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EFFECT OF THE AROMATASE INHIBITOR, 4 HYDROXY-ANDROSTENEDIONE, ON THE ENDOTOXIN-INDUCED CHANGES IN STEROID HORMONES IN MALE RATS

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Summary

The increase in circulating estrogen concentrations that follows injection of Escherischia coli endotoxin (Endo) may be due to increased aromatase activity. We have therefore analysed the effect of the aromatase inhibitor, 4 hydroxyandrostenedione (4OHA) on the steroid hormone response of male rats, particularly the dramatic increase in estrogens and decrease in androgens, induced by Endo.
The concentrations of corticosterone (B), progesterone (P4), 17α hydroxy-progesterone (17αOHP4), androstenedione (Δ4), testosterone (T), estrone (E1) and estradiol (E2) were determined 2 hours after injection of increasing doses of 4OHA with and without Endo.
The increase in serum estrogen concentrations and drop in serum androgen levels in response to Endo were blocked by a single dose of 4OHA. The effect of 4OHA appeared to be dose dependent. Low doses (30 mg/kg and 50 mg/kg) induced significant changes in the estrogen and androgen responses, but the high dose (100 mg/kg) blocked all changes in sex steroids induced by Endo. 4OHA did not alter the Endo-induced changes in other steroids.

The circulating concentration of estrone increases dramatically in men during septic shock, while that of serum testosterone decreases (1, 2). These changes in sex hormone concentrations have been confirmed experimentally in intact adult male rats acutely treated with non-lethal, non-hypotensive doses of Escherichia coli endotoxin (Endo), a constituent of the bacterial cell wall released during septic shock (3). Acute injection of male rats with 2 mg/kg Endo induces within 2 hours a very large increase in estrogen, particularly estradiol, and a parallel fall in testosterone (3). Adrenalectomized or orchidectomized male rats do not show these hormonal changes in response to Endo (3). These results suggest that the sex steroid hormone response to acute Endo

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injection involves adrenal-testicular cooperation and potentiation of adrenal and/or testicular androgen aromatisation. The adrenal-testicular cooperation was confirmed by studies on the hormonal changes in male rats during development (4). The lack of change in serum estrogen concentrations in young 8-week-old rats in response to Endo may be due to the relative immaturity of the gonads in these animals.

The present study confirms that the high estrogen levels observed during Endo agression result from potentiation of aromatase activity. Further investigations are needed to determine the effect, particularly on lethality, of this aromatase inhibitor during Endo agression. A knowledge of the biological role of the high estrogen and low androgen levels, and the normalization of these steroid levels by 4OHA, may well form the basis for a new therapeutic approach to septic shock.

Methods

Animal treatments

Ninety eight intact male Wistar rats, 12-weeks old weighing 305 ± 10 g, were randomly assigned to one of four groups. Group I, 29 rats were given vehicle alone ; group II, 17 rats were given 4OHA alone (5, 6 or 6 rats were given respectively with 30, 50 or 100 mg/kg of 4OHA) ; Group III, 25 rats were given Endo (2 mg/kg) alone and group IV, 27 rats were given 4OHA (10, 10 or 7 rats were given respectively with 30, 50 or 100 mg/kg of 4OHA) followed by a single injection of Endo (2 mg/kg).

4 hydroxyandrostenedione and endotoxin administration

4OHA was dissolved in propylene glycol and given at 30, 50 or 100 mg/kg S.C. (0.15 to 0.20 ml propylene glycol per rat) alone (group II) or 5 min. before injection of Endo (group IV).

Escherichia coli endotoxin (0127 B8 Sigma) was dissolved in saline and administred (2 mg/kg I.V.) at 10 a.m. (groups III and IV). This dose of Endo was selected as it induces major steroid hormone changes. None of the rats died or developed the shock syndrome of systemic hypotension within the 4 hours of the experiment when this dose of Endo was used (5). Control rats (group I) were given vehicle alone (0.9% saline, 1.0 ml/kg and/or propylene glycol 0.5 ml/kg).

Blood sampling

Rats were anesthetised with urethane (1.2 g/kg i.p.) 2 h after injection of vehicle alone, 4OHA with or without Endo. This anesthetic is without effect on the response to Endo (4). Rats were bled and killed 2 to 5 min. after anesthesia.

A single blood sample was taken from each animal to minimize hemodynamic changes resulting from repeated stress or blood loss. The samples were allowed to coagulate and the serum was separated by centrifugation (1500 g for 10 min. at 4°C) and stored at -20°C until assayed.

Steroid extraction and chromatographic fractionation

1. Serum samples (1 ml) were extracted for 30 min. with 5 ml of a solvent mixture (ethyl acetate / cyclohexane, 1/1) and the aqueous phase was removed by freezing (-20°C). The organic phase was evaporated to dryness, taken up in 1 ml of solvent system I (benzene/ethanol, 95/5) and placed on a Sephadex LH20 microcolumn (0.5x6 cm). Progesterone (P4) was first eluted with 2.6 ml of solvent I. Estrone (E1) and corticosterone (B) were then eluted with 3.5 ml of solvent I followed by 1.0 ml of solvent II (benzene/ethanol, 90/10). Finally, estradiol (E2) was eluted with 6 ml of solvent II.

2. Serum samples (0.5 ml) were extracted as above and placed on a Sephadex LH20 microcolumn : 17α hydroxyprogesterone was eluted with 5.5 ml of solvent I.

The fractions were evaporated to dryness and dissolved in RIA buffer for steroid hormone assays.The yields from these extraction and purification steps were between 70 and 95%.

Radioimmunoassay (RIA) of steroids

Samples of E1, E2, P4, 17α OH P4, B were assayed using rabbit antisera from Miles, Yeda Ltd, Israel (anti estrone 6-thyroglobulin serum, anti-17β-estradiol-6-BSA serum, anti-17α-hydroxyprogesterone-7-BSA serum, anti-corticosterone-21 thyroglobulin serum) and rabbit antisera from Biosys, France (anti-progesterone-11HS-BSA). The detection limit was 18 pmol/L in all cases.The tritiated steroids 2,4,6,7^{3}H E2 (100 Ci/mmol) ; 2,4,6,7^{3}H E1 (91 Ci/mmol) ; 1,2,6,7^{3}H P4 (82 Ci/mmol) ; 1,2,6,7^{3}H B (85 Ci/mmol), 1,2,6,7^{3}H 17α OH P4 (56 Ci/mmol) were purchased from the Radiochemical Center, Amersham. All were 99% pure ; purity was determined by thin layer chromatography. Radioactivity was determined on samples dissolved in 4 ml PCSII (Amersham) by counting in Packard 1500 liquid scintillation analyzer using the internal standard for quench correction.

Determination of androstenedione and testosterone by isotope dilution-gas chromatography-mass spectrometry

Isotopic internal standards [19,19,19-^{2}H$_3$] androstenedione (0.5 ng) and [3,4-^{13}C$_2$] testosterone (2 ng) were added to 0.5 ml plasma to obtain a ratio of labeled to native compound close to 1. When this ratio was higher than 2, a second assay was performed with a reduced sample size. Equilibration (1 h. at room temperature) preceded extraction (3 ml hexane/diethyl ether, 4/1) and chromatography on columns (20 x 0.5 cm) of Sephadex LH20 with dichloromethane/methanol/acetic acid (95/5/1) as the mobile phase. The first 2.5 ml were discarded and the next two 1 ml-fractions

contained androstenedione and testosterone, respectively. The heptafluorobutyrate (HBF) derivatives were injected into a fused silica capillary column (25 m x 0.32 mm), coated with 0V-1701 stationary phase, and directly coupled to the source (electron impact mode) of a quadrupole mass spectrometer (R 10. 10 T, Delsi Nermag Instruments, France). Selected ion monitoring was performed at the nominal masses of the molecular ions of androstenedione-HBF (482 and 485) and testosterone -bis HBF (680 and 682). Concentrations were calculated according to equations outlined previouly (6).

Accuracy was tested by adding standard to samples of pooled plasma and the inter-assay coefficient of variation was 3.3%.

Statistics

All data were analyzed by analysis of variance (ANOVA) followed by the Duncan Kramer test (7, 8). Results were considered significant when the probalities were :
* $p<0.05$; ** $p< 0.01$; *** $p<0.001$

Results

Effect of 4 hydroxyandrostenedione treatment on non endotoxin-injected male rats.

Serum hormone levels were measured 2 hours after injection of single doses (30 or 50 or 100 mg/kg) of 4-OHA or the propylene glycol vehicle (control). The results obtained are shown in Fig. 1, Fig. 2 and Fig. 3 (open columns).

Increasing doses of 4OHA produced no significant changes in serum B, P4, Δ4, T, E1 and E2. But there was a significant increase in 17αOHP4 concentration after administration of the 50 mg/kg (35%, $p<0.05$) and 100 mg/kg (3 fold, $p<0.001$) doses of 4OHA.

Effect of 4 hydroxyandrostenedione on endotoxin-injected male rats.

Corticosterone and progestins

The serum B, P4 and 17αOHP4 concentrations of untreated and 4OHA-treated rats injected with Endo or vehicle are shown in Fig. 1.
The serum B and P4 concentrations were elevated 3-4 fold ($p<0.001$) in all endotoxin-injected rats.The 17αOHP4 levels were 2 fold ($p<0.001$) and 3-4 fold ($p<0.001$) higher in the untreated and 4OHA-treated (30 mg/kg) endotoxin-injected rats that in the controls. High doses of 4OHA (50 mg and 100 mg/kg) blocked the increase in serum 17αOHP4 induced by Endo.

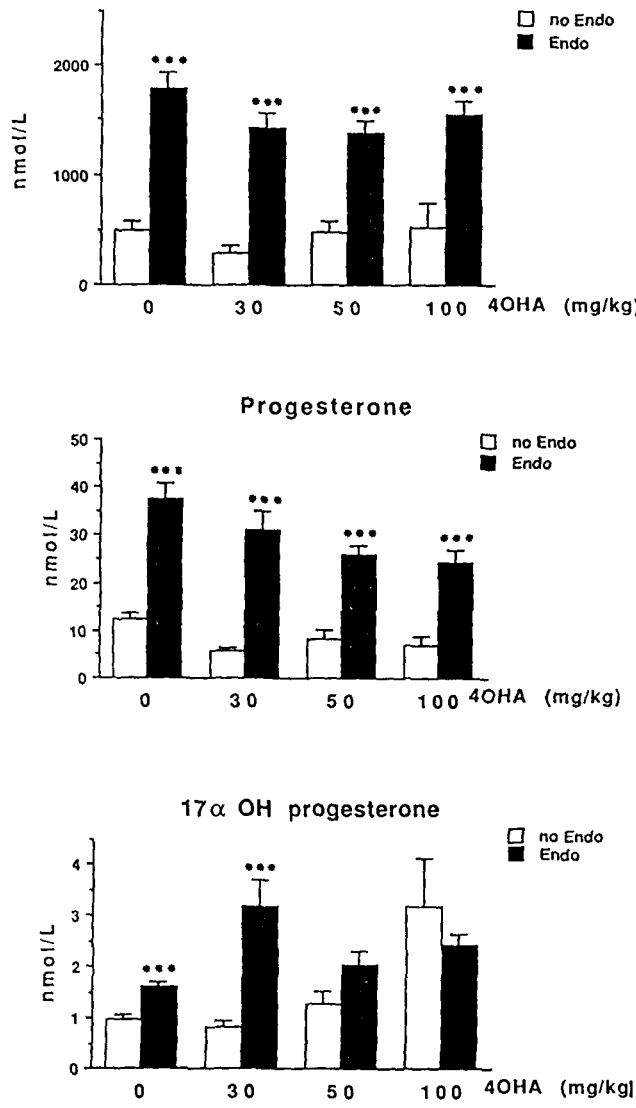

Fig. 1.

Corticosterone, progesterone and 17α hydroxyprogesterone levels of untreated and 4 hydroxyandrostenedione (4OHA) treated male rats 2h after injection with a non-lethal (2 mg/kg) dose of Endo. Mean (±SEM) serum concentrations were determined by RIA.*** $p<0.001$

Androgens and estrogens

Fig. 2 shows the serum concentrations of Δ4 and T in untreated or 4OHA-treated male rats injected with Endo or its vehicle.

The serum of Δ4 and T levels of rats not given 4OHA decreased 2-3 fold ($p<0.001$) after Endo injection. In contrast, 4OHA blocked the Endo-induced drop in serum Δ4 and T concentrations, except at the lowest (30 mg/kg) dose.

The changes in serum E1 and E2 are shown in Fig. 3. The E1 level doubled ($p<0.001$) and E2 increased 7-8 fold ($p<0.001$) in untreated rats after Endo injection. 4OHA treatment inhibited the Endo- induced increase in estrogen. This effect of 4OHA appeared to be dose-dependant; 100 mg/kg of 4OHA completely blocked the Endo-induced increase in serum estrogen.

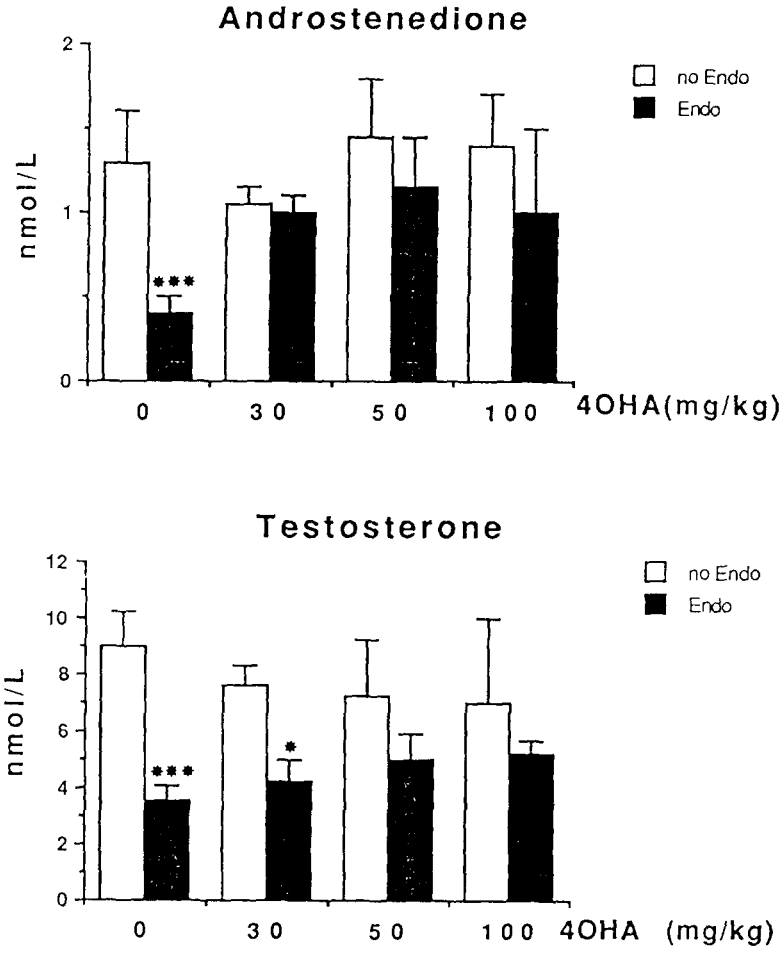

Fig. 2.

Androstenedione and testosterone levels of untreated and 4 hydroxyandrostenedione (4OHA) treated male rats 2h after injection with a non-lethal (2 mg/kg) dose of Endo. Mean (±SEM) serum concentrations were determined by isotope dilution- gas chromatography-mass spectrometry * $p<0.05$ *** $p<0.001$

Discussion

These results indicate that treating 12-week-old male rats with the aromatase inhibitor, 4OHA, modifies only the estrogen and androgen response to acute injection of non-lethal doses of Endo. The large increase in serum estrogen concentrations and the drop in serum androgen in response to Endo were blocked by a single dose of 4OHA.

The effect of 4OHA appears to be dose dependent. The lower doses (30 and 50 mg/kg) induced significant changes in the estrogen and androgen responses, but the high dose (100 mg/kg) blocked all changes in sex steroids induced by Endo.

Previous studies have shown that the estrogen response to Endo is due to adrenal-testicular cooperation and stimulation of testicular aromatase activity (3). The adrenal-testicular interaction is complete in 12 week-old-male rats (4). The lack of increase in serum estrogen concentrations in 4OHA- treated male rats after Endo injection reflects a low aromatase activity, due to the 4OHA treatment.

In normal adult men, the major sources of estrogen include conversion of androstenedione and testosterone to estrone and estradiol via aromatization in muscle and adipose tissue (9, 10) and direct secretion by the adrenals (11) and testes (12). This conversion is carried out by the microsomal cytochrome P-450-dependant enzyme system, generally referred to as aromatase. The largest group of aromatase inhibitors studied to date are steroid substrate analogues (13). Of these, androstenedione derivatives are the most effective (14, 15) and 4OHA is a potent aromatase inhibitor (16-18). It causes rapid competitive inhibition followed by irreversible inactivation of aromatase (14). In vitro, 4OHA inhibits ovarian estrogen synthesis in rats (17, 19) and peripheral aromatization in rhesus monkeys (20). The response of postmenopausal patients with advanced metastatic breast cancer to 4OHA-treatment indicates that it inhibits peripheral aromatization (21- 24).

The lack of change in the serum estrogen concentrations of 4OHA-treated rats in response to Endo indicates that the high estrogen levels result from potentiation of aromatase activity by Endo. The aromatase activity may be stimulated by elevated glucocorticoids (25-27) induced by Endo (2, 3, 28).

The decrease in serum $\Delta 4$ and T induced by Endo in male rats was blocked by 4OHA, confirming it as an effective aromatase inhibitor. This interpretation is supported by the fact that overestimation of $\Delta 4$ and T in the presence of pharmacological doses of 4OHA can be ruled out here. Indeed, serum $\Delta 4$ and T were assayed by a reference analytical method based on stable isotope dilution plus high resolution gas chromatography-mass spectrometry. Similar results have been obtained with the nonsteroid inhibitor of aromatase activity, CGS 16949A (29).

4 OHA did not alter the concentrations of P4 and B in either untreated or Endo-treated rats.Thus, 4OHA acts specifically on aromatase even when Endo is given. This result also indicates that the bacterial toxin acts on both P4 and B synthesis and on the conversion of androgens to estrogens.

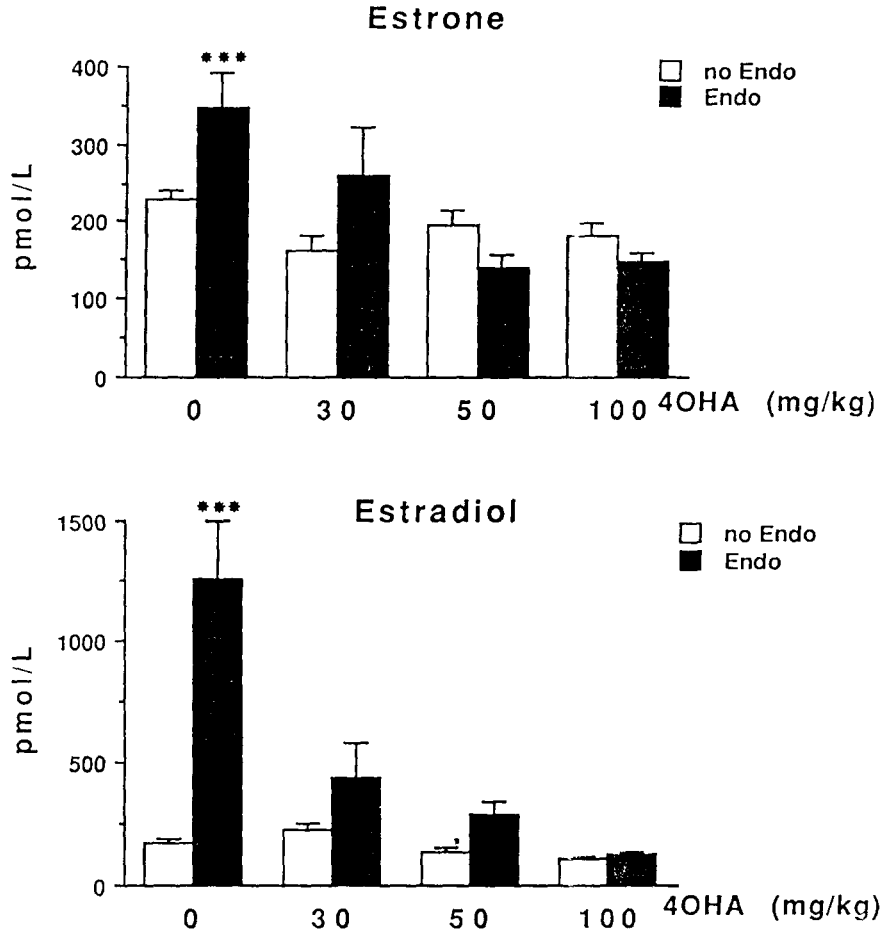

Fig. 3

Estrone and estradiol levels of untreated and 4 hydroxyandrostenedione (4OHA) treated male rats 2h after injection with a non-lethal (2 mg/kg) dose of Endo. Mean (±SEM) serum concentrations were determined by RIA.*** $p<0.001$

The Endo-induced increase in 17αOHP4 was blocked by high dose (100 mg/kg) of 4OHA.The rise in serum 17αOHP4 following injection of high doses of 4OHA and the lack of difference between the serum 17αOHP4 levels of control and Endo-injected rats require further investigation to determine the way in which 17αOHP4 metabolism in the adrenals and testes is blocked.

These results confirm that the high estrogen levels observed during Endo agression result from direct stimulation of aromatase activity in various tissues. This indicates that 4OHA could be useful in studies on Endo or not agressed animals to determine the role of various steroids (progestins, glucocorticoids, androgens) in male physiology. These experiments will also help elucidate the respective roles of the adrenals and testes in

the steroid response to Endo. Parallel studies of the relationship between Endo, aromatase and steroid hormones in females could provide additional insight into the female response to toxin agression. Further investigations are needed to determine the effect, particularly on lethality of this aromatase inhibitor during Endo agression. A knowledge of the biological role of the high estrogen and low androgen, and the normalization of these steroid levels by 4OHA may well form the basis for a new therapeutic approach to septic shock.

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