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ANDROGEN METABOLISM IN RAT L6 MYOBLAST CELLS; HIGH FORMATION OF 5α-ANDROSTANE-3α,17β-DIOL FROM TESTOSTERONE

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Summary—We have studied androgen metabolism in L6 rat myoblasts. 4-androstene-3,17-dione (Adione), testosterone, 5α-dihydrotestosterone (DHT), and 5α-androstane-3α,17β-diol (3α-diol) were used for substrates and the amounts of metabolites formed from the respective substrates in the medium were determined. Conversion of Adione to testosterone was dominant over the reverse conversion. DHT formation from testosterone was low and did not change with the duration of incubation, whereas 3α-diol formation increased in a time-dependent manner. Major metabolite of testosterone was not DHT but 3α-diol. A large amount of 3α-diol was formed from DHT, however DHT formation from 3α-diol was very low. These data indicate that L6 cells have high 5α-reductase activity and suggest that DHT formed from testosterone is rapidly metabolized to 3α-diol in these cells.

INTRODUCTION

Secreted testosterone is converted to bioactive 5α-dihydrotestosterone (DHT) by 5α-reductase in the genital organs and acts on the nucleus [1, 2]. The skeletal muscle is a major extragenital target organ of androgen action. Testosterone stimulates nitrogen retention [3, 4], and also increases muscle mass *in vivo* [5]. In male patients with myotonic dystrophy, plasma testosterone levels decreased [6, 7], and testosterone administration increased muscle protein synthesis significantly in these patients [8]. It is fact that the development of the skeletal muscle occurs in patients with 5α-reductase deficiency [9], and 5α-reductase levels in the skeletal muscle are very low, resulting in poor conversion of testosterone to DHT [10]. These findings suggest that DHT is not needed for the development of the skeletal muscle. Androgen action in the skeletal muscle appears to differ from its effect on the genital organs.

There are few papers reporting on the androgen metabolism in primary culture of skeletal muscle. L6 cells are a clonal strain of myoblasts, originally derived from new born rat thigh muscle, and these cells possess the capacity to fuse and differentiate into multinucleated skeletal muscle myotubules [11]. After differentiation, L6 cells bear similar biochemical and histological properties to normal skeletal muscle cells [12, 13]. We have therefore studied the androgen metabolism (4-androstene-3,17-dione⇌testosterone →DHT⇌5α-androstane-3α,17β-diol) using L6 cells as a model of the skeletal muscle and found the high formation of 5α-androstane-3α,17β-diol from testosterone in L6 cells.

EXPERIMENTAL

Steroids

[1,2,6,7-^{3}H]4-androstene-3,17-dione (SA, 85 Ci/mmol), [1,2-^{3}H]testosterone (SA, 60 Ci/mmol), [1,2-^{3}H]5α-dihydrotestosterone (SA, 55.5 Ci/mmol), [1,2-^{3}H]5α-androstane-3α,17β-diol (SA, 30.1 Ci/mmol), [4-^{14}C]4-androstene-3,17-dione, [4-^{14}C]testosterone, [4-^{14}C]5α-dihydrotestosterone were obtained from New England Nuclear (Boston, Mass), and these radiolabeled steroids were purified before use by thin-layer chromatography using silica gel plates 60F254 (Merck, Darmstadt, West Germany). Non-radioactive steroids were obtained from Sigma Chemical Co. (St Louis, Mo.). [4-^{14}C]androstane-3α,17β-diol was made by reduction [4-^{14}C]5α-dihydrotestosterone [14], and purified by thin layer-chromatography as described below.

Cell culture

L6 cells [11] were maintained in a humidified 5% CO_2 incubator at 37°C in flasks containing 10 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 μ/ml), streptomycin (0.1 mg/ml), and 10% fetal bovine serum (FBS). On day 0, the cells were dissociated from the stock flasks with 0.05% trypsin–0.05 M EDTA and were seeded at a concn of 1.0×10^4 cells into each well

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Table 1. Systems of thin layer chromatography

	Solvent system	Plate
System 1	Choloroform–methanol (98:1.75)	Silica gel*
System 2	Choloroform–ethylacetate–ethanol (85:15:3)	Silica gel*
System 3	Benzen–ethanol (96:3.5)	Aluminiumoxide**
System 4	Dichloromethane–diethylether (90:10)	Aluminiumoxide

*Silicagel plate; 60F254 (Merck, Darrmstadt, West Germany), **aluminiumoxide plate; 60F254 (Merck, Darrmstadt, West Germay).

of 24-well Coster tissue culture plates with 0.5 ml culture medium. On day 3, the medium was exchanged with fresh culture medium, and on day 6, the medium was replaced with DMEM without FBS.

Determination of androgen metabolism in L6 cells

On day 7, the medium was removed, and washed twice with Hanks' solution, and DMEM (0.2 ml) containing the respective ^{3}H-radiolabeled steroids for substrate was added to each well to evaluate metabolite formations in L6 cells. After incubation, a known amount (approximately 3000 dpm) of ^{14}C-radiolabeled steroids was added to each well for recovery collection, and the medium then removed, and extracted twice with 3 ml diethylether. The cells were disrupted with 0.1 ml NaOH (0.1 N), and an aliquot was assayed for protein content (Bio-Rad, Calif.).

Separation and quantification of steroid metabolites

After extraction of the medium, the extracts were evaporated to dryness and redissolved in ethanol containing 20 μg of non-radioactive steroids and isolated by thin-layer chromatography (TLC) described in Table 1. DHT and 4-androstene-3,17-dione (Adione) converted from testosterone were separated by chromatography of System 1 followed successively by System 2. Testosterone converted from Adione was also separated by System 1 and 2 TLC. 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol (3α-diol and 3β-diol) converted from testosterone or DHT were separated by System 1 chromatography followed successively by System 3. DHT converted from 3α-diol was separated by System 1 chromatography followed successively by System 4. Testosterone and Adione were detected by u.v. light. DHT, and 3α-diol plus 3β-diol separated by System 1 were detected by spraying plates with water. 3α-diol plus 3β-diol was separated to 3α-diol and 3β-diol respectively by System 3. 3α-diol and 3β-diol separated by System 3, and DHT separated by System 4 were detected by spraying with 30% methanolic sulfuric acid, and heating to 110°C. The area of each steroid was scraped off, and each steroid was then eluted with 3 ml ethylacetate. The purity of each steroid was confirmed by obtaining the constancy of the ^{3}H to ^{14}C ratio after acetylation and crystalization. This quantification of products was performed using the specific activity of the radiolabeled substrate and the ^{3}H to ^{14}C ratio of the purified metabolites as previously described [5].

RESULTS

Metabolite formations as a function of incubation time

L6 cells were incubated with 0.1 μM [^{3}H]testosterone for up to 6 h as substrate to evaluate the

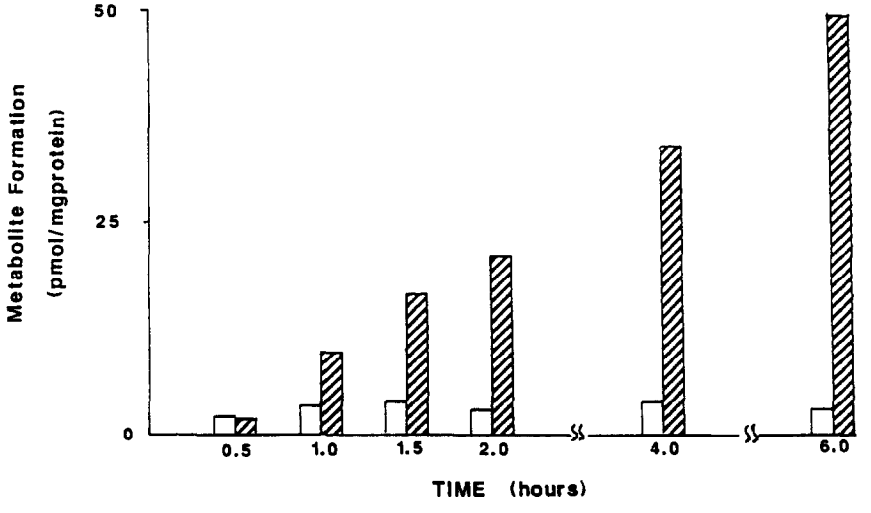

Fig. 1. L6 cells were incubated with [^{3}H]testosterone (0.1 μM) for up to 6 h. After incubation with [^{3}H]testosterone, metabolites in medium were extracted and separated as described in the Experimental. The activity of each radiolabeled metabolite was counted by β-scintillation counter (Aloka, LSC 903 Japan). The formation of DHT (open bar) and 3α-diol (solid bar) from testosterone are shown. The Adione formation was not detectable (data not shown). The data were shown the values obtaining from using duplicate wells of cells in the representative experiment.

respective formations of Adione, DHT, 3α-diol, and 3β-diol. DHT formation did not change with the duration of the incubation, whereas 3α-diol formation increased in a time-dependent manner. 3α-diol formation was greater than DHT formation after 1 h, and by 6 h had increased 20-fold over compared to its formation at 30 min. Adione formation was not detected (Fig. 1). [^{3}H]Adione was used as a substrate to evaluate the formation of testosterone, and [^{3}H]DHT and [^{3}H]3α-diol were for 3α-diol and DHT respectively. After the incubation with each substrate, the formation of testosterone from Adione increased in a time-dependent manner plateauing after 4 h. A similar result was observed in 3α-diol formation from DHT. DHT formation from 3α-diol, however, was barely detectable (Fig. 2). 3β-diol formation was very low after incubation with testosterone or DHT (data not shown).

Metabolite formations as a function of substrate concentration

L6 cells were incubated for 1 h with increasing concentrations of each substrate. The formations of DHT and 3α-diol from testosterone are shown in Fig. 3. 3α-diol formation was greater than DHT formation at all substrate concentrations tested. The formations of testosterone from Adione, 3α-diol from DHT, and DHT from 3α-diol increased depending on the concentration of the respective substrates (Fig. 4).

Activities of androgen metabolism in L6 cells

Activities of androgen metabolism in L6 cells were determined by using the respective metabolite formations after incubating with 0.1 μM of each substrate for 1 h as summarized in Table 2 and

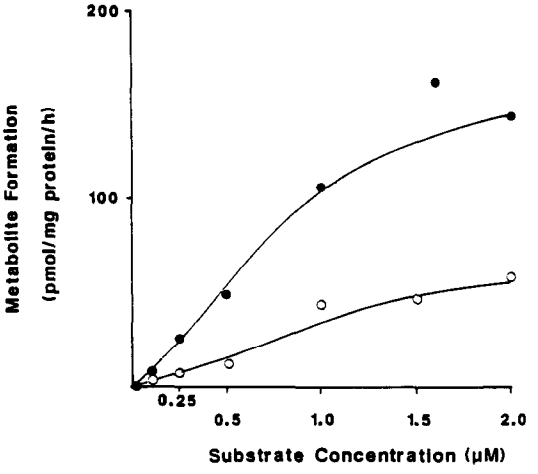

Fig. 3. L6 cells were incubated for 1 h with increasing concentrations of testosterone. DHT formation (open circle) was obtained from the conversion from testosterone, 3α-diol formation (closed circle) was obtained simultaneously by the conversion from testosterone. The data were obtained using duplicate wells of cells in the representative experiment.

Fig. 5. The formation of testosterone from Adione, which was a parameter of 17β-hydroxysteroid oxidoreductase (HSOR) reductase activity, was 9.1 ± 0.6 pmol/mg protein/h (mean ± SD). In contrast, 17β-HSOR oxidase activity was undetectable. Since 5α-reductase activity was determined by the formation of DHT plus 3α-diol from testosterone, our data demonstrated high activity of 5α-reductase in L6 cells (13.8 ± 0.8 pmol/mg protein/h), even though DHT formation was very low (2.1 ± 0.1 pmol/mg protein/h). 3α-diol formation

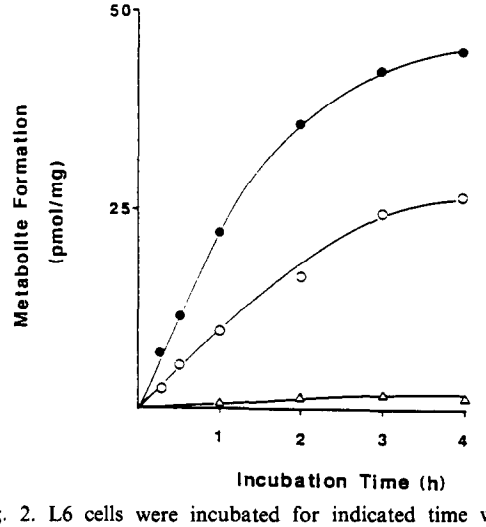

Fig. 2. L6 cells were incubated for indicated time with 0.1 μM Adione, DHT, and 3α-diol respectively. The formation of testosterone from Adione (open circle), 3α-diol from DHT (closed circle), and DHT from 3α-diol (triangle) are shown. The data were shown obtained using a duplicate wells of cells in the representative experiments.

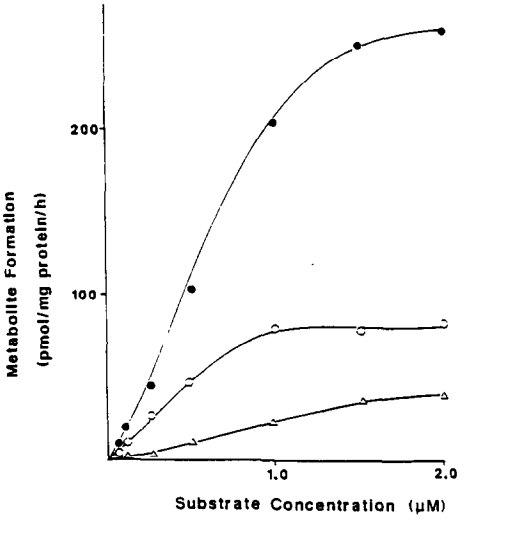

Fig. 4. L6 cells were incubated for 1 h with increasing concentrations of Adione, DHT and 3α-diol respectively. The formation of testosterone from Adione (open circle), 3α-diol from DHT (closed circle), and DHT from 3α-diol (triangle) are shown. The data are shown obtained using duplicate wells of cells in the representative experiment.

Table 2. Activities of androgen metabolism in L6 cells

Enzyme	Substrate	Metabolite	Activity (pmol/mg protein/h)
17β-HSOR* reductase	Adione	Testosterone	9.1 ± 0.6
17β-HSOR oxidase	Testosterone	Adione	Undetectable
5α-reductase	Testosterone	DHT + 3αdiol	13.8 ± 0.8
3α-HSOR-reductase	DHT	3αdiol	28.8 ± 5.8
3α-HSOR oxidase	3αdiol	DHT	1.4 ± 0.2

*HSOR: hydroxysteroid oxidoreductase. Activities were obtained from respective metabolite formation after incubating with 0.1 μM each substrate for 1 h. Values are expressed as the mean ± SD of 6 experiments.

from DHT as a parameter of 3α-HSOR reductase activity was 28.8 ± 5.8 pmol/mg protein/h, and was the greatest of the androgen metabolism in the present study. 3α-HSOR oxidase activity was very low (1.4 ± 0.2 pmol/mg protein/h).

DISCUSSION

In L6 cells, Adione was metabolized to testosterone, however, the reverse conversion was not observed. In the study of the *in vivo* interconversion between Adione and testosterone in human skeletal muscle, Longscope *et al.* [16] reported that the conversion of Adione to testosterone was dominant over the reverse conversion. These data are similar to our findings using rat myoblasts. Testosterone was metabolized to DHT in L6 cells, while quantities of DHT formation were minimal, similar to previous studies [10, 17–21]. In the study of DHT degradation, DHT was mainly metabolized to 3α-diol in L6 cells, while 3β-diol formation was minimal. 3α-diol formation from DHT was high compared with the other androgen metabolism in our study and these results indicate that these cells have high activity of 3α-HSOR reductase. These data are similar to previous findings *in vivo* [10, 20, 22] and *in vitro* [23, 24] studies utilizing the skeletal muscle. The reverse conversion was very low in L6 cells compared to the conversion of DHT to 3α-diol, and this is also similar to previous studies in the skeletal muscle [10]. Low formations of DHT from testosterone and 3α-diol in L6 cells suggest that DHT may not play an important role on these cells. These observations of androgen metabolism in L6 cells indicate that these cells possess similar androgen metabolism to those found in human and rat skeletal muscle.

The androgen metabolism (testosterone→DHT⇆3α-diol) in rat L6 myoblasts differs from the prostate and human genital skin fibroblasts. In previous study utilizing human genital skin fibroblasts [15], we observed that DHT formation from testosterone increased in a time-dependent manner, while 3α-diol formation from testosterone was very low. However, in L6 cells, 3α-diol formation from testosterone was higher than DHT formation, and DHT formation was constant after changing the incubation time, whereas 3α-diol formation increased in a time-dependent manner. Dionne *et al.* [10] demonstrated that 3α-diol content was higher than DHT in rat skeletal muscle cytosol after 1 h perfusion with [^{3}H]testosterone. Our data suggest that DHT formed from testosterone was rapidly metabolized to 3α-diol

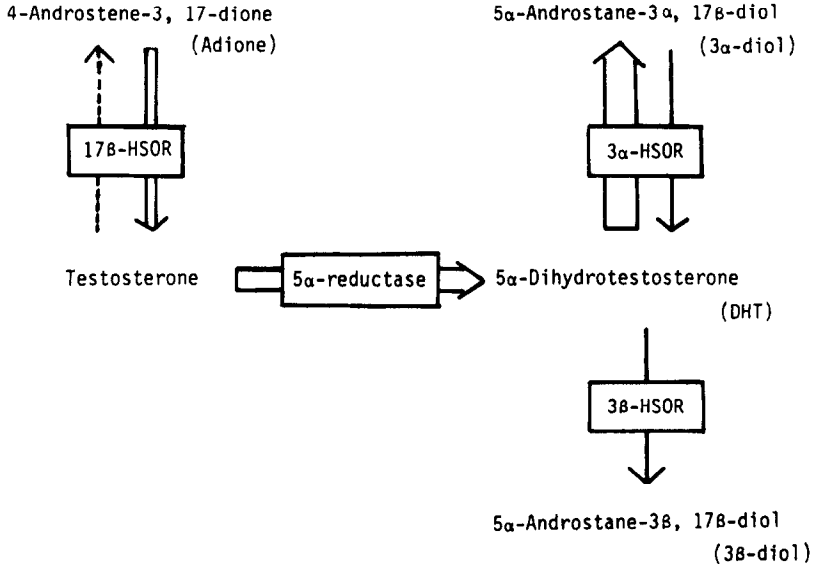

Fig. 5. The pathways of androgen metabolism in rat L6 myoblasts. The dotted line (---→) is expressed as the undetectable activity. The formation of DHT from 3β-diol was not determined.

in L6 cells. Determination of 3α-diol formation from testosterone as well as DHT formation therefore provides a useful parameter of 5α-reductase activity [25]. The data in the present study indicate that rat L6 myoblasts has a high activity of 5α-reductase because DHT plus 3α-diol formation from testosterone was high. In contrast with our conclusion, the previous studies [2, 17, 19, 21] indicated that the activity of 5α-reductase in the skeletal muscle was very low because they examined only DHT formation from testosterone, not DHT plus 3α-diol formation.

In summary, androgen metabolism in L6 cells is similar to normal skeletal muscle, and these cells provide a useful model to study mechanisms of androgen action of on the skeletal muscle. Furthermore, the data in the present study suggest that the skeletal muscle have high 5α-reductase activity, and the low formation of DHT is not due to low 5α-reductase activity, but due to a rapid conversion of DHT to 3α-diol.

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