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TESTOSTERONE ENHANCES $[^{14}\text{C}]$ 2-DEOXYGLUCOSE UPTAKE BY STRIATED MUSCLE

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Running Title: Sex Hormones and Muscle

Abbreviations: LAM - levator ani muscle

$[^{14}\text{C}]$ 2DG - $[^{14}\text{C}]$ 2-deoxy-D-glucose

2DG6P - 2-deoxy-D-glucose-6-phosphate

TP - testosterone propionate

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Abstract

We studied the effect of testosterone propionate (TP) on $[^{14}C]2$-deoxyglucose ($[^{14}C]2$DG) uptake in the rat levator ani muscle in vivo using the autoradiographic technique of Sokoloff et al. (J. Neurochem., 28:897-916, 1977). Following a delay of 1-3 h after injecting TP, the rate of $[^{14}C]2$DG uptake in experimental animals began to increase and continued to increase for at least 20 h. The label, which corresponds to $[^{16}C]2$-deoxyglucose-6-phosphate, as demonstrated by chromatographic analysis of muscle extracts, was uniformly distributed over the entire muscle and was predominantly in muscle fibers, although non-muscular elements were also labeled. The 1-3 h time lag suggests that the TP effect may be genomic, acting via androgen receptors, rather than directly on muscle membranes. Acceleration of glucose uptake may be an important early event in the anabolic response of the rat levator ani muscle to androgens.
INTRODUCTION

Mechanisms underlying the effects of androgenic steroids on muscle remain inchoate in spite of over 50 years of active research. It is clear, however, that striated muscle is a target tissue for androgens. Thus, skeletal muscle (22), as well as hormone-sensitive striated muscles such as the rat levator ani (LAM) (25), contain a cytosolic receptor with binding characteristics reminiscent of those of "classical" androgen target tissues (2,31). In addition, testosterone exerts apparently direct effects on the mitotic index of myoblast cultures (28) and on RNA polymerase activity in skeletal muscle (7). Reports have also appeared concerning metabolic effects of androgens on muscle. For example, Bergamini and co-workers (4-6,27) reported enhanced uptake of \(^{14}\)C xylose and increased phosphorylation of 2-deoxyglucose in vitro following administration of TP in vivo. These studies, carried out on whole muscles, convey little information concerning relative effects of hormone administration on muscular and non-muscular elements. We and others have shown androgen-induced regulation of a number of enzymes associated with intermediary metabolism (3,9,16,17,20-22,24). These effects were manifested within hours to days following hormone administration, and are likely to be several steps removed from the primary events of hormone action.

We have addressed the problem of elucidating the mechanism of action of androgens by measuring the effects of testosterone on \(^{14}\)C 2-deoxy-D-glucose (\(^{14}\)C 2DG) uptake by the rat levator ani muscle, employing the autoradiographic technique of Sokoloff et al. (32), adapted for use in muscle by Toop et al. (34). The results to be
described show a striking increase in $^{14}$C 2DG uptake by muscle fibers in the LAM of testosterone-treated rats. Some of these data have appeared in abstract form (29).

MATERIALS AND METHODS

Autoradiographic Studies

Immature (60-85 gm) male rats (Charles River Breeding Labs., Wilmington, MA) were used because of their low levels of endogenous sex hormones (10). Experimental animals were injected, i.p., with 2.5 mg testosterone propionate (TP) per 100 gm body weight in 0.1 ml dimethylsulfoxide, while controls received only dimethylsulfoxide (14,21). Pairs of rats (1 experimental and 1 control) were used at each time point. The animals were then returned to their cages. Food was withdrawn for about 16 h before injection of $^{14}$C 2DG; water was provided ad libitum. After 1, 3, 10 or 20 hrs following injection, the rats were again briefly removed from their cages for administration of $^{14}$C 2DG. $^{14}$C 2DG (New England Nuclear, specific activity 280 mCi/mmol) was injected i.p. in 0.1-0.25 ml of sterile saline or 90% ethanol. The animals were then returned to their cages for a further 45 min. At the end of this period, they were decapitated, and samples of trunk blood were taken for serum glucose analysis (vide infra). The LAM as well as bulbocavernosus, extensor digitorum longus, biceps, and soleus muscles were rapidly removed, trimmed free of fat and connective tissue, and frozen in isopentane cooled in liquid nitrogen.

Autoradiography of LAMs labelled with $^{14}$C 2DG was carried out as described by Toop et al. (34). Briefly, acid-cleaned microscope slides were washed and subbed with 0.5% gelatin, 0.05% chrome alum, and then,
under a sodium vapor safelight (Thomas duplex super safelight, with ortho vanes in place), covered with Kodak AR.10 stripping film such that the emulsion was outermost. After drying, the film-covered slides were cooled to cryostat temperature. Frozen 10 μm sections of muscle were picked up onto the surface of the film, thawed with a fingertip, and dried on a warm plate at approximately 45°C for at least 1 min. Slides were placed in black plastic boxes, which were then sealed with tape, and exposed at -80°C for up to 120 days. After the slides warmed to room temperature, they were placed sequentially in Wohlmann's fixative (5% acetic acid in 95% ethanol) for 1 min (to prevent loss of sections), gently running tap water for 5 mins, D-19 for 5 mins, tap water briefly, and 30% sodium thiosulphate for 2 mins; the slides were finally washed in running tap water for 5 mins. After drying, the slides were either dehydrated and mounted unstained, or were mounted after staining with 0.1% toluidine blue (1 min), Ehrlich's hematoxylin diluted 1:3 with distilled water (1 hr), and 1% aqueous eosin (2 min) (1).

Analysis of the autoradiograms was carried out by counting numbers of silver grains over several areas of each muscle cross-section. Under darkfield illumination grains were counted using an eyepiece reticle marked with squares, each of which was 70 μm². In areas with many grains 20 squares were counted, while in lightly labelled areas or in background areas, 80 squares were studied. Grain counts over tissue sections were corrected by subtraction of background readings taken from areas of emulsion outside the section. Frozen 10 μm sections of muscle serial to those used for autoradiography were stained to demonstrate either NADH-tetrazolium reductase (NADH-TR) (11) or myosin ATPase (14) in alkaline (pH 9.4; ATPase 9.4) or acid (pH 4.35; ATPase 4.35) media.
Data for the 20 h point (autoradiograms exposed for 108 days) were multiplied by a factor of 1.11 to make the readings comparable to results at the other time points (autoradiograms exposed for 120 days). Because the animals used for the 1, 3 and 20 hr points were given 12.5 μCi of \([^{14}\text{C}]\) 2DG and the animals at the 10 h point were given 25 μCi, the grain counts for the 10 h point were halved to normalize this value.

Thin Layer Chromatography

If the \([^{14}\text{C}]\) 2DG given to the experimental animals is being actively metabolized, as the experiments reported here assume, then the majority of the label at the end of the experiment should be in 2-deoxyglucose-6-phosphate (2DG6P). Skeletal muscle (tibialis anterior, diaphragm, biceps brachii, EDL and SOL), taken from rats whose LAM was also used for autoradiography with \([^{3}\text{H}]\) 2DG (New England Nuclear, specific activity 38 Ci/μmol) were homogenized in 100% ethanol using a Virtis model 45 tissue grinder. The extract was centrifuged and the supernatant fraction was evaporated to dryness with nitrogen and redissolved in a small volume of ethanol. The material was then applied to cellulose (Avicel) TLC plates (Analtech, Wilmington, DE) along with unlabeled 2-deoxyglucose and 2DG6P. The plate was then developed in isobutyric acid: \(\text{NH}_4\text{OH}: \text{H}_2\text{O}, 66:1:33\ (v/v/v)\). After chromatography, the standards were visualized by spraying the plates with 2', 2'-dichlorofluorescein spray reagent, 0.1% (EM Reagents); the standards appear as dark spots on a bright background when the plate is illuminated with ultraviolet light. Sites of radioactivity on the chromatograms were visualized by autoradiography after covering the area containing the spots with 2, 5-diphenyloxazole (PPO) dissolved in acetone. When this solution had saturated
the TLC plates and the acetone had evaporated, the plate was placed against a pre-exposed sheet of Kodak x-ray film (NS-2T) and exposed at -80°C for 2 weeks. The use of PPO, pre-exposure of the film, and exposure at -80°C have all been reported to increase the sensitivity of ³H autoradiography (22,29). An experiment (not shown) in the present case confirmed this. In addition to this method, spots on the TLC were scraped into small columns, eluted with ethanol and the eluates were counted in a liquid scintillation spectrometer at about 30% efficiency. It was not feasible to use the LAM for the chromatographic experiments because of paucity of muscle and, therefore, of amount of isotope.

Serum Glucose Determination

Serum glucose concentration was determined by a glucose oxidase assay, obtained in kit form from Sigma (St. Louis, MO).

Biochemical Assay of [¹⁴C] 2DG Uptake

This was accomplished by injecting rats with TP and [¹⁴C] 2DG as described above. However, instead of processing for autoradiography, the muscles were dissolved in 1.0 ml Protosol (New England Nuclear) for 24 h at 25°C, after which liquifluor was added and radioactivity was determined by liquid scintillation spectrometry at about 84% efficiency.

RESULTS

At each of the time points studied, silver grains were found over the whole cross-section of each muscle, in an apparently uniform distribution. The majority of the label was in the muscle fibers
themselves, although there was also labeling of connective tissue, nerves and blood vessels (Fig. 1, a-h).

The density of developed silver grains over experimental and control muscles is shown graphically in Fig. 2. The data show enhancement of $[^{14}\text{C}]$ 2DG uptake by TP-treated rats. This increase was apparent at 3 h after TP injection and continued to increase linearly up to 20 h after TP injection.

Biochemical studies, in which labeled muscles (at least 5 experimental and 5 control muscles per time point) were counted in their entirety instead of analyzed autoradiographically, showed a similar trend of time-related stimulation of $[^{14}\text{C}]$ 2DG uptake by TP (data not shown). This result rules out sampling errors in the choice of material for autoradiography.

Histological observations showed that the muscle fibers of immature rat LAMs are clearly differentiated, albeit small (10-15 μm average diameter) (Fig. 3). Study of histochemically stained frozen sections revealed the majority of the fibers in the LAM to have intermediate staining for ATPase 9.4 and either light or dark staining for NADH-TR. The remainder of the fibers stained darkly for ATPase 9.4 and also darkly for NADH-TR. Thus the immature LAM is not entirely uniform in its pattern of staining, in contrast to LAM from adult rats (15). The autoradiograms did not show a correlation between grain density and histochemical fiber type.

Autoradiographic results suggested a similar enhancement of $[^{14}\text{C}]$ 2DG uptake by TP in the hormone-sensitive bulbocavernosus muscle. However, there was no discernible effect of testosterone on the acceleration of $[^{14}\text{C}]$ 2DG uptake in skeletal muscle demonstrable either
by biochemical assay (extensor digitorum longus, soleus, or biceps muscles) or by autoradiography (biceps brachii).

Blood glucose concentration was the same in hormone-treated (27.7 ± 6.5 mg/ml, n = 29) and control rats (29.8 ± 8.6 mg/ml, n = 28).

Thin-layer chromatography of muscle extracts showed that all incorporated radioactivity was \(^{14}C\)2DG6P. This is shown by autoradiography in Fig. 4. The same result was obtained by eluting the spots and counting in a liquid scintillation spectrometer (data not shown).

**DISCUSSION**

The results described above provide evidence for an effect of testosterone on glucose metabolism in striated muscle. Thus, one i.p. injection of TP resulted in a striking increase in the utilization of \(^{14}C\)2DG by muscle fibers of the rat levator ani muscle. This point is important because previous studies of sugar uptake into whole muscles do not permit cellular localization of metabolic effects (4-6, 27). It is not possible to discern without autoradiography whether observed changes are in muscle fibers or connective tissue elements. Furthermore, the autoradiographic technique has the advantage of sensitivity, which can be enhanced by prolonging exposure time.

The mechanism of the effect of TP on \(^{14}C\)2DG uptake is not known. It may be mediated by interaction of the hormone with its cytosolic receptor followed by nuclear changes and protein synthesis as described in other androgen target tissues (30). Indeed, the time-lag of 1-3 h before a discernible enhancement of \(^{14}C\)2DG uptake by TP may indicate a receptor-mediated effect of TP, rather than a direct effect on the muscle membrane.
The studies of Bergamini and co-workers on $^{14}$C xylose uptake by the rat LAM suggest that the effects of androgen on sugar transport are mediated via the insulin-sensitive glucose transport system (4). The capacity of the system to transport $^{14}$C xylose in vitro is apparently enhanced by androgen treatment (4). On the other hand, Mills and Spaziani (26) found no enhancement of $^{14}$C 2DG uptake by the LAM in vitro after in vivo administration of testosterone. Mills and Spaziani used muscle from adult rats; such large muscles are not suitable for in vitro study due to considerable ischemia in their central portions (13). Thus, the work of Mills and Spaziani (26) appears invalid. Our data therefore confirm and extend the findings of Bergamini and co-workers (3-6,27). It should be noted that we measured $^{14}$C 2DG uptake in vivo, in contrast to the procedure of Bergamini (4).

We found no effect of TP on $^{14}$C 2DG uptake by "ordinary" skeletal muscles. This result may mean that only specialized "hormone-sensitive" muscles such as the LAM and bulbocavernosus are susceptible. It is possible, however, that the dose and/or route of administration of TP was not appropriate for the skeletal muscles examined.

Glucose transport in muscle, the limiting step in its utilization, is a facilitated diffusion process that is stimulated by insulin. The glucose carrier is regulated by "supply" e.g., when substrate becomes available, or by "demand", e.g., contraction (12). Since TP promotes hypertrophy of the LAM, it may increase the demand for substrates and may, therefore, enhance the glucose transport system. That all the $^{14}$C 2DG is phosphorylated in muscle (Fig. 4) suggests that the present studies are measuring uptake by the glucose transport system, and that this system is enhanced by TP.
TP has a well-documented anabolic influence on the rat levator ani muscle (35). It is therefore likely that important metabolic changes would occur prior to hypertrophy. Enhanced glucose utilization may be a precursor of these metabolic events. Indeed, it is possible to speculate that enhanced glucose utilization may be necessary to initiate the events leading to hypertrophy.

The [14C] 2DG technique will be valuable for future studies of effects of sex steroids on muscle metabolism.
FIGURES LEGENDS

Figure 1 - Autoradiograms (viewed by darkfield) of LAMs from pairs of experimental animals. a,b - 1 h; c,d - 3 hs; e,f - 10 hs; g,h - 20 hs; a,c,e,g - experimental; b,d,f,h - controls.

Figure 2 - Histograms of autoradiographic grain density over LAMs of animals given TP (E) or dimethylsulfoxide only (C) from 1-20 hs before killing. All grain densities have been normalized to a dose of 12.5 Ci of $^{14}\text{C}$ 2DG and an exposure time of 120 days (see text). Background counts (shown negative) are for areas of emulsion outside the section. The autoradiograms used for this analysis are shown in Fig. 1.

Figure 3 - Transverse sections of LAM used for the 1 hour experimental point. A: NADH-TR; B: ATPase 9.4 All fibers intermediate for ATPase 9.4 are either pale or dark for NADH-TR, while all fibers dark for ATPase are also dark for NADH-TR. Scale bar - 100 μm.

Figure 4 - A: Thin layer chromatogram showing the migration of samples of unlabelled 2DG and 2DG6P. B: Autoradiogram of $^{3}\text{H}$-2DG standard and of the labelled compound extracted from frozen skeletal muscle. The migration of the label in the muscle extract corresponds to the position of 2DG6P.
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