Cellular and Molecular mechanisms responsible for the action of testosterone on human skeletal muscle. A basis for illegal performance enhancement.

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Tuesday, June 16, 2020 1:45 PM

Introduction

This non-data review summarizes a series of studies on testosterone's physiological role in muscle growth, but also how that physiological role is carried out, molecularly and cellularly (mechanisms).

Conclusions

Testosterone increases muscle mass and strength and while type 2 cells end up larger from testosterone administration, the greatest increase in muscle cell size is in type 1. Type 2 only grow with higher testosterone concentrations.

Testosterone increases protein synthesis, but does not decrease protein degradation - it also increases amino acid recycling.

Testosterone encourages greater cell division in satellite cells on the musculature.

Testosterone may promote lipolysis and discourages pre-adipocytes from turning into adipocytes.

Convinces mesenchymal stem cells to turn myogenic rather than adipogenic.

Amendments

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Article in British Journal of Pharmacology · July 2008

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REVIEW

Cellular and molecular mechanisms responsible for the action of testosterone on human skeletal muscle. A basis for illegal performance enhancement

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The popularity of testosterone among drug users is due to its powerful effects on muscle strength and mass. Important mechanisms behind the myotrophic effects of testosterone were uncovered both in athletes using steroids for several years and in short-term controlled studies. Both long-term and short-term steroid usage accentuates the degree of fibre hypertrophy in human skeletal muscle by enhancing protein synthesis. A mechanism by which testosterone facilitates the hypertrophy of muscle fibres is the activation of satellite cells and the promotion of myonuclear accretion when existing myonuclei become unable to sustain further enhancement of protein synthesis. Interestingly, long-term steroid usage also enhances the frequency of fibres with centrally located myonuclei, which implies the occurrence of a high regenerative activity. Under the action of testosterone, some daughter cells generated by satellite cell proliferation may escape differentiation and return to quiescence, which help to replenish the satellite cell reserve pool. However, whether long-term steroid usage induces adverse effects of satellite cells remains unknown. Testosterone might also favour the commitment of pluripotent precursor cells into myotubes and inhibit adipogenic differentiation. The effects of testosterone on skeletal muscle are thought to be mediated via androgen receptors expressed in myonuclei and satellite cells. Some evidence also suggests the existence of an androgen-receptor-independent pathway. Clearly, testosterone abuse is associated with an intense recruitment of multiple myogenic pathways. This provides an unfair advantage over non-drug users. The long-term consequences on the regenerative capacity of skeletal muscle are unknown.
British Journal of Pharmacology (2008) **154,** 522–528; doi:10.1038/bjp.2008.118; published online 14 April 2008

Keywords: satellite cells; anabolic steroids; exercise training; androgen receptor; hypertrophy; hyperplasia; muscle fibre; regeneration; myonuclei; protein synthesis

Abbreviations: AR, androgen receptor; GnRH, gonadotropin-releasing hormone; PCNA, proliferating cell nuclear antigen; T/E ratio, testosterone/epitestosterone ratio

Introduction

The use of testosterone and related steroids is a widespread phenomenon among top athletes, amateurs, school-age children and a large part of the population who simply desire to improve their appearance. The popularity of testosterone and related steroids among drug users is due to the powerful effects of these substances on muscle strength and mass. Recent reports have uncovered important cellular and molecular mechanisms behind the myotrophic action of anabolic steroids. The effects of testosterone might be mediated via several myogenic pathways. This review starts with a brief description of the reasons for testosterone usage and the methods used in the detection of its abuse. The main focus is the description of the cellular and structural changes observed in human skeletal muscle in response to testosterone administration. Important cellular and molecular pathways by which testosterone might exert its action on skeletal muscle are discussed.

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Received 30 July 2007; revised 11 January 2008; accepted 7 March 2008; published online 14 April 2008

Reasons for testosterone abuse and side effects

Testosterone is a 19-carbon steroid with powerful androgenic and anabolic effects. Testosterone is primarily produced by the Leydig cells in the testes and a small quantity comes from the adrenal cortex and the peripheral conversion of androstenedione. The anabolic action of testosterone and

related steroids on skeletal muscle is the reason for their popularity among drug users. Anabolic androgenic steroids are taken orally, by intramuscular injection, and as gels and creams. These drugs are used to increase lean body mass, to decrease fat mass, to enhance performance, to sustain intensive training periods and, finally, to improve the appearance (Yesalis, 1993; Hartgens and Kuipers, 2004). Through case reports, androgenic–anabolic drugs have been associated with a wide range of adverse effects: deleterious changes in risk factors associated with cardiovascular disease, alterations in liver structure and function, and in the reproductive system and changes in behaviour (Wilson, 1988; Yesalis, 1993; Hartgens and Kuipers, 2004). In this respect, treatment of human neuroblastoma cells with high doses of testosterone decreases cell viability by activation of cell death programmes indicating possible long-term effects of drug abuse on brain function (Estrada *et al.*, 2006). Athletes often use doses that are far beyond those used in controlled studies, which implies that serious adverse effects of drug abuse may be under-recognized and more pronounced than what is currently described in scientific studies (Hartgens and Kuipers, 2004).

Detection of testosterone abuse

The fight against doping was initiated by the International Olympic Committee in the 1960s and androgenic–anabolic steroids were added on the list of banned substances in 1976. Doping by means of testosterone is difficult to uncover due to the fact that the hormone is also produced endogenously. Therefore, the critical issue in doping control is to establish the origin of testosterone found in human urine. Doping by testosterone can be indirectly tested using the urinary testosterone/epitestosterone (T/E) ratio (WADA, 2004). In normal healthy individuals, testosterone and epitestosterone are produced in a ratio of 1:1. Therefore, it is assumed that the urinary T/E ratio increases in athletes taking exogenous testosterone. If an athlete has a T/E of more than 4:1, the sample is submitted to GC-C-IRMS (gas chromatography–combustion–isotope ratio mass spectrometry) for determination of the $^{13}C/^{12}C$ ratio (WADA, 2004). This is because exogenous compounds contain less ^{13}C than their endogenous homologue (Shackleton *et al.*, 1997; Aguilera *et al.*, 2001). However, if GC-C-IRMS does not conclusively indicate exogenous administration despite a T/E > 4.0, longitudinal monitoring of the T/E–time profile is required. In this respect, the T/E ratio is characterized by a larger inter- than intra-individual variability (Sottas *et al.*, 2007), and a T/E ratio in the range of 4:1 can be found even in individuals not taken testosterone. Therefore, a twofold increase of the T/E ratio might go undetected. For this reason, it is suggested that a population-based T/E reference is not sensitive to individual variations. According to Sottas *et al.* (2007), the problem can be statistically defined as the detection of an outlier out of series of individual test results using a Bayesian analysis of T/E ratio, that is, comparing a value to both a population-based reference range and a subject-based reference range.

Testosterone, muscle strength and mass

A main reason behind the popularity of testosterone among drug users is its effects on athletic performance and on muscle size. Suppression of endogenous testosterone production in young men by a gonadotropin-releasing hormone (GnRH) analogue resulted in marked decreases in the rates of whole-body protein synthesis, in muscle strength and in fat oxidation together with an increased adiposity (Mauras *et al.*, 1998). Manipulation of the circulating testosterone levels by simultaneous treatment with GnRH analogues and exogenous testosterone showed the existence of a positive relationship between testosterone concentration and fat-free mass, muscle size and strength (Bhasin *et al.*, 2001). Moreover, a randomized placebo-controlled and double-blinded intervention showed that the physiological response to a period of 12 weeks strength training is attenuated in a group of subjects receiving a GnRH analogue once every 4th week (Kvorning *et al.*, 2006). The attenuation of the response to strength training included a reduced increase in lean leg mass and no changes in maximum isometric knee extensor strength. However, it is important to note that in the same study, the progression of training load during the 12 weeks training period in the group treated with a GNRH analogue was similar to the placebo-treated group (Kvorning *et al.*, 2006). Moreover, suppression of testosterone does not seem to blunt mRNA expression of some members of the myogenic regulatory factors (MyoD and myogenin), insulin-like growth factor-1(IGF-1), myostatin and androgen receptors (ARs; Kvorning *et al.*, 2007). This elucidates the complexity of the regulation of the signalling pathways behind the hypertrophy of human skeletal muscle in response to resistance training.

Improvements in muscle strength have been observed in response to the administration of testosterone. The amplitude of the effects of testosterone on muscle strength depends upon the initial muscle strength of the subjects, the doses used and the period of administration. Accordingly, consistent strength gains occurred in young healthy individuals receiving testosterone enanthate (300 mg week^{-1}) for 6 weeks (Friedl *et al.*, 1991). Similarly, the administration of supraphysiological doses of testosterone (600 mg week^{-1}) for 10 weeks in untrained and trained men produced a significant increase in muscle strength and in the cross-sectional area of the quadriceps (Bhasin *et al.*, 1996). It was also shown that moderate doses of testosterone combined with weight training induced short-term changes in upper body strength and body composition (Giorgi *et al.*, 1999). Data also suggest that the effects of testosterone administration on human skeletal muscle mass is dose dependent (Bhasin *et al.*, 2001). Interestingly, the use of testosterone in conjunction with heavy resistance training seems to be associated with changes in muscle pennation angle and possibly fascicle length (Blazevich and Giorgi, 2001).

Testosterone and the hypertrophy of muscle fibres

Important mechanisms behind the strong myotrophic effects of testosterone were first uncovered in a population

Testosterone, muscle strength and mass

Reducing testosterone leads to reduction in whole body protein synthesis, muscle strength, and increases in fatness. Re-introducing testosterone re-introduces muscle mass and strength, and favorable body composition. Loss of testosterone does not lead to a decrease in IGF-1 and other muscle promoting signals. Testosterone administration (exogenous) increases muscle size and strength in a dose dependent manner (more testosterone, more size).

Testosterone and the hypertrophy of muscle fibres

Long term use of testosterone increases the size of muscle fibers in well trained lifters. Testosterone induces increases in muscle size for all muscle types (Type 1 and 2). While type 2 fibers tend to be larger, in general, the type 1 fibers increase the most drastically in size (over 50%) compared to non-steroid/testosterone users. Type 2 fibers only enlarged with higher testosterone administration (600 vs 300 mg).

of high-level powerlifters who reported the use of testosterone (100–500 mg week^{-1}) for a period of 9 ± 3.3 years (Kadi *et al.*, 1999; Kadi, 2000). Long-term administration of testosterone accentuates the degree of fibre hypertrophy in already well-trained powerlifters (Kadi *et al.*, 1999; Kadi, 2000). Testosterone induces the hypertrophy of both type I and type II muscle fibres. Type II muscle fibres are the largest muscle fibres in powerlifters both in steroid users and non-users. However, there is evidence suggesting that the largest difference in muscle fibre size between steroid users and non-users is observed in slow type I muscle fibres (Kadi *et al.*, 1999; Kadi, 2000; Eriksson *et al.*, 2005). In the trapezius muscle of steroid users, the area of type I muscle fibres is 58% larger than in non-users, whereas the area of type II muscle fibres is 33% larger than in non-users (Kadi *et al.*, 1999). The same tendency is observed in the vastus lateralis (Eriksson *et al.*, 2005). Accordingly, it has been shown that type I muscle fibres are more sensitive to anabolic agents than type II muscle fibres (Hartgens *et al.*, 1996). Subsequently, it has been shown that the administration of 300 and 600 mg testosterone induced an increase in the area of type I muscle fibres, whereas type II muscle fibres enlarge only after administration of 600 mg testosterone (Sinha-Hikim *et al.*, 2002).

Testosterone, protein synthesis and myonuclear content

Enhanced contractile protein synthesis is an important mechanism by which testosterone can enhance the size of muscle fibres. Intramuscular injection of 200 mg testosterone enanthate in healthy individuals induced a significant twofold increase in net protein synthesis, whereas protein breakdown was unchanged (Ferrando *et al.*, 1998). Testosterone did not affect inward amino-acid transport to muscle but increased re-utilization of intracellular amino acids in skeletal muscle (Ferrando *et al.*, 1998).

Adult muscle fibres contain hundreds of myonuclei, where each myonucleus sustains the protein synthesis over a finite volume of cytoplasm, a concept called 'nuclear domain' (Cheek, 1985). In this respect, significant enlargement of muscle fibres (36% increase in fibre cross-sectional area) is accompanied by a significant increase in the myonuclear number, whereas no alterations in the number of myonuclei are observed when the increase in fibre area does not exceed approximately 26% (Kadi, 2000; Kadi *et al.*, 2004b, 2005). Therefore, it is suggested that existing myonuclei are able to support a certain level of fibre hypertrophy. However, when the transcriptional activity of existing myonuclei reaches its maximum, the enhancement of the number of myonuclei is thought to become involved in the enhancement of protein synthesis, a concept termed the ceiling theory (Kadi *et al.*, 2004b, 2005; Petrella *et al.*, 2006). This is further supported by the relationship between the cross-sectional area of muscle fibres and the number of myonuclei per fibre cross-section (Kadi, 2000; Kadi *et al.*, 2006). In this respect, a mechanism by which testosterone facilitates the hypertrophy of muscle fibres seen in drug users is to promote myonuclear accretion (Kadi *et al.*, 1999; Kadi, 2000; Sinha-Hikim *et al.*, 2002). In high-level powerlifters, the mean number of myonuclei per fibre cross-section is significantly higher in steroid users compared with non-users, and myonuclear accretion is greater in type I fibres (+23%) compared with type II muscle fibres (+14%) (Kadi, 2000). This is in accordance with the larger hypertrophy of type I muscle fibres seen in steroid users.

Testosterone and centrally located myonuclei

In steroid-using powerlifters, the number of muscle fibres with internal myonuclei reaches 25% in trapezius muscle and 29% in vastus lateralis (Kadi *et al.*, 1999; Eriksson *et al.*, 2005). In non-steroid-using powerlifters, the number of fibres with internal myonuclei is 5% in trapezius and 9% in the vastus lateralis (Kadi *et al.*, 1999; Eriksson *et al.*, 2005). This indicates that testosterone is associated with a three- to fivefold increase in centrally located myonuclei in the vastus lateralis and trapezius, respectively. In steroid users, centrally located myonuclei are encountered in both type I and type II muscle fibres. In contrast, centrally located myonuclei in non-steroid users are mainly located in type II muscle fibres (Kadi *et al.*, 1999). The presence of internal myonuclei is traditionally recognized as an indication of ongoing muscle regeneration. The activation of satellite cells can lead to proliferation and differentiation into new myotubes that might fuse with existing muscle fibres. During the fusion process, some myonuclei might be trapped in the central part of the resulting new and larger fibre. This physiological positioning of the myonuclei might be required for fibre growth when new myotubes fuse with the existing parent muscle fibre. Centrally located myonuclei might remain in their position for a period after the fusion and that would reduce the diffusion distance from a nucleus to the central part of the myofibre.

Testosterone and satellite cells

Satellite cells are located between the basal lamina and the plasma membrane of muscle fibres (Mauro, 1961). In human skeletal muscle, satellite cell content varies between muscles with different functional properties and between individuals with different physical activity levels and ages (Kadi *et al.*, 2004a, 2005). In human vastus lateralis muscle of young adults, the number of satellite cells per fibre cross-section does not differ between type I and type II muscle fibres (Kadi *et al.*, 2006). Existing myonuclei in adult muscle fibres are post mitotic, and muscle satellite cells are the major source for the addition of new myonuclei into the hypertrophying muscle fibre (Moss and Leblond, 1971). A variety of alterations in the surrounding environment of the satellite cell, including mechanical and growth factors and also hormonal signalling might regulate the activation and proliferation of satellite cells (Kadi, 2005; Kadi *et al.*, 2005; Mackey *et al.*, 2007). Satellite cells can proliferate and withdraw from differentiation to return to quiescence to replenish satellite cell pool, or to enter differentiation to provide new myonuclei or to generate new muscle fibres

Testosterone, protein synthesis and myonuclear content

Testosterone enhances overall (body) protein synthesis, but does not affect degradation. It does not increase uptake of amino acids, but it does increase the re-utilization of amino acids found in the cell (from the degradation machinery). With initial size increases, the myonuclei (nucleus of the muscle cell) that make up the muscle cells are sufficient to increase the size of the cell; however, after a point of muscle cell growth, more myonuclei are necessary to maintain rapid growth - this effect is more heavily seen in type 1 muscle cells.

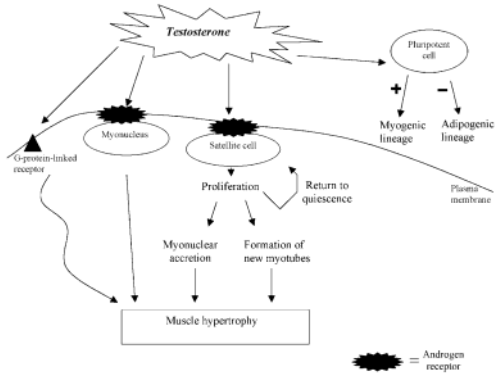

Figure 1 Mechanisms of testosterone action on skeletal muscle.

(Figure 1) (Kadi, 2000; Kadi *et al.*, 2005). The mechanisms regulating the fate of daughter cells generated by satellite cell activation are currently not understood. Testosterone is able to stimulate the mitotic activity of satellite cells in myoblast culture systems (Powers and Florini, 1975). Furthermore, in response to the administration of testosterone, an increase in the number of PCNA- (proliferating cell nuclear antigen-positive) satellite cells occurs in human skeletal muscle. The presence of satellite cells expressing the PCNA indicates that testosterone can promote the entry of satellite cells into the cell cycle (Sinha-Hikim *et al.*, 2006). This highlights the role of satellite cells as mediators of the myotrophic action of testosterone on skeletal muscle. When satellite cells are forced to enter the cell cycle, some daughter cells escape differentiation and can return to quiescence, which ultimately lead to the generation of new satellite cells. In this respect, a significant increase in the number of satellite cells has been reported in men receiving testosterone (300 and 600 mg week^{-1}) during 20 weeks (Sinha-Hikim *et al.*, 2003). The number of satellite cells in skeletal muscle of power-lifters using anabolic steroids for a period of 9 ± 3.3 years is higher than in healthy young men, but it remains similar to that seen in non-steroid-using powerlifters (Kadi, 2000). It might be hypothesized that short-term administration of steroids favours the generation of new satellite cells, whereas in long-term users the generation of new satellite cells might not be the main fate of the newly generated daughter cells. Alternatively, using steroids for many years attenuates their action on satellite cells.

Muscle satellite cells are the stem cells of skeletal muscle. As such, they have the ability to maintain a ready source of muscle fibre precursors. Several mechanisms are proposed to explain the self-renewal of satellite cells in skeletal muscle. Asymmetric and/or symmetric cell divisions would lead to one or two daughter cells to become new satellite cells. Notch is a transmembrane receptor that can activate transcription factors involved in the regulation of cell fate. It has been proposed that the asymmetric expression of Numb (membrane-associated inhibitor of the Notch signalling) in dividing satellite cells *in vitro* might account for the occurrence of asymmetric cell divisions (Conboy and Rando, 2002). Numb-positive cells would enter differentiation, whereas Numb-negative cells would escape differentiation and become a satellite cell. It is hypothesized that Numb-negative cells would continue to proliferate and re-populate the satellite cell pool (Conboy and Rando, 2002). Thus, the interaction between Notch and its antagonist Numb might represent one pathway involved in the control of the fate of newly generated cells. In this respect, it is suggested that testosterone might influence the fate of newly generated daughter cells by enhancing the expression of the activated form of Notch, thus promoting cell proliferation and generation of new satellite cells (Sinha-Hikim *et al.*, 2006).

Testosterone and the commitment of pluripotent precursor cells into myogenic lineage

It is suggested that the formation of new myotubes can also be achieved via the contribution of stem cells from sources other that satellite cells (Figure 1). However, the contribution of these muscle precursor cells in both physiological and supraphysiological adaptations of human skeletal muscle remains unclear. Nevertheless, it has been shown that mouse C3H 10T1/2 pluripotent mesenchymal cells (cells capable of differentiating into muscle, fat, cartilage and bone cells)

treated with testosterone start to express specific myogenic markers (MyoD and myosin heavy chains) (Singh *et al.*, 2003). This can be an indication of the ability of testosterone to recruit mesenchymal pluripotent cells into the myogenic lineage.

Testosterone and the adipogenic differentiation

Testosterone can also reduce body fat, as it is a potent regulator of lipolysis by influencing catecholamine signal transduction in fat cells (Arner, 2005) (Figure 1). It has been suggested that testosterone inhibits lipid uptake in adipocytes and stimulates lipolysis (De Pergola, 2000). Similarly, evidences suggest that testosterone can inhibit differentiation of adipocyte precursor cells (De Pergola, 2000). In agreement with these data, treatment of mouse C3H 10T1/2 pluripotent cells with testosterone inhibits adipogenic differentiation assessed by adipocyte counting and the expression of two adipogenic inhibitory factors ($PPAR_{\gamma 2}$ (peroxisomal proliferator-activated receptor) and CCAAT/enhancer-binding protein α) (Singh *et al.*, 2003).

Androgen receptors: mediators of testosterone effects

The hypertrophy of muscle fibres is a process under the complex control of several myogenic pathways (Figure 1). In this respect, the blockade of ARs by oxendolone, an AR antagonist, suppressed the hypertrophy of rat muscle fibres (Inoue *et al.*, 1994). This experiment clearly elucidates the role played by ARs as potential mediators of the exercise-induced muscle fibre hypertrophy. Experiments also demonstrated substantial increases in the concentration of AR in response to exercise (Deschenes *et al.*, 1994; Bamman *et al.*, 2001). ARs belong to the super family of ligand-responsive transcription regulators. When androgenic hormones bind to the receptor, it becomes activated and the androgen-receptor complex is translocated to the hormone-responsive element within the nucleus. The binding to selective genes increases rates of transcription (Luke and Coffey, 1994). The number of binding sites per mg of protein is much lower in skeletal muscle than in the prostate in rats (Krieg, 1976). Two reports failed to identify a positive immunostaining in myonuclei of human muscle fibres (Ruizeveld de Winter *et al.*, 1991; Janssen *et al.*, 1994). The lack of staining using conventional immunohistochemical methods is probably due to the low level of AR in skeletal muscle. However, when immunohistochemistry is performed using a signal amplification technique, positive immunolabeling is observed in human vastus lateralis and trapezius muscles (Kadi, 2000; Kadi *et al.*, 2000). Immunolabeling is also observed in capillary endothelium as well as in intramuscular nerve bundles (Kadi *et al.*, 2000). Interestingly, not all myonuclei express AR and muscles of different sites may vary in AR content (Kadi *et al.*, 2000). Quantification of AR-containing myonuclei per fibre cross-section revealed significant differences between two different muscles: in untrained subjects, the proportion of AR-containing myonuclei in the trapezius was nearly 60% higher than in the vastus lateralis (Kadi *et al.*, 2000). These results are supported by data suggesting that androgen sensitivity may vary between different muscle groups (Kochakian and Tillotson, 1957; Wilson, 1988).

Self-administration of androgenic–anabolic steroids can alter the proportion of AR-containing myonuclei in human skeletal muscle. In the trapezius of steroid-using athletes, the myonuclear number and the percentage of AR-positive myonuclei are higher than in non-steroid users. In the vastus lateralis of steroid-using athletes, the number of myonuclei is higher but the percentage of AR-containing myonuclei is similar to non-steroid using athletes. (Kadi *et al.*, 2000). Another study showed that AR expression is enhanced after 1 month of treatment with testosterone (Ferrando *et al.*, 2002). However, when testosterone is administrated for 6 months AR expression returns to pretreatment levels (Ferrando *et al.*, 2002). It has been suggested that the expression of AR can reach a steady state in response to the treatment paradigm (Ferrando *et al.*, 2002). Altogether these results show the complexity of AR regulation in human skeletal muscle both under physiological and supraphysiological conditions.

Satellite cells express androgen receptors

The effect of testosterone on satellite cells is supported by the immunological detection of AR in porcine myogenic satellite cells *in vitro* (Doumit *et al.*, 1996) (Figure 1). Furthermore, immunoreactive AR increased in response to testosterone treatment (Doumit *et al.*, 1996). Thus, satellite cells are direct targets for testosterone action. Similar results were subsequently found in human muscle cells (Sinha-Hikim *et al.*, 2004). The increase in androgen-binding sites might be important for the regulation of pathways involved in the control of satellite cell activity.

Androgen-receptor-independent pathway: mediators of testosterone effects

Recent studies suggest the existence of a rapid intracellular AR-independent mode of action for testosterone (Figure 1). This rapid non-genomic testosterone action may be exerted via increased intracellular Ca^{2+} concentration through the activation of a G-protein-linked receptor at the plasma membrane of myoblasts obtained from rat neonatal hind limbs (Estrada *et al.*, 2003). This would result in an early but transient ERK1/2 (extracellular signal-regulated kinases) activation, which can potentially lead to the phosphorylation of transcription factors associated with cellular growth (Estrada *et al.*, 2003). In the context of human skeletal muscle, the physiological significance of the rapid non-genomic action of testosterone remains unclear.

Conclusion

Testosterone has a powerful effect on human skeletal muscle. Data gathered on the muscular effects of testosterone clearly

Androgen-receptor independent pathway: mediators of testosterone effects

Testosterone could also be acting androgen receptor independent and affecting calcium levels within the muscle cell by activating G protein receptors, leading to ERK (a signaling protein) signaling that would phosphorylate factors that are in the nucleus associated with cell growth genes.

demonstrate that drug abuse is associated with an intense recruitment of multiple myogenic pathways. Clearly, testosterone administration in sports provides an unfair muscular advantage over non-drug users. The long-term consequences of the heavy recruitment of satellite cells on their proliferative potential and the regenerative capacity of skeletal muscle are unknown.

Conflict of interest

The author states no conflict of interest.

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