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Rapid Stimulatory Effects of Testosterone Upon Myotubule Metabolism and Sugar Transport, as Assessed by Silicon Microphysiometry

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A considerable number of studies have revealed behavioral circumstances that give rise to small or transient differences in circulating testosterone concentrations; however, careful consideration of androgen physiology leads to the disquieting conclusion that these differences are often unlikely to have much physiologic or behavioral significance. In the present report, we observe that small transients of testosterone secretion could have very rapid anabolic effects on a cultured muscle-derived cell line. Specifically, we have examined the effects of testosterone on metabolism in cultured C2C12 myotubules, using a silicon microphysiometer. The instrument monitors cellular extrusion of protons and acidic metabolites, and such extrusion is directly linked to ATP hydrolysis, thus providing a real-time measure of cellular metabolism. Testosterone caused a small but significant increase in metabolism. The most striking feature of this effect was its rapidity, in that it occurred within 3 hr. This rapid enhancement of metabolism suggested that testosterone might be enhancing substrate uptake even more rapidly. Indeed, we found that testosterone increased 2-deoxyglucose uptake within 1 min. The rapidity of this effect seemed to preclude mediation by classical intracellular steroid receptors. In support of this, we were unable to detect specific intracellular binding of testosterone. These findings show that testosterone can exert rapid anabolic effects on substrate transport and metabolism in myotubules. Should this finding general to muscle in vivo, it suggests that relatively small individual differences in testosterone profiles, in response to various social interactions, may have very real consequence for subsequent muscle physiology.

Key words: androgens, testosterone, muscle metabolism, microphysiometry, glucose transport

INTRODUCTION

A vast number of psychoendocrine studies have examined the effects of various behaviors, or of social or psychological experiences upon testosterone secretion. Far fewer

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studies have tested whether the differences in testosterone concentrations uncovered are of any actual physiologic consequence. Some of the more careful of investigators have emphasized how the often small or transient changes in concentrations, for example, in response to aggressive or sexual behavior, are unlikely to have meaningful effects upon reproductive physiology, or subsequent aggressive or sexual behavior [reviewed in Brain and Haugh, 1992; Monaghan and Glickman, 1992; Rose, 1992; however, see Sacks et al., 1988, or Erskine et al., 1992, for some of the few cases of rapid testosterone effects on behavior or behaviorally-relevant physiology].

Less attention has been paid to the possibility of effects of transient bursts of testosterone exposure on muscle metabolism. Surprisingly, there is far from consensus as to even the effects of sustained exposure on muscle. Androgens in general are profoundly anabolic to muscles such as the levator ani. However, when considering various skeletal muscles, some investigators have reported that androgens increase muscle wet weight, work capacity, protein synthesis [Exner et al., 1973; Menschikowski et al., 1988; Viru, 1984], and induce the activity of a number of metabolic enzymes [Exner et al., 1973; Pastoris et al., 1983; Saborido et al., 1985], while others have failed to support these findings [Bates et al., 1987; Kuhn and Max, 1985; Pastoris et al., 1983; Stone and Lipner, 1978].

We examined the effects of testosterone upon the metabolism of a muscle-derived cell line, using the recently-developed silicon microphysiometer. The instrument monitors changes in extracellular pH as a result of extrusion of protons and acidic metabolites such as lactic acid and CO_2; proton extrusion reflects the hydrolysis of ATP, thus providing a real-time measure of cellular metabolism [McConnell et al., 1992; Parce et al., 1989; Raley-Susman et al., 1992; Redish et al., 1993]. We also examined testosterone effects on sugar transport in these cells. These two approaches indicate enhancement of both transport and metabolism by testosterone. While the effects were quite small, their most striking feature was their speed. While these findings might initially be thought to be most appropriate to a physiology readership, we think they will inform discussions as to the consequences of altered testosterone profiles in different behavioral settings.

MATERIALS AND METHODS

Materials

Mouse C2C12 cells, an immortalized muscle-derived cell line, were obtained from American Type Culture Collection. Fetal bovine serum (FBS), horse serum (HS), defined supplemented calf serum (DSCS), and defined supplemented FBS (DSFBS) were obtained from HyClone (Logan, UT), and Dulbecco's modified Eagle's medium (DMEM, containing 5 mM glucose) from Grand Island Biological Company (New York). All microphysiometric supplies were from the Molecular Devices Corporation (Menlo Park, CA). Testosterone and corticosterone were from Sigma (St. Louis, MO).

Cell Culturing

C2C12 cells were passaged (grown to confluence and then split) once, stored in liquid nitrogen until ready for use, then thawed and plated in DMEM supplemented with 5% FBS and 15% DSCS (48-well plates for binding assays and 2-DG uptake, and 12-well plates with glass coverslips for microphysiometry). Cells were grown to 80–90%

confluence (3 days). Myoblast studies were conducted at that time. For myotubule studies, cells were rinsed and refed with DMEM supplemented with 2% HS. Cells were allowed to fuse (forming long, multinucleated fibers) for 3 additional days. Experimental manipulations were performed on the sixth day.

Microphysiometry

Details of microphysiometry are described elsewhere [McConnell et al., 1992; Parce et al., 1989; Raley-Susman et al., 1992]. Briefly, cover slips containing cells were placed in a low-volume flow chamber, one side of which is a silicon-based light-addressable potentiometric sensory that measures small changes in extracellular medium pH. The other side of the chamber is the cell-bearing coverslip, which is coated with indium-tin oxide to create a conductive surface. Cultures were perfused with a low-buffering-capacity (1 mM) medium at 15 µl/min for 150 seconds, followed by a 100 sec period of halted flow. The perfusion/halt cycle was controlled by an IBM PC.

"Metabolic rate," as used throughout this paper, was defined as the rate of acidification of the external medium during the brief halt (100 sec) in the perfusion. Previous studies have shown that the rate of acidification of the external medium is a sensitive index of metabolic rate in a wide variety of cell types [McConnell et al., 1992] and correlates well with other indices of metabolism, including lactate production and oxygen consumption. Acidification rates of the medium were measured at regions of the silicon-electrolyte interface using a light-emitting diode. The rate of acidification was determined as the slope of a linear least-squares fit to the relation of pH versus time (sec). The perfusion then resumed, allowing the pH of the medium to return to basal levels (within 10–15 sec of perfusion onset). Multiple determinations of the acidification rate were obtained, and the data are expressed as the average of 5–10 such determinations at a given time point. All studies were carried out on a prototype microphysiometer made available by the Molecular Devices Corporation.

2-Deoxyglucose (2-DG) Uptake

2-DG uptake was determined as described previously [Klip and Ramel, 1987] with minor modifications. Briefly, cells were refed with serum-free media containing 1 µM testosterone or vehicle alone (ethanol). At indicated timepoints, cells were rinsed once with hexose-free uptake solution (140 mM NaCl, 20 mm HEPES, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$; pH 7.4), then incubated in uptake solution containing 0.5 µCi 14C-2DG (New England Nuclear, 304 mCi/mmol) and 0.005 mM cold 2-DG for 10 min at room temperature. After incubation, cells were rinsed three times with ice-cold PBS to terminate uptake. Cells were lysed with 0.05 N NaOH. Aliquots of lysates were used for scintillation counting and protein determination by the Bradford method.

Assay of Intracellular Testosterone Receptors

A binding assay was performed according to the method of Kontula et al. [1980]. Cells were incubated for 4 h in DMEM containing 40 nM 3H-testosterone (a saturating concentration) at 37°C. Nonspecific binding was determined by parallel incubations with 400 nM cold testosterone plus tracer. Following incubation, cells were washed three times with DMEM, lysed with sterile water, and the lysate were used for scintillation counting. Aliquots of lysates were used for protein determination by the Bradford method.

Data Analysis and Statistics

Data are expressed as mean ± SEM. Data were compared by two-way ANOVA and Newman-Keuls post-hoc tests. Receptor binding data were analyzed by *t*-test.

RESULTS

Basal metabolic rate in myotubules, as defined in the Materials and Methods section, typically ranged from 20–50 uV/sec (0.3–0.8 mpH units/sec).

The effects of 1 μM testosterone were examined. A small but significant increase in metabolic rate was seen over the first 4 hr (Fig. 1 and Table I) with the peak difference occurring at approximately the 4 hr mark. The effect had abated by 8 hr after testosterone exposure. As a test of steroidal specificity, cultures were also exposed to 1 μM corticosterone, which failed to alter metabolic rate (Table II).

While the magnitude of the testosterone effect was quite small, the speed was striking. This raised the possibility of an increase in uptake of energy substrates that preceded this enhancement of metabolism. Thus, the effects of testosterone on sugar uptake in myotubule cultures were examined. The steroid enhanced uptake within 1 min (Fig. 2).

A steroid effect of this speed raised the possibility of an unconventional mechanism of hormone action. In support of this possibility, we were unable to detect specific androgen binding above background (n = 3).

Microphysiometric measures in myoblasts revealed basal metabolic rates ranging from 120–175 uV/sec (1.8–2.8 mpH units/sec), more than four times the rate in myotubes. However, neither testosterone nor corticosterone had any effect on metabolism (data not shown).

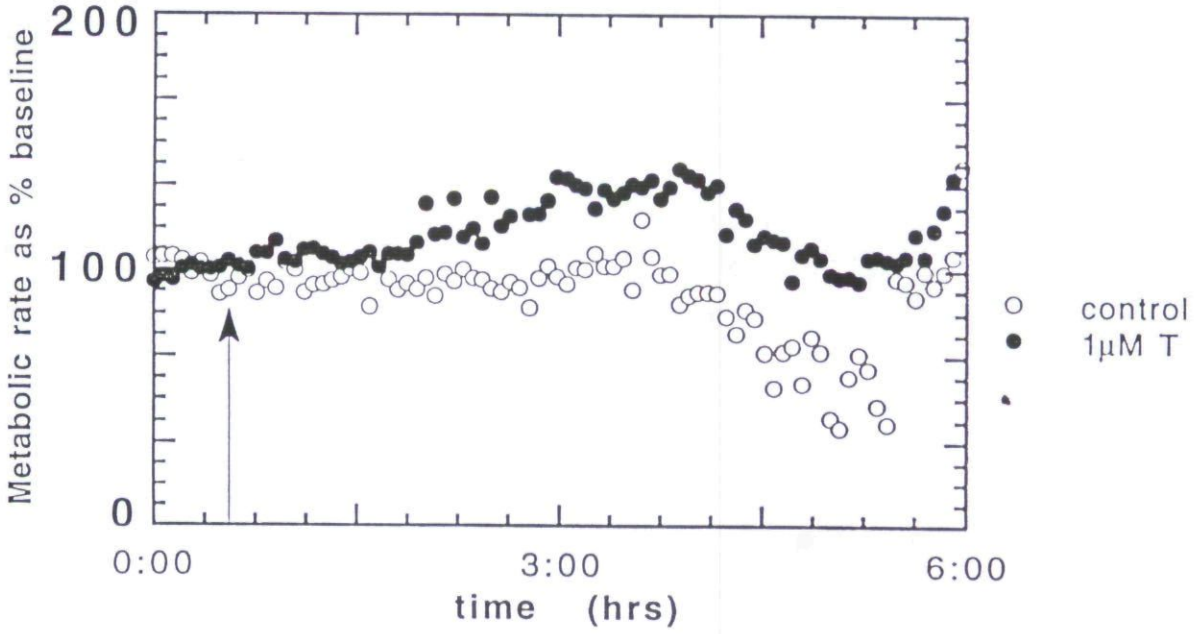

Fig. 1. Representative microphysiometric data showing the transient enhancement of metabolic rate beginning a few hours after testosterone exposure.

TABLE I. Effects of 1 μM Testosterone on Metabolism in Myotubules*

Time after testosterone treatment (hr)	Controls	Testosterone
1	57 ± 1	59 ± 1
2	56 ± 3	60 ± 2
3	60 ± 6	68 ± 3
4	58 ± 6	69 ± 4
8	40 ± 8	27 ± 8

*Data represents area under the curve, expressed as normalized metabolic rate/min. Mean ± SEM. Testosterone caused a significant enhancement at the 4 hr mark ($P < .05$, Newman-Keuls post-hoc test following two-way ANOVA). n = 14-16 for 1–4 hr, 7 for 8 hr.

DISCUSSION

We have observed that testosterone enhances metabolism in cultured C2C12 myotubes, as assessed by microphysiometric measures of proton efflux secondary to ATP hydrolysis. Furthermore, this increase in metabolism was preceded by a transient increase in glucose uptake.

Both effects were quite small. For example, the approximate 25% increase in 14C-2DG uptake contrasts with the approximate four-fold increase in uptake by muscle following testosterone administration in vivo [Max and Toop, 1983]. The most salient feature of these testosterone actions was its rapidity. The effect on metabolism occurred within a few hours, while the effect on 14C-2DG uptake was demonstrable by a minute. The classical picture of testosterone action involves genomic regulation by the hormone/receptor complex with a latency incompatible with the extremely rapid effects we observed. Furthermore, we could not demonstrate specific binding of testosterone by intracellular receptors in these cultures. While skeletal muscle can contain intracellular androgen receptors [Snowchowski et al., 1981], their concentrations are quite variable and far below the concentrations found in primary androgen target tissues such as prostate or perineal muscles [Celotti and Hegrilesi, 1992].

Our findings suggest, instead, non-genomic testosterone actions. There are precedents for androgen action occurring in this manner. For example, in rat heart myocytes, testosterone enhances glucose and amino acid uptake within 30 sec, and stimulates ornithine decarboxylase activity within 5 sec [Koenig et al., 1989]. Similarly, 17α-methyltestosterone enhances glucose and amino acid uptake in fish intestine within 20 min [Habibi et al., 1984; Hazzard and Ahearn, 1992]. Erulkar and Wetzel [1989] used patch-clamp techniques to demonstrate that 5α-DHT modulates acetylcholine-activated

TABLE II. Effects of 1 μM Corticosterone on Metabolism in Myotubules*

Time after corticosterone treatment (hr)	Controls	Corticosterone
1	64 ± 1	64 ± 1
2	70 ± 2	72 ± 3
3	74 ± 3	76 ± 4
4	76 ± 3	79 ± 4
8	79 ± 5	80 ± 5

*Data represents area under the curve, expressed as normalized metabolic rate/min. Mean ± SEM. There were no differences between control and corticosterone-treated cultures. n = 6–8/timepoint.

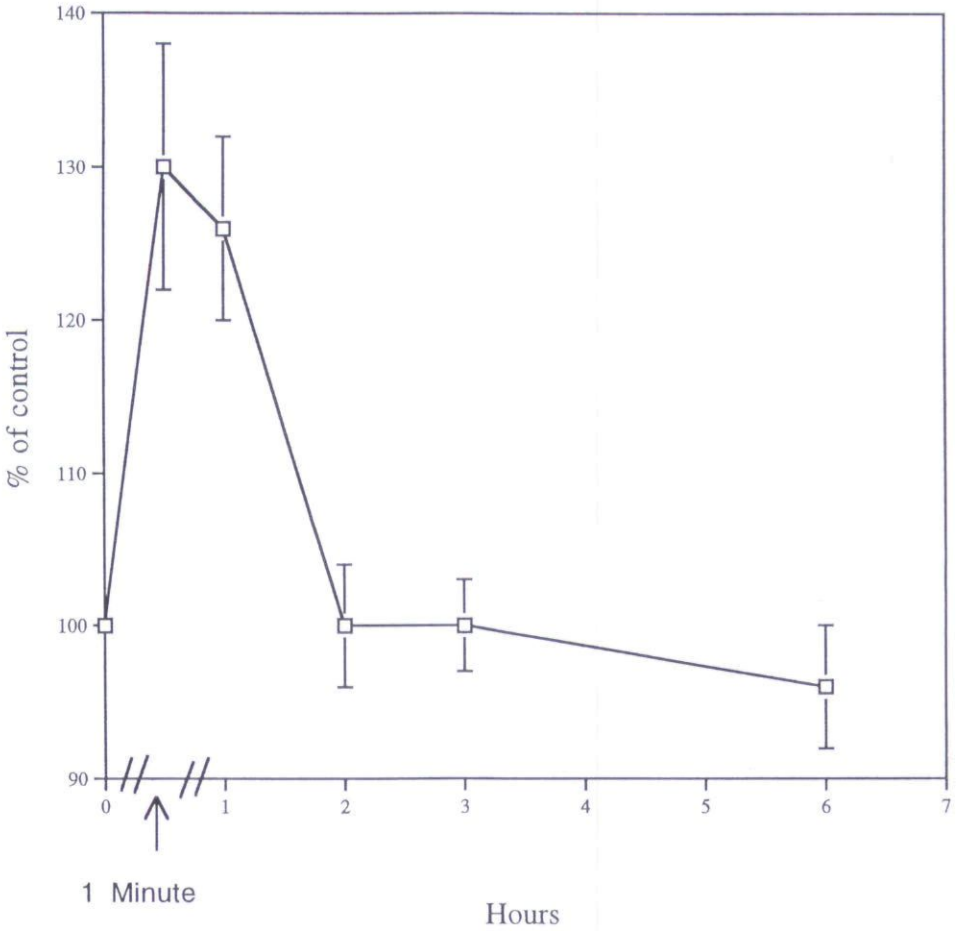

Fig. 2. Enhancement of 14C-2DG uptake into myotubules by testosterone. Uptake was significantly enhanced, relative to controls, 1 min and 1 h after testosterone exposure ($P < .05$ in both cases, Newman-Keuls post-hoc test following two-way ANOVA).

channels in *Xenopus* myotubes within 2 min. The failure of corticosterone to alter metabolism in our study suggests that the stimulation is not due to generic steroidal fluidization of membrane (a possible mechanism of action of all steroids, independent of interacting with a receptor). Beyond that, the mechanism underlying the present effect is not clear.

In conclusion, we have observed a small stimulatory effect of testosterone on metabolism and hexose transport in cultured myotubes. The generalizability of this finding is speculative at this point. This is in part because of the necessary reliance on in vitro data. In addition, the use of an immortalized, muscle-derived cell line, rather than muscle itself must add caution to any conclusions. Such cell lines, of which C2C12 is a standard example, are typically used in in vitro studies, as muscle itself is notoriously difficult to culture; however, it should be noted that we observed our effect only when the lines were in the myotube, rather than myoblast stage (i.e., only when they had a

muscle phenotype). Should these results be generalizable, we feel they have a number of implications that justify the rather unorthodox decision to seek a readership concerned with psychoendocrinology, rather than with endocrine physiology. For example, we have observed that among wild baboons living freely in East Africa, stress causes a prompt decline in testosterone concentrations among socially subordinate individuals, but a transient (approximate 2 hr) rise in dominant males [Sapolsky, 1991]. Should the present findings apply to a situation such as that, it predicts rank-related differences in muscle substrate stores and metabolism in response to a stressor such as male-male aggression.

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