Investigation of Putative Androgen-like Activity of α_{2u}-Globulin in Castrated and Estrogen-Treated Male Rats[1] (41826)

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Abstract. α_{2u}-Globulin, a male sex-dependent protein of the rat, was tested for its ability to produce androgenic or anabolic responses. Castrated rats were injected with α_{2u}-globulin isolated from urine or kidney and the effects on urinary nitrogen excretion, body weight, and weight of ventral prostate, seminal vesicles, and levator ani muscle were studied. α_{2u}-Globulin had no effect but testosterone propionate gave the expected stimulation of nitrogen retention, body growth, and growth of androgen-responsive tissues. α_{2u}-Globulin injected into estrogen-treated male rats (animals with suppressed serum levels of α_{2u}-globulin, gonadotropins, and testosterone) was also found to be without effect on body weight and on the weight of ventral prostate, seminal vesicles, levator ani muscle, and testes. We conclude that the α_{2u}-globulin molecule, by itself, is incapable of producing, directly or indirectly, androgenlike responses.

α_{2u}-Globulin is the major protein excreted in the urine of adult male rats (1). The major site of synthesis of α_{2u}-globulin in adult males is the liver (2) and α_{2u}-globulin secreted from this organ, is filtered by the kidneys where about half of the protein is reabsorbed and the remainder is excreted in the urine at quantities of about 20 mg/day (1, 3). Androgen, glucocorticoid, growth hormone, thyroxine, and insulin are required to maintain maximal levels of hepatic α_{2u}-globulin synthesis (4–7).

The biological function of α_{2u}-globulin has received only limited attention. It is known that estrogen suppresses the hepatic production of α_{2u}-globulin in male rats (8) and Roy *et al.* (9) showed that the loss of spermatogenesis in estrogen-treated male rats could be prevented with injections of α_{2u}-globulin. Recently, Biswas *et al.* (10) demonstrated that injections of α_{2u}-globulin prevented the decline in serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in estrogen-treated male rats as well as protecting spermatogenesis. In addition, α_{2u}-globulin injections resulted in a stimulation of growth of androgen-responsive tissues.

Since hepatic α_{2u}-globulin is normally made only in adult male rats, we wondered whether this protein might be a mediator of some actions normally attributed to testosterone. We tested whether α_{2u}-globulin isolated from urine or kidney had anabolic or androgenic properties in castrated rats. We also investigated whether α_{2u}-globulin could stimulate the growth of androgen-responsive tissues in estrogen-treated male rats.

Materials and Methods. *Animals and diets.* Male Holtzman rats (Holtzman Co., Madison, Wisc.) were used in all experiments and were fed the 20% casein AIN-76 diet (11) *ad libitum* except where noted.

Purification of α_{2u}-globulin. α_{2u}-Globulin for biological testing was isolated from either mature male rat urine or kidney. Urine (500 ml batches) was filtered through Whatman No. 1 paper and was dialyzed against three 6-liter changes of 10 m*M* Tris(hydroxymethyl)-aminomethane (Tris) (pH 7.4). Kidneys (100 g batches) were homogenized in 2 vol of 10 m*M* Tris (pH 7.4) in a Potter–Elvehjem homogenizer and the supernatant fraction was prepared by centrifuging the homogenate at 140,000*g* for 60 min at 1°C. Kidney supernatant was dialyzed as described for urine.

Dialyzed urine or kidney supernatant was applied to a 2.5 × 30-cm DEAE–cellulose column equilibrated with 10 m*M* Tris, pH 7.4. After the sample was loaded, the column was washed with 500 ml of the Tris buffer and protein was eluted with a linear 700-ml gradient of 0 to 0.4 *M* NaCl in 10 m*M* Tris (pH 7.4) at a flow rate of 50 ml/hr. Column frac-

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tions (10 ml) were monitored for protein by the absorbance at 280 nm and by the Bradford assay (12). When urine was chromatographed, a single large protein peak eluted which was almost entirely α_{2u}-globulin. Large amounts of contaminating proteins eluted with renal α_{2u}-globulin from the DEAE–cellulose column; therefore prior to sample application, a small amount (50,000 cpm) of ^{3}H-labeled α_{2u}-globulin (previously purified from kidneys of [^{3}H]leucine injected rats) was added to the supernatant to act as a tracer.

The partially purified preparations of α_{2u}-globulin were concentrated approximately eightfold with an ultrafiltration cell containing a YM-5 membrane (Amicon Corp., Danvers, Mass.). These samples (20 ml) were chromatographed on a 5.0 × 50-cm Sephadex G-75 column equilibrated with 0.9% NaCl (saline) at a flow rate of 50 ml/hr; 10-ml fractions were collected and assayed for protein as described above. A large peak of α_{2u}-globulin eluted at an apparent mol wt of 15,000. Urinary and renal α_{2u}-globulin thus obtained were electrophoresed on 15% polyacrylamide–SDS gels (13) and were found to be greater than 90% pure as determined by densitometric scanning of the Coomassie blue-stained gels. We observed that the major form of renal α_{2u}-globulin was smaller than the major form of urinary α_{2u}-globulin (mol wt of 14,400 and 15,800, respectively) (details in manuscript submitted for publication). The percentage contribution of the two different mol wt forms of α_{2u}-globulin in the preparations used for biological testing are listed in the respective experiments.

α_{2u}-Globulin radioimmunoassay. α_{2u}-Globulin was measured in selected samples by radioimmunoassay. The major form of urinary α_{2u}-globulin was used for iodination and standards and was also used to produce antibodies. α_{2u}-Globulin was enzymatically radioiodinated with sodium [^{125}I]iodide (New England Nuclear, Boston, Mass.) to a specific activity of 15 μCi/μg of protein using the Enzymobead lactoperoxidase–glucose oxidase system (Bio-Rad, Rockville Centre, N.Y.). The radioimmunoassay procedure of Ying (14) was used (18 hr incubation period). Under the conditions employed, α_{2u}-globulin excretion of 2 mg/day or greater could be measured. Urinary and renal forms of α_{2u}-globulin were found to react with equal efficacy in this radioimmunoassay.

Experiment 1. The ability of α_{2u}-globulin to stimulate body growth and the growth of androgen-responsive tissues of castrated rats was examined. Rats were castrated (15) at 36 days of age. At 55 days of age the castrated rats were divided into three groups: a vehicle-injected control (0.05 ml of corn oil/rat/day), a testosterone propionate-injected group (100 μg/0.05 ml corn oil/rat/day), and a urinary α_{2u}-globulin (63% 15,800 mol wt form)-injected group (1.7 mg/0.8 ml saline/rat/day) (all injections in this report were sc). The rats were sacrificed at 80 days of age and, thus, received a total of 25 injections. An uninjected noncastrated group of rats was carried through the entire scheme. Upon sacrifice, the ventral prostate, seminal vesicles, and levator ani muscle were dissected and weighed.

Experiment 2. To further explore possible anabolic actions of α_{2u}-globulin, the effect of the protein on the urinary nitrogen excretion of castrated rats was studied. Rats were castrated at 47 days of age and, over a period of 3 months, were fed gradually decreasing amounts of a 10% casein AIN-76 diet (1.4% nitrogen) until a constant body wt was maintained. Initially the rats were fed daily an amount of diet equal to 10% of their body wt and the food ration had decreased to an average of 3.4% of their body wt by the end of this regimen. Measurement of urinary and fecal nitrogen by the Kjeldahl method (16) indicated that these animals were in a slight positive nitrogen balance of approximately 15 mg of nitrogen/day (average intake was 164 mg of nitrogen/day). Kochakian (17) and Arnold *et al.* (18) have shown that such a regimen is necessary if one is to detect nitrogen-retaining effects of suspected anabolic compounds. α_{2u}-Globulin isolated from urine (57% 15,800 mol wt form) or kidney (84% 14,400 mol wt form), or testosterone propionate were administered at dosages listed in the Results for two 5-day treatment periods. The urinary nitrogen of pooled urine from the last 3 days of each treatment period was measured (triplicate samples) and was compared with the urinary nitrogen of a pretreatment 3-day pooled sample. Fecal nitrogen has been shown to be unaltered by androgens (17) and, hence, was not measured. At the end of the last treatment period, the rats were sacrificed and the ventral prostate and seminal vesicles were dissected and weighed.

Experiment 3. Estrogen treatment of male rats completely suppresses the hepatic synthesis of α_{2u}-globulin (8); therefore, we tested the ability of α_{2u}-globulin to stimulate the growth of androgen-responsive tissues in estrogen-treated male rats. Male rats (56-days-old) were injected with 50 μg of 17β-estradiol/100 g body wt/day for 7 days (17β-estradiol was suspended in 0.1 ml of 0.4% Tween 80, 10% propylene glycol, and 89.6% 0.1 *M* sodium phosphate, pH 7.2). Twenty-four hours after the last estrogen treatment, the rats were separated into three groups: a saline-injected control (0.2 ml/rat/day), a low α_{2u}-globulin-injected group (1.3 mg/0.2 ml saline/rat/day), and a high α_{2u}-globulin-injected group (8.5 mg/0.4 ml saline/rat/day) (urinary α_{2u}-globulin of which 57% was the 15,800 mol wt form). These injections were given for a period of 14 days. A vehicle-injected control was carried through the entire protocol. Twenty-four hours after the last injection, the rats were sacrificed and the ventral prostate, seminal vesicles, levator ani muscle, and testes were dissected and weighed.

Statistical analysis. Values were expressed as the mean ± SEM. Differences between means were analyzed by Student's paired or unpaired, two-tailed *t* test.

Results. *Experiment 1.* Injection of castrated rats with testosterone propionate resulted in a body weight gain equal to that of the noncastrated rats and also caused a significant increase in the weight of the ventral prostate, seminal vesicles, and levator ani muscle compared to the castrated controls (Table I). Injection of α_{2u}-globulin into castrated rats did not cause a stimulation of body growth or growth of androgen-responsive tissues. In this experiment, the rats were castrated before puberty and we did not measure an index of the endogenous production of α_{2u}-globulin since it is well established that prepubescent castration largely prevents the hepatic synthesis of the protein (4).

Experiment 2. The serum concentration of α_{2u}-globulin was measured by radioimmunoassay in a group of 12 rats castrated at the same time as those used in this experiment and was found to be 2.5 ± 0.5 μg/ml compared to 20.1 ± 2.4 μg/ml in a group of 9 chow-fed male rats. This correlates well with the results of Kurtz *et al.* (19) showing that hepatic production of α_{2u}-globulin decreased by 88% after castration of postpubescent rats. Treatment of castrated rats with dosages of urinary α_{2u}-globulin as high as 32 mg/day for 5 days failed to significantly affect urinary nitrogen excretion or body weight (Table II). The cause of the slightly (but not significantly) depressed urinary nitrogen excretion during the second

TABLE I. EFFECT OF α_{2u}-GLOBULIN AND TESTOSTERONE PROPIONATE ON THE GROWTH OF ANDROGEN-RESPONSIVE TISSUES IN CASTRATED RATS[a]

	N	Body wt change[b] (g)	Tissue wt (mg/100 g body wt)		
			Ventral prostate	Seminal vesicles	Levator ani
Castrated	(8)	103 ± 5	3.2 ± 0.2	3.8 ± 0.4	11.1 ± 0.7
Castrated plus α_{2u}-globulin	(8)	92 ± 10	3.5 ± 0.2	4.0 ± 0.2	12.9 ± 1.1
Castrated plus testosterone propionate	(7)	143 ± 9[c]	58.8 ± 4.4[c]	53.5 ± 3.3[c]	43.4 ± 3.6[c]
Noncastrated	(8)	135 ± 6[c]	93.1 ± 5.0[c]	55.1 ± 2.7[c]	46.4 ± 2.8[c]

[a] Rats (186 ± 2 g) were castrated at 36 days of age. Nineteen days later the castrated rats weighed 301 ± 4 g compared to 351 ± 4 g for the noncastrated rats ($P < 0.001$). For the next 25 days the rats were injected daily with either 0.05 ml of corn oil (castrated group), 1.7 mg of urinary α_{2u}-globulin/0.8 ml saline, or 100 μg of testosterone propionate/0.05 ml corn oil (the noncastrated rats received no injections). Twenty-four hours after the last injection, the ventral prostate, seminal vesicles, and levator ani muscle were dissected and weighed. Values are expressed as means ± SEM.

[b] Listed are the body weight changes during the 25-day injection period.

[c] Indicates statistical significance of at least $P < 0.05$ by Student's unpaired *t* test when compared to castrated values.

TABLE II. EFFECT OF α_{2u}-GLOBULIN AND TESTOSTERONE PROPIONATE ON URINARY NITROGEN EXCRETION IN CASTRATED RATS[a]

Substance tested	N	Urinary nitrogen excretion (mg/day)			Body wt change[b] (g)
		Pretreatment	First treatment period	Second treatment period	
Saline[c]	(4)	130.0 ± 7.9	110.4 ± 9.0	115.4 ± 8.1	5.3 ± 1.8
Urinary α_{2u}-globulin[d]	(4)	141.3 ± 7.6	142.2 ± 9.9	116.9 ± 1.6	2.3 ± 2.2
Renal α_{2u}-globulin[e]	(4)	134.8 ± 5.5	131.0 ± 5.8	—	3.0 ± 3.2
Testosterone propionate[f]	(4)	138.0 ± 4.6	81.1 ± 4.8[g]	73.2 ± 5.4[g]	13.8 ± 3.3[h]

[a] Rats were castrated at 47 days of age and were brought to near nitrogen balance by gradually decreasing their feed intake over a period of 3 months. Urinary nitrogen excretion before treatment was determined from a 3-day pooled sample. The test substances were injected daily for two 5-day treatment periods; urinary nitrogen excretion was measured from a pooled sample collected over the final 3 days of treatment in each period. Values are expressed as means ± SEM.

[b] There were no significant differences in pretreatment body weight between groups (all rats 361 ± 4 g).

[c] Saline was injected at 0.5 ml/rat.

[d] Urinary α_{2u}-globulin was injected sc at 3.4 mg/day during the first treatment period and at 32 mg/day during the second treatment period.

[e] Renal α_{2u}-globulin was injected sc at 3.4 mg/day during the first treatment period. In the second treatment period, two rats received 3.4 mg/day, one rat received 14 mg/day, and the remaining rat received 34 mg/day; the results for the second treatment period are not shown but did not appear different from those of the first treatment period.

[f] Testosterone propionate was injected sc at 400 μg/day during both treatment periods.

[g] Indicates statistical significance of at least $P < 0.01$ by Student's paired t test when compared to pretreatment values.

[h] Indicates statistical significance of $P < 0.05$ by Student's paired t test.

treatment period in the urinary α_{2u}-globulin-injected rats and during the first and second treatment periods in the saline-treated rats is unknown. Renal α_{2u}-globulin also had no significant effect on urinary nitrogen excretion or body weight. Testosterone propionate injection significantly depressed urinary nitrogen excretion and significantly increased body weight. The rats were sacrificed at the end of the second treatment period and it was found that testosterone propionate treatment, compared to the saline-injected rats, significantly increased the weight of the ventral prostate (76.0 ± 14.5 versus 2.8 ± 0.3 mg/100 g body wt, $P < 0.001$) and seminal vesicles (77.6 ± 5.3 versus 6.2 ± 0.2 mg/100 g body wt, $P < 0.001$) while urinary or renal α_{2u}-globulin treatment had no significant effect on the weight of these tissues (results not shown).

Experiment 3. α_{2u}-Globulin in the urine was measured by radioimmunoassay in a group of six estrogen-treated rats housed in metabolism cages. The urinary excretion of α_{2u}-globulin decreased from an initial value of 15.3 ± 3.2 mg/day to undetectable after 4 days of estrogen treatment and remained at an undetectable level for the duration of the experiment. Estrogen treatment resulted initially in a decline in body weight but after the last estrogen injection, the rate of body growth in all groups of estrogen-treated rats was the same as that of the vehicle-injected rats (Table III). Injection of estrogen-treated rats with either 1.3 or 8.5 mg of α_{2u}-globulin/day for 2 weeks had no effect on body weight or weight of the ventral prostate, seminal vesicles, levator ani muscle, or testes.

Discussion. In our experiments we found no evidence indicating that α_{2u}-globulin had anabolic or androgenic properties. In one experiment we injected castrated rats with dosages of α_{2u}-globulin approaching the daily hepatic output and observed no effects on urinary nitrogen excretion or on the weight of androgen-responsive tissues. We even considered that molecular weight differences of α_{2u}-globulin forms might result in an activation or inactivation of the protein and, therefore, tested α_{2u}-globulin isolated from urine (mol wt of predominant form = 15,800) and kidney (mol wt of predominant form = 14,400) but observed negative results with either prepa-

TABLE III. EFFECT OF α_{2u}-GLOBULIN ON THE GROWTH OF ANDROGEN-RESPONSIVE TISSUES IN ESTROGEN-TREATED MALE RATS[a]

			Tissue wt (mg/100 g body wt)			
	N	Body wt change[b] (g)	Ventral prostate	Seminal vesicles	Levator ani	Testes
Estrogen-treated	(8)	92 ± 8	48.4 ± 4.7	50.1 ± 2.4	36.6 ± 2.9	915 ± 52
Estrogen-treated plus low α_{2u}-globulin	(8)	88 ± 11	50.7 ± 7.6	47.5 ± 4.2	40.7 ± 2.8	935 ± 27
Estrogen-treated plus high α_{2u}-globulin	(4)	77 ± 17	41.5 ± 14.1	38.7 ± 7.4	39.3 ± 8.3	964 ± 111
Vehicle-injected	(8)	89 ± 3	124.3 ± 8.7[c]	69.9 ± 3.7[c]	64.4 ± 3.2[c]	952 ± 23

[a] Rats (266 ± 1 g) were injected daily with 50 μg of 17β-estradiol/100 g body wt for 7 days; at this time rats treated with estrogen weighed 241 ± 6 g compared to 317 ± 3 g for the vehicle-injected rats ($P < 0.001$). Three groups of estrogen-treated rats then received the following injections daily for 2 weeks: 0.2 ml saline (estrogen-treated), 1.3 mg urinary α_{2u}-globulin/0.2 ml saline (estrogen-treated plus low α_{2u}-globulin), and 8.5 mg urinary α_{2u}-globulin/0.4 ml saline (estrogen-treated plus high α_{2u}-globulin). Twenty-four hours after the last injection the rats were sacrificed and the ventral prostate, seminal vesicles, levator ani muscle, and testes were dissected and weighed. Values are expressed as means ± SEM.

[b] Listed are the body weight changes during the final 14-day injection period.

[c] Indicates statistical significance of $P < 0.001$ by Student's unpaired t test when compared to estrogen-treated values.

ration on urinary nitrogen excretion and weight of androgen-responsive tissues in castrated rats. Testosterone propionate produced anabolic and androgenic effects in our experiments. Thus, the design of the experiments should have allowed the detection of putative androgen-like activities of α_{2u}-globulin. We conclude that α_{2u}-globulin, by itself, cannot produce anabolic or androgenic responses.

Our observation of a lack of an effect of α_{2u}-globulin on the weight of prostate, seminal vesicles, levator ani muscle, and testes in estrogen-treated male rats contrasts sharply with the results of Biswas *et al.* (10) who demonstrated a stimulatory effect of α_{2u}-globulin on these tissues in estrogen-treated rats. This is confusing since the experimental protocols in these two studies were essentially identical and one group of rats in our experiment was given α_{2u}-globulin at a level six times higher than that in the studies of Biswas *et al.* (10). A possible explanation of this discrepancy is that the α_{2u}-globulin preparations used by Biswas *et al.* contained a substance in addition to α_{2u}-globulin which was responsible for the stimulatory effects that were observed. Evidence for such an additional substance is presented below.

A hypothesized function of α_{2u}-globulin is that it may act as a pheromone since such large quantities of the protein are excreted in the urine (20, 21). It is known that the urine of male rodents contains a factor which accelerates the onset of puberty in female rodents (22) and that this factor appears to act by stimulating the release of LH or FSH into the blood (22, 23). The puberty accelerating pheromone is under androgenic control (24) and appears either to be protein bound or to be a portion of a large protein molecule (25). Perhaps either α_{2u}-globulin, a portion of the α_{2u}-globulin molecule, or a substance bound to α_{2u}-globulin may be the puberty-accelerating pheromone and is also the factor which results in the stimulation of androgen-responsive tissues in estrogen-treated rats as described by Biswas *et al.* (10).

If an α_{2u}-globulin-associated factor is the puberty-accelerating pheromone, one must wonder whether α_{2u}-globulin serves a normal physiological function within the male rat. Biological effects of α_{2u}-globulin in male rats have not been demonstrated in the absence of estrogen treatment. Thus, the stimulation of growth of androgen-responsive tissues and prevention of loss of spermatogenesis caused by α_{2u}-globulin in estrogen-treated male rats (9, 10) may represent the ability of α_{2u}-globulin to reverse a "pathological" depression of FSH and LH caused by estrogen. Estrogen-treated male rats may simply be a convenient system where a stimulation of LH and FSH by α_{2u}-globulin (or more likely a factor associated with the protein) can be measured either directly by radioimmunoassay of serum LH and FSH, or indirectly by bioassay of androgen-responsive tissues.

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