Acute effect of androgens on maximal force-generating capacity and electrically evoked calcium transient in mouse skeletal muscles

Bodvael Fraysse a, Alban Vignaud b, Bourama Fane a, Mélanie Schuh c, Gillian Butler-Browne a, Daniel Metzger c, Arnaud Ferry a,d,⇑

a Université Pierre et Marie Curie-Paris, Sorbonne Universités, UMR 7455, INSERM U974, Institut de Myologie, Paris, France
b Génethon, 1 Rue de l’Internationale, Evry, France
c Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS UMR7104/INSERM U964, Université de Strasbourg, Illkirch, France
d Université Paris Descartes, Sorbonne Paris Cité, Paris, France

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A B S T R A C T
As androgens might have rapid androgen-receptor (AR) independent action on muscle cells, we analysed the in vivo acute effect of androgens on maximal force generation capacity and electrically evoked calcium transient responsible for the excitation–contraction coupling in skeletal muscle from wild-type male mice and muscle fibre androgen receptor (AR) deficient (AR+/−) male mice. We tested the hypothesis that acute in vivo androgen treatment improves contractility and modifies calcium transient in mouse hindlimb muscles. In addition, we determined whether the reduced maximal force generation capacity of AR+/− mice is caused by an alteration in calcium transient. We found that acute dehydrotestosterone (DHT) and testosterone treatment of mice does not change in situ maximal force, power or fatigue resistance of tibialis anterior muscles. In agreement with this observation, maximal force and twitch kinetics also remained unchanged when both whole extensor digitorum longus (EDL) muscle or fibre bundles were incubated in vitro with DHT. Electrically evoked calcium transient, i.e. calcium amplitude, time to peak and decay, was also not modified by DHT treatment of EDL muscle fibre bundles. Finally, we found no difference in calcium transient between AR+/− and wild-type mice despite the reduced maximal force in EDL fibre bundles of AR+/− mice. In conclusion, acute androgen treatment has no ergogenic effect on muscle contractility and does not affect calcium transient in response to stimulation. In addition, the reduced maximal force of AR+/− mice is not related to calcium transient dysfunction.

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1. Introduction

Testosterone, a male sex steroid hormone that is converted to dihydrotestosterone (DHT), notably in skeletal muscle, binds the androgen receptor (AR) in the cytosol. This complex translocates into the nucleus where it either activates or represses target genes. The expression of many genes is modified within hours to days after androgen exposure. Chronically administrated androgens can exert an ergogenic action, i.e. increases skeletal muscle maximal force production via muscle growth. It has also been reported that androgens can exert fast actions that are thought to be too rapid to be explained by this process [1,2]. For example incubation of fast skeletal muscle fibre bundles isolated from mice of both sex with DHT at 630 µg/ml for 30 min increases maximal force production by 24–30% [3]. Moreover, treatment of such fibre bundles with an AR inhibitor did not block DHT-induced maximal force increase, suggesting that this effect of DHT does not involve AR signaling. Several other studies also support the possibility of signal transduction pathways independent of AR signalling [2–9]. In the present study we would like to confirm if this rapid response to acute androgen treatment is observed in skeletal muscle in vivo. If an increase in maximum force is observed we will then look at the effect on calcium handling since this rapid AR response does seem to not involve AR signalling. Calcium is known to play a crucial role in skeletal muscle physiology, and several lines of evidence indicate that androgens modulate calcium homeostasis [2,10–12]. Chronic androgen administration in orchidectomized rodent has been shown to interfere with many aspects of calcium release and uptake of the sarcoplasmic reticulum of the myocardium [11,12], responsible for the excitation–contraction–relaxation cycle.
Moreover, it was recently shown that acute testosterone treatment increases intracellular calcium in myotubes [4–6]. However, acute effects of androgens on calcium transient initiating the contraction of the skeletal muscle are poorly characterised. It is possible that the reduced maximal force generation capacity that was observed in skeletal muscle from ARskm−/− mice, in which the AR was selectively ablated in skeletal muscle fibres [13], is related to an alteration of the calcium transient. The selective ablation of AR in muscle fibres from ARskm−/−y mice leads to a 20–25% reduction in the maximal force generation capacity in mouse fast muscle, with no change in muscle weight [13].

The main purpose of this study was to examine the acute effect of androgens on the skeletal muscle of wild-type (WT) and ARskm−/− mice. To our knowledge, the acute effect of androgens in vivo on muscle performance has not yet been studied. Our first hypothesis was that maximal force would be increased by acute in vivo androgen administration in both WT and ARskm−/−y mice. A second hypothesis is that electrically evoked calcium transient would be affected by acute androgen administration in mice. A third hypothesis is that reduced force generation capacity of the ARskm−/− mice would be related to altered calcium transient. Such findings would be of benefit to antidoping agencies, as they would indicate that acute androgen administration might have ergogenic effects in athletes. Moreover, they would open new avenues to develop treatments to combat the muscle weakness observed in different contexts (ageing, neuromuscular diseases, etc.).

2. Materials and methods

2.1. Animals

All procedures were performed in accordance with national and European legislations and all experimental protocols have been approved by the French Departmental Direction of Animal Protection (agreement 75-1102). Male CD1 mice (referred to below as WT), ARskm−/− mice [13] and age-matched control (referred to below as WT) mice were analysed. Testosterone and DHT (Sigma, St. Louis) were used to replace androgens in vivo.

2.2. In situ TA muscle contractility

We analysed in situ tibialis anterior (TA) muscle contraction in response to nerve stimulation as described previously [14,15]. Mice were anesthetized using pentobarbital (80 mg/kg ip). Body temperature was maintained at 37 °C using radiant heat. The knee and foot were fixed with pins and clamps and the distal tendon of the muscle was attached to a lever arm of a servomotor system (305B, Dual-Mode Lever, Aurora Scientific) using a silk ligature. The sciatic nerve was proximally crushed and distally stimulated by a bipolar silver electrode using supramaximal square wave pulses of 0.1 ms duration. Absolute maximal force (P0) generated during isometric contractions in response to electrical stimulation (frequency of 75–150 Hz, train of stimulation of 500 ms) was determined at L0 (length at which maximal tension was obtained during the tetanus). Absolute maximal power (Pmax, mW) was calculated from force–velocity data. The isometric contraction was initiated during the first 300 ms, then muscle shortening was allowed during the last 200 ms. Five shortening contractions (15–50% of P0) were performed, each separated by a 45 s rest period. The corresponding shortening speeds were measured during the first 50 ms. Finally, the fatigue resistance was assessed. The fatigue resistance protocol consisted of 40 contractions (100 Hz for 300 ms, evoked once every second), and the force produced at the end of the protocol was measured (% of initial force).

2.3. In vitro EDL muscle contractility

Isometric contractile properties of extensor digitorum longus (EDL) muscles were studied in vitro. Measurements were performed according to methods previously detailed [16]. Briefly, muscles were dissected and connected at one end to an electromagnetic puller and at the other end to a force transducer. Muscles were dissected free from adjacent connective tissue and soaked in an oxygenated Tyrode solution (95% O2 and 5% CO2) containing (mM): 118 NaCl, 25 NaHCO3, 5 KCl, 1 KH2PO4, 2.5 CaCl2, 1 MgSO4, 5 glucose, maintained at a temperature of 20 °C. After equilibration (45 min), stimulation (frequency of 125 Hz, train of stimulation of 300 ms) was delivered through electrodes running parallel to the muscle. P0, the absolute twitch force (Pt), the contraction time and the half-relaxation time during the twitch were measured.

2.4. In vitro EDL fibre bundle contractility and calcium transient

EDL muscles were pinned in SYLGARD coated dishes containing normal physiological solution (in mM: 148 NaCl, 4.5 KCl, 2.5 CaCl2, 1 MgCl2, 10 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 5.5 glucose (pH = 7.4). Small bundles of 10–15 fibres arranged in a single layer were dissected lengthwise, tendon-to-tendon, with the use of microscissors. Muscle preparations were loaded during 1 h with 3 µM of the Fura-2 AM fluorescent calcium probe. After Fura-2 loading, muscle fascicles were mounted in an experimental chamber allowing isometric force and cytosolic calcium measurement and electrical stimulation. One extremity was attached to a hook linked to a micromanipulator and the other extremity was fixed to an electromagnetic force-transducer device described elsewhere [17]. Muscle preparations were stretched until the twitch amplitude elicited by efficient electrical stimuli (5 ms duration; 14 V; 0.2 Hz) reached the maximum value. The diameter of each contracting fibre was measured to calculate the cross sectional area. The sum of the areas was used to calculate the cross-sectional area (mm2) and to normalise force amplitude (mN). Electrical triggered force development was acquired under two stimulus protocols: (1) 5 ms duration/14 V/0.2 Hz, protocol designed to elicit single separated twitches (contraction related to a unique action potential); (2) 20 pulses of 5 ms duration/14 V/100 Hz, protocol designed to elicit perfect tetanus. The evaluation of the contractile responses was done by measuring the amplitude (mN/mm2), the time to peak (ms) and the constant of relaxation (s−1) of the twitch, and the amplitude (mN/mm2) and the time constant of relaxation (s−1) of the tetanus. All experiments were conducted at room temperature.

Ratiometric Fura-2 fluorescence measurements were made using optical excitation filters of 380 and 360 nm and an IonOptix microStepper Switch integrated system. Emitted fluorescence (510 nm) was background subtracted. Cytosolic [Ca] was calculated at rest according to a modified method from Grynkiewicz and collaborators [18]. A pseudo ratiometric approach was used to acquire calcium transients. Preparations were electrically stimulated with a pace protocol (0.5 Hz; 4 ms duration; 14 V) designed to induce a single action potential. Fura-2 fluorescence was first recorded at 1000 Hz under 380 nm excitation during 10 electrical stimulations. Thereafter, Fura 2 fluorescence was recorded at 360 nm. The 10 records were averaged at each excitation wavelength. Finally the calcium transient was calculated by making the ratio of the means (360/380) and transformed in [Ca] values using the method described for resting [Ca] determination. The time to peak, the amplitude and the rate constant of the calcium decay (by fitting a monoexponential on the recovery phase of the calcium transient) were calculated.
2.5. Statistical analysis

Groups were statistically compared using variance analysis or Student’s t-test. If necessary, subsequent Bonferroni post hoc test was performed. For groups that did not pass the tests of normality and equal variance, non-parametric tests were used (Kruskal Wallis and Wilcoxon). Values are means ± SEM.

3. Results

3.1. Does an acute in vivo androgen treatment increases muscle force?

We examined the acute effect of androgens in vivo by analysing in situ TA muscle force production in response to electric nerve stimulation. Mice were injected ip with androgens dissolved in ethanol/PBS or vehicle (ethanol/PBS) and muscle performance was evaluated 30, 60 and 90 min after injection. Since we found no difference between the different times ($p > 0.05$), only 30 min data are shown. Fig. 1 shows that treatment with 10 mg/kg DHT (Fig. 1A and B), 100 mg/kg DHT (Fig. 1C) and testosterone (1, 10 and 100 mg/kg) (Fig. 1D, and data not shown) did not change the absolute maximal force and absolute maximal power in WT and ARskm-y mice ($p > 0.05$). Fatigue resistance was not increased (Fig. 1A, C and D) ($p > 0.05$). In addition, 100 mg/kg of testosterone dissolved in DMSO did not alter muscle performance after 90 min (Fig. 1E) ($p > 0.05$). To ensure the drug is rapidly available in muscle, we locally applied androgens and measurement were carried out 15 min after administration. Since we found no difference between dosages, 0.04, 0.4 and 4 mg/ml DHT data were pooled ($p > 0.05$). Fig. 1F shows that locally applied DHT did not affect the absolute maximal force, absolute maximal power or fatigue resistance in WT mice ($p > 0.05$).

To complete our study, we investigated the in vitro effect of androgens on EDL muscle contraction in response to electric stimulation. The EDL muscle similarly to the TA muscle is mainly composed of fast fibres. Whole EDL muscle was incubated with DHT dissolved in ethanol/thyrode and measurements were performed before, and 15, 30 and 45 min after treatment. Treatment with different DHT doses (63 and 630 pg/ml, and 6.3 ng/ml) did not alter maximal force of the EDL muscle (Fig. 2A) in WT mice ($p > 0.05$).

Fig. 1. Absolute maximal force ($P_0$), absolute maximal power ($P_{max}$) and fatigue resistance of TA muscle from WT and ARskm-y mice after in vivo acute androgen treatment. (A) 10 mg/kg DHT treatment of WT mice. (B) 10 mg/kg DHT treatment of ARskm-y mice. (C) 100 mg/kg DHT treatment of WT mice. (D) 1, 10 and 100 mg/kg testosterone treatment of WT mice. Data from 1, 10 and 100 mg/kg testosterone treatments were pooled. In A, B, C and D drugs were dissolved in ethanol/PBS. WT and ARskm-y control mice were treated with appropriate vehicle (ethanol/PBS). Mice were ip injected, then anesthetized and muscle performance was studied 30 min after. (E) 100 mg/kg testosterone treatment in WT mice. Drug was dissolved in DMSO and muscle performance was studied 90 min after ip injection. WT control mice were similarly treated with vehicle (70 μl DMSO). (F) Drug was applied locally. Drug was dissolved in ethanol/PBS. Control mice were treated with appropriate vehicle (ethanol/PBS) and data were pooled. Measurements were done 15 min after. $a$: significant difference between treatment ($p < 0.05$) using variance analysis followed by Bonferroni post hoc analysis. $P_0$: absolute maximal force, $P_{max}$: absolute maximal power. $n = 6–12/group$. Data are means ± SEM.
We also found no effect of these in vitro DHT treatments on submaximal force (force of the twitch) or the kinetic of the twitch (contraction time, half-relaxation time) (Fig. 2B) \( p > 0.05 \). Similarly, DHT dissolved in DMSO had no effect on absolute maximal force (Fig. 2C and D) 15, 30, 45, 60, 75 and 90 min after treatment \( p > 0.05 \).

3.2. Do androgens affect calcium transient?

The effect of DHT on electrically evoked calcium transient was analysed in vitro on fibre bundles loaded with a calcium indicator. The magnitude and the kinetic of the evoked calcium transient were assayed in either WT or ARskm\(^{-/-}\) mice. We found that amplitude (Fig. 4A and B), time to peak (Fig. 4C and D) and the decay (Fig. 4E and F) of the calcium transient did not differ between DHT treated and untreated fibre bundles from both mouse strains \( p > 0.05 \).

3.3. Is calcium transient alteration responsible for the reduced maximal force in ARskm\(^{-/-}\) mice?

In a previous study, we reported that the specific maximal force of TA and EDL muscles was reduced in ARskm\(^{-/-}\) mice [13]. Similarly, in the present study we found that the specific maximal force of EDL fibre bundles was decreased in such mice as compared to WT mice (Fig. 5A) \( p < 0.05 \). However, this reduced muscle force capacity generation was no related to calcium transient dysfunction. Since, there was no difference in calcium amplitude, time to peak and decay between ARskm\(^{-/-}\) mice and WT mice (Fig. 5B) \( p > 0.05 \).
4. Discussion

Results from several recent studies support the possibility that sex hormones may have a rapid action via signal transduction pathways independently of AR [3–9,19,20]. Moreover, it was reported that they might alter various aspects controlling the muscle excitation–contraction–relaxation cycle: increased muscle excitability, calcium homeostasis and maximal force-generation capacity [2,5,6,10–12,19]. We first tested whether maximal force generation by skeletal muscle is increased by in vivo acute androgen administration in both WT and AR<sub>skm-/y</sub> mice. Our study demonstrates that in vivo acute androgen treatment does not affect maximal force (and maximal power) of mouse muscle expressing or not AR. In addition, we found no effect of in vitro androgen treatment on maximal force generation capacity of whole muscle and fibre bundles, eliminating the potential problems of hormone bioavailability. Therefore, our results do not support an ergogenic action of acute androgens systemically administered on muscle maximal performance. An ergogenic effect of androgens on motor behaviour [21] in vivo can however not be excluded.

In addition, acute androgen treatment did not alter the calcium transient evoked electrically, in both WT and AR<sub>skm-/y</sub> mice. This result is in agreement with the absence of an effect of acute androgen treatment on maximal force, since calcium transient plays a key role in the contraction. Our results indicate that the potential acute effect of androgens on intracellular calcium on skeletal muscle cells [4] has no major impact on the evoked calcium release during excitation–contraction coupling.

A reduction in the amplitude of the calcium signal delivered to the contractile apparatus would lead to a reduced maximal force generation capacity. A reduced calcium signal may be achieved via a reduced release from the ryanodine receptor, the calcium channel of the reticulum sarcoplasmic, or increased calcium buffering such as that performed via the sarcoplasmic reticulum calcium ATPase pumps. However, muscle weakness induced by selective AR ablation in skeletal muscle fibres ([13], and present study) cannot be explained by such effects, since electrically calcium transient were similar in AR<sub>skm-/y</sub> and WT mice. The marked alterations in force production observed in AR<sub>skm-/y</sub> mice, both at the levels of the whole muscle and the fibre bundles, must
therefore be related to altered sarcomere structure and posttranslational modifications of contractile proteins, but not to a reduced function/expression of reticulum sarcoplasmic proteins.

5. Conclusion

Our results show that muscle contractility and electrically evoked calcium transients are not affected by acute androgen treatment, indicating that androgens have no major rapid action on either intact fast skeletal muscle or isolated muscle fibres. In contrast, they suggest that acutely administered androgens cannot improve muscle performance in athletes or muscle weakness in patients with neuromuscular diseases. Moreover, the present study shows that an altered electrically evoked calcium transient is not responsible for the reduced maximal force observed in AR-deficient fast muscles.

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References