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Testosterone and its precursors and metabolites enhance guanylate cyclase activity

(progesterone/5α-dihydrotestosterone/pregnenolone/cyclic GMP)

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ABSTRACT **Both testosterone and cyclic GMP stimulate DNA synthesis. Because cyclic GMP and testosterone seem to have similar actions, the objective of this investigation was to determine if testosterone and its precursors might have part of their mechanism of action through stimulation of guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2.], the enzyme that catalyzes the formation of cyclic GMP from GTP. The precursors—namely, progesterone, pregnenolone, 17α-progesterone, 17α-hydroxypregnenolone, androstenedione, and dehydroepiandrosterone—caused a 2- to 3½-fold enhancement of guanylate cyclase activity in rat liver, kidney, skeletal muscle, and ventral prostate at a concentration of 1 μM. These precursors are generated from cholesterol, which had no effect itself on guanylate cyclase activity. Testosterone, 19-nortestosterone, 17-methyltestosterone, and 5α-dihydrotestosterone enhanced guanylate cyclase activity 2- to 5-fold in the same tissues at 1 μM. Etiocholanolone, androsterone, and epiandrosterone, metabolites of testosterone metabolism, enhanced guanylate cyclase activity 1½- to 2-fold at this same concentration. Dose–response relationships revealed that testosterone and its precursors and metabolites had their maximal effect at 1 μM but still had some effect at 0.001 μM. The data in this investigation suggest that the guanylate cyclase–cyclic GMP system plays a role in the mechanism of action of testosterone and its precursors.**

Since Burkhart (1) demonstrated in 1942 that a single injection of 0.1 mg of testosterone to 40-day-old castrated rats caused a wave of mitotic activity, androgens have been known to have an important role in growth and development of certain tissues such as muscle and prostate. In 1965 Sheppard *et al.* (2) described the stimulatory action of androgens on DNA synthesis in target organs. These findings have been confirmed by others (3–5). The exact mechanism by which testosterone stimulates DNA synthesis and growth is unknown.

Guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] and its product guanosine 3′,5′-monophosphate (cyclic GMP) are throught to be involved in cell growth (6–10). Cyclic GMP itself has been shown to stimulate DNA synthesis (6, 11, 12). Because cyclic GMP and androgens seem to have similar actions, the effects of testosterone and androgen precursors and degradation products were tested on the guanylate cyclase–cyclic GMP system. Progesterone, pregnenolone, 17α-progesterone, 17-α-hydroxypregnenolone, androstenedione, dehydroepiandrosterone, testosterone, 17-methyltestosterone, 19α-nortestosterone, 5α-dihydrotestosterone, androsterone, epiandrosterone, and etiocholanolone all had stimulatory effects on guanylate cyclase activity. Cholesterol, from which the precursors are generated, had no effect on guanylate cyclase activity.

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MATERIALS AND METHODS

Materials. Tissues used in these experiments were obtained from male 150- to 200-g Sprague–Dawley rats that had been maintained ad lib on Purina Laboratory Chow. Progesterone (4-pregene-3,20-dione), 17α-hydroxyprogesterone (4-pregnen-17α-ol-3,20-dione), androstenedione (4-androsten-3,17-dione), pregnenolone (5-pregen-3β-ol-20-one), 17α-hydroxypregnenolone (3β-17α-dihydroxy-5-pregnen-20-one), dehydroepiandrosterone (5-androsten-3β-ol-17-one), cholesterol (5-cholesten-3β-ol), testosterone (4-androsten-17β-ol-3-one), 17-methyltestosterone (17α-methyl-4-androsten-17-β-ol-3-one), 19-nortestosterone (17β-hydroxy-4-estren-3-one), 5α-dihydrotestosterone, androsterone (5α-androstan-3α-ol-17-one), epiandrosterone (5α-androstan-3β-ol-17-one), and etiocholanolone (etiocholan-3-ol-17-one) were obtained from Sigma. All of the above were dissolved in dimethyl sulfoxide, which has no effect on guanylate cyclase activity by itself in the concentrations used. Alumina, neutral activity I for column chromatography, was obtained from E. Merck (Darmstadt, West Germany). The [^{32}P]GTP was from ICN.

Guanylate Cyclase Assay. Guanylate cyclase activity was measured as described (13–15). The various tissues were homogenized in cold 0.03 M Tris·HCl, pH 7.6, and centrifuged at 37,000 × g at 4°C for 15 min. The supernatant, to which the above hormones had been added to the final concentrations noted in the text, was then assayed at 37°C for 10 min for guanylate cyclase activity, using a 0.075-ml reaction mixture consisting of 20 mM Tris·HCl (pH 7.6), 4 mM $MnCl_2$, 2.67 mM cyclic GMP (used to minimize destruction of cyclic [^{32}P]GMP) a GTP-regenerating system (5 mM creatine phosphate/11.25 units of creatine kinase), 100 μg of bovine serum albumin, 20 mM caffeine, 1.2 mM [^{32}P]GTP (approximately 5×10^5 cpm), and enzyme preparation having 0.2–0.4 mg of protein. The final pH of the reaction mixture was 7.6. The reaction was terminated by the addition of 10 μl of EDTA, pH 7.6, containing about 30,000 cpm of cyclic [^{3}H]GMP (to estimate recovery in the subsequent steps) and boiling for 3 min. After cooling in an ice bath, the cyclic [^{32}P]GMP formed was isolated by sequential chromatography on Dowex-50 H^+ and alumina by using the modification described in detail (15). The overall recovery of cyclic GMP after the two-stage chromatographic procedure was 95%. Blank ^{32}P counting rates averaged 40–50 cmp. With this assay system, production of cyclic GMP was linear with time for at least 20 min and with added protein from 50 to 400 μg. All of the ^{32}P-containing material was identifiable as cyclic GMP as determined by thin-layer chromatography on PEI-cellulose (Brinkmann) with 1 M LiCl as solvent and Chromar sheets (Mallinckrodt) developed with absolute alcohol and concentrated NH_4OH (5:2 vol/vol). Each assay was conducted in triplicate and the results were confirmed in three separate experiments. Protein was determined by the method of Lowry *et al.* (16).

RESULTS

Progesterone, 17α-hydroxyprogesterone, and androstenedione, the precursors in the dominant pathway of testosterone synthesis in the testis (Fig. 1), all stimulated guanylate cyclase activity in four tissues in which testosterone is known to have a positive anabolic effect (Table 1). These precursors increased guanylate cyclase activity 2- to 3½-fold in gracilis anticus skeletal muscle, liver, ventral prostate, and kidney *in vitro* (Table 1). Likewise, the precursors in the alternate pathway—namely pregnenolone, 17-hydroxypregnenolone, and dehydroepiandrosterone—enhanced guanylate cyclase activity in these same tissues (Table 1). These six precursors are generated from cholesterol, which, as opposed to the above, had no effect on guanylate cyclase activity (Table 1). The dose–response curves for these seven precursors on hepatic guanylate cyclase activity are shown in Table 2. Maximal stimulation was seen at a concentration of 1 μM with all the above precursors except cholesterol, and nonstimulated levels were reached when these concentrations were decreased to 0.0001 μM.

5α-Dihydrotestosterone and 19-nortestosterone each caused a 5-fold increase in guanylate cyclase activity in gracilis anticus skeletal muscle, compared to a 3-fold increase for 17-methyltestosterone and a 2-fold increase with testosterone at the same concentration of 1 μM (Table 1). Similar results were found in kidney, liver, and ventral prostate at 1 μM (Table 1). Maximal stimulation of hepatic guanylate cyclase activity was seen at 1 μM with testosterone, 17-methyltestosterone, 5α-dihydrotestosterone, and 19-nortestosterone; guanylate cyclase activity approached nonstimulated levels at a concentration of 0.001 μM with all four agents (Table 2).

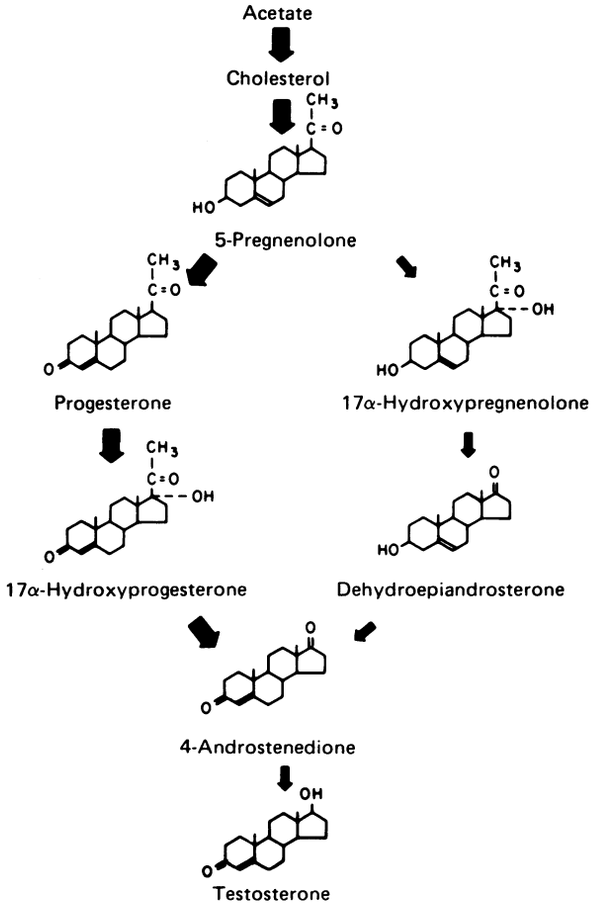

FIG. 1. The two major pathways for testosterone synthesis in the testis. The heavy arrows indicate the preferred steroid pathway.

Etiocholanolone, androsterone, and epiandrosterone—the metabolic breakdown products of testosterone—also caused an enhancement of guanylate cyclase activity in these various tissues (Table 1). The amount of enhancement of guanylate cyclase activity was less with these metabolic products than for testosterone and its precursors, with maximal enhancement being 1½ to 2 times basal activity with the metabolites. These three metabolic products of testosterone metabolism caused their maximal enhancement of hepatic guanylate cyclase activity when utilized at 1 μM concentrations and approached nonstimulated levels when the concentrations were decreased to 0.001 μM (Table 2).

DISCUSSION

Androgens as well as the precursors of testosterone except for cholesterol stimulated guanylate cyclase activity in the present investigation. The precursors that did stimulate guanylate cyclase activity—namely, progesterone, 17α-hydroxy progesterone, androstenedione, pregnenolone, 17α-hydroxypregnenolone, and dehydroepiandrosterone—do have some properties similar to those of testosterone. The anabolic properties of progestins are well known: they have been shown to increase the growth of uterine tissues (17–20) and mammary gland follicles (18) *in vivo*. In addition, progesterone has been reported to increase the multiplication of HeLa cells in tissue culture (21). Of all the steroid compounds reported herein, only progesterone appears to have been examined in regards to the possibility that cyclic GMP might be mediating some of the effects of these steroids. Progesterone was shown to increase cyclic GMP in uterine tissues (22). In recent years investigators have studied the regulation of the components of cyclic GMP metabolism in order to obtain insight into the biologic role of cyclic GMP, and this approach encompasses research (*i*) on guanylate cyclase, the enzyme that catalyzes the formation of cyclic GMP from GTP, and (*ii*) on cyclic 3′,5′-GMP phosphodiesterases, which cause the breakdown of cyclic GMP to 5′-GMP. In the present investigation the phosphodiesterases were blocked with 20 mM caffeine, which in our laboratory blocks over 95% of measured phosphodiesterase activity (D. L. Vesely, D. C. Lehotay, and G. S. Levey, unpublished observations). Each of the respective steroids was not tested on phosphodiesterase activity in the present investigation. The effect of progesterone on guanylate cyclase activity shown in this investigation has not been previously reported. The stimulation of the guanylate cyclase–cyclic GMP system by the other precursors of testosterone synthesis has not been previously demonstrated, but these findings were not completely unexpected, because many of these precursors have been shown to stimulate protein synthesis (23), and cyclic GMP also has been shown to stimulate protein synthesis (24, 25).

In regards to testosterone effects on another second messenger, cyclic AMP, and the enzyme that catalyzes its production, adenylate cyclase, Rosenfeld and O'Malley (26) have shown that testosterone does not stimulate adenylate cyclase in ventral prostate either *in vitro* or *in vivo*. Others have confirmed the finding that testosterone does not stimulate adenylate cyclase activity (27–28), and Liao *et al.* have shown that 5α-dihydrotestosterone also does not significantly affect adenylate cyclase activity (28). Because both testosterone (2–5) and cyclic GMP (6, 11, 12) have been shown to stimulate DNA synthesis, one might have suspected that testosterone, 5α-dihydrotestosterone, 19-nortestosterone, and 17-methyltestosterone would enhance the activity of guanylate cyclase, as was seen in the present investigation. This correlation between the guanylate cyclase–GMP system and DNA synthesis was considerably strengthened recently when a specific inhibitor of guanylate cyclase activity was isolated (14) and shown to result

Table 1. Effect of testosterone and its precursors and metabolites on guanylate cyclase activity

Addition (each 1 μM)	Guanylate cyclase specific activity, pmol cyclic GMP/mg protein per 10 min* Skeletal muscle†	Liver	Kidney	Ventral prostate
None	136 ± 8	284 ± 6	290 ± 8	38 ± 6
Dimethyl sulfoxide‡§	140 ± 12	280 ± 8	286 ± 6	40 ± 6
Precursors				
Cholesterol§	143 ± 6	287 ± 6	293 ± 6	42 ± 8
Progesterone¶	410 ± 12	860 ± 8	1015 ± 15	114 ± 12
17α-Progesterone¶	346 ± 8	586 ± 8	890 ± 12	84 ± 8
Androstenedione¶	398 ± 12	738 ± 6	920 ± 16	86 ± 6
Pregnenolone¶	402 ± 6	648 ± 12	870 ± 12	96 ± 8
17α-Pregnenolone¶	378 ± 8	628 ± 8	852 ± 12	90 ± 12
Dehydroepiandrosterone¶	392 ± 12	820 ± 12	865 ± 8	98 ± 8
Testosterones and analogues				
Testosterone¶	293 ± 8	584 ± 8	605 ± 12	98 ± 8
17-Methyltestosterone¶	464 ± 12	631 ± 12	672 ± 12	116 ± 8
19-Nortestosterone¶	703 ± 12	698 ± 8	721 ± 8	129 ± 12
5α-Dihydrotestosterone¶	721 ± 16	703 ± 12	698 ± 2	135 ± 8
Metabolites				
Etiocholanolone¶	289 ± 8	571 ± 12	625 ± 12	92 ± 8
Androsterone¶	201 ± 12	489 ± 8	586 ± 8	79 ± 6
Epiandrosterone¶	243 ± 6	504 ± 8	593 ± 12	87 ± 6

* Each value is the mean ± SEM of triplicate samples from three animals in each group in three separate experiments.
† Gracilis anticus muscle.
‡ All of the steroids were dissolved in dimethyl sulfoxide. Final concentration in the assay was 10 mM.
§ No significant difference, $P > 0.01$, for all tissues compared to controls by Student's t test for unpaired values.
¶ Significant difference, $P < 0.001$, for all tissues compared to controls.

in decreased DNA synthesis both in the basal state and during stimulation by mitogens (15). The metabolic products of testosterone degradation also caused some stimulation of the guanylate cyclase–cyclic GMP system, but less than either testosterone or its precursors. Thus, the data in the present investigation suggest that the guanylate cyclase–cyclic GMP system plays a role in the mechanism of action of testosterone and its precursors.

A general characteristic of steroid hormones is that they passively diffuse into cells, where they are thought to interact with cytoplasmic receptors (29). When one centrifuges any tissue homogenate at 37,000 × g, one finds that 90–95% of the guanylate cyclase is in the supernatant (30), indicating that this enzyme is cytoplasmic, as opposed to adenylate cyclase, which is bound to the plasma membrane of the cell, as evidenced by the fact that adenylate cyclase activity is found in the pellet from centrifugation at the same speed (31). This evidence alone would disfavor the possibility that adenylate cyclase is involved

Table 2. Dose–response relationships between hepatic guanylate cyclase activity and testosterone, its precursors, and its metabolites

Addition	Guanylate cyclase specific activity, pmol cyclic GMP/mg protein per 10 min* 0 μM	0.0001 μM	0.001 μM	0.01 μM	0.1 μM	1 μM	10 μM
Dimethyl sulfoxide	281 ± 6	285 ± 8ns	287 ± 6ns	283 ± 12ns	288 ± 10ns	280 ± 8ns	278 ± 15ns
Precursors							
Cholesterol	283 ± 8	286 ± 6ns	281 ± 8ns	289 ± 6ns	291 ± 8ns	287 ± 6ns	290 ± 8ns
Progesterone	284 ± 6	292 ± 8ns	583 ± 12†	697 ± 8†	747 ± 8†	860 ± 8†	880 ± 12†
17α-Progesterone	278 ± 6	303 ± 12ns	523 ± 8†	549 ± 12†	557 ± 12†	587 ± 8†	603 ± 12†
Androstenedione	286 ± 6	287 ± 8ns	556 ± 8†	613 ± 6†	673 ± 6†	738 ± 6†	758 ± 8†
Pregnenolone	279 ± 8	301 ± 6ns	568 ± 8†	584 ± 8†	602 ± 8†	641 ± 12†	628 ± 8†
17α-Pregnenolone	284 ± 8	285 ± 6ns	543 ± 12†	564 ± 8†	597 ± 8†	628 ± 8†	603 ± 12†
Dehydroepiandrosterone	282 ± 6	307 ± 8	597 ± 6†	688 ± 8†	713 ± 8†	820 ± 12†	810 ± 8†
Testosterones and analogues							
Testosterone	278 ± 6	284 ± 8ns	539 ± 8†	555 ± 16†	566 ± 8†	584 ± 8†	596 ± 12†
17-Methyltestosterone	277 ± 8ns	288 ± 8	556 ± 8†	576 ± 16†	611 ± 8†	631 ± 12†	626 ± 12†
19-Nortestosterone	285 ± 6	279 ± 8ns	536 ± 6†	613 ± 8†	666 ± 16†	698 ± 16†	684 ± 12†
5α-Dihydrotestosterone	283 ± 8	280 ± 12ns	540 ± 12†	593 ± 16†	601 ± 12†	673 ± 8†	679 ± 12†
Metabolites							
Etiocholanolone	286 ± 6	302 ± 6ns	383 ± 8†	398 ± 6†	432 ± 8†	571 ± 12†	575 ± 8†
Androsterone	280 ± 6	292 ± 8ns	366 ± 8†	387 ± 8†	421 ± 11†	489 ± 8†	493 ± 8†
Epiandrosterone	280 ± 6	299 ± 6ns	377 ± 8†	402 ± 12†	443 ± 8†	504 ± 8†	507 ± 12†

* Each value is the mean ± SEM of triplicate samples from three animals in each group in three separate experiments. ns, No significant difference, $P > 0.01$, compared to 0 μM control by Student's t test for unpaired values.
† Significant difference, $P < 0.001$, compared to control.

in the mechanism of action of steroid hormones. Estrogens also passively diffuse into cells and, like androgens, have anabolic actions (23, 32) and stimulate protein synthesis (33–35). Estrogens have recently been reported to increase cyclic GMP *in vivo* (22, 36). Estrogens do enhance guanylate cyclase *in vitro* to an extent similar to that found with testosterone (D. L. Vesely and D. E. Hill, unpublished observation). Thus, at the cellular level it appears that the mechanism of action of steroid hormones may include a final common pathway that involves enhancement of guanylate cyclase activity.

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