TESTOSTERONE-INDUED FOCAL MYOCARDITIS IN RATS II.
MORPHOLOGICAL AND BIOCHEMICAL OBSERVATIONS OF THE ADRENAL IN RELATION TO THE PATHOGENETICAL MECHANISM OF CARDIAC LESIONS

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Abstract……Correlative morphological and biochemical studies were made on the adrenal of rats treated with daily subcutaneous injections of testosterone propionate in a dose of 50 mg/kg for 30 consecutive days.

Histologically, there is a thinning of the zona fasciculata with a decrease of cell numbers, swelling of the cell body and the occurrence of large lipid droplets in the cytoplasm. Electron microscopic examination revealed swelling of mitochondria with reduction of cristae and hypertrophy of smooth-surfaced endoplasmic reticulum with cluster formation or whirl-like arrangement. In in vitro biochemical experiments, it was noted that conversion of deoxycorticosterone to corticosterone or 18-hydroxydeoxycorticosterone was decreased to 45% of the control level in the testosterone-treated group.

Keyword: Testosterone, deoxycorticosterone, 11β-hydroxylation, adrenal cortex, mitochondria, smooth-surfaced endoplasmic reticulum.

INTRODUCTION

In a previous report (Imai et al 1978), it was shown that consecutive administrations of testosterone propionate to rats could induce focal myocarditis in association with elevation of deoxycorticosterone in blood plasma. It was also shown that similar cardiac lesions were produced in rats by administration of large amounts of deoxycorticosterone acetate. In addition, myocarditis did not appear in adrenalectomized rats after treatment with testosterone while repeated treatment with deoxycorticosterone acetate invariably produce cardiac lesions both in adrenalecto-
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mized and sham-operated rats.

From these results, the possibility was raised that in relation to the pathoge-
netical mechanism of myocarditis by testosterone, administration of a large amount
of androgen to rats interfered with adrenocortical steroidogenesis in some fashion,
including high blood levels of deoxycorticosterone, which eventually affected the
myocardium. To ascertain this possibility, correlative morphological and biochemical
studies were made on the adrenals from rats after consecutive administration of
testosterone propionate.

MATERIALS AND METHODS

Five-week-old male Sprague-Dawley rats, weighing around 100 g, were used.
Testosterone propionate was dissolved in sesame oil in a concentration of 5 %, and
subcutaneously injected into ten rats in a dose of 50 mg/kg for 30 consecutive days.
As the control, 10 additional rats were administered 0.1 ml/kg of sesame oil for 30
days. On the 31st day, the adrenals were removed from each animals under
Nembutal anesthesia for morphological and biochemical examinations.

Morphological examination: Small pieces of the left adrenal cortex were fixed
in phosphate-buffered 3% glutaraldehyde for one hour and post-fixed in phosphate-
buffered 2% osmium tetroxide for 30 minutes. These samples were then dehy-
drated through a series of graded alcoholic concentrations and embedded in Epon
812. Thick and thin sections were cut on a porter-blum MT-2-B ultramicrotome.
Thick sections were stained with 0.5% toluidine blue for light microscopy. Thin
sections were stained with lead citrate-uranium acetate and observed with a HS-9
electron microscope. Remnant pieces of the left adrenal were fixed in 10% formalin
for histological examinaton.

Biochemical examination: Samples of the right adrenal were homogenized in
0.05 M tris-buffered 0.25 M sucrose (pH 7.4) and centrifuged at 600 G for 10 minutes.
The supernatant fraction was kept at 0°C and used as an enzyme source to evaluate
the activity of corticosteroidgenesis. The supernatant samples of an amount equiva-
 lent to 30-50 mg wet weight of the adrenal tissue were incubated for one hour at
37°C with 250 µg of pregnenolone or progesterone in a medium consisting of 0.1 M
ethanol and 3 ml of 0.05 M tris-buffered 0.25 M sucrose (pH 7.4) which contained
0.5 mg of NADP, 3 mg of sodium glucose-6-phosphate, 1 unit of glucose-6-phosphate
dehydrogenase, 40 µ moles of MgSO₄, 5 mg of bovine albumin and 3.5 mg of sodium
malate. After incubation, the medium was shaken twice with 20 ml and 10 ml of
ethyl acetate, respectively. The ethyl acetate fractions were combined together,
washed with 3 ml of distilled water, dehydrated with NaSO₄ and evaporated in
vacuo. The dried materials were dissolved in dichloromethane, transferred to Silica
gel GF thin layer plates and developed with benzen-acetate (3:1). Spots of each
steroid were marked under a U.V. absorption at 220-260 m.
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RESULTS

Morphological changes of the adrenal:

The adrenals of treated rats did not exhibit any remarkable gross changes other than a weight decrease when compared to adrenals of control rats. In histological examination, these was a slight thinning of adrenal cortex with a moderate atrophy of glomerulosa cells and a decrease in the number of fasciculata and reticularis cells. In the zona glomerulosa, lipid droplets appeared to be depleted. In the transitional zone between the zona glomerulosa and zona fasciculata, most of the cells appeared cuboidal, exhibited an eosinophilic and slight granular cytoplasm and lacked lipid droplets (Fig. 2).

Zona fasciculata cells were swollen and shown to contain large lipid droplets in the cytoplasm (Fig. 3). These findings were especially prominent in the area adjacent to the zona reticularis, where the greater part of the cytoplasm of almost all of the cells appeared to be occupied by large lipid droplets and the nucleus was dislocated to the cellular periphery. Cell cords were distorted, and sinusoidal spaces were narrowed. Zona reticularis cells had decreased both in size and number.

No remarkable changes were noted in the medulla.

Electron microscopic examination revealed an increase of atrophic clear cells

Fig. 1. A: An area of the adrenal cortex from a control rat, B: An area of the adrenal cortex from a rat administered \(50\text{mg/kg}\) of testosterone propionate for one month. In the testosterone treated rat, thinning of adrenal cortex is noted. In this rat, large lipid droplets are noted in an area adjacent to the zona reticularis. (Hematoxylin-Eosin ×80)
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Fig. 2. An area of the adrenal cortex from a rat administered 50mg/kg of testosterone propionate for one month. The zona glomerulosa cells appear to be atrophic and lipid droplets have decrease in these cells. In the transitional zone between the zona glomerulosa and zona fasciculata, most of the cells appear cuboidal in shape. (Toluidine blue ×320)

Fig. 3. An area of the adrenal cortex from a rat administered 50mg/kg of testosterone propionate for one month. Zona fasciculata cells are swollen and shown to contain large lipid droplets in the cytoplasm. (Toluidine blue ×320)

characterized by a moderate depletion of lipid droplets and a slight decrease of ribosomal granules in the zona glomerulosa. In these cells, smooth-surfaced endoplasmic reticulum appeared to have decreased, and mitochondria were swollen in association with bulging of the cristae (Fig. 4).

In the zona fasciculata cells, lipid droplets were larger in size and had decreased in electron density (Fig. 5). Whorled membranous bodies (Rhodin 1971) with a myelin-like lamellar structure occurred in connection with the outer membrane of the mitochondria or the limiting membrane of large lipid droplets (Fig. 8),...
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Fig. 4. An electron micrograph of the glomerulosa cells in the adrenal cortex from a rat administered 50mg/kg of testosterone propionate for one month. Depletion of lipid droplets and a decrease of ribosomal granules have noted in the glomerulosa cells. Smooth-surfaced endoplasmic reticulum appear to decreased and mitochondria are swollen in association with the bulging of cristae in these cells. ×16,000

Fig. 5. An electron micrograph of the fasciculata cells in the adrenal cortex from a rat administered 50mg/kg of testosterone propionate for one month. Extremely large lipid droplets are noted in cytoplasm of the fasciculata cells and mitochondria appear to be swollen in association with a decrease of cristae. ×9,000

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Fig. 6. An electron micrograph of the fasciculata cells in the adrenal cortex from a rat administered 50 mg/kg of testosterone propionate for one month. A hyperplasia of smooth-surfaced endoplasmic reticulum is noted surrounding in several to a greater number of layers a large lipid droplet and mitochondria. Mitochondria are swollen showing a decrease of cristae and a diminution of electron density in the matrix. ×22,900

as well as, inside the lipid droplets. Mitochondria were swollen and their cristae were decreased in number and shortened in length (Fig. 5). The mitochondrial matrix was decreased in electron density, with large spherical inclusion bodies of high electron density sometimes being recognized therein (Fig. 7). These inclusions were more numerous in the transitional zone between the zona glomerulosa and the zona fasciculata. In the testosterone-treated group, an increase of smooth-surfaced endoplasmic reticulum was also seen surrounding in several to a greater number of layers the mitochondria and lipid droplets (Fig. 6 and 8). Hypertrophy of smooth-surfaced endoplasmic reticulum showing cluster formation or whirl-like arrangement was also noted at several distinct locations in the cytoplasm (Fig. 9 and 10). The Golgi complex was well developed and lysosomal granules appeared to have increased in number around the Golgi complex. Occasionally, necrosis of fasciculata cells with condensation of nuclear components was noted in the area adjacent to the zona reticularis. Nuclear debris were also noted in the sinusoidal spaces of the inner fasciculata zone (Fig. 11). Zona reticularis cells appeared to be slightly atrophic in association with a moderate decrease of lipid droplets. Smooth-surfaced endoplasmic reticulum appeared vesicular in these cells and numerous myelin bodies were also noted (Fig. 12).

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Fig. 7. An electron micrograph of the fasciculata cells in the adrenal cortex from a rat administered 50 mg/kg of testosterone propionate for one month. Large spherical inclusion bodies of high electron density are noted in the mitochondria. ×6,900

Fig. 8. An electron micrograph of the fasciculata cells in the adrenal cortex from a rat administered 50 mg/kg of testosterone propionate for one month. Whirled membranous bodies are noted in connection with the outer membrane of the mitochondria and the limiting membrane of the lipid droplets, and inside the lipid droplets. ×21,000
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Fig. 9. An electron micrograph of the fasciculata cells in the adrenal cortex from a rat administered 50 mg/kg of testosterone propionate for one month. Hyperplasia of smooth-surfaced endoplasmic reticulum showing cluster formation or whirl-like arrangement is noted at several distinct locations in the cytoplasm. Lysosomal granules appear to have increased in number around the Golgi complex. ×16,000

Fig. 10. An electron micrograph of the fasciculata cells in the adrenal cortex from a rat administered 50 mg/kg of testosterone propionate for one month, showing a whirl-like arrangement of hypertrophic smooth-surfaced endoplasmic reticulum. ×45,800

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Fig. 11. An electron micrograph of the fasciculata cells in the adrenal cortex from a rat administered 50 mg/kg of testosterone propionate for one month. Necrosis of fasciculata cells is noted in association with condensation of the nuclear component. ×6,900

Fig. 12. An electron micrograph of the reticularis cells in the adrenal cortex from a rat administered 50 mg/kg of testosterone propionate for one month. Smooth-surfaced endoplasmic reticulum appear vesicular and myelin bodies are noted in association with a decrease of lipid droplets. ×6,900
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Table 1. Corticosteroid production in adrenal homogenate of rats administered testosterone propionate after incubation with progesterone

<table>
<thead>
<tr>
<th>Group</th>
<th>Adrenal Weight (mg)</th>
<th>Product (n moles/100 mg tissue/hr)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prog. DOC Comp. B 18-OH-DOC Δ^4-3-one 21-OH</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>46.50±1.31*</td>
<td>83 390 149 109 731 648</td>
<td></td>
</tr>
<tr>
<td>TP 50 mg/kg</td>
<td>45.05±1.42*</td>
<td>246 389 65 44 744 498</td>
<td></td>
</tr>
</tbody>
</table>

Prog.: progesterone  DOC: deoxycorticosterone  Comp. B: corticosterone  18-OH-DOC: 18-hydroxydeoxycorticosterone

* Mean±standard error

Table 2. Corticosteroid production in adrenal homogenate of rats administered testosterone propionate after incubation with progesterone

<table>
<thead>
<tr>
<th>Group</th>
<th>Product (n moles/100 mg tissue/hr)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DOC Comp. B 18-OH-DOC 11-OH-Prog.</td>
<td>11-OH 21-OH</td>
</tr>
<tr>
<td>Control</td>
<td>640 133 92 51</td>
<td>184 866</td>
</tr>
<tr>
<td>TP 50 mg/kg</td>
<td>458 53 36 0</td>
<td>53 574</td>
</tr>
</tbody>
</table>

As shown in Table 1, the total amount of Δ^4-3-ketosteroids between the control group and the testosterone-treated group was comparable. In regard to each steroid's amount, progesterone was higher in the treated group and deoxycorticosterone was comparable between the two groups, while corticosterone and 18-hydroxydeoxycorticosterone were lower in the treated group. As shown in Table 2, the corticosterone level was also estimated to be lower in the treated group than in the control group when progesterone was used as the substrate. All these date indicate that testosterone treatment results in inhibition of the 11β-hydroxylation and 18-hydroxylation.

DISCUSSION

We have previously shown that repeated administration of testosterone to rats can induced focal myocarditis accompanied by an increase of the blood deoxycorticosterone level (Imai et al. 1978) and that there may be a causal relationship between these two phenomena. We have also shown that adrenalectomy can protect rats from such effects of testosterone. From these results, it is postulated that testosterone primarily interferes with the steroid metabolism in the adrenal cortex to induce a high blood level of deoxycorticosterone which secondarily affects the myocardium.

This possibility can be supported by the present findings which indicate that testosterone treatment resulted in a significant inhibition of 11β-hydroxylation activity and 18-hydroxylation activity in the adrenal cortex concomitant with morphological changes of fasciculata cells such as decrease in cell numbers, swelling of mito-
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Mitochondria and hypertrophy of smooth-surfaced endoplasmic reticulum.

Biochemical effects of androgen on the adrenal cortex have been reported by several authors. Kitay et al (1963) showed a decrease of corticosterone secretion in rats by administration of large amount of testosterone phenylacetate. Sharma et al (1963) indicated, in in vitro experiments, that dehydroepiandrosterone inhibits 11β-hydroxylation of deoxycorticosterone in the bovine adrenal cortex. Colby et al (1970) and Skeleton et al (1969) noted that administration of testosterone or methyltestosterone to uninephrectomized rats results in a decrease of 11β-hydroxylation activity and reduction of mitochondrial p-450 content in the adrenal cortex. All these data are quite consistent with the present findings.

The present electron microscopic study showed that two kinds of ultrastructural alterations occurred in adrenal cortical cells: Mitochondrial alterations characterized by swelling, reduction of cristae or diminution of electron density in the matrix and alterations of smooth-surfaced endoplasmic reticulum such as hypertrophy, cluster formation or whirl-like arrangement. Dodge et al (1970) demonstrated that the 11β-hydroxylation system of steroids was localized in the inner membrane fraction of mitochondria in the adrenal of rats. Therefore, the present electron microscopic findings of mitochondria might be related to the reduction of 11β-hydroxylation activity shown in the biochemical examination. In a study of adrenal regeneration hypertension in rats, Nickerson et al (1969) also demonstrated that similar alterations of mitochondria occur in the adrenal cortical cells in association with reduction of 11β-hydroxylation activity and decrease of mitochondrial p-450 content. In regard to the biochemical function of the smooth-endoplasmic reticulum of adrenal cortical cells, although still debated in detail, it is known that the steroidal 3β-dehydrogenase is localized in this membrane structure (Rhodin 1971 and Frübling 1976). Therefore, the hypertrophy of this membrane system shown in our experiment might be related in part to the increase of 3β-dehydrogenation activity which could be deduced from the biochemical data that indicated the production of progesterone from pregnenolone was significantly higher in the testosterone-treated group than in the control group (Table 1).

In our experiments, large lipid droplets were frequently noted in fasciculata cells of the adrenal in testosterone-treated rats. Selye et al (1950) and Roy et al (1964) also noted similar morphological alterations in the adrenal cortex of rats treated with testosterone propionate or methyltestosterone. At present, however, it is uncertain whether such droplets indicate a type of degenerative process or represent a cellular function such as the storage of metabolites.

SUMMARY

We have previously shown that repeated administration of testosterone to rats induced focal myocarditis in association with elevation of the deoxycorticosterone
level in blood plasma and that adrenalectomy can prevent rats from experiencing such effects of testosterone. The present study was attempted to examine the primary effects of testosterone on the adrenal in rats.

Five-week-old male Sprague-Dawley rats were treated with daily subcutaneous injections of testosterone propionate in a dose of 50mg/kg for 30 consecutive days, and the adrenal was subjected to histological, electron microscopic and biochemical examinations. Histologically, there was a thinning of the zona fasciculata with a decrease in cell number, swelling of the cell body and occurrence of large lipid droplets in the cytoplasm. Electron microscopic examination revealed mitochondrial alterations of fasciculata cells such as swelling, reduction of cristae and diminution of the electron density of the matrix. Hypertrophy of the smooth-surfaced endoplasmic reticulum with cluster formation or whirl-like arrangements was also noted in these cells. In in vitro biochemical experiments in which the adrenal homogenate was incubated with either progesterone or progrenolone, it was found that 11β-hydroxylase activity and 18-hydroxylation activity were significantly decreased in the testosterone-treated group as compared to the control group.

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