Effects of Steroid 5α-Reductase Inhibitor ONO-9302 and Anti-Androgen Allylestrenol on the Prostatic Growth, and Plasma and Prostatic Hormone Levels in Rats

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ABSTRACT—ONO-9302 [epristeride; (−)-17β-(*tert*-butylcarbamoyl)androsta-3,5-diene-3-carboxylic acid] is a novel inhibitor of steroid 5α-reductase. We studied in vitro and in vivo effects of ONO-9302 on the rat prostatic tissue in comparison with those of the anti-androgen allylestrenol. ONO-9302 inhibited the rat prostatic enzyme with an IC_{50} value of 11 nM, whereas allylestrenol was about 80,000-fold less potent. The growth of ventral prostate, which was induced by the subcutaneous injection of testosterone propionate in the castrated rats, was significantly reduced by ONO-9302 at oral doses of 1–100 mg/kg/day. Allylestrenol showed a significant effect only at a dose of 100 mg/kg/day. In mature male rats, ONO-9302 significantly reduced the ventral prostate weight at doses of 10–100 mg/kg/day and decreased prostatic 5α-dihydrotestosterone (DHT) content associated with a rise in testosterone (T) content at doses of 0.1–100 mg/kg/day. Plasma hormone levels (i.e., T, DHT, luteinizing hormone (LH) and follicle stimulating hormone (FSH)) were not altered significantly. Allylestrenol significantly reduced the ventral prostate weight at doses of 10–100 mg/kg/day. However, unlike ONO-9302, allylestrenol reduced both the prostatic DHT and T contents and also lowered plasma T, DHT, LH and FSH levels at a dose of 30 mg/kg/day. These results suggest that ONO-9302 reduces the prostatic growth by inhibiting the conversion of T to DHT in the prostate without lowering blood T level unlike anti-androgen drugs.

Keywords: Steroid 5α-reductase, Dihydrotestosterone, Testosterone, Prostate, ONO-9302

Development of prostatic hyperplasia is an almost universal phenomenon in aging men (1, 2). The prostate surrounds the urethra, so any enlargement of this tissue is a potential cause of urinary tract obstruction; indeed, benign prostatic hyperplasia (BPH) is the most common cause of urinary outflow obstruction in men (3). It is considered that growth of the prostate gland is dependent on tissue androgen contents (4). Many of the clinical studies have clearly demonstrated that BPH could regress with anti-androgen therapies (5–10). The mode of action of such drugs is presumed to be due to their ability to inhibit prostatic uptake of testosterone (T), to antagonize androgen receptors, to inhibit T production or combination of these actions (7–10). However, the major problem of these drugs is side effects on sexual function, including impotence, decreased libido, sterility, hot flashes, increased breast tenderness and loss of the anabolic effects of androgen on muscle mass, which is caused by plasma T deprivation through gonadotropin release suppression (7–12). By the way, it has been suggested that the primary intracellular androgen is not T but 5α-dihydrotestosterone (DHT) in the prostate. Evidence for the importance of DHT in human prostatic growth may be derived from the following observations. In subjects with a 5α-reductase deficiency, the prostate is vestigial despite their high plasma T levels (13–15). The intracellular concentrations of DHT exceed that of T or any other androgen metabolite in the prostate (16–20). Furthermore, DHT binds to the intracellular androgen receptor protein about ten times more tightly than T (21–23). Binding of DHT to androgen receptors releases the DNA-binding domain of the receptor protein, enabling it to associate with the genome, thereby modulating the transcription of specific genes and the regulation of particular biologic responses (24, 25). Steroid 5α-reductase is a NADPH-dependent enzyme responsible for the irreversible conversion of T to the potent androgen DHT (21, 26). Accordingly, specific inhibitors of 5α-reductase may be useful in controlling pathological conditions dependent on DHT, such as BPH, without adverse effects on sexual function

(27). ONO-9302 has been reported to be a potent and specific inhibitor of 5α-reductase (28–30). The present studies were carried out to obtain preclinical evidence for the efficacy of ONO-9302 by comparing it with the anti-androgen allylestrenol, which is currently used in the treatment of BPH.

MATERIALS AND METHODS

Chemicals

ONO-9302 (epristeride) was synthesized at the Smith Kline Beecham Pharmaceuticals (Philadelphia, PA, USA). Allylestrenol was extracted and purified from the commercially available drug product (Perselin®; Japan Organon, Tokyo) by Ono Pharmaceutical Co., Ltd. (Osaka). These drugs were dissolved in dimethyl sulfoxide (DMSO) for the in vitro study, and they were suspended in 0.5% methylcellulose (MC) containing 1% polyethylene glycol 400 (PEG 400) solution for the in vivo study. [4-^{14}C]Testosterone (59.8 mCi/mmol) was purchased from DuPont Co. (Wilmington, DE, USA). T and testosterone propionate (TP) was purchased from Nacalai Tesque, Inc. (Kyoto). TP was dissolved in ethanol and then diluted in sesame oil when given subcutaneously. DHT was purchased from Fulka (Buchs, Switzerland). β-NADPH, dithiothreitol (DTT), MC and PEG 400 were purchased from Sigma (St. Louis, MO, USA). Other chemicals used were of analytical grade.

Animals

Adult male Sprague-Dawley rats and immature male Sprague-Dawley rats were obtained from Japan SLC, Inc. (Shizuoka) and Charles River Japan, Inc. (Kanagawa), respectively. Animals were maintained under controlled temperature (23±2℃), humidity (55±10%) and lighting conditions (12 hr of light, 12 hr of darkness). Animals were fed a commercially available chow (MM-5; Keari Co., Ltd., Osaka), and water was available ad libitum.

Preparation of 5α-reductase from rat prostates

Adult male rats (12-weeks-old, 360–430 g body weight) were anesthetized with diethyl ether and sacrificed by exsanguination from the abdominal aorta. The ventral prostates of rats were dissected free of their capsules, washed with saline, and stored at −80℃. Prostatic enzyme fractions were prepared as previously described by Liang et al. (31). Frozen tissues were thawed on ice and minced with scissors. The following procedures were carried out at 4℃. The tissues were homogenized with a glass-glass homogenizer in 3 tissue vol. of 20 mM potassium phosphate, pH 6.5, containing 0.32 M sucrose and 1 mM DTT. The homogenate was centrifuged at 140,000×*g* for 60 min, and then the pellet was washed with 3 tissue vol. of 20 mM potassium phosphate, pH 6.5, containing 0.32 M sucrose and 1 mM DTT. The washed pellet was suspended in 20 mM potassium phosphate, pH 6.5, containing 20% glycerol and 1 mM DTT, and then filtered through a layer of gauze. The suspension (approx. 4 mg protein/ml) was stored at −80℃ until use.

In vitro inhibition of 5α-reductase

5α-Reductase activities were assayed as previously described (31). The reaction mixture contained varied concentration of inhibitor, 40 mM potassium phosphate (pH 6.5), 1 μM [^{14}C]T, 1 mM DTT, 50 μM NADPH and the prostatic enzyme fractions (≅0.2 mg) in a final volume of 0.5 ml. The reaction was initiated by adding the enzyme fraction, incubated at 37℃ for 30 min, and terminated by mixing with 2 ml of ethyl acetate. After centrifugation at 2000 rpm for 10 min, the organic phase was transferred to a tube containing unlabeled DHT and T (10 μg each) as carriers and markers, and then evaporated to dryness under a nitrogen stream. The residue was dissolved in 50 μl ethyl acetate. The solution was applied to a plate for thin layer chromatography (Kieselgel $60F_{254}$ plate; Merck, Darmstadt, Germany), and the plate was developed in ethyl acetate-cyclohexane (1 : 1, v/v) at room temperature. The plate was air dried, and DHT and T were located with iodine vapor and under UV light, respectively. The plate was contacted with an imaging plate (IP) in a brass chamber for 30 min. Radioactivity (photo stimulated luminescence, PSL) on the IP was quantified using a BAS-2000 Bio-Image Analyzer (Fuji Photo Film Co., Ltd., Tokyo) (32–35). Enzyme activity was calculated from the ratio of the radioactivity of DHT to the total radioactivity. The concentration of test compound required to inhibit 5α-reductase activity by 50% (IC_{50}) was determined from the regression curve (Hill plot).

In vivo effects in castrated immature rats

Immature male rats (4-weeks-old, 70–100 g body weight) were anesthetized by pentobarbital (50 mg/kg, i.p.) and castrated. Castration was performed by a scrotal incision. The castrated animals were randomized into 9 groups of 8 rats each on the day following castration. Eight of the groups were given ONO-9302 (0.1, 1, 10 and 100 mg/kg/day), allylestrenol (1, 10 and 100 mg/kg/day) or an equivalent volume of vehicle (0.5% MC containing 1% PEG 400, 10 ml/kg; control group) orally once daily for 14 days. TP (25 μg/250 μl/rat) in sesame oil was subcutaneously injected to the animals immediately after each drug administration. The remaining one was given vehicle orally and sesame oil without TP during the same period (castrated group). In addition, an age-matched,

non-castrated group was prepared as the normal group. The normal group was given vehicle and sesame oil as well as the castrated group. Animals were sacrificed by decapitation 24 hr after the last dosing, and the ventral prostate and seminal vesicles were removed and weighed.

In vivo effects in mature male rats

Mature male rats (11-weeks-old, 330–360 g body weight) were randomized into 11 groups of 8 rats each. One of these groups was given no treatment (normal group). Nine of the groups were given ONO-9302 (0.1, 1, 10 and 100 mg/kg/day), allylestrenol (1, 3, 10 and 30 mg/kg/day) or an equivalent volume of vehicle (0.5% MC containing 1% PEG 400, 5 ml/kg; control group) orally once daily for 21 days. The remaining one was castrated on the day of assigning and also given vehicle during the same period (castrated group). Animals were anesthetized with diethyl ether and sacrificed by exsanguination 8 hr after the last dosing, and the following organs were removed and weighed: ventral prostate, seminal vesicles, testes, epididymides, liver, kidneys and adrenals. The prostates were stored at −80℃ for androgen determinations. Blood was collected from the abdominal aorta and immediately transferred to a plastic tube containing sodium heparin. Plasma was collected and stored at −80℃ for gonadotropin and androgen determinations.

Tissue and plasma androgen determinations

The ventral prostate was homogenized with a glass-glass homogenizer in 1 ml purified water. The prostatic homogenate and plasma were extracted twice with 3 ml diethyl ether. The combined ether phases were evaporated to dryness under nitrogen stream. T content in the residue was measured by radioimmunoassay (RIA) using a Coat-A-Count® Total Testosterone RIA kit (Diagnostic Products Corporation, Los Angeles, CA, USA). For the DHT determination, the residue was dissolved in 100 μl EtOH, and further purification was performed by HPLC (36–40) using a Capcell Pak C18 column (Shiseido, Tokyo) with acetonitrile-water (45 : 55, v/v) at a flow rate of 0.8 ml/min. An aliquot of DHT-containing eluate (8–12 min) was assayed by RIA using a Testosterone /Dihydrotestosterone [^{3}H] Assay System® (Amersham, Buckinghamshire, England). The calculated value of each sample from the standard curve was corrected with the recovery count of [^{3}H]DHT, which was added to each sample before ether extraction (approx. 15,000 dpm).

Plasma gonadotoropin determinations

Plasma luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured by RIA using a Rat Luteinizing Hormone [^{125}I] Assay System® and a Rat Follicle Stimulating Hormone [^{125}I] Assay System® (Amersham), respectively. RIA was performed at SRL, Inc. (Tokyo).

Protein determination

Protein concentration was determined by the method of Bradford (41) using a Bio-Rad® Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard.

Statistical analyses

Experimental values are each expressed as a mean±S.E. Evaluation of the results was performed by

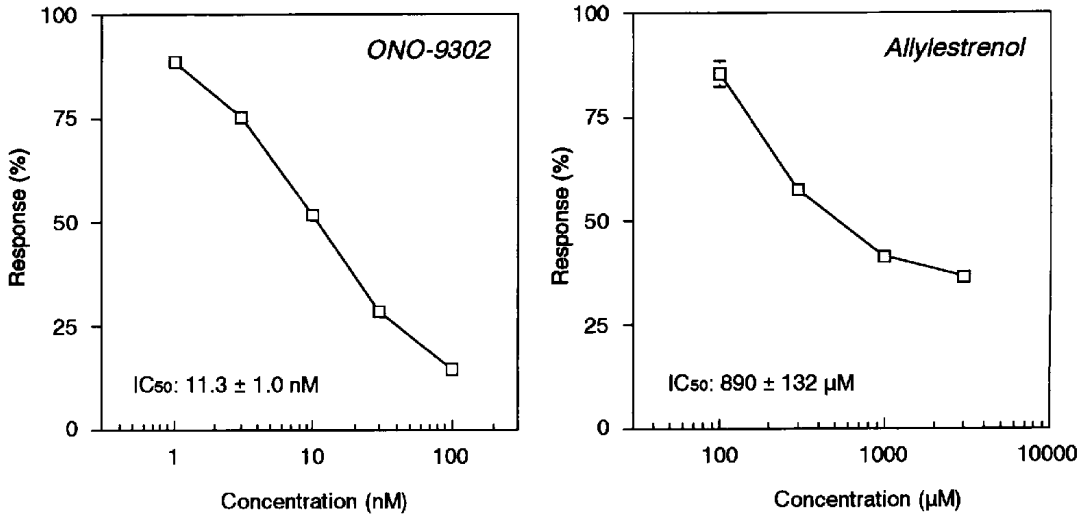

Fig. 1. Inhibitory effects of ONO-9302 and allylestrenol on rat prostatic 5α-reductase activity. Values are the mean±S.E. of 4 separate experiments.

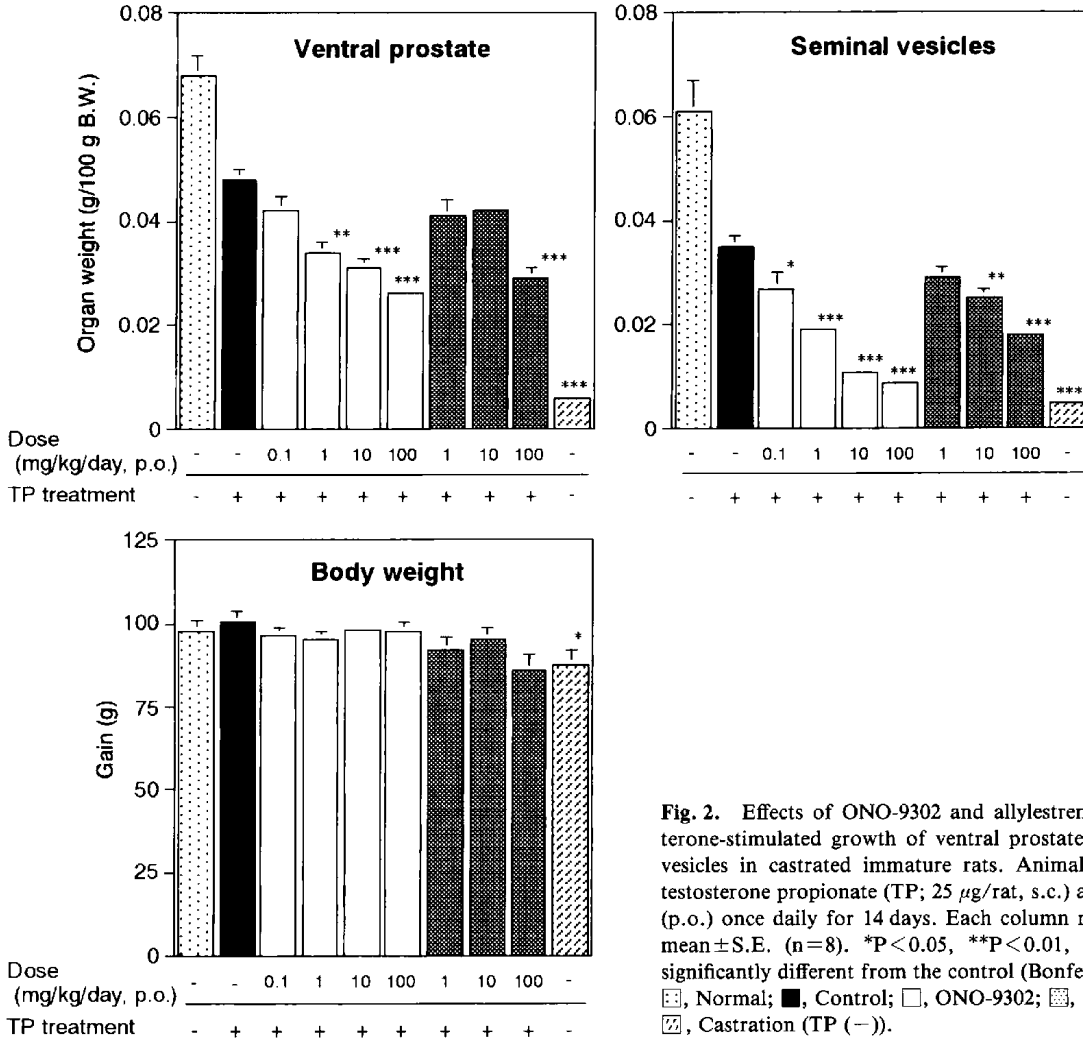

Fig. 2. Effects of ONO-9302 and allylestrenol on testosterone-stimulated growth of ventral prostate and seminal vesicles in castrated immature rats. Animals were given testosterone propionate (TP; 25 μg/rat, s.c.) and each drug (p.o.) once daily for 14 days. Each column represents the mean±S.E. (n=8). *P<0.05, **P<0.01, ***P<0.001, significantly different from the control (Bonferroni/Dunn). ⊡, Normal; ■, Control; □, ONO-9302; ▨, Allylestrenol; ▨, Castration (TP (−)).

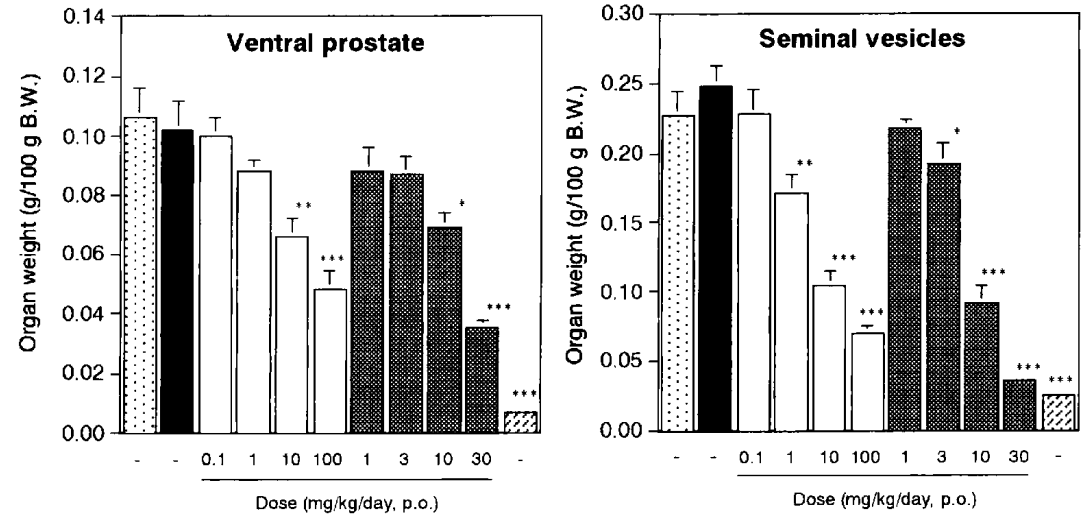

Fig. 3. Effects of ONO-9302 and allylestrenol on the weights of ventral prostate and seminal vesicles in mature male rats. Animals were orally administered with each drug once daily for 21 days. Each column represents the mean±S.E. (n=8). *P<0.05, **P<0.01, ***P<0.001, significantly different from the control (Bonferroni/Dunn). ⊡, Normal; ■, Control; □, ONO-9302; ▨, Allylestrenol; ▨, Castration.

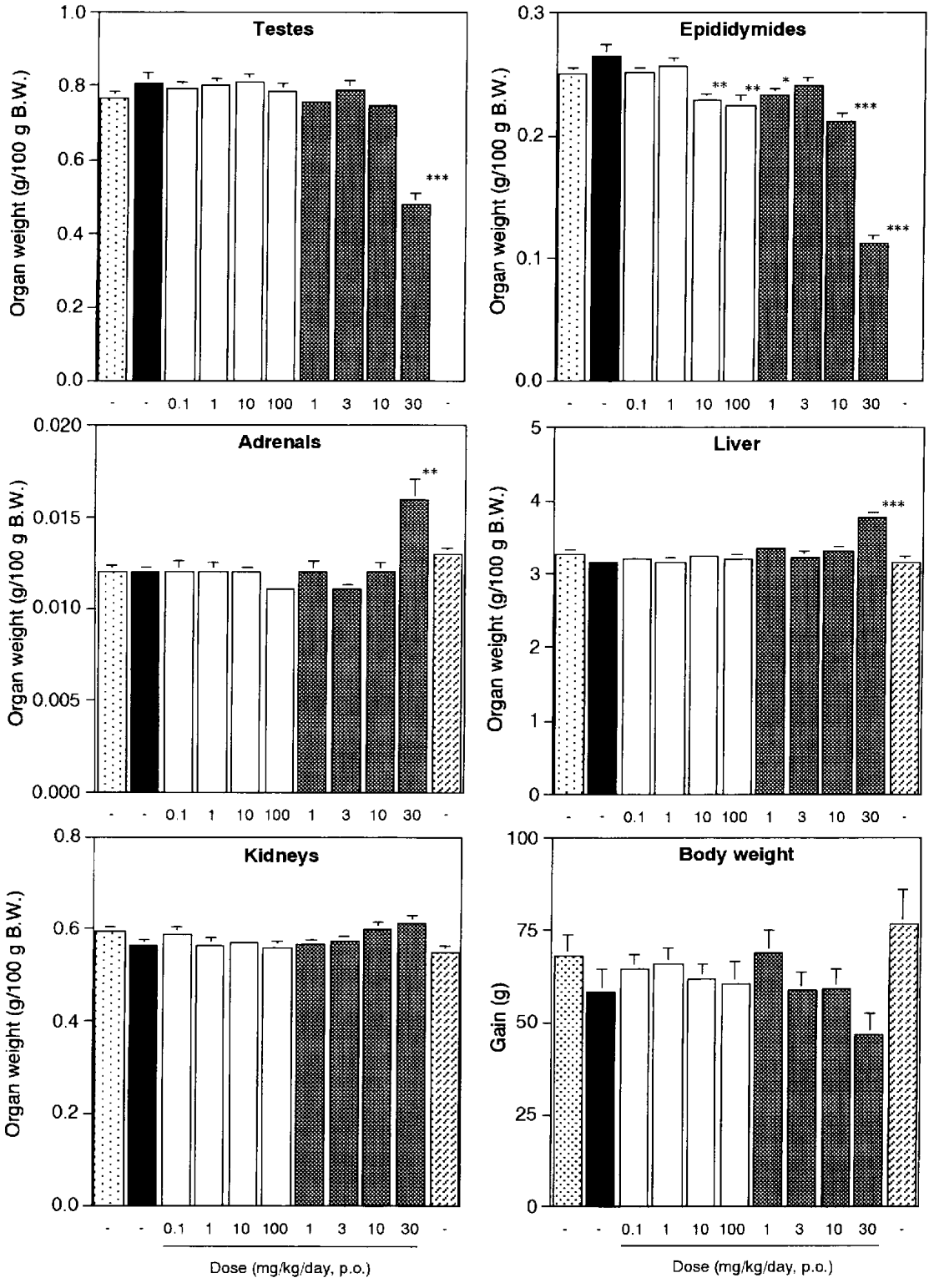

Fig. 4. Effects of ONO-9302 and allylestrenol on the organ weights in mature male rats. Animals were orally administered with each drug once daily for 21 days. Each column represents the mean±S.E. (n=8). *P<0.05, **P<0.01, ***P<0.001, significantly different from the control (Bonferroni/Dunn). ⊡, Normal; ■, Control; □, ONO-9302; ▨, Allylestrenol; ▨, Castration.

analysis of variance (ANOVA) followed by the test of Bonferroni/Dunn. P-values less than 0.05 were considered to be significant.

RESULTS

Effects on rat prostatic 5α-reductase in vitro

The abilities of ONO-9302 and allylestrenol to inhibit the 5α-reductase from rat prostatic tissues are shown in Fig. 1. ONO-9302 inhibited rat 5α-reductase activity with an IC_{50} value of 11.3 ± 1.0 nM, whereas allylestrenol was extremely less potent than ONO-9302 (i.e., $IC_{50} = 890 \pm 132$ μM).

Effects on T-stimulated prostatic growth in castrated immature rats

To exclude the possibility that compounds act through endocrine glands such as the pituitary or testis, the effects of compounds on T-stimulated prostatic growth were evaluated in the castrated rats. The growth of ventral prostate and seminal vesicles were inhibited by surgical castration (Fig. 2). Treatment of castrated rats with exogenous TP resulted in substantial recoveries of both organ weights (i.e., 71% and 57% of the normal level for the ventral prostate and seminal vesicles, respectively). Under these conditions, ONO-9302 (0.1, 1, 10 and 100 mg/kg/day) dose-dependently reduced the weights of the ventral prostate (14%, 33%, 41% and 52%) and seminal vesicles (27%, 53%, 80% and 87%), and significant differences from the control were seen at doses equal to or greater than 1 and 0.1 mg/kg/day, respectively (Fig. 2). Allylestrenol (1, 10 and 100 mg/kg/day) also reduced the weights of the ventral prostate (17%, 14% and 45%) and seminal vesicles (20%, 33% and 57%) in a dose-dependent manner. However, it required about 100-fold higher dose compared with ONO-9302 to exhibit the similar magnitude of inhibitory action. Neither compounds caused any significant change in body weight (Fig. 2).

Effects on organ weights and hormone levels in mature male rats

To evaluate the effects of compounds in intact animals, mature male rats were treated with test compounds for 21 days. ONO-9302 (0.1, 1, 10 and 100 mg/kg/day) dose-dependently reduced the weights of the ventral prostate (2%, 15%, 38% and 57%) and seminal vesicles (9%, 35%, 65% and 80%), and significant differences from the control were seen at doses equal to or greater than 10 and 1 mg/kg/day, respectively (Fig. 3). ONO-9302 exerted no effect on the weights of other organs except for the epididymis. The weight of the epididymis, which is one of the androgen-target organs (42–46), was slightly but significantly decreased at doses equal to or greater than 10 mg/kg/day (Fig. 4). Allylestrenol (1, 3, 10 and 30 mg/kg/day) also reduced the weights of the ventral prostate (15%, 16%, 35% and 71%) and seminal vesicles (13%, 25%, 70% and 96%), and significant differences from the control were seen at doses equal to or greater

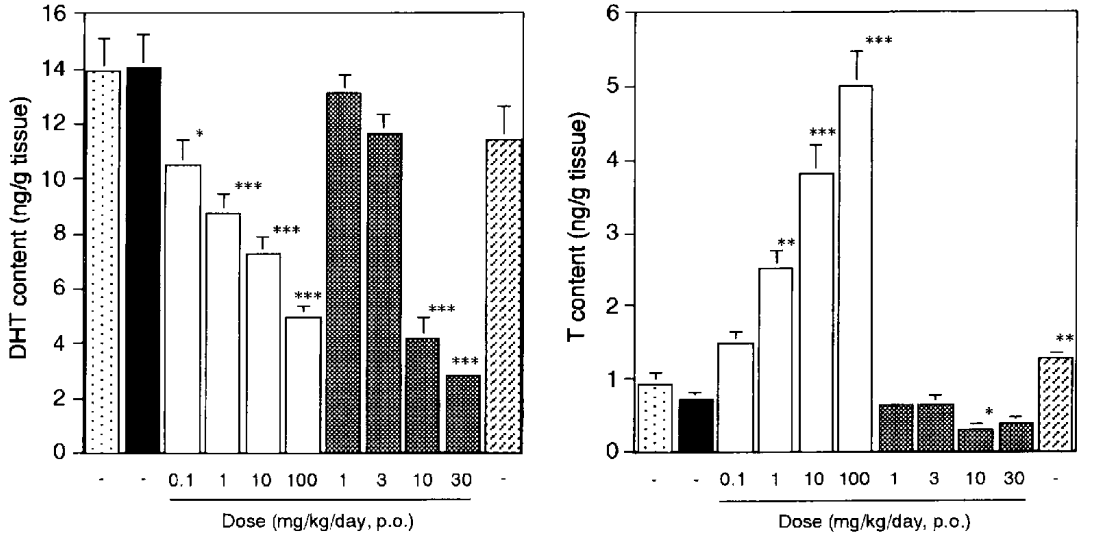

Fig. 5. Effects of ONO-9302 and allylestrenol on the 5α-dihydrotestosterone (DHT) and testosterone (T) levels in the ventral prostate of mature male rats. Animals were orally administered with each drugs once daily for 21 days. Each column represents the mean±S.E. (n=8). *P<0.05, **P<0.01, ***P<0.001, significantly different from the control (Bonferroni/Dunn). In cases where tissue concentrations of DHT and T were below the quantitative limit, the limit value of quantitation for each animal was used in the calculation of a mean value and statistical analysis. ⁝⁝, Normal; ■, Control; □, ONO-9302; ▨, Allylestrenol; ▨, Castration.

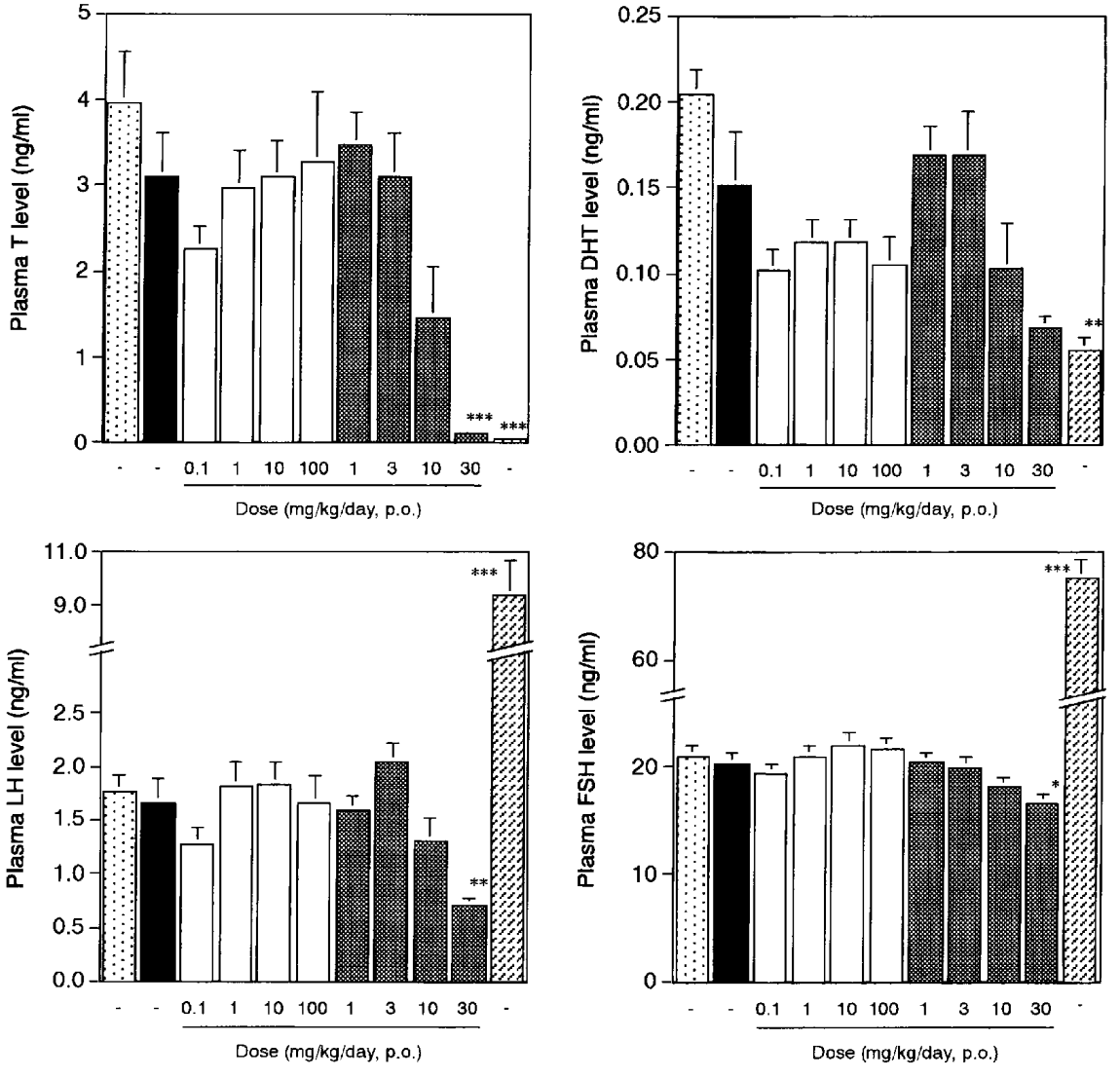

Fig. 6. Effects of ONO-9302 and allylestrenol on the testosterone (T), 5α-dihydrotestosterone (DHT), luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels in the plasma of mature male rats. Animals were orally administered with each drug once daily for 21 days. Each column represents the mean±S.E. (n=8). *P<0.05, **P<0.01, ***P<0.001, significantly different from the control (Bonferroni/Dunn). In cases where plasma concentrations of T, DHT and LH were below the quantitative limit, the limit value of quantitation for each animal was used in the calculation of mean value and statistical analysis. ⁚⁚, Normal; ■, Control; □, ONO-9302; ▨, Allylestrenol; ▨, Castration.

than 10 and 3 mg/kg/day, respectively (Fig. 3). The inhibitory potency of allylestrenol seemed to be equal to or greater than that of ONO-9302 in this model. However, allylestrenol showed signs of toxicity such as a marked decrease in the weight of testes and significant increases in the weight of liver and adrenals at a dose of 30 mg/kg/day (Fig. 4). Neither compounds caused any significant change in body weight (Fig. 4). The direct evidence of 5α-reductase inhibition by ONO-9302 was demonstrated by measurement of prostatic DHT and T contents (Fig. 5). The prostatic DHT contents were significantly and dose-dependently decreased at the all tested doses of ONO-9302 (25%, 38%, 48% and 65%), while T contents were conversely increased (109%, 255%, 439% and 608%). ONO-9302 lowered plasma DHT by 22–33%, which, however, did not reach statistical significance (Fig. 6). No effect on plasma T, LH or FSH level was observed. On the other hand, allylestrenol caused marked reductions in both the prostatic DHT and T contents (Fig. 5). In addition, it significantly decreased not only the plasma T level but also the plasma LH and FSH levels, both of which regulate T production (Fig. 6). Castration markedly decreased the plasma T level by 98% and caused compensatory increases in the plasma LH and

FSH levels by 482% and 269%, respectively (Fig. 6).

DISCUSSION

The aim of this study was to obtain experimental evidence that would suggest the clinical relevance of ONO-9302 by simultaneous comparison with allylestrenol, one of the common anti-androgen drugs currently used in the treatment of BPH. A number of different studies have attempted to characterize the efficacy of 5α-reductase inhibitors, but there are few reports in which those 5α-reductase inhibitors and other common anti-androgen drugs were evaluated simultaneously. The present study clearly reveals the considerable advantage of ONO-9302 over allylestrenol, especially in the aspect of side effects.

In an in vitro study, we examined the effects of ONO-9302 and allylestrenol on rat prostatic 5α-reductase. ONO-9302 inhibited rat prostatic 5α-reductase activity with an IC_{50} value of 11 nM, which was similar to that reported by Levy et al. (28, 30); thus ONO-9302 was confirmed to be a potent 5α-reductase inhibitor in vitro. Allylestrenol inhibited rat prostatic 5α-reductase activity with an IC_{50} value of 890 μM. This inhibitory potency was weaker than that reported by Yamanaka et al., who showed allylestrenol caused about 70% inhibition on rat prostatic 5α-reductase at a concentration of 60 μM (7). This may be due to different experimental conditions such as enzyme content, substrate concentration and incubation time. In spite of these differences, it was clearly demonstrated that the inhibitory ability of allylestrenol on rat prostatic 5α-reductase was much weaker than that of ONO-9302.

In in vivo studies, we compared the effects of both drugs using castrated immature rats that were treated with exogenous T and intact mature rats. ONO-9302 showed significant and dose-dependent reductions of the ventral prostate weight in both models. On the other hand, allylestrenol showed a significant reduction only at the highest dose (100 mg/kg/day) in castrated rats, and a more pronounced effect was observed in intact animals. ONO-9302 markedly lowered the prostatic DHT content while it increased the prostatic T content, making a sharp contrast with the finding that allylestrenol lowered both prostatic androgen contents. These results indicate that ONO-9302 exhibits its efficacy by inhibiting 5α-reductase activity in vivo as well as in vitro, and it appears that much of allylestrenol's effect may be the result of its ability to inhibit T production, thus leading to the lowering of both prostatic androgen contents. When plasma hormone levels were measured in mature rats, it was found that allylestrenol significantly lowered not only the T level but also LH and FSH levels, both of which regulate T production in the testis. Although the castration also caused a marked reduction in plasma T level, the compensatory increases in plasma LH and FSH levels were observed in castrated rats. These facts suggest that allylestrenol inhibits T production through the hypothalamus-pituitary-testicular axis as well as other anti-androgen drugs (8–12). Namely, it lowers the plasma T level by inhibiting LH and FSH production in the anterior pituitary rather than by directly acting on the T-producing organs. By contrast, ONO-9302 did not lower plasma T, LH and FSH levels. Since the inhibition of T production is thought to be deeply associated with clinically common adverse effects of anti-androgen drugs on sexual function (8–11), it is anticipated that ONO-9302 will not cause such undesirable side effects in clinical studies.

It has been demonstrated that ONO-9302 did not affect the DHT-induced growth of the prostate at all in castrated rats (47). This suggests that ONO-9302 does not act as an agonist to or as an antagonist against androgen receptors. This postulate is further supported by the findings that ONO-9302 showed no affinity to various hormone receptors in vitro (48). Taken altogether, it appears that ONO-9302 does not act otherwise than by inhibiting 5α-reductase activity. It has recently been reported that 5α-reductase has two isoforms and that the functional characteristics and the pattern of tissue distribution differ from one isoform to the other (49–52). It has also been found that type 2 is predominantly expressed in human prostate. The study with human recombinant enzymes shows that ONO-9302 is a selective inhibitor of type 2 5α-reductase (48), so that it seems to have an ideal pharmacological profile from the standpoint of the inhibition of enzyme activity in the prostate.

In addition, ONO-9302 has been shown to inhibit 5α-reductase in an uncompetitive manner versus T (28), while most steroidal compounds including finasteride (53) function as the competitive substrate analogue. Inhibition of 5α-reductase leads to not only a decrease in DHT, but also an increase in the prostatic T content (54). Such an increase in prostatic T could overcome some portion of the initial inhibition by the competitive inhibitors, but not in the case of the uncompetitive inhibitors; thus ONO-9302 may consequently demonstrate an advantage in clinical studies.

In conclusion, ONO-9302 exhibits the prostatic involution by selectively inhibiting 5α-reductase activity, notably type 2 5α-reductase, which accounts for most of the enzyme activity in human prostate. ONO-9302 does not lower the circulating T level unlike anti-androgen drugs. Accordingly, ONO-9302 looks promising as a new type of drug with efficacy for the treatment of BPH without adverse effects on sexual function.

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