direct acute effects in adult mammalian skeletal muscle fibres. Indeed, it has been suggested that the effects of T in skeletal muscles may not be direct but due to the release of another hormone such as IGF-1. Here, I propose that this secondary hormone is DHT. However, further studies to confirm this are necessary.

The results in Chapter 5 also showed that the acute effects of DHT on amino acid uptake are mediated through the MAPK pathway. Thus, pre-treating the muscle fibre bundles with the MEK inhibitor PD98059 completely abolished the DHT-induced increased in amino acid uptake. The increase in amino acid uptake was also abolished by cyclohexamide and actinomycin D suggesting that it involved both translation and transcription. The suggestion that the increase in amino acid uptake was coupled to an increase in protein synthesis was further supported by my observation that DHT treatment of the muscle fibre bundles completely abolished the phosphorylation of eEF2. Indeed, as stated in section 1.9.2, the unphosphorylated eEF2 is essential for the elongation of the polypeptide chain.

Another important finding in chapter 5 was the observation that DHT treatment increased the activity of the sodium-dependent neutral amino acid transporter (SNAT) 2 and the expression of the L-type amino acid transporter (LAT) 2. Indeed, the DHT-induced amino acid uptake was completely abolished by the system A amino acid transporter inhibitor MeAB and the archetypal system L amino acid transporter inhibitor BCH. To me these results suggest that the DHT-induced amino acid transport is mediated through these two amino acid transport systems. Thus by activating LAT2, DHT also indirectly activates SNAT2. This type of coupling, commonly referred to as tertiary active transport, which 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 and his co-workers

suggest that it may also exist between SNAT2 and LAT1 in human skeletal muscle (Drummond *et al.* 2010); suggesting that this type of coupling may be widespread in skeletal muscle. However, further studies to confirm this are necessary.

7.4 The receptor mediating the physiological effect of DHT on amino acid uptake

In chapter 6, I investigated the receptor mediating the acute/non-genomic effect of DHT and T. As the results reported in that chapter show pre-treating small muscle fibre bundles with inhibitors of androgen receptor had no effect on the DHT-induced increase in the phosphorylation of ERK1/2 (Fig. 6.1) and the 20kDa RMLCs (Fig. 6.2). In the chronic/genomic mechanism, testosterone exerts its effects by activating the cytosolic androgen receptor. The hormone-receptor complex then translocates to the nucleus where the hormone-receptor complex acts as a transcription factor (Simental *et al.* 1991). However, as this mechanism of steroid action involves gene transcription and mRNA translation, its effects usually take several hours to days to be manifested (Florini, 1970; Beato, 1989; Beato *et al.* 1996). In chapter 6 of this thesis, the effects of DHT were evident within 1hour and were insensitive to androgen receptor inhibitors cyproterone and flutamide. Therefore, from these I suggest that the non-genomic effects of DHT on phosphorylated ERK1/2 and RMLCs reported here are not exerted through the androgen receptor. Furthermore, they were also insensitive to inhibitors of src

(Fig. 6.5), IGF-1R (Fig. 6.6) and PDGFR (Fig. 6.7); suggesting that they were not mediated via these receptors either.

Previously, I 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 activation of ERK1/2 module of the MAPK pathway (Hamdi & Mutungi, 2010). The results presented in Chapter 6 suggest that the acute/non-genomic actions of DHT on amino acid uptake in adult mammalian skeletal muscle fibres are also mediated through this receptor too (Fig.6.9). Thus pre-treating the muscle fibre bundles with the EGFR inhibitor tyrphostin AG1478 completely abolished the DHT-induced increase in amino acid uptake and the phosphorylation of the receptor (EGFR). It also abolished the activation of ERK1/2 and RSK 1/2. However, how DHT stimulates the EGFR is uncertain and further research to confirm is needed.

7.5 **Summary**

In summary, the results I have presented in this thesis suggest that the acute/non-genomic actions of DHT in adult mammalian skeletal muscle fibres are to increase the uptake of essential amino acids. As Fig. 7.1, shows, DHT somehow seems to activate the EGFR leading to increase in the phosphorylation of ERK 1/2. The activated ERK1/2 then phosphorylates RSK1/2 which in turn increases the expression of the L-type amino acid transporter LAT2. LAT2 then increases the uptake of isoleucine. DHT also increases protein synthesis and eventually to skeletal muscle hypertrophy.

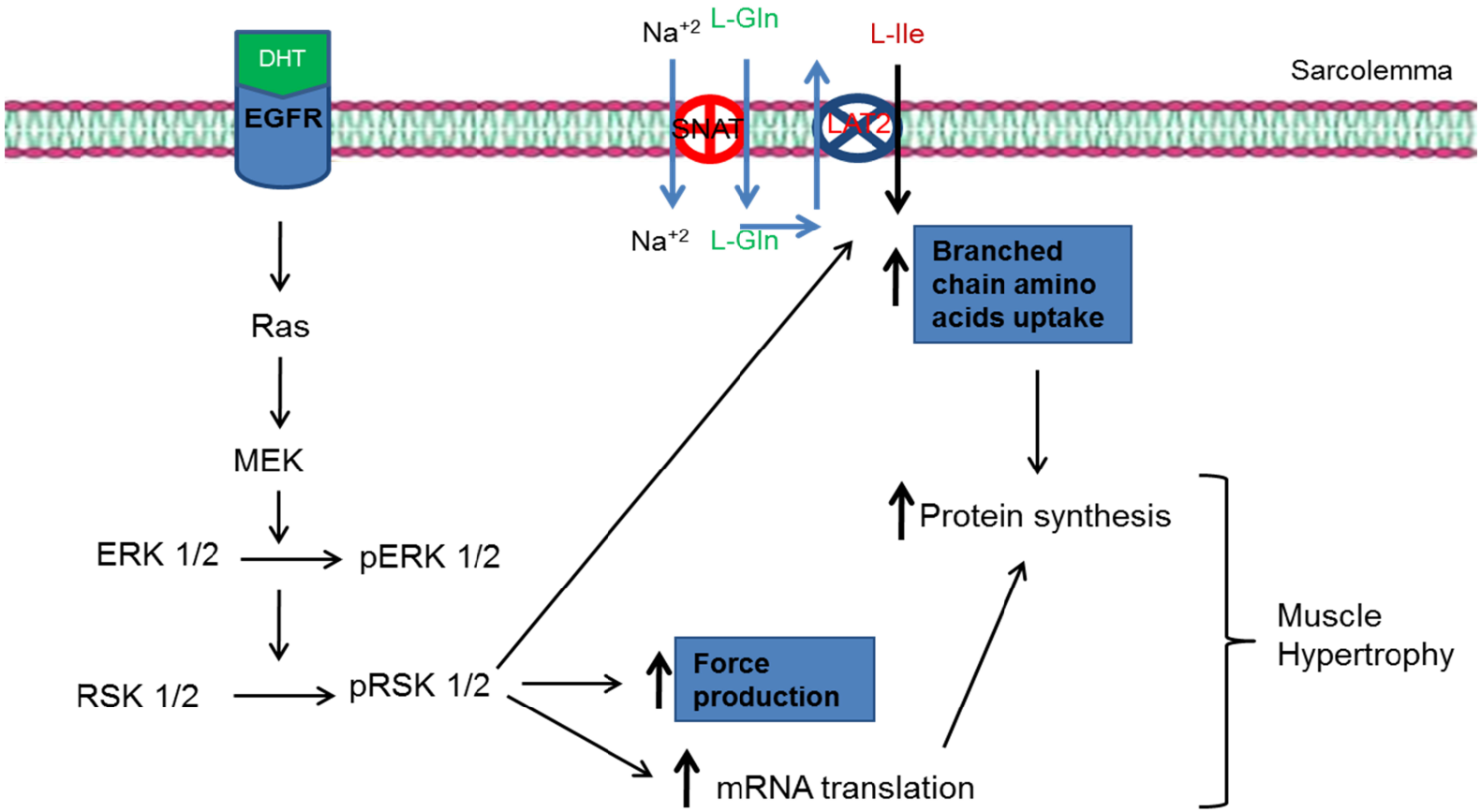


***Figure 7.1**: Proposed cellular signalling pathway mediating the non-genomic actions of DHT in adult mammalian skeletal muscle fibres*

A schematic diagram illustrating the cellular signalling mechanism that I think mediates the acute effects of DHT in adult mammalian skeletal muscle fibre bundles. My hypothesis is that DHT somehow activates the EGFR leading to an increase in the phosphorylation of ERK 1/2. The activated ERK1/2 then phosphorylates RSK1/2 which in turn increases the expression of the L-type amino acid transporter LAT2 on the cell surface. LAT2 then increases the uptake of branched chain amino acids such as isoleucine. The end product of all this I think is an increase in skeletal muscle hypertrophy.

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APPENDIX 1

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Dihydrotestosterone activates the MAPK pathway and modulates maximum isometric force through the EGF receptor in isolated intact mouse skeletal muscle fibres

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It is generally believed that steroid hormones have both genomic and non-genomic (rapid) actions. Although the latter form an important component of the physiological response of these hormones, little is known about the cellular signalling pathway(s) mediating these effects and their physiological functions in adult mammalian skeletal muscle fibres. Therefore, the primary aim of this study was to investigate the non-genomic actions of dihydrotestosterone (DHT) and their physiological role in isolated intact mammalian skeletal muscle fibre bundles. Our results show that treating the fibre bundles with physiological concentrations of DHT increases both twitch and tetanic contractions in fast twitch fibres. However, it decreases them in slow twitch fibres. These changes in force are accompanied by an increase in the phosphorylation of MAPK/ERK1/2 in both fibre types and that of regulatory myosin light chains in fast twitch fibres. Both effects were insensitive to inhibitors of Src kinase, androgen receptor, insulin-like growth factor 1 receptor and platelet-derived growth factor receptor. However, they were abolished by the MAPK/ERK1/2 kinase inhibitor PD98059 and the epidermal growth factor (EGF) receptor inhibitor tyrphostin AG 1478. In contrast, testosterone had no effect on force and increased the phosphorylation of ERK1/2 in slow twitch fibres only. From these results we conclude that sex steroids have non-genomic actions in isolated intact mammalian skeletal muscle fibres. These are mediated through the EGF receptor and one of their main physiological functions is the enhancement of force production in fast twitch skeletal muscle fibres.

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Abbreviations AAS, anabolic androgenic steroids; AR, androgen receptor; DHT, dihydrotestosterone; EDL, extensor digitorum longus; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinase1/2; IGF-1R, insulin-like growth factor 1 receptor; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK/ERK kinase1/2; MLCK, myosin light chain kinase; PDGFR, platelet-derived growth factor receptor; RMLCs, regulatory myosin light chains; src, Src kinase.

Introduction

Testosterone, the principle sex hormone in males, is a C19 steroid synthesised from cholesterol in Leydig cells of the male testes. In healthy young adult men, its plasma concentration ranges from 300 to 1000 ng dl^{-1} (Basaria *et al.* 2001). In plasma, most of the testosterone is bound to either albumin (∼54%) or the sex hormone-binding globulin (∼44%) and only 2% (∼1.5–20 ng dl^{-1}) is free (Pardridge, 1986; Evans, 2004). In certain target tissues such as the liver, brain, prostate and pubic skin testosterone is irreversibly converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase (Bruchovsky & Wilson, 1968). Although, in healthy young men, the concentration of DHT (normal range 4–57.5 ng dl^{-1}) in the plasma is similar to that of free testosterone, DHT is considered to be the more potent hormone because it has a higher receptor binding affinity and a lower dissociation constant than testosterone (Saartok *et al.* 1984). However, as skeletal muscle is thought to lack 5α-reductase (Thigpen *et al.* 1993), it is uncertain whether DHT has any physiological functions in this tissue.

Testosterone and DHT are lipid-soluble hormones and can cross the cell membrane freely. Once inside

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the cell, they bind the androgen receptor (AR) leading to a conformational change in its tertiary structure, the release of the chaperones bound to it and exposure of nuclear localisation sequences (Gelmann, 2002; Heinlein & Chang, 2002*a*). The hormone–receptor complex then translocates into the nucleus where it binds to the hormone-responsive elements on the promoter regions of its target genes. Depending on the co-activators/co-repressors recruited, the hormone–receptor complex leads to either the activation or repression of these genes (Rahman & Christian, 2007). This mechanism of steroid action is known as the classical or genomic pathway and because it involves gene transcription and mRNA translation, its effects usually take several hours to days to be manifested (Florini, 1970; Beato, 1989, 1996).

In addition to their genomic actions, it is now generally accepted that steroid hormones can also exert actions that are too rapid to be explained by the classical/genomic pathway (Falkenstein *et al.* 2000; Heinlein & Chang, 2002*b*; Simoncini & Genazzani, 2003). These actions occur within seconds to minutes after administration of the hormone and are generally insensitive to inhibitors of the androgen receptor (Estrada *et al.* 2003), suggesting that they are regulated by cellular signalling pathways involving surface membrane receptors and second messengers (Heinlein & Chang, 2002*b*). This mechanism of steroid action is referred to as non-genomic or non-classical (Lösel *et al.* 2003).

Although the non-classical actions of other steroid hormones such as oestrogen, progesterone and aldosterone have received a lot of attention and their actions in many tissues are well characterised (for details see review by Lösel *et al.* 2003), little is known about the non-genomic actions of steroid hormones in adult mammalian skeletal muscles. Furthermore the two studies published so far were performed on cultured muscle cells and used supra-physiological levels of testosterone (Estrada *et al.* 2000, 2003). Therefore, it is uncertain whether physiological levels of testosterone or its metabolite DHT have any rapid actions in isolated intact mammalian skeletal muscle fibres. It is also uncertain whether these actions have a physiological role.

Therefore, the primary aims of this study were to investigate: (1) the rapid actions of DHT in adult mammalian skeletal muscle fibres, (2) the cellular signalling pathway(s) mediating these actions, and (3) their physiological role. Our results show that the rapid actions of DHT in adult mammalian skeletal muscles include the modulation of force production. These effects are mediated through the EGF receptor (EGFR) and involve the phosphorylation of the 20 kDa regulatory myosin light chains (RMLCs) by the extracellular signal-regulated kinases (ERK) 1/2.

Methods

Preparation of intact skeletal muscle fibre bundles

All the experiments reported here were performed at $20 \pm 0.1^{\circ}$C on small 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 ($n = 20$) and male ($n = 4$) CD1 mice aged 49 ± 5 days (range 40–84 days). The mice were killed by cervical disarticulation as recommended in the Animals (Scientific Procedures) Act 1986 (for details of the regulations see Drummond, 2009) and all the experiments conformed to the local animal welfare committee guidelines. The EDL and soleus muscle from both hind limbs were isolated. Small muscle fibre bundles (~10–15 fibres, mean cross-sectional diameter 230.6 ± 17.7 μm, $n = 24$) were dissected under dark-field illumination and care was taken to ensure that the fibres in a bundle were electrically excitable.

Determination of the effects of dihydrotestosterone (DHT) and testosterone on force

The procedure used was basically similar to that described previously by Mutungi & Ranatunga (2000). Briefly, the fibre bundles were mounted horizontally between two stainless steel hooks, one attached to a force transducer (Model 400A, Aurora Scientific Inc., Ontario, Canada) and the other to a servo-motor (Model 322C, Aurora Scientific Inc.), in a muscle chamber with a glass bottom. The bundles were then perfused, at the rate of 1 ml min^{-1}, with the standard Ringer solution or the standard Ringer solution plus the various compounds whose effects were being tested. The standard Ringer solution contained (in mM): 109 NaCl, 5 KCl, 1 $MgCl_2$, 4 $CaCl_2$, 24 $NaHCO_3$, 1 $NaHPO_4$, 10 sodium pyruvate plus 200 mg l^{-1} bovine calf serum; the pH was maintained at ~7.24 by continuously bubbling with 95% O_2 and 5% CO_2. The temperature of the muscle chamber was maintained using a thermoelectric controller (Model 825A, Aurora Scientific Inc.) and a Peltier device placed next to the muscle chamber.

At the beginning of each experiment, the sarcomere length of each preparation was set at 2.4 μm using a He-Ne laser (Laser Lines Ltd, Oxon, UK). The preparations were then electrically stimulated once every 90 s using a single supra-maximal stimulus to elicit twitch contractions and once every 5 min with trains of stimuli (30–60 Hz in slow twitch fibres and 90–120 Hz in the fast twitch fibres) to elicit fully fused tetani. The fibre bundles were then perfused with either the standard Ringer solution or the standard Ringer solution plus 630 ρg ml^{-1} androstanolone (17β-hydroxy-5α-androstan-3-one, DHT) or testosterone propionate (Sigma, Gillingham, Dorset, UK) dissolved in

Table 1. The receptors/proteins tested and the inhibitors used

	Inhibitors			
Receptor/protein	Scientific name	Common name	Concentration	Source
Androgen receptor (AR)	6-Chloro-1β,2β-dihydro-17-hydroxy-3′*H*-cyclopropa(1,2)-pregna-1,4,6-triene-3,20-dione acetate	Cyproterone acetate	1 μM	Sigma-Aldrich
	2-Methyl-*N*-[4-nitro-3-(trifluoromethyl) phenyl]-propanamide	Flutamide	3 μM	Sigma-Aldrich
Epidermal growth factor receptor (EGFR)	(*N*-[3-Chlorophenyl]-6,7,-dimethoxy-4-quinazolamine)	Tyrphostin AG 1478	100 nM	Sigma-Aldrich
Myosin light chain kinase (MLCK)	1-(5-Iodonaphthalene-1-sulfonyl) homopiperazine, HCl	ML-7 hydrochloride	500 nM	Sigma-Aldrich
MAP kinase kinase (MEK)	2′-Amino-3′-methoxyflavone	PD98059	20 μM	Alexis biochemicals
Platelet-derived growth factor receptor (PDGFR)	4-(6,7-Dimethoxy-4-quinazolinyl)-*N*-(4-phenoxyphenyl)-1-piperazinecarboxamide	PDGFR tyrosine kinase inhibitor III	200 nM	Calbiochem
Insulin-like growth factor 1 receptor (IGF-1R)	α-Cyano-(3-methoxy-4-hydroxy-5-iodocinnamoyl)-(3′,4′-dihydroxyphenyl) ketone	Tyrphostin AG 538	100 μM	Sigma-Aldrich
Src kinase (src)	4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4,d]pyrimidine	PP_2	10 μM	Alexis biochemicals

absolute ethanol for at least 30 min. Twitch and tetanic contractions were then recorded in the presence or absence of DHT and testosterone.

In another experiment, the fibre bundles were perfused for 15 min with Ringer solution containing the inhibitors listed in Table 1. The Ringer solution was then switched to one containing the inhibitor plus either DHT or testosterone for at least 30 min. Twitch and tetanic contractions were then recorded. Finally, the fibre bundles were switched back to the standard Ringer solution.

Most of the compounds were dissolved in either DMSO or absolute ethanol. Therefore, to ensure that the vehicle (absolute ethanol or DMSO) had no affect on maximum isometric force (P_o), some of the fibre bundles were treated for 30 min with Ringer solution containing the vehicle only. Twitch and tetanic contractions were then recorded. Analysis of these data showed that, at the concentration used, the vehicle had no effect on either twitch or tetanus.

At the end of the experiments, some of the muscle fibre bundles were processed for Western blot analysis and their myosin heavy chain isoforms were analysed using monoclonal antibodies from Sigma-Aldrich (Fig. 2*D*).

Determination of the cellular signalling pathways mediating the rapid actions of DHT and testosterone

To investigate the cellular signalling pathway(s) mediating the effects of DHT and testosterone on force, small muscle fibre bundles (~200 μm in diameter) were used. The fibre bundles were divided into two equal groups. One half was treated with the standard Ringer solution plus the vehicle (absolute ethanol/DMSO) for 1 h. Proteins from these fibres acted as controls. The other half was treated for the same period of time with Ringer solution containing 630 ρg ml^{-1} DHT or testosterone. In some experiments, the fibre bundles were pre-incubated for 15 min in Ringer solution containing the inhibitors listed in Table 1. They were then treated with DHT or testosterone plus the inhibitor for a further 1 h. During these experiments, the fibre bundles were subjected to twitch contractions once every 90 s.

Immunoblotting

At the end of the experiments described above, the muscle fibre bundles were snap frozen in liquid nitrogen, pulverised and proteins extracted using NP40 lysis buffer. The proteins were then immunoblotted as previously described by Mutungi (2008). Briefly, equal amounts of the proteins (10 μg per lane) were resolved in 10% SDS-polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked for non-specific antibody binding with 5% milk for at least 30 min. They were then incubated overnight with antibodies against ERK1/2 phosphorylated at threonine 202 and serine 204 (Santa Cruz Biotechnology, CA, USA), the

20 kDa regulatory myosin light chains phosphorylated at serine 20 (Abcam, Cambridge, UK), the C-terminus of the AR (Santa Cruz Biotechnology) and the N-terminus of the AR (Abcam). The following day the membranes were incubated with species-specific secondary antibodies conjugated to horseradish peroxidase. Finally, they were visualised using SuperSignal WestPico (Perbio Science UK Ltd, Cramlington, UK) chemiluminescence substrate and exposure of the membranes to film.

On the third day, the membranes were stripped and re-probed for total ERK1/2 (Santa Cruz Biotechnology) and total regulatory myosin light chains (Abcam). In some experiments, the membranes were probed for actin using a pan-actin antibody from Abcam.

Data handling and analysis

The force (produced by the muscle fibres) and the temperature (from the thermocouple) signals were collected via a CED 1401 Micro laboratory interface using Signal 2.11 software (Cambridge Electronic Design Ltd, Cambridge, UK) and stored in a computer. The amplitude of the twitch and tetanus was determined using the Signal 2.11 software. The amplitude of the tension (= force) records was normalised to the cross-sectional area of the bundles. The tension recorded from each bundle under the various experimental conditions was averaged and divided by the mean tension recorded from the same fibre bundle in the standard Ringer solution. The data are presented as a percentage ± S.E.M. of the control tension.

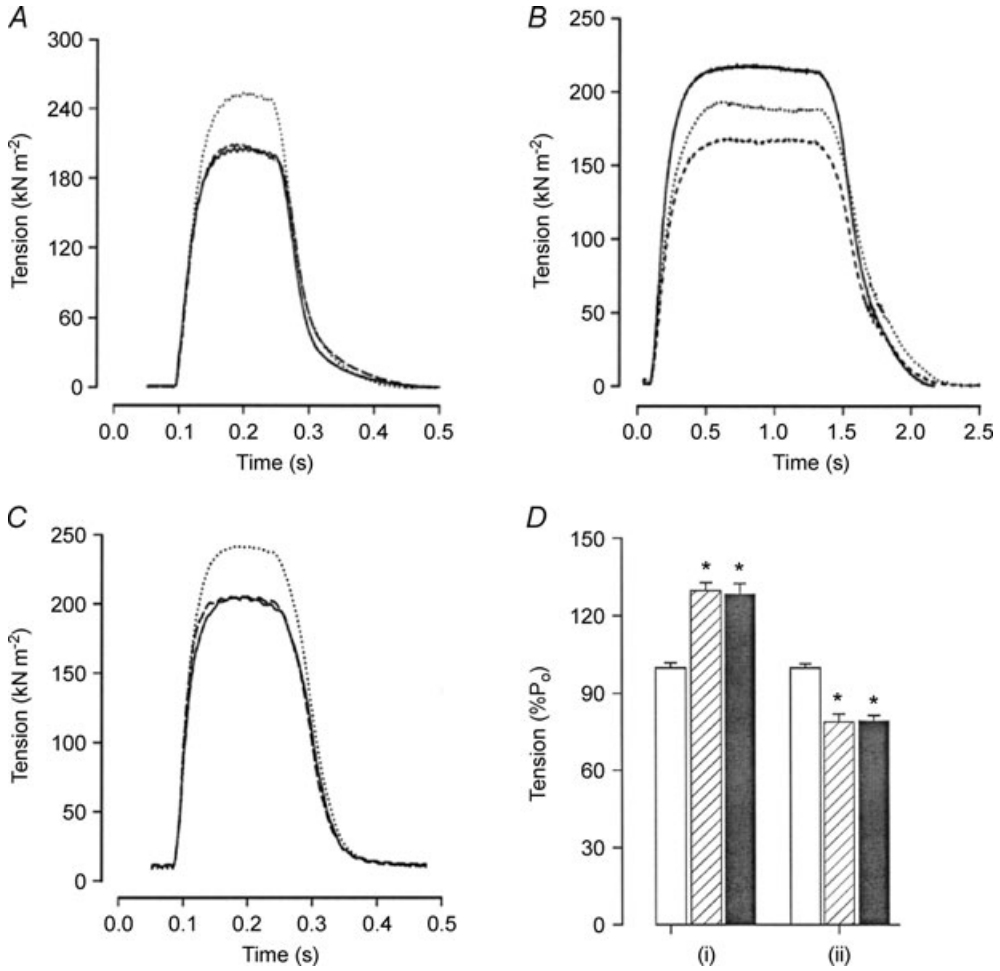

Figure 1. The effects of DHT on maximum isometric force (P_o) in isolated intact mammalian skeletal muscle fibre bundles are fibre type dependent

Representative force records obtained from a female fast twitch (*A*), a female slow twitch (*B*) and a male fast twitch (*C*) muscle fibre bundle before (continuous line traces), during (dotted line traces) and after (dashed line traces) exposure to DHT. Note that DHT leads to a reversible increase in P_o in the fast twitch fibre bundles and to an irreversible decrease in P_o in the slow twitch muscle fibre bundles irrespective of whether they are from a male or female mouse. *D*, data summarising the effects of DHT on P_o in fast (i) and slow (ii) twitch muscle fibre bundles isolated from 10 female (hatched bars) and 4 male (filled bars) mice. The data are presented as a percentage of the P_o recorded from the same fibre bundles perfused with Ringer solution containing no added DHT (open bars). Note that DHT induces a statistically significant ($*P = 0.02$) increase in P_o in the fast twitch fibre bundles but to a statistically significant ($*P = 0.03$) decrease in P_o in the slow twitch fibre bundles.

All the Western blots were run in triplicate and each experiment was repeated at least three times. The intensity of the protein bands from an experiment were analysed using Scion Image from NIH and are presented as mean ± S.D. Statistical analysis of the data was performed using Statistica 5.0 (StatSoft. Inc., Tulsa, USA). Comparison of the various data sets was performed using two-way ANOVA and $P < 0.05$ was considered statistically significant.

Results

The effects of treating small muscle fibre bundles with DHT and testosterone on maximum isometric tension (P_o)

Figures 1 and 2 show the basic observations from this study. As the results show, treating small skeletal muscle fibre bundles with physiological levels of DHT for at least 30 min increased P_o in the fast twitch fibre bundles (Fig. 1*A*) but decreased it in the slow twitch fibre bundles (Fig. 1*B*) irrespective of whether they were from a female (Fig. 1*A* and *B*) or male (Fig. 1*C*) mouse. The increase in P_o observed in the fast twitch fibres was transitory and P_o reverted back to its control values within 1–2 h after the fibres were transferred back into the standard Ringer solution. In contrast, the decline in P_o recorded in the slow twitch fibres was considered to be permanent as none of the fibres fully recovered after they were transferred back into the standard Ringer solution.

The summary data from 10 female and 4 male mice is shown in Fig. 1*D*. As the data shows, DHT treatment led to a 29.6 ± 3% ($n = 8$ fibre bundles) and to a 24.1 ± 3% ($n = 4$ fibre bundles) increase in P_o in the female and male fast twitch muscle fibre bundles, respectively. Conversely, it

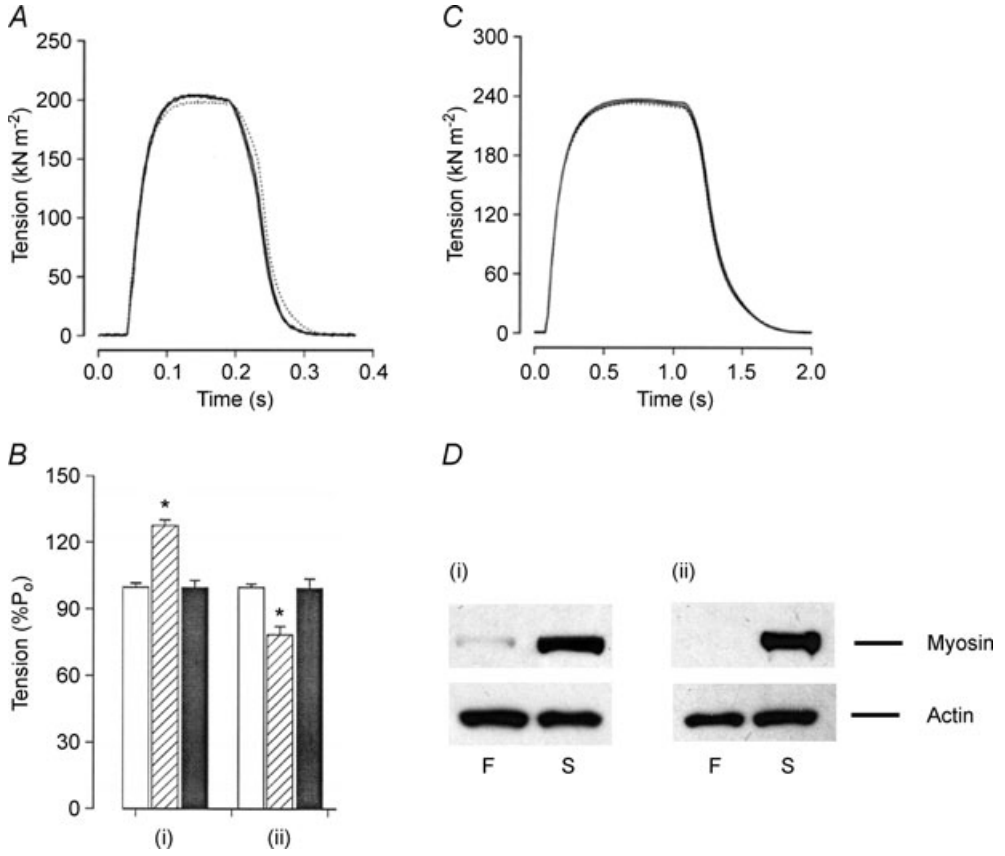

Figure 2. Testosterone has no effect on P_o in isolated intact mouse skeletal muscle fibre bundles
Force records obtained from a female fast (*A*) and a female slow (*C*) twitch muscle fibre bundle before (continuous line traces), during (dotted line traces) and after (dashed line traces) treatment with 630 ρg ml^{-1} testosterone. Note that testosterone has no effect on P_o in either fibre type. *B*, summary data showing the effects of DHT (hatched bars) and testosterone (filled bars) on P_o in 5 fast (i) and 4 slow (ii) twitch muscle fibre bundles isolated from 6 female mice. The open bars show data recorded from the same fibre bundles in Ringer solution containing no testosterone. Note that while DHT significantly (*$P = 0.02$) increases P_o in the fast twitch fibres and significantly (*$P = 0.025$) decreases it in the slow twitch fibre bundles, testosterone had no effect on P_o in either fibre type. *D*, representative Western blots showing the myosin heavy chain (MHC) isoform composition of female fast (F) and female slow (S) twitch muscle fibre bundles probed using monoclonal antibodies against fast (i) and slow (ii) MHC. Note that each bundle consists mainly of a single MHC isoform.

led to a 21.1 ± 3% ($n = 7$ fibre bundles) and to a 19.8 ± 3% ($n = 4$ fibre bundles) decrease in P_o in the female and male slow twitch fibre bundles, respectively. Statistical analysis of the data showed that the increase or decrease in force was statistically significant. However, no statistical difference was observed between the effects of DHT in the fibres isolated from male and female mice.

On the other hand, when the muscle fibre bundles were treated with testosterone propionate, no changes in P_o were observed in either fibre type (Fig. 2).

The effects of treating small muscle fibre bundles with DHT and testosterone on twitch tension

The effects of treating small muscle fibre bundles with DHT and testosterone on twitch tension are displayed in Fig. 3. As the results show, the effects of these hormones on twitch tension were similar to those on P_o. Thus, treating the fibre bundles with DHT led to a 23.6 ± 1.3% ($n = 6$ fibre bundles) increase in peak twitch tension in fast twitch muscle fibre bundles and to a 15.3 ± 2.1% ($n = 6$ fibre bundles) decrease in twitch tension in slow twitch fibre bundles isolated from adult female mice (Fig. 3*B*). However, DHT did not alter the characteristics of the isometric twitch (Fig. 3*A* and *C*). For example, the half rise time of twitch tension in the fast fibres was 9.3 ± 0.8 ms and 9.6 ± 0.4 ms in the absence and presence of DHT, respectively. The corresponding values in the slow twitch fibres were 20.5 ± 3 ms and 21.2 ± 6 ms in the presence and absence of DHT, respectively. Treating the fibre bundles with testosterone had no effects on the amplitude of the isometric twitch tension in both fibre types (Fig. 3*D*). Similar effects were seen in muscle fibre bundles isolated from male mice (results not shown).

The effects of treating small muscle fibre bundles with DHT and testosterone on the activation of the mitogen-activated protein kinase (MAPK) pathway

It has been previously shown that treating cultured muscle cells with supra-physiological doses of testosterone increases the phosphorylation of ERK1/2 (Estrada *et al.* 2003). However, from these results it is uncertain whether similar changes occur in adult mammalian skeletal muscle fibres treated with physiological levels of either DHT or testosterone. Therefore, in another experiment we investigated the effects of treating small skeletal muscle fibre bundles isolated from adult female mice with either

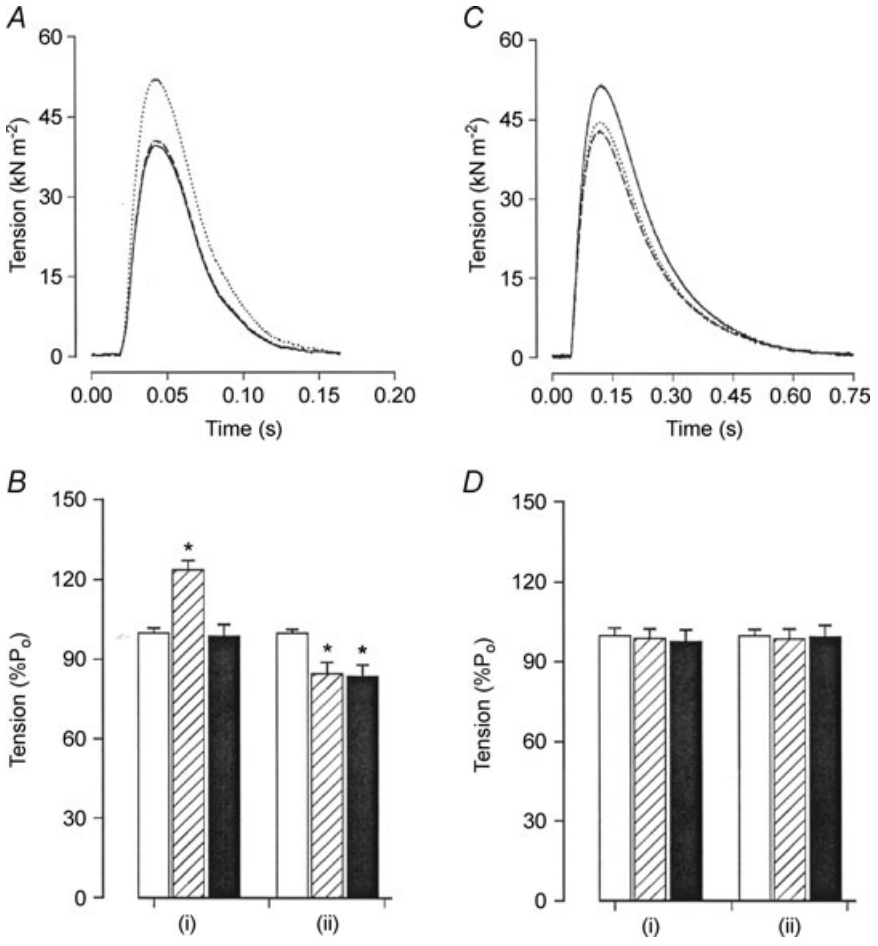

Figure 3. The effects of DHT on isometric twitch tension in isolated intact mammalian skeletal muscle fibre bundles are fibre type dependent
Representative twitch myographs recorded from a female fast (*A*) and a female slow (*C*) twitch muscle fibre bundle before (continuous line traces), during (dotted line traces) and after (dashed line traces) treatment with DHT. Note that DHT reversibly increases twitch tension in the fast twitch fibre bundle and irreversibly decreases it in the slow twitch muscle fibre bundle. Summary data illustrating the effects of DHT (*B*, hatched bars) and testosterone (*D*, hatched bars) on isometric twitch tension in 6 fast (i) and 6 slow (ii) twitch muscle fibre bundles isolated from 6 adult female mice. The open and filled bars show the twitch tension recorded from the same fibre bundles before and after treatment with DHT, respectively. Note that DHT induces a statistically significant (*$P = 0.021$) increase in P_o in the fast twitch fibre bundles and a statistically significant (*$P = 0.034$) decrease in P_o in the slow twitch fibre bundles.

DHT or testosterone on the phosphorylation of ERK1/2. As the results in Fig. 4 show, treating small muscle fibre bundles with physiological concentrations of DHT for 1 h led to a 2- to 3-fold increase in the phosphorylation of ERK1/2 in both fibre types (Fig. 4*A*). In contrast, testosterone led to an increase in the phosphorylation of ERK1/2 in slow twitch fibres only (Fig. 4*B*).

To determine whether there was a link between the phosphorylation of ERK1/2 and the changes in tension reported above, some of the fibre bundles were pre-treated with Ringer solution containing 20 μM of the mitogen-activated protein kinase kinase (MEK)1/2 inhibitor PD98059. They were then treated with Ringer solution containing DHT and the inhibitor for 1 h. As the results in Fig. 4*C* show, pre-treating the fibre bundles with PD98059 completely abolished the effects of DHT on the phosphorylation of ERK1/2.

Androgen receptor density in fast and slow twitch skeletal muscles

Previously, it has been suggested that the diverse effects of steroid hormones on specific muscle tension observed in animal experiments arise from differences in their androgen receptor (AR) densities (Egginton, 1987; Salmons, 1992). Therefore, to test this hypothesis and to determine whether the different effects of DHT on P_o in fast and slow twitch muscle fibre bundles reported above arose from differences in their AR densities, we examined

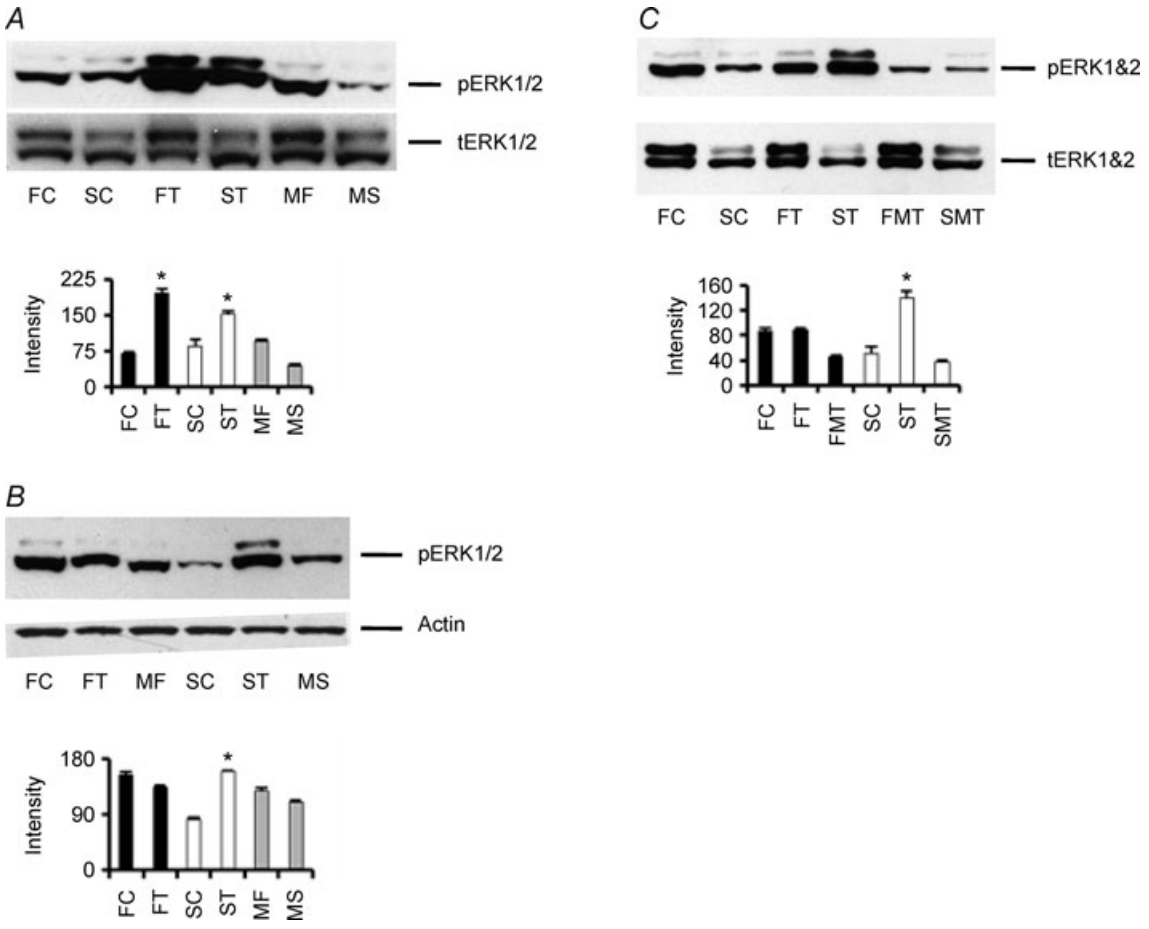

Figure 4. DHT increases the phosphorylation of ERK1/2
Representative Western blots showing the concentration of phosphorylated ERK1/2 (pERK1/2; *A*, *B* and *C*) in female fast twitch (F) and female slow twitch (S) skeletal muscle fibre bundles treated with either the standard Ringer solution (FC and SC) or the standard Ringer solution plus 630 ρg ml^{-1} DHT (*A*, FT, ST) or testosterone (*B*, FT, ST). In *C*, the fibre bundles were pre-treated with the MEK1/2 inhibitor PD89059, followed by DHT plus the inhibitor (FMT, SMT). Note that treating the muscle fibre bundles with DHT significantly ($^*P = 0.02$) increased the phosphorylation of ERK1/2 in both fibre types, whereas testosterone increased the phosphorylation of ERK1/2 in slow twitch fibres only. Moreover, the effects of DHT on the phosphorylation of ERK1/2 were abolished by PD89059 and fast twitch muscle fibre bundles expressed higher concentrations of total ERK1/2 (tERK1/2) than slow twitch fibres.

the concentration of the AR in EDL (fast twitch) and soleus (slow twitch) muscles of adult male and female mice. As the results displayed in Fig. 5 show, one of the antibodies (from Santa Cruz) identified two proteins: one with a molecular mass of 110 kDa and the other ∼67 kDa in all the samples probed (Fig. 5*A*). On the other hand, a second antibody (from Abcam) identified only one protein with a molecular mass of ∼110 kDa (Fig. 5*B*). Moreover, the concentration of both proteins was similar in the fast and slow twitch muscles isolated from each animal irrespective of sex. From these results, we think that the protein with a molecular mass of ∼110 kDa corresponds to the full-length AR (also known as AR-B), whereas that with a molecular mass of ∼67 kDa represents the truncated or AR-A isoform (Wilson & McPhaul, 1996).

The effects of DHT and testosterone on the phosphorylation of the 20 kDa regulatory myosin light chains

Previously, a number of studies have shown that the motility of non-muscle cells results from an ERK1/2-dependent increase in the phosphorylation of the 20 kDa RMLCs (Klemke *et al.* 1997; Iwabu *et al.* 2004). However, it is uncertain whether the changes in the phosphorylation of ERK1/2 induced by DHT in intact mammalian skeletal muscles have similar effects on the phosphorylation of RMLCs. Therefore, in another experiment, we investigated the effects of DHT and testosterone on the phosphorylation of the 20 kDa RMLCs. As the results displayed in Fig. 6 show, treating the muscle

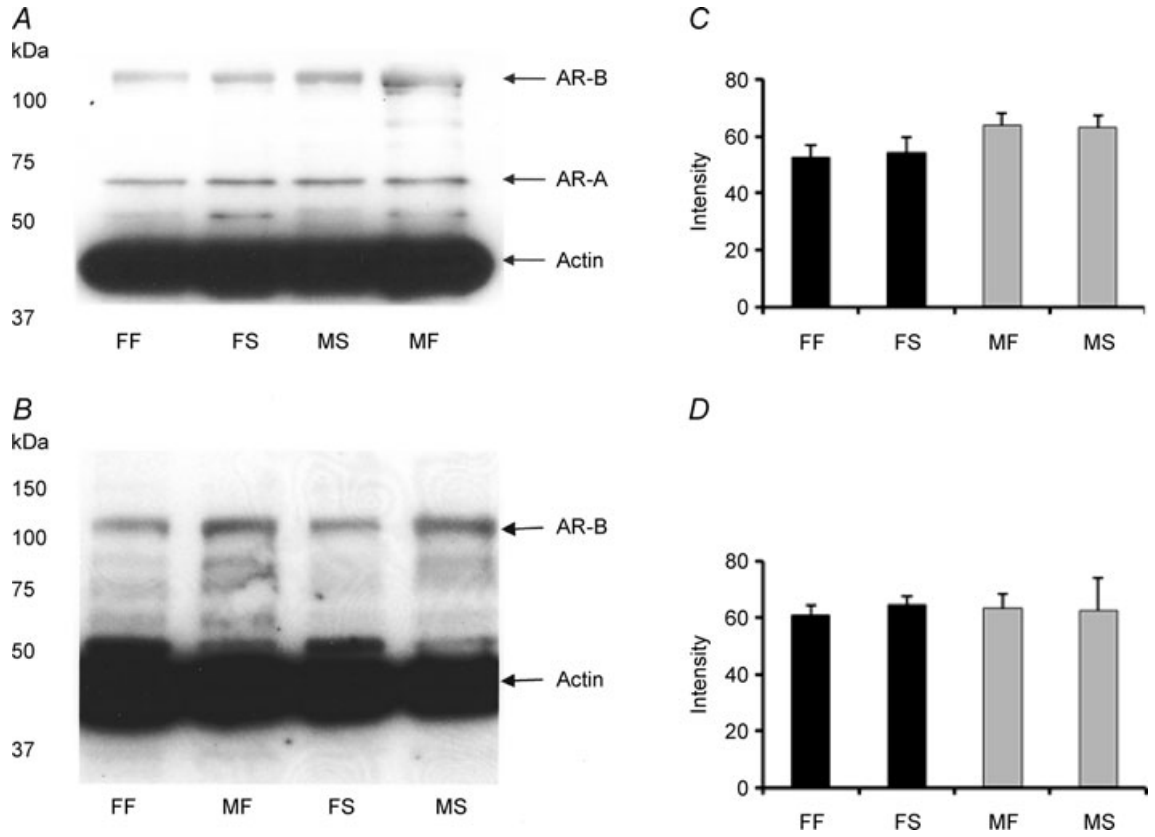

Figure 5. Fast twitch and slow twitch muscles express similar concentrations of the androgen receptor
Western blots showing the androgen receptor expression in female fast (FF), female slow (FS), male fast (MF) and male slow (MS) twitch skeletal muscles immunoblotted with a polyclonal antibody against the C-terminus of the human AR (*A*) or a monoclonal antibody against the N-terminus of the human AR (*B*). Note that the polyclonal antibody identified two proteins, one with a molecular mass of ∼110 kDa (AR-B) and the other with a molecular mass of ∼67 kDa (AR-A), whereas the monoclonal antibody identified only a single band with a molecular mass of ∼110 kDa. *C* and *D*, summary data showing the relative concentrations of AR-B (*C*) and AR-A (*D*) in fast (F) and slow (S) twitch muscles isolated from male (M) and female (F) mice. Note that fast and slow twitch muscles from the same sex animal express similar concentrations of the AR and that no significant difference ($P = 0.12$) was observed when the concentration of the receptor in the male and female muscles were compared.

fibre bundles with DHT led to a 20–40% increase in the phosphorylation of RMLCs in fast twitch fibre bundles only (Fig. 5*A*). In contrast, testosterone, which had no effect on P_o led to a decrease in the phosphorylation of RMLCs in both fibre types (Fig. 6*C*). Furthermore, treating the fibre bundles with the myosin light chain kinase inhibitor ML-7 did not significantly affect P_o (Fig. 6*D*) but completely reversed the effects of DHT on force (Fig. 6*B*).

Identity of the surface membrane receptor mediating the rapid actions of DHT in mammalian skeletal muscle fibres

As stated in the Introduction, the genomic/classical actions of anabolic androgenic steroids (AAS) are mediated through the androgen receptor. Therefore, to determine whether the effects of DHT described above were exerted through this mechanism, small muscle fibre bundles were treated with Ringer solution containing the AR inhibitors cyproterone (a steroidal inhibitor) and flutamide (a non-steroidal inhibitor). As the results in Fig. 7 show, treating the fibre bundles with cyproterone had no effect on the DHT-induced changes in force (Fig. 7*A*), ERK1/2 phosphorylation (Fig. 7*B*) and the phosphorylation of the RMLCs (Fig. 7*C*). Similar results were obtained when the muscle fibre bundles were treated with flutamide (results not shown), the insulin-like growth factor 1 receptor (IGF-1R) inhibitor tyrphostin AG 538 (Fig. 8*A* and *B*), the Src kinase (src)-specific inhibitor PP2 (results not shown) and the platelet-derived growth factor receptor (PDGFR) inhibitor (Fig. 8*C* and *D*).

A number of studies have recently suggested that the ERK1/2-dependent phosphorylation of MLCs and hence the motility of non-muscle cells is mediated through the EGFR (Klemke *et al.* 1997; Iwabu *et al.* 2004). Therefore, in another experiment we used pharmacological interventions to investigate whether the rapid actions of DHT in adult mammalian skeletal muscle fibres were mediated through the EGFR. As the results displayed in Fig. 9 show, treating the fibres with the EGFR-specific inhibitor tyrphostin AG 1473 completely abolished the effects of DHT on P_o in both fibre types (Fig. 9*A*). It also blocked the DHT-induced increase in the phosphorylation of ERK1/2 (Fig. 9*B*) as well as that of RMLCs (Fig. 9*C* and *D*).

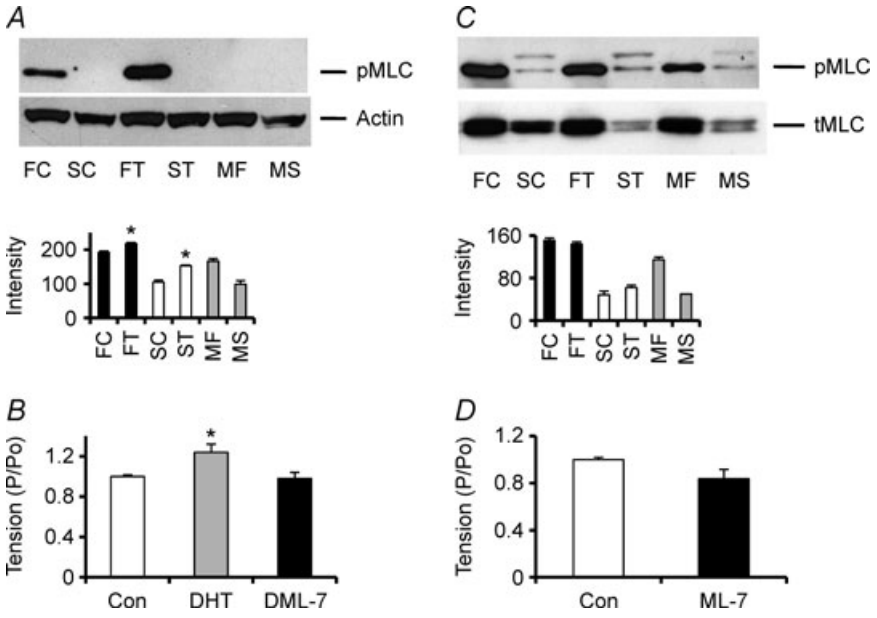

Figure 6. DHT increases the phosphorylation of regulatory myosin light chains in fast and slow twitch muscle fibre bundles

Representative Western blots showing the effects of treating female fast twitch (F), female slow twitch (S), male fast twitch (MF) and male slow twitch (MS) skeletal muscle fibre bundles treated with Ringer solution containing either DHT (*A*) or testosterone (*C*) on phosphorylation of the 20 kDa regulatory myosin light chains (pMLC; *A* and *C*). Note that treating the muscle fibre bundles with DHT significantly ($*P = 0.018$) increases the phosphorylation of the RMLCs in fast twitch fibre bundles (*A*). In contrast, treating the bundles with testosterone led to a small decrease in the phosphorylation of the RMLCs which was not statistically different from that in control fibres ($P = 0.08$). Furthermore, fast twitch fibres contained 15–20% more unphosphorylated myosin light chains (tMLC) than slow twitch fibres. *B* and *D* show the effects of treating small muscle fibre bundles with the standard Ringer solution (Con), the standard Ringer solution plus DHT (DHT), or the standard Ringer solution containing the myosin light chain kinase inhibitor ML-7 alone (*D*, ML-7) or with DHT (*B*, DML-7) on P_o. Note that treating the fibre bundles with ML-7 alone has no effect on P_o (*D*) but reversibly abolishes the effects of DHT on P_o.

Discussion

One of the key findings in the present study is the observation that treating small skeletal muscle fibre bundles with Ringer solution containing physiological levels of male sex steroids can modulate P_o in a fibre type- and hormone-dependent manner. Thus, DHT increased P_o in fast twitch fibres but decreased it in slow twitch fibres. In contrast, testosterone had no effect on P_o in both fibre types. Although the effects of treating animals with AAS for several weeks have been the subject of a number of previous studies and some of their effects are generally accepted (Exner *et al.* 1973*a*,*b*; Egginton, 1987; Salmons, 1992), their effects on specific muscle force are still controversial. For example, treating female rats (Egginton, 1987), male rats (Exner *et al.* 1973*a*) and female rabbits (Salmons, 1992) with nandrolone for 4–6 weeks has been shown to have no effect on specific muscle force in the EDL and soleus muscles. On the other hand, it increased specific muscle force in the flexor hallucius muscle of female rats (Exner *et al.* 1973*b*) and the tibialis anterior of female rabbits (Salmons, 1992). Interestingly, a similar increase in specific muscle force could be induced by exercise alone or exercise plus nandrolone (Exner *et al.* 1973*b*) suggesting that the effects of the AAS may have been

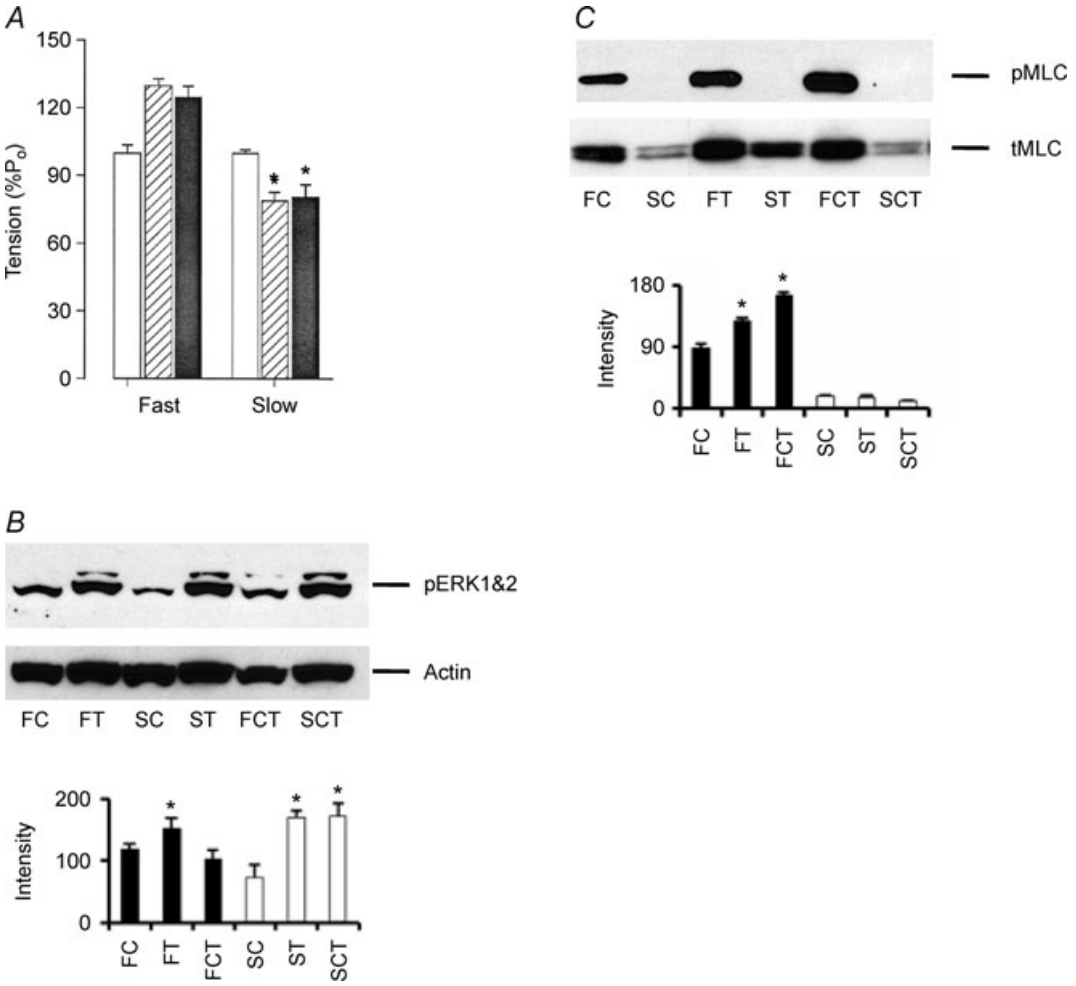

Figure 7. Inhibiting the AR does not abolish the effects of DHT in isolated intact mammalian skeletal muscle fibres

A, summary data showing the effects of treating small skeletal muscle fibre bundles with the standard Ringer solution (open bars), the standard Ringer solution plus DHT (hatched bars) and the standard Ringer solution containing DHT plus the AR inhibitor cyproterone (filled bars) on P_o. Note that treating the muscle fibres bundles with cyproterone does not reverse the effects of DHT on P_o in either fibre type. *B* and *C*, representative Western blots and summary data showing the effects of treating fast (F) and slow (S) twitch skeletal muscle fibre bundles isolated from female mice with the standard Ringer solution (FC, SC), DHT alone (FT, ST) or DHT plus cyproterone (FCT, SCT) on the phosphorylation of ERK1/2 (*B*) and regulatory myosin light chains (*C*). Note that treating the muscle fibre bundles with DHT significantly ($^*P = 0.015$) increases the phosphorylation of ERK1/2 in both fibre types (*B*) and the regulatory myosin light chains in fast twitch fibre bundles only (*A*) and that treating the fibre bundles with cyproterone does not reverse these effects.

non-specific. It is not just in animal experiments where the effects of AAS on specific muscle force are controversial, the results from human studies are as variable (Bhasin *et al.* 2001).

Previously, this variability has been attributed to factors such as the generalised increase in muscle mass that accompanies chronic AAS treatment (Egginton, 1987), the sensitivity of different muscles/species to the AAS (Salmons, 1992), the androgen receptor density in the muscles (Salmons, 1992), route of administration and variability in the resorption of the drugs from muscles and fat depots (van der Vies, 1970), study design (Bhasin *et al.* 2001) and the effects of the AAS on the nervous system (Blanco *et al.* 1997; Nguyen *et al.* 2005). In the present study, most of these variables were eliminated by the use of small muscle fibre bundles isolated from age- and weight-matched mice kept under the same laboratory conditions. Furthermore, each fibre bundle acted as its own control and the AAS was delivered directly to the fibres. Additionally, fast and slow twitch muscles contained

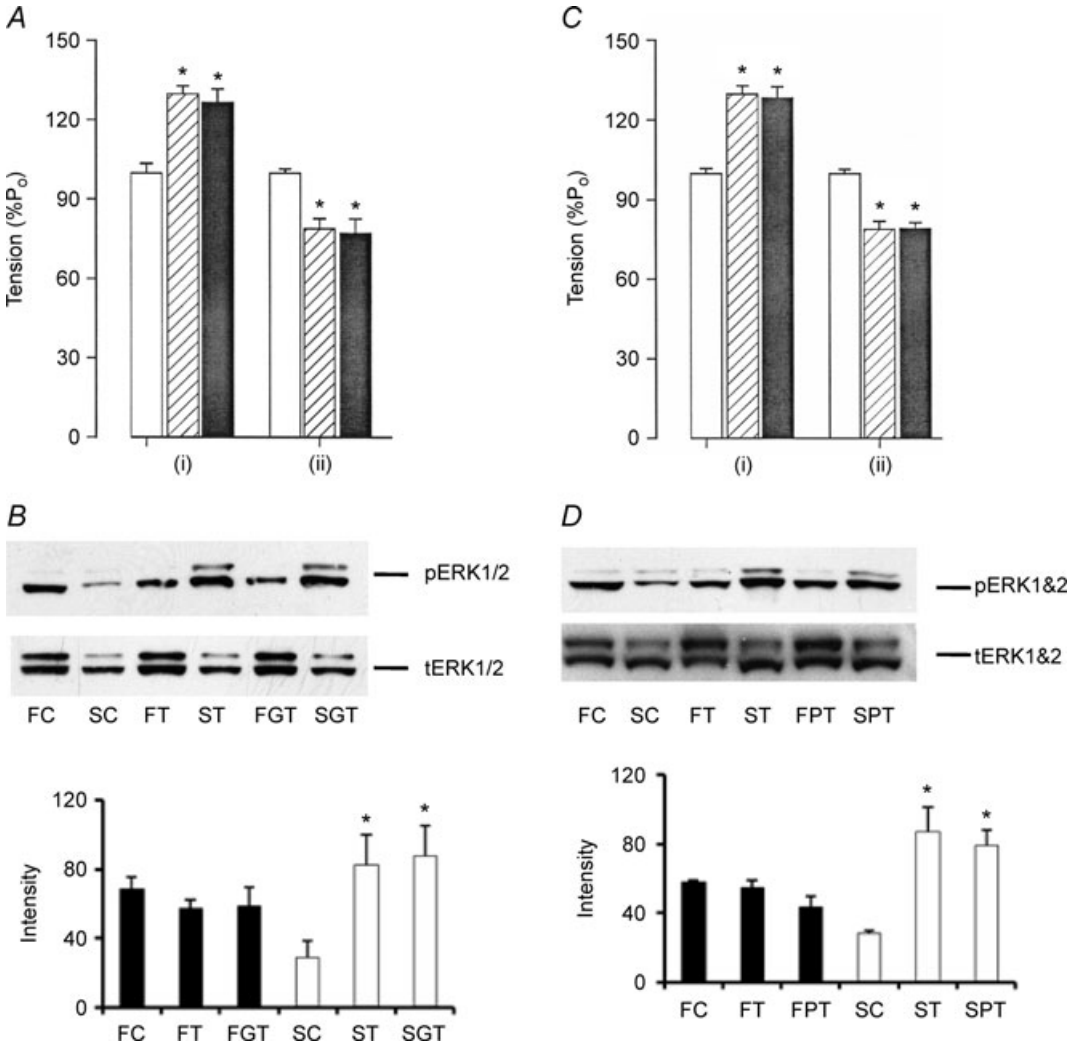

Figure 8. Inhibiting the IGF-1 and the PDGF receptors does not abolish the effects of DHT in isolated intact mammalian skeletal muscle fibres

Summary data showing the effects of treating small skeletal muscle fibre bundles with the standard Ringer solution (open bars), the standard Ringer solution plus DHT (hatched bars) and the standard Ringer solution containing both DHT and the IGF-1R inhibitor tyrphostin AG 538 (*A*) or the PDGFR inhibitor tyrosine kinase inhibitor III (*C*) on P_o in fast (i) and slow (ii) twitch muscle fibre bundles. Note that treating the muscle fibres bundles with both inhibitors does not reverse the effects of DHT on P_o in either fibre type. *B* and *D*, representative Western blots and summary data showing the effects of treating fast (F) and slow (S) twitch skeletal muscle fibre bundles isolated from female mice with the standard Ringer solution (FC, SC), DHT alone (FT, ST) or DHT plus AG 538 (FGT, SGT) or tyrosine kinase inhibitor III (FPT, SPT) on the phosphorylation of ERK1/2. Note that treating the muscle fibre bundles with DHT significantly ($^*P = 0.015$) increases the phosphorylation of ERK1/2 in the slow twitch muscle fibre bundles and that adding the inhibitors does not abolish these effects.

similar levels of AR (Fig. 5). Thus, the results we report here provide the first unequivocal evidence suggesting that AAS may have direct effects on force in isolated intact mammalian skeletal muscle fibre bundles, and that these effects may depend on both the fibre type as well as the AAS used. Therefore, it is possible that the variable effects of AAS on specific muscle force reported previously in both animal and human experiments may have arisen from the fibre type composition of the various muscles examined, the AAS used, or a combination of both factors.

In addition to the changes in force reported above, treating small muscle fibre bundles with DHT led to an increase in the phosphorylation of ERK1/2 in both fibre types and the 20 kDa RMLCs in fast twitch fibres. It is important to note that testosterone, which had no effect on maximum isometric tension in either fibre type, did not affect the phosphorylation of RMLCs in both fibre types and increased the phosphorylation of ERK1/2 in the slow twitch fibres only. In both muscle and non-muscle cells, force generation results from the interaction of myosin and actin leading to the formation of the

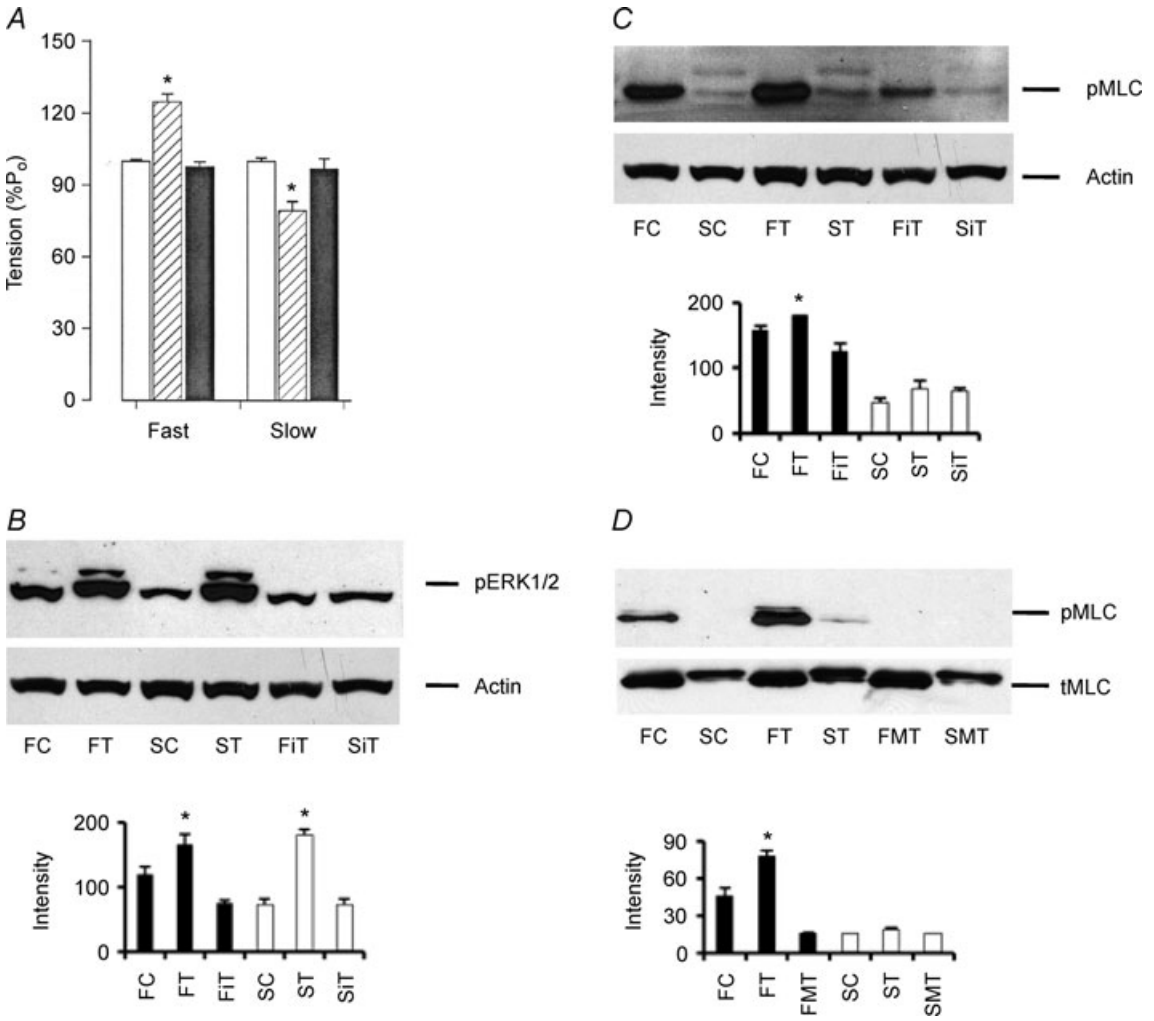

Figure 9. Inhibiting the EGF receptor abolishes the effects of DHT in isolated intact mammalian skeletal muscle fibres

A, summary data showing the effects of treating small skeletal muscle fibre bundles with the standard Ringer solution (open bars), the standard Ringer solution plus DHT (hatched bars) and the standard Ringer solution containing DHT plus the EGFR inhibitor tyrphostin AG 1478 (filled bars) on P_o. Note that treating the muscle fibre bundles with tyrphostin AG 1478 reverses the effects of DHT on P_o in both fibre types. *B*, *C* and *D*, representative Western blots and summary data showing the effects of treating fast (F) and slow (S) twitch skeletal muscle fibre bundles isolated from female mice with the standard Ringer solution (FC, SC), DHT alone (FT, ST), DHT plus tyrphostin AG 1478 (FiT, SiT) or DHT plus PD89059 (FMT, SMT) on the phosphorylation of ERK1/2 (*B*) and regulatory myosin light chains (*C* and *D*). Note that treating the muscle fibre bundles with DHT significantly ($^*P = 0.01$) increases the phosphorylation of ERK1/2 (*B*) and the regulatory myosin light chains (*C* and *D*) in fast fibres and that treating the fibre bundles with tyrphostin AG 1478 and PD89059 abolishes these effects.

independent force-generating units known as crossbridges (Gordon *et al.* 2000). Although this mechanism is basically similar in all cells, its regulation in striated muscles and other cells is different. Thus, in striated muscle, the interaction between myosin and actin is regulated mainly through the thin (actin) filament, whereas, in smooth muscles and non-muscle cells it is controlled via the thick (myosin) filament (Adelstein, 1983). The thick filament regulation of actino-myosin interaction involves the phosphorylation and dephosphorylation of the 20 kDa regulatory light chains located within the myosin molecule by myosin light chain kinase and myosin phosphatase, respectively (Adelstein, 1983; Bresnick, 1999). Thus, in smooth muscle and non-muscle cells the phosphorylation of the RMLCs is essential for actino-myosin interaction to occur, whereas in striated muscles it is not. In striated muscle, the phosphorylation of these light chains seems to have a modulatory rather than a regulatory function on force production (Manning & Stull, 1979). Therefore, from our observations we suggest that the changes in force induced by DHT in mouse skeletal fibres and the phosphorylation of the RMLCs are interlinked. This suggestion is further supported by the observations that treating fast twitch muscle fibre bundles with the myosin light chain kinase inhibitor ML-7 led to a 10% decrease in P_o and completely abolished the effects of DHT on P_o (Fig. 6).

Why DHT increases maximum isometric force in fast twitch fibres and decreases it in slow twitch fibres is uncertain. Previously, it has been suggested that these differences may arise from the androgen receptor (AR) density in the muscles (Egginton, 1987; Salmons, 1992). However, as our results show, fast and slow twitch muscles from the same animal contain similar concentrations of the androgen receptor (Fig. 5). Furthermore, treating muscle fibre bundles with the androgen receptor inhibitors cyproterone and flutamide does not abolish the effects of DHT on force, the phosphorylation of ERK1/2 as well as that of the 20 kDa RMLCs (Fig. 7). Therefore, it is unlikely that the opposing effects of DHT on force in fast and slow twitch muscle fibres arise from their androgen receptor densities.

In mammalian skeletal muscles, a brief period of repetitive stimulation leads to a transitory increase of the isometric twitch in fast twitch muscles and its depression in slow twitch muscles (Close & Hoh, 1968, 1969). Furthermore, this post-tetanic twitch potentiation is accompanied by an increase in the phosphorylation of the 20 kDa RMLCs (Klug *et al.* 1982; Moore & Stull, 1984; Houston *et al.* 1985). Although the phosphorylation of the RMLC does not seem to affect maximum Ca^{2+}-activated force in chemically skinned skeletal muscle fibre bundles (Metzger *et al.* 1989), our results suggest that under certain circumstances it may. It is generally believed that the phosphorylation of RLMCs potentiates the isometric twitch by increasing the sensitivity of the contractile apparatus to Ca^{2+} and freeing crossbridges from the surface of the thick filament (Sweeny *et al.* 1993). Here we speculate that in addition to the phosphorylation of RMLCs, DHT treatment increases the phosphorylation of other proteins of the contractile system such as troponin I and myosin binding protein C that are involved in the regulation of actino-myosin interaction; and that this accentuates its effects on force production. However, the

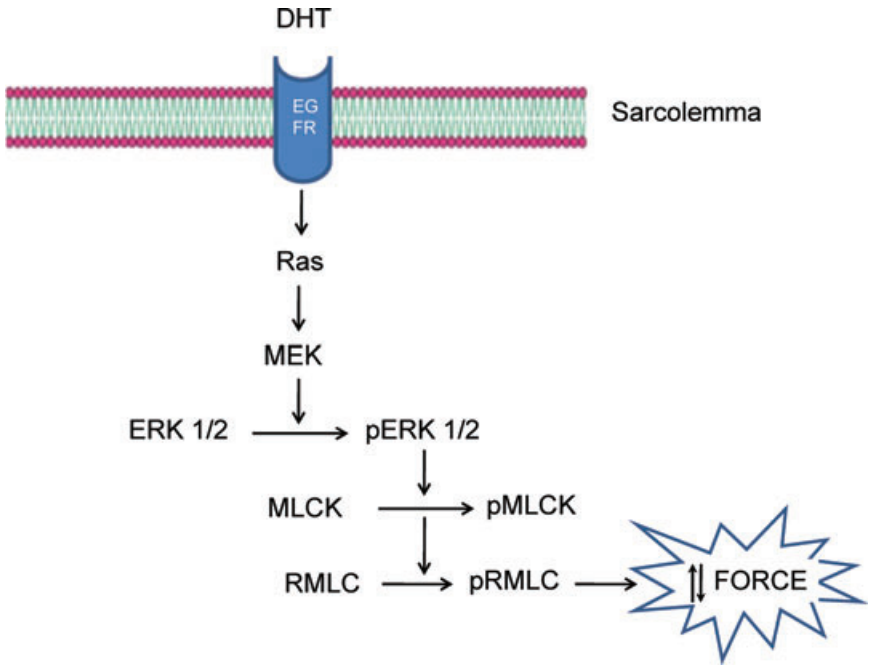

Figure 10. Proposed cellular signalling pathway mediating the non-genomic actions of DHT in adult mammalian skeletal muscle fibres
Schematic diagram illustrating the molecular mechanism that we suggest underlies the effects of DHT in adult mammalian skeletal muscle fibre bundles. Our hypothesis is that DHT activates the epidermal growth factor receptor (EGFR), either directly or indirectly, and this leads to an increase in the phosphorylation of ERK1/2. The activated ERK1/2 then phosphorylates MLCK which in turn phosphorylates the 20 kDa RMLCs and this increases force production in fast twitch fibres but decreases it in slow twitch fibres.

exact mechanism underlying the effects of DHT in isolated mammalian skeletal fibres is still uncertain and further studies to elucidate this are necessary.

In the classical or genomic pathway, testosterone exerts its effects by activating the cytosolic androgen receptor. The hormone–receptor complex then translocates to the nucleus where the hormone–receptor complex acts as a transcription factor (Simental *et al.* 1991). However, as this mechanism of steroid action involves gene transcription and mRNA translation, its effects usually take several hours to days to be manifested (Florini, 1970; Beato, 1989, 1996). In the present study, the effects of DHT were evident within 30 min after its application and were insensitive to both cyproterone and flutamide, both inhibitors of the intracellular androgen receptor. Therefore, from these results we suggest that the effects of DHT reported here are not exerted through the androgen receptor. They were also insensitive to inhibitors of src, IGF-1R and PDGFR; suggesting that they were not mediated via these receptors either. Instead, our results suggest that these effects are mediated through EGFR. Our hypothesis, shown in Fig. 10, is that DHT binds to the EGFR on the sarcolemma leading to the activation of the MAPK pathway. The activated MAPK pathway then drives the phosphorylation of the regulatory myosin light chains by myosin light chain kinase, culminating in the changes in force reported in this study. This mechanism of action is similar to that previously reported in Sertoli cells (Cheng *et al.* 2007). However, unlike that study, no evidence was observed to suggest that Src kinase is involved. In the only other study of muscles cells, it was suggested that testosterone and nandrolone exert their rapid actions through an unidentified G-protein-coupled receptor located on the cell membrane (Estrada *et al.* 2003). From the results presented here, we suggest that this receptor is probably the EGFR. However, whether the effects of DHT are direct or indirect remain uncertain and further studies are necessary.

Besides the phosphorylation of myosin light chain kinase (MLCK) the activation of the MAPK pathway in most non-muscle cells leads to the transcriptional regulation of genes important for cell proliferation and differentiation (Marshall, 1995). Therefore, it is likely that in addition to its effects on force, the activation of ERK1/2 by DHT may have other functions such as the transformation of slow to fast twitch muscle fibres. Our speculation is that the irreversible decline of force induced by DHT in slow twitch fibre bundles marks the onset of this process. The atrophying slow twitch fibres are then slowly replaced by fast fibres generated from satellite cells. Indeed, in animal models, the prolonged administration of AAS is accompanied by satellite cell activation (see review by Chen *et al.* 2005). Thus, the rapid actions of AAS in mammalian skeletal muscles may be multifaceted and their main physiological function may be to prepare the muscles for their genomic actions. However, whether this is the case is uncertain and further studies are needed.

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Author contributions

G.M. conceptualised and designed the study. He also performed the force measurement experiments. M.M.H. performed the Western blot experiments and the initial analysis of the data.

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APPENDIX 2

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

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Lay Summary

In a recent study we showed that 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. ^{14}C-labelled amino acids were used to investigate the effects of DHT and T on amino acid uptake; whereas, 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 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 EGFR, system A, system L, mTOR and MEK. However, it was relatively insensitive to those 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.

Abbreviations list: AAS, anabolic-androgenic steroid; ATF2, activating transcription 2; Akt, protein kinase B; BCH, 2-aminobicyclo-(2,2,1)heptane-2-carboxylic acid; DHT, dihydrotestosterone; 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, α-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, 90kDa ribosomal s6 kinase; SNAT2, sodium-coupled neutral amino acid transporter 2.

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 5α-reductase (Bruchovsky & Wilson, 1968). Although the free plasma concentration of DHT and T (normal range 1.5-20ng $dL^{-}1$ and 4-57.5ng 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 5α-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; AAS) 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 is the largest pool of amino acids in the body with the branched chain amino acids leucine, isoluecine 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 transport systems 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 et al. 1994; Wagner et al., 2003). 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.

Materials & Methods

Preparation of the small intact skeletal muscle fibre bundles

All the experiments reported in this study were performed at room temperature (~20°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 ± 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 hind limbs were then isolated and small muscle fibre bundles, containing approximately 10-15 fibres (mean cross-sectional diameter 350±17µm, 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 5ml and the other 25mls 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's solution alone or the standard Ringer's solution plus the various compounds/inhibitors listed in Table 1. The composition of the Ringer's solution was (in mM); 109 NaCl, 5 KCl, 1 $MgCl_2$, 4 $CaCl_2$, 24 $NaHCO_3$, 1 $NaHPO_4$, 10 sodium pyruvate plus 200mg l^{-1} bovine calf serum; and its pH was maintained at 7.42 by constantly bubbling it with 95% O_2 and 5% CO_2.

Determination of the effects of Dihydrotestosterone (DHT) and testosterone (T) on the uptake of isoleucine and α-(Methylamino)isobutyric acid

To determine the uptake of the amino acids, 8 fast-twitch and 8 slow-twitch muscle fibre bundles were divided into two groups. Half were treated with the standard Ringer's solution containing 6.3µl/100ml (107.9µM) ethanol (the vehicle used to dissolve both DHT and T) plus either 2mM C^{14} isoleucine (L-[U-^{14} C] Ile; radioactivity level 3.46µCi ml $^{-1}$) or 68.3µM C^{14} α-methylaminoisobutyric acid (α-[1-^{14}C]-MeAIB; 2-(Methylamino)-2-methylpropionic acid; radioactivity level 0.8µCi ml $^{-1}$) (both purchased from PerkinElmer, Buckinghamshire, UK) for 1hr. These preparations acted as controls. The other half were

treated with the Ringer's solution containing 630ρgml^{-1} 4,5α-Dihydrotestosterone (2.17ηM; DHT; 5α-Androstan-17β-ol-3-one) or testosterone propionate (1.83ηM; T; 17β-Hydroxy-4-androsten-3-one 17-propionate) (both from Sigma, Gillingham, Dorset, UK) plus the radio-labelled amino acids for the same duration (1hr). These fibre bundles acted as the experimental/treatment group. Radio-labelled 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's solution was aspirated and the fibre bundles were washed twice in ice cold phosphate buffer saline (PBS). The excess buffer was blotted out and the bundles were then snap frozen in liquid nitrogen, 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, Buckinghamshire, UK) and the level of radioactivity in the mixture was determined using a liquid scintillation counter (Tri-Carb 2250 CA, Canberra-Packard) 1hr and 24hrs 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 8 fast- and 8 slow- twitch muscle fibres bundles. Briefly, half of the fibre bundles were treated with the standard Ringer's solution containing 107.9µM ethanol plus the radio-labelled amino acids for 1 hr. The other half was pre-incubated for 20 minutes in the standard Ringer's solution containing one of the inhibitors listed in Table 1. They were then treated, for 1hr, with the Ringer's solution containing DHT plus either 2mM L-[U-^{14} C] Ile or 68.3µM α-[1-^{14}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.

Table 1 near here

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 2 fast- and 2 slow-twitch muscle fibre bundles. Each group was then treated for at least 20 minutes as follows; Group 1 (control) was treated with the standard Ringer's solution plus 107.9µM ethanol, Group 2 was treated with the Ringer's solution plus DHT alone, Group 3 was treated with Ringer's solution containing 10ηM deguelin (a specific (PI3K)/protein kinase B (Akt) inhibitor) (Chun et al. 2003) or 100ηml^{-1} rapamycin (an mTOR specific inhibitor) (Shepherd et al. 1995) plus 1 mM ethanol; whereas, Group 4 was treated with the Ringer's 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 2mM L-[U-^{14} C] Ile. The fibre bundles were then incubated in these solutions for 1hr after which the uptake of L-[U-^{14} C] Ile was determined as described above.

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, 6 fast-twitch and 6 slow-twitch fibre bundles were divided into two equal groups. Half (controls) were treated with the standard Ringer's

solution containing 107.9µM ethanol plus 2mM L-[U-^{14} C] Ile for 1hr. These preparations acted as controls. The other half (experimental) were treated with the Ringer's solution containing 630pgml^{-1} DHT plus the radio-labelled amino acid for the same duration (1hr). 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 2-3hrs. The lysate/TCA mixture was then centrifuged at 15000g for 20 minutes and the supernatant was discarded. The pellet was washed twice in ice-cold acetone, centrifuged at 15000g for 10 minutes and the supernatant was discarded. Finally, the pellet was dried, pulverised and re-dissolved in 50-100µl urea buffer depending on the size of the pellet. The buffer contained 7M urea, 2M thiourea and 20mM 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 and 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 and Erickson 2004) and the sodium-independent L-type amino acid transporter (LAT) 2 (Segawa et al., 1999; Pineda 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 α-(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 4 fast- and 4 slow-twitch fibre bundles. Each group was then treated for at least 20 minutes as follows; Group 1 (control) was treated with the standard Ringer's solution plus 107.9µM ethanol, Group 2 was treated with the Ringer's solution plus DHT alone, Group 3 was treated with the Ringer's solution containing either BCH or MeAIB alone plus 107.9µM ethanol; whereas, Group 4 was treated with the Ringer's solution containing DHT plus either 10mM BCH or 100µM MeAIB. At the end of this period, the solutions were then replaced with the same solutions but this time 2mM L-[U-^{14} C] Ile was added. The bundles were then incubated in these solutions for 1hr 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 minutes with Ringer's solution containing 1:500 dilution of a rabbit polyclonal antibody against LAT2 (Santa Cruz Biotechonology, Inc, CA, USA). They were then incubated for 1hr in the Ringer's solution containing 2mM L-[U-^{14} 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's solution plus the vehicle (107.9µM ethanol) or the standard Ringer's solution plus DHT. Cytosolic and crude membrane proteins were then extracted and immunoblotted for the expression of SNAT2 and LAT2 (Santa Cruz Biotechonology, Inc, CA, USA) 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's solution plus the vehicle or the standard Ringer's solution plus DHT for 1hr. In some of the experiments, the fibre bundles were pre-incubated with the standard Ringer's solution plus the EGFR inhibitor tyrphostin AG1478 or the MEK inhibitor PD98059 for 20 minutes. They were then treated with the Ringer's solution containing the inhibitor whose effects were being investigated plus DHT for 1hr. 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 10min at 15000rpm. The supernatant was then collected and stored at -80°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, pulverized and proteins were extracted as described above. Equal amounts (10µg) 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 minutes. They were then incubated overnight at 4°C with primary antibodies against pEGFR, pJNK, pp38 and pATF2 (from Cell Signalling Technology, Inc, MA, USA); pEK1/2, cmyc, pcJUN, MyoD , SNAT2 and LAT2 (from Santa Cruz Biotechnology); pRSK1/2, pMSK1/2 and peEF2 (Abcam,

Cambridge, UK). The following day they were washed and incubated for 2hr with species specific secondary antibodies conjugated with horse-radish peroxidase. Finally, they were visualised using SuperSignal WestPico (Perbio Science UK Ltd, Cramlington, Northumberland) 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, Gillingham, Dorset, UK).

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 (Bq/µg). The radioactivity was then normalized to that recorded under control conditions and all the data are presented as the mean percentage change in uptake ±S.D.

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 analyzed using Scion Image® from 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., London, UK). The data obtained from the two fibre types under control and from the various experimental conditions were then compared using a 3-way ANOVA and a $p<0.05$ was considered statistically significant.

Fig. 1 near here

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 (630ρgml^{-1}) of DHT significantly (p=0.001) increased the uptake of L-[U-^{14} C]Ile and α-[1-^{14}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±14% (n=4 fibre bundles) increase in the uptake of L-[U-^{14} C]Ile in the fast-twitch fibre bundles but only to a 5.9 ± 4.5% (n=4 fibre bundle) increase in the slow-twitch ones (Fig. 1A). The corresponding values for α-[1-14C]-MeAIB were 53.9±12.8% (n=4 fibres) and 7.0 ± 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} C]Ile and α-[1-^{14}C]-MeAIB in either fibre type (Fig. 1C and 1D). For example, it led to 3.2 ± 4.9% (n=4 fibres) increase and to an 4.4±5% (n=4 fibres) decrease in the uptake of L-[U-^{14} C]Ile in the fast- and slow-twitch muscle fibres, respectively (Fig. 1C). The corresponding values for α-[1-14C]-MeAIB) were 6.0±5% (n=4 fibres) increase and 6.5±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

Fig. 2 shows the effects of pre-incubating another set of fast- and slow-twitch muscle fibre bundles with 100μM MeAiB (a system A inhibitor) on the basal and DHT-induced uptake of L-[U-^{14} 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} 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} 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.

Fig. 3 near here

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} C]Ile in both fibre types. Also, it completely abolished the DHT-induced increase in L-[U-^{14} 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} C]Ile in both fibre types. Also, it completely abolished the DHT induced uptake of L-[U-^{14} C]Ile (Fig.3C) in the fast-twitch fibre bundles..

Receptor(s) mediating the effects of DHT 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 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-^{14} 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±4%) increase in the uptake of L-[U-^{14} 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-^{14} C]Ile in the fast-twitch fibre bundles only; whereas, pre-treating the fibre bundles with it completely reversed the DHT-induced amino acid uptake seen in the fast-twitch fibre bundles.

Fig. 4 near here

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).

Fig. 5 near here

Upon stimulation, the EGFR dimerises and then undergoes autophosphorylation (Mendelsohn & Baselga, 2000). Therefore, in another experiment we examined the effects of DHT on the phosphorylation of the EGFR. As the results displayed in Fig. 4 B 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's solution containing 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-^{14} C]Ile as well as the phosphorylation of the extracellular-signal regulated kinases (ERK)1/2, the 90kDa 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-^{14} 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-^{14} 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).

Fig. 6 near here

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).

Although 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).

Fig. 7 near here

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-^{14} 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-^{14} 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 cyclohexamide. The uptake of L-[U-^{14} 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-^{14} 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-^{14} C]Ile uptake without completely abolishing it (Fig. 8A). In contrast, treatment of the fibre bundles with

cyclohexamide significantly ($p<0.05$) reduced the basal uptake of L-[U-^{14}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-^{14}C]Ile observed in the fast-twitch fibre bundles (Fig. 9A).

Fig. 8 near here

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 fibre bundles 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 (9.8±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.

Fig. 9 near here

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 (630pgml^{-1}) of DHT, for 1hr, 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 (Bashin et al. 1997; Ferrado 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 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 5α-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 (Ferrado 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. 1973a & 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; Bhasin et al. 2001; Herrera & Regnier, 1993). Therefore, we speculate that the presence of these hormones in males may be the main reason male muscles are larger and stronger muscle 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 Fig. 2 and Fig.3 shows, 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; Segawa et al. 1999; Pineda et al. 1999; Yao et al. 2000; Hyde et al. 2001; Sugawara et al. 2000), 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 we display in Fig.2 show, the

untreated slow-twitch muscle fibre bundles express more (~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 (Yao et al. 2000; Sugawara et al. 2000), osmolarity (Horio et al. 1997), intracellular amino acid levels (Gazzola et al. 1972; Drummond et al. 2010), hormones (Edmonson and Lumeng, 1980; Barber et al. 1982), growth factors (Moule and 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 Fig. 2 and Fig. 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 Ringers 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 and his co-workers suggest that it may also exist between SNAT2 and LAT1 in human skeletal

muscle (Drummond et al. 2010); 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 degulin blunted 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 cyclohexamide completely abolished the DHT-induced increase in amino acid uptake. Cyclohexamide 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 hypothesis 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 (Avruch et al. 2009; Beugnet et al. 2003). 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: GM was responsible for the conception and design of the experiments. He also dissected most of the small muscle fibre bundles and contributed to the writing of the manuscript. MMH performed most of the experiments and analysis of the data.

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

PROTEIN/PROCESS	INHIBITORS			
	Scientific name	Common name	Concentration	Source
Transcription	Actinomycin IV	Actinomycin D	1µgml^{-1}	Sigma-Aldrich
Translation	3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide	Cyclohexamide	10µgml^{-1}	Sigma-Aldrich
LAT2	2-Aminobicyclo[2,2,1]heptane-2-carboxylic acid	BCH	100µM	Sigma-Aldrich
Androgen receptor	6-Chloro-1β,2β-dihydro-17-hydroxy-3′H-cyclopropa(1,2)-pregna-1,4,6-triene-3,20-dione acetate	Cyproterone	1µM	Sigma-Aldrich
	2-Methyl-N-(4-nitro-3 [trifluoromethyl]phenyl)propanamide	Flutamide	3µM	Sigma-Aldrich
SNAT2	2,N-Dimethylalanine,2 (Methylamino)-2-methylpropionic acid	MeAIB	10µM	Sigma-Aldrich
EGFR	(N-[3-chlorophenyl]-6,7,-dimethoxy-4-quinazolamine)	AG 1478	100nM	Sigma-Aldrich
MEK	2´-amino-3´-methoxyflavone	PD98059	20µM	Alexis Biochemicals
Akt	(7aS,13aS)-13,13a-Dihydro-9,10-dimethoxy-3,3-dimethyl-3H-bis[1]benzopyrano[3,4-b:6′,5′-e]pyran-7(7aH)-one	Deguelin	10nM	Sigma-Aldrich
mTOR	23,27-Epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine	Rapamycin	100ngml $^{-1}$	Sigma-Aldrich

Figure legends

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- (clear bars) twitch fibre bundles with physiological levels of DHT (A, B) and T (C,D) on the uptake of L-[U-^{14} C]Ile (A,C) and α-[1-14C]-MeAIB (B,D). Note that treating the fibre bundles with DHT, but not T, significantly (p=0.001) increases the uptake of both L-[U-^{14} C]Ile and α-[1-^{14}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 the data indicated by the asterisk was compared to that recorded under control conditions.*

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 (clear bars) muscle fibre bundles with DHT and MeAIB, as shown, on the uptake of L-[U-^{14} 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, clear 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 weight (MW) of ~60kDa and that DHT treatment has no effect on the expression of SNAT2. FT and ST are the fast- and slow-twitch preparations treated with DHT, respectively. *$ indicate p<0.05 when the data shown was compared to that recorded under control conditions (*) or after DHT treatment ($).*

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- (clear bars) twitch muscle fibre bundles with DHT and BCH, as shown, on the uptake of L-[U-^{14} 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, clear 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 MW of ~60kDa 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. *$ indicate $p<0.05$ when the data shown was compared to that recorded under control conditions (*) or after 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's solution or the Ringer's 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. *$ indicate p<0.05 when the data shown was compared to that recorded under control conditions (*) or following DHT treatment ($).*

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 tyrphostin 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 phosporylation of the EGFR in membrane proteins. Note that the antibody labels a single band with a MW of ~170kDa and that treating the bundles with DHT increases the phosphorylation of EGFR and this effect is completely reversed by AG1478. *$ indicate p<0.05 when the data shown was compared to that recorded under control conditions (*) or after DHT treatment ($).*

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- (clear bars) twitch muscle fibre bundles with either DHT or the MEK inhibitor PD98059, as shown in the figure, on the uptake of L-[U-^{14} 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 MWs of ~42 and 44kDa; whereas, that of pRSK labels two bands with MWs of ~78 and 80kDa. 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 MW ~60kDa; whereas, that of pp38 labels a band with a MW of ~42kDa. Furthermore, DHT treatment has no effect on the phosphorylation of either kinase. *$ indicate p<0.05 when the data shown was compared to that recorded under control conditions (*) or after 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- (clear bars) twitch muscle fibre bundles, with the compounds shown below each panel, on the uptake of L-[U-^{14} 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-^{14} C] Ile in both fibre types. Furthermore, rapamycin but not deguelin abolished the DHT-induced increase in Ile uptake. *$ indicate p<0.05 when the data shown was compared to that recorded under control conditions (*) or after 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- (clear bars) twitch muscle fibre bundles with Ringer's solution containing either DHT or actinomycin D

*on the uptake of L-[U-^{14} 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, cmyc and pATF2. Note that DHT does not significantly (p>0.05) affect the phosphorylation/expression of any of the transcriptional factors except ATF2. *$ indicate p<0.05 when the data shown was compared to that recorded under control conditions (*) or after DHT treatment ($).*

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- (clear bars) twitch muscle fibre bundles with Ringer's solution containing either DHT or cyclohexamine on the uptake of L-[U-^{14} C]Ile. Note that treating the muscle fibre bundles with cyclohexamide 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 phosphorylationof eEF2. Note that the antibody labels a single band with a MW of ~100kDa. 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 uptake of Ile in the fast-twitch fibre bundles. In contrast it leads to a slight decrease (9.8 ± 5.6%, n=3 fibres) in the incorporation of Ile in the slow-twitch fibre bundles.$ indicate p<0.05 when the data shown was compared to that recorded under control conditions (*) or after DHT treatment ($).*

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.

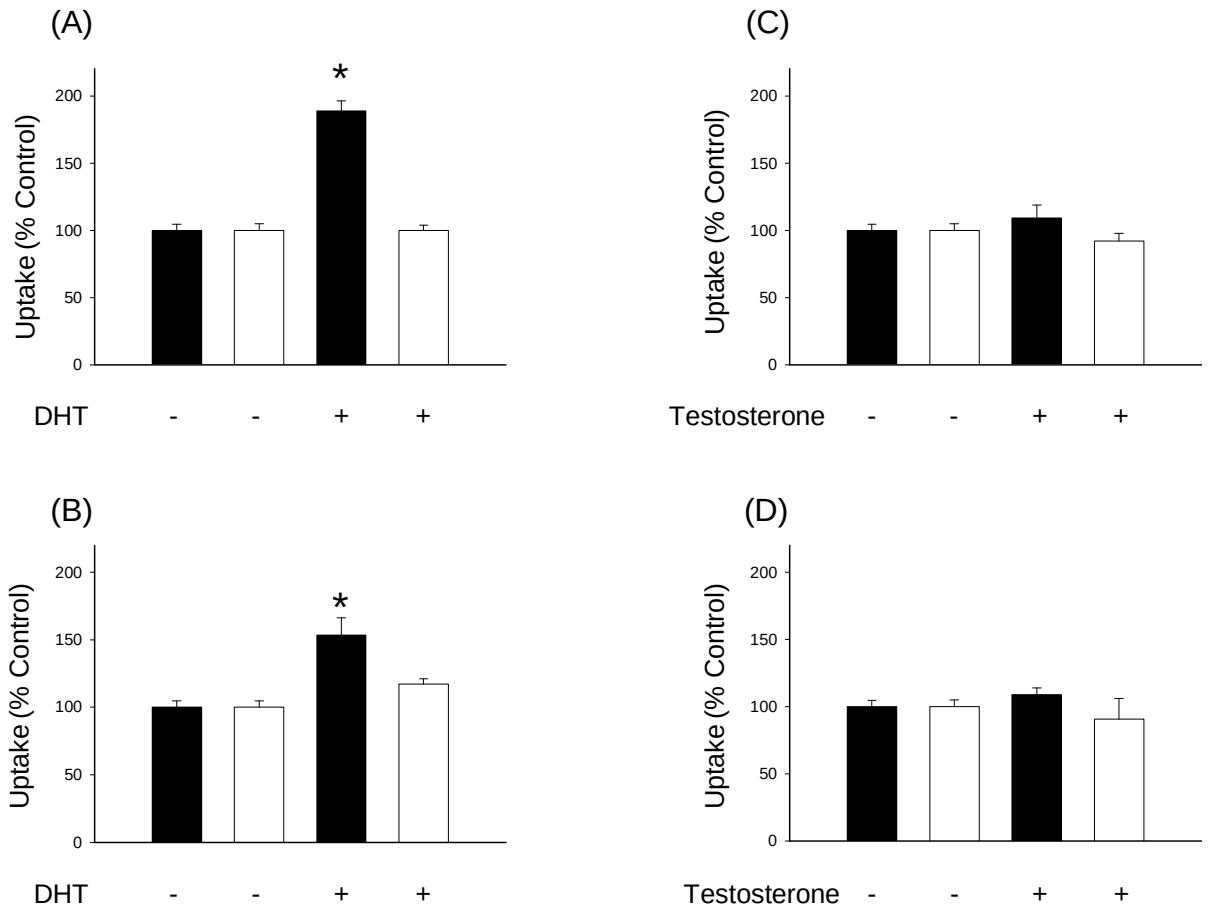
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Figure 1

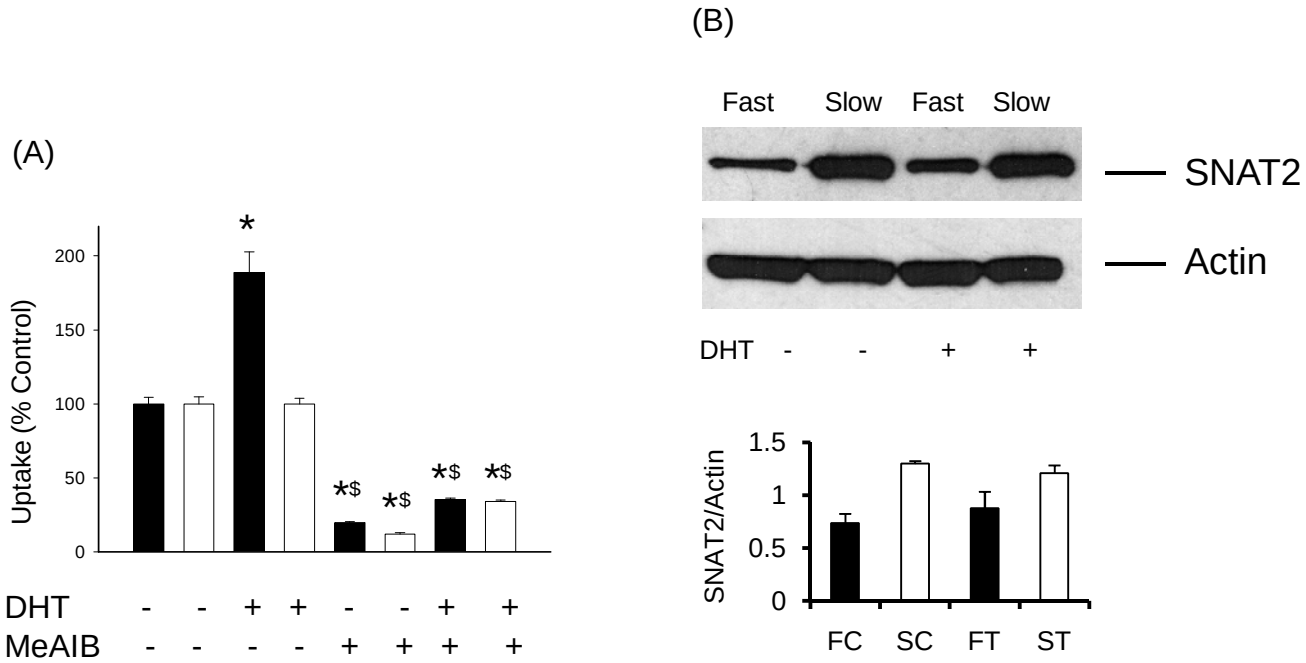
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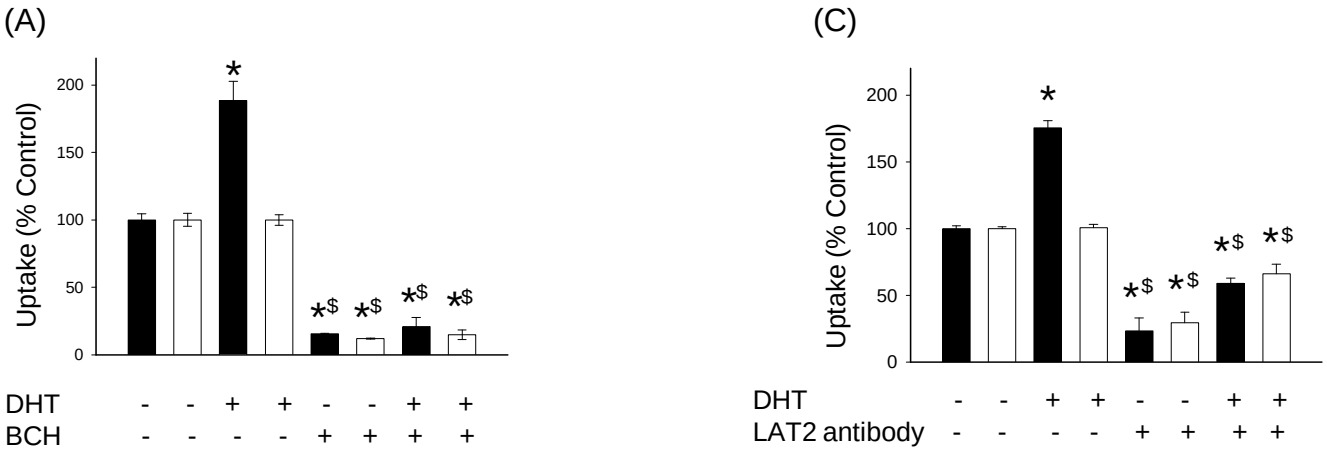
Figure 2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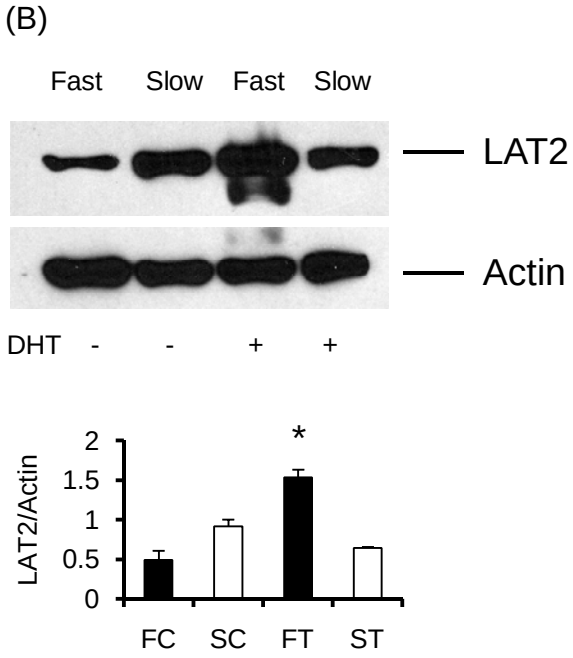
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Figure 3

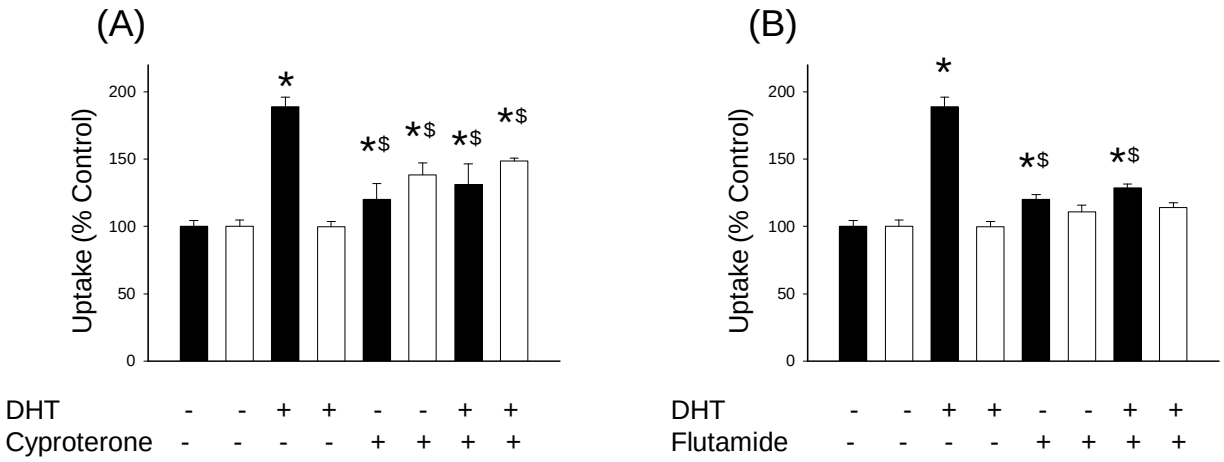
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Figure 4

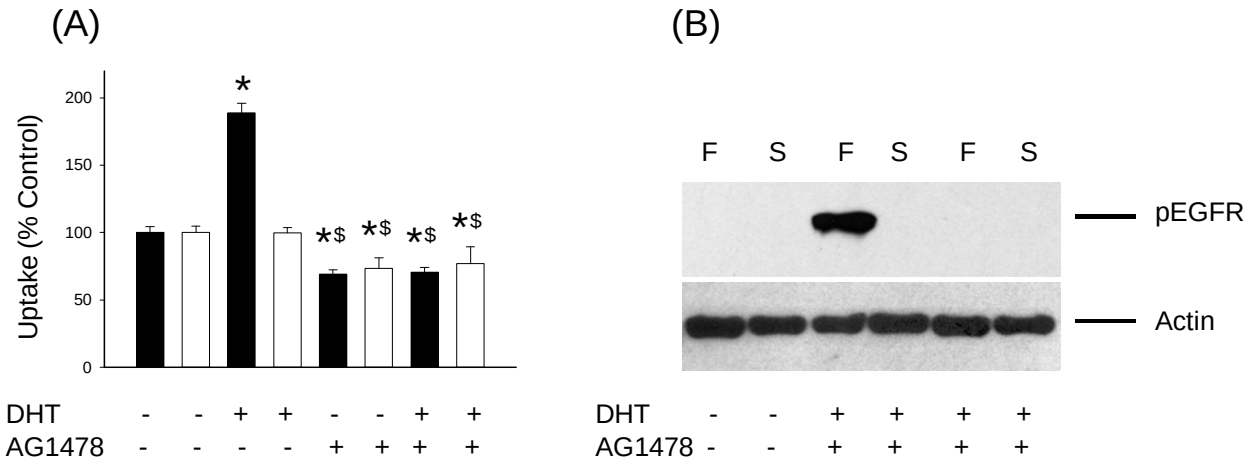
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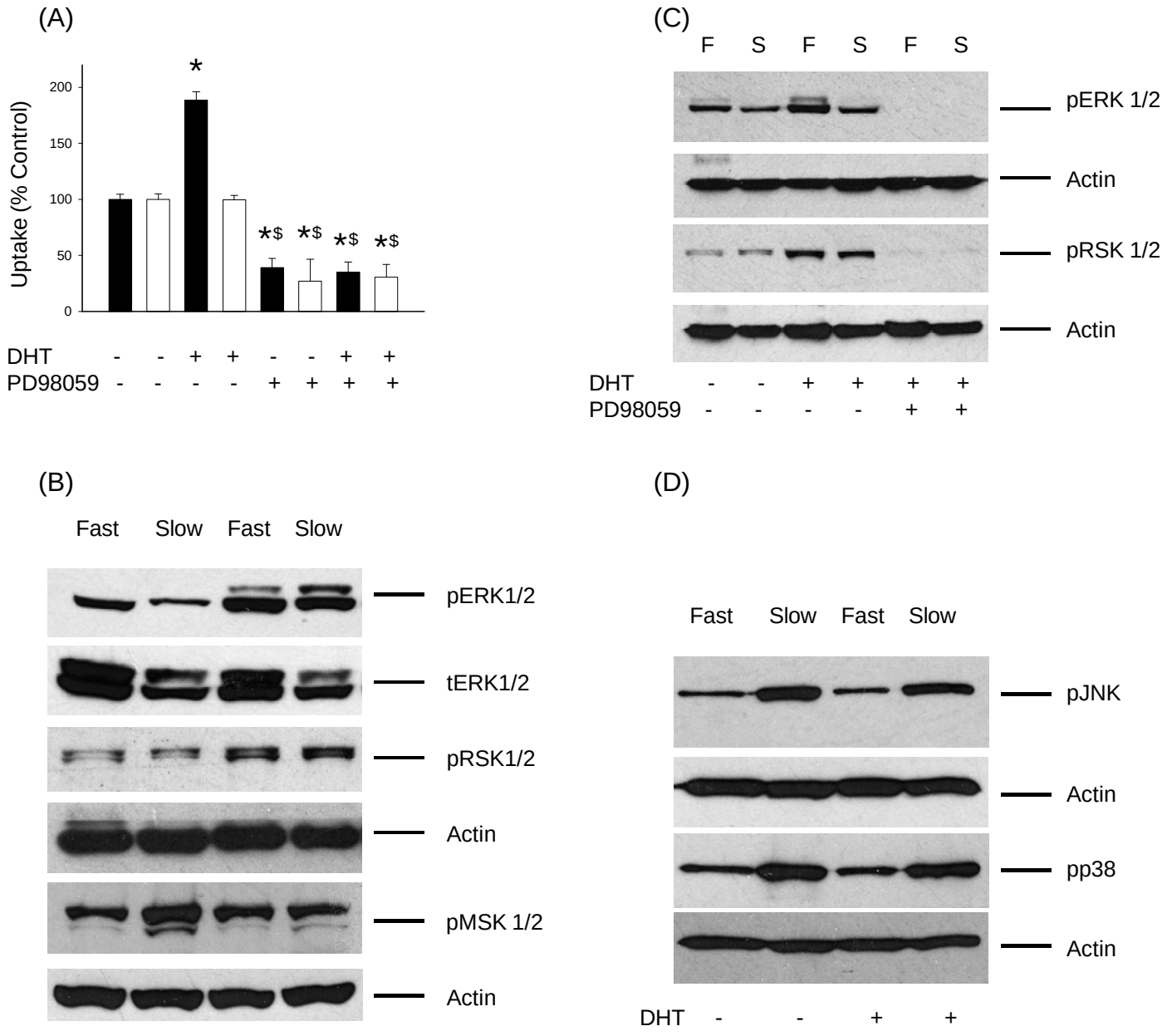
Figure 5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DHT - - + +

Figure 6

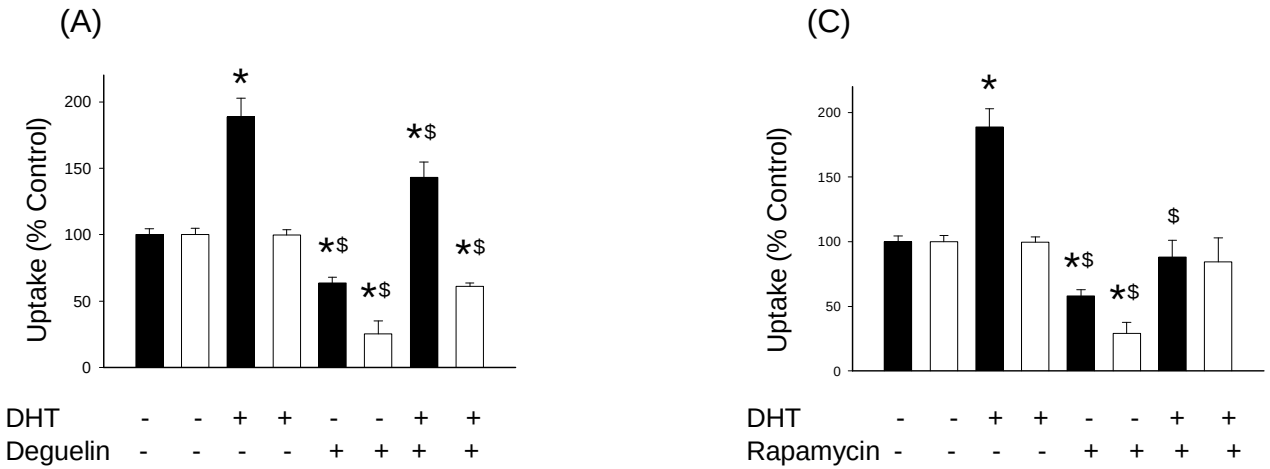

(A)
Uptake (% Control)
0
50
100
150
200
*
*$
*$
*$
DHT - - + + - - + +
Deguelin - - - - + + + +
(C)
Uptake (% Control)
0
50
100
150
200
*
*$
*$
$
DHT - - + + - - + +
Rapamycin - - - - + + + +

Figure 7

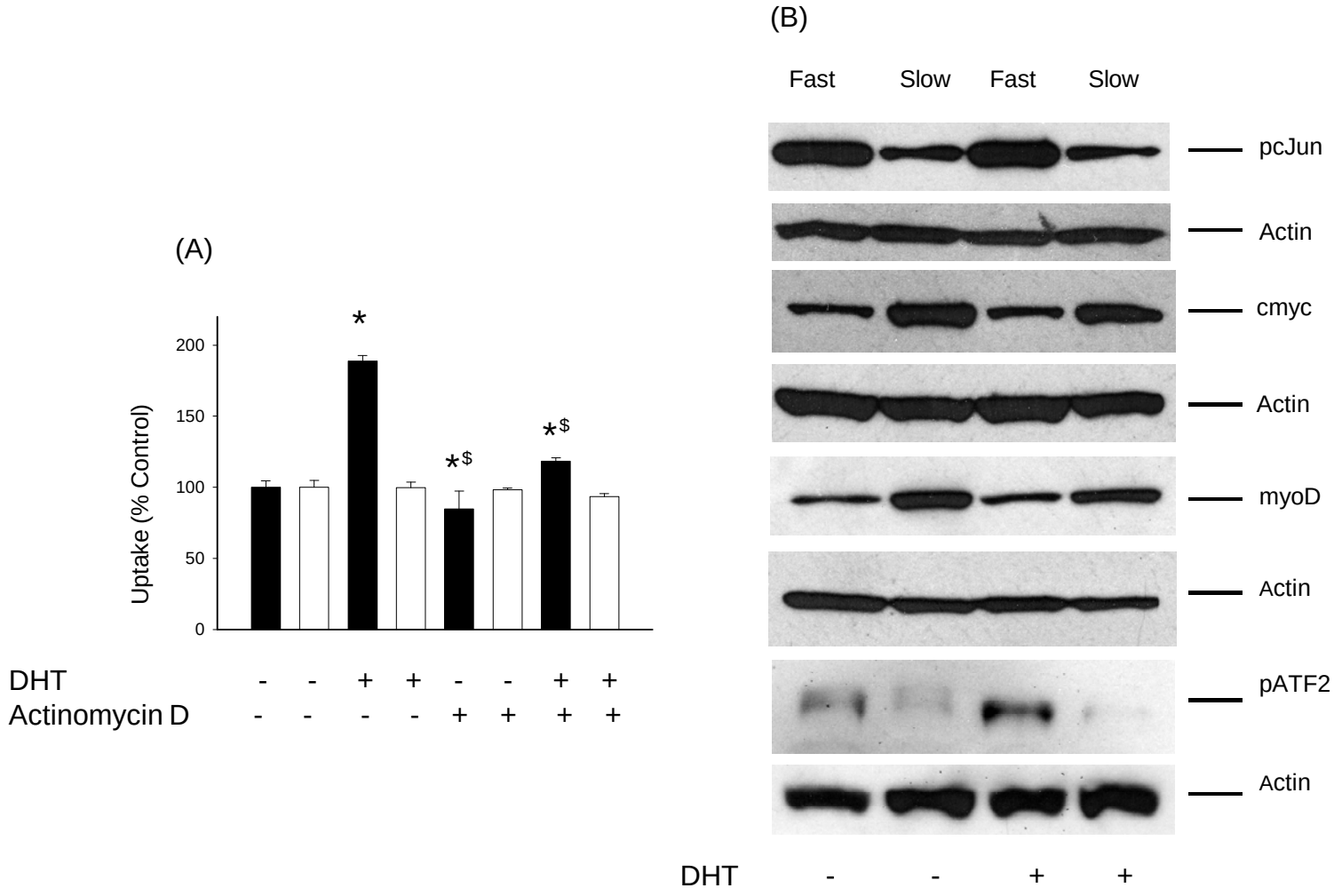
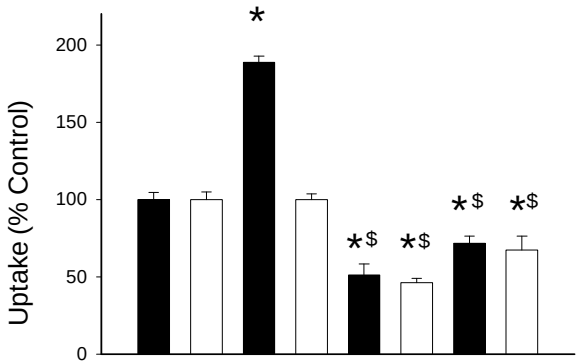

(A)
Uptake (% Control)
0
50
100
150
200
*
*$
*$
DHT - - + + - - + +
Actinomycin D - - - - + + + +
(B)
Fast
Slow
Fast
Slow
pcJun
Actin
cmyc
Actin
myoD
Actin
pATF2
Actin
DHT - - + +

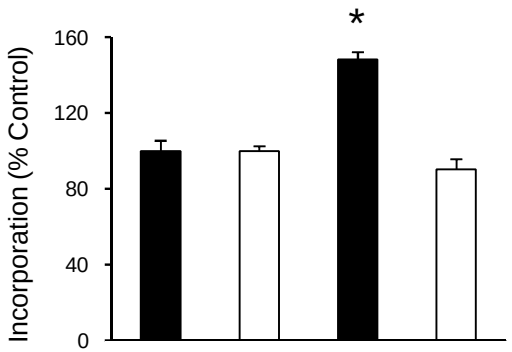
Figure 8

(A)



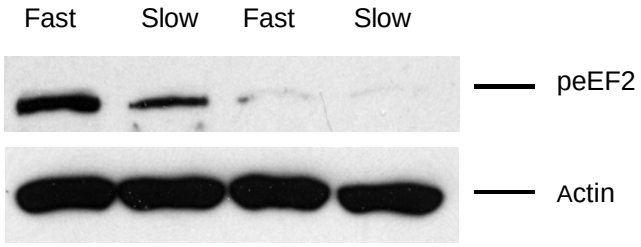
DHT	-	-	+	+	-	-	+	+
Cyclohexamide	-	-	-	-	+	+	+	+

(C)



DHT	-	-	+	+

(B)



DHT	-	-	+	+

Figure 9

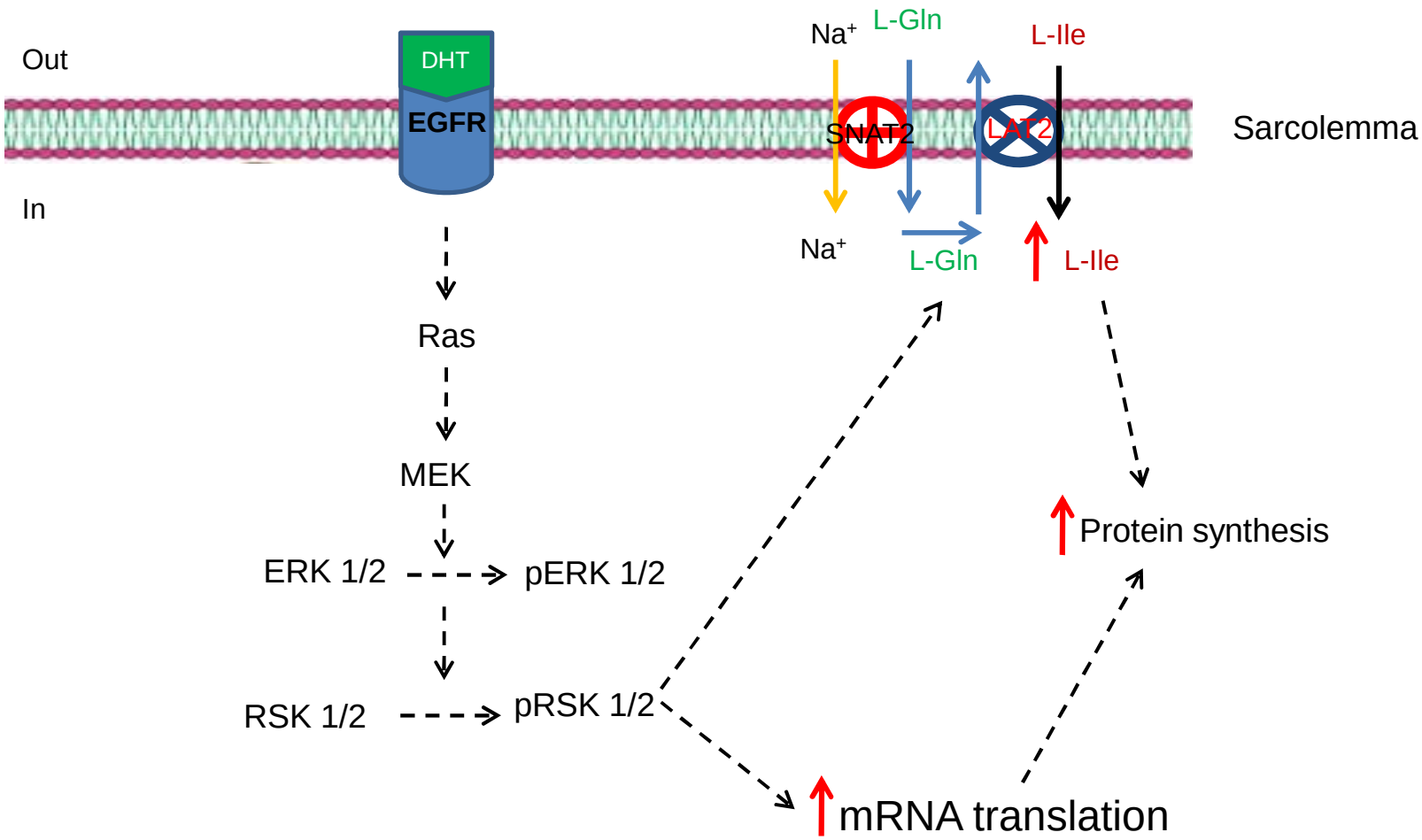

Out
In
DHT
EGFR
Na+
L-Gln
L-Ile
SNAT2
LAT2
Sarcolemma
Na+
L-Gln
L-Ile
Ras
MEK
ERK 1/2
pERK 1/2
RSK 1/2
pRSK 1/2
Protein synthesis
mRNA translation

Figure 10