Peripheral Serum Corticosteroid Concentrations in Relation to the Rat Adrenal Cortical Circadian Rhythm in Androgen-Induced Hypertension

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SUMMARY Adrenal function was assessed in control rats and in rats treated for 2 and 4 weeks with 17α-methylandrosterol (MAD; 17α-methyl-5-androstene-3β,17β-diol), a synthetic androgen known to produce hypertensive cardiovascular disease. In both groups and at both time periods, a circadian rhythm of blood corticosteroid concentrations was observed. The high point for serum corticosterone (C), 18-hydroxy-11-deoxycortisol (18-hydroxy-DOC), and 11-deoxycorticosterone (DOC) concentrations occurred at the beginning of the dark period (1800 hours), and the low point occurred at the onset of the light period (0600 hours). Serum concentrations of DOC were always found to be higher in MAD-treated rats as compared with controls. The serum concentrations of C and 18-hydroxy-DOC were lower than control values at 1800 hours but higher than control concentrations at 0600 hours. The in vitro 11β- and 18-hydroxylation of DOC was markedly reduced with MAD treatment. In contrast, cholesterol side-chain cleavage activity was higher in animals treated with MAD. These in vitro findings correlated with spectral studies that showed a decreased binding of DOC to cytochrome P450 and increased binding of cholesterol to cytochrome P450. These studies suggest that MAD treatment selectively decreases 11β- and 18-hydroxylation in adrenal mitochondria, and this results in an increased serum concentration of DOC, a hypertensinogenic steroid. This effect of MAD on peripheral serum DOC concentration is most readily observed in quiescent animals at the high point of the circadian rhythm. (Hypertension 2: 617-622, 1980)

KEY WORDS • androgen-induced hypertension • adrenal cortex • serum corticosteroids • 17α-methylandrosterol • circadian rhythm

SKELTON1 found that hypertensive cardiovascular disease results from chronic treatment of rats with the synthetic androgen, 17α-methylandrosterol (MAD; 17α-methyl-5-androstene-3β,17β-diol). An important observation that allows one to propose a mechanism for this form of experimental hypertension is that of Salgado and Selye2 who demonstrated that the adrenal gland had to be present for the development of hypertension. In addition, Brownie and Skelton3 found that steroid 11β-hydroxylase activity was low in adrenal homogenates and mitochondria from MAD-treated rats, leading to an accumulation of 11-deoxycorticosterone (DOC). MAD is converted to 17α-methyltestosterone (MT; 17α-methyl-4-androstene-17β-ol-3-one) in the adrenal4 and MT is similar to testosterone5 in being an inhibitor of the 11β-hydroxylation of DOC. This has led to general agreement that increased secretion of the hypertensinogenic steroid DOC6 is involved in the pathogenesis of MAD-induced hypertension. Indeed, there is evidence7 for increased secretion of DOC in the adrenal venous effluent of MAD-treated rats at the same time as there is decreased secretion of corticosterone (C)8 and 18-hydroxy-DOC.9 These studies in which adrenal venous blood was collected, however, of necessity involve rats subjected to the stress of anesthesia. Furthermore, effects of MAD upon other enzymes of the corticosteroid biosynthetic pathway have not been described. Accordingly, the present studies were designed to measure concentrations of DOC, 18-hydroxy-DOC, and B in peripheral blood of control and MAD-treated rats, with blood being collected under quiescent conditions and at 6-hour intervals during a 24-hour period in order to take into account the circadian rhythm of adrenal cortical activity.10 In addition, adrenal mitochondria were isolated for the determination of cholesterol side-chain cleavage activity as this enzyme catalyzes what is recognized as the rate-limiting step in corticosteroidogenesis.11,12

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Methods

Animals and Treatment

Male Sprague-Dawley rats weighing approximately 200 g (Holzman Co.; Madison, Wisconsin) were singly caged in three rooms with controlled temperature (22 ± 1°C) and lighting (fluorescent illumination from 0600-1800 hours). All rats were uninjected, given 1% NaCl as drinking solution, and fed Charles River Rat Chow that contained 0.40% sodium, ad libitum. The experimental groups received 10 mg MAD (Sigma), suspended in 0.2 ml corn oil, by subcutaneous injection 6 days per week. Control groups received the vehicle alone.

Blood Pressures

Systolic blood pressures were measured indirectly under light ether anesthesia with a Physiograph Four (E and M Instrument Co., Inc., Houston, Texas).

Collection of Rat Serum and Serum Steroid Assays

After 2 or 4 weeks of treatment, seven control and eight MAD-treated rats were killed by decapitation at each of the following times: 0600, 1200, 1800, and 2400 hours. Exposure to stress was minimized by handling the rats twice daily during the treatment period and by having five people enter each room in turn and begin blood collection within 12 seconds of entry and removal of the rat from its cage. Blood was individually collected from the trunk and allowed to clot on ice. The serum was separated by centrifugation and stored in the frozen state until assayed.

Corticosterone was measured by the direct radioimmunoassay (RIA) procedure of Gomez-Sanchez et al. following methylene dichloride extraction of a 0.1 ml aliquot of serum. Serum 18-hydroxy-DOC was measured by direct RIA according to the method of Gallant. Serum DOC concentrations were determined by RIA. The serum samples were extracted and then chromatographed in a solvent system of cyclohexane:methanol:water (100:50:25 v/v) by a similar method previously described. In this chromatography system, DOC had an Rf value of 0.29 and was well separated from MT, MAD, B, 18-hydroxy-DOC, and progesterone, which had Rf values of 0.46, 0.11, 0.01, 0.001 and 0.81 respectively. The cross-reactivities of these steroids with the DOC antibody were: MT (0.2%), MAD (0.1%), B (2%), 18-hydroxy-DOC (0.01%), and progesterone (95%).

Collection of Adrenal Tissue and Enzyme Assays

Adrenal zona fasciculata-reticularis tissue was obtained by adrenal enucleation in situ. Adrenal tissue was homogenized in cold buffered 0.25 M sucrose, and mitochondria were obtained by differential centrifugation. The concentration of adrenal mitochondrial protein used for each assay ranged between 1.5-2.5 mg/ml and was determined by the method of Lowry et al. Cytochrome P450 concentrations in the adrenal mitochondrial preparations were determined by using an Aminco DW2 Spectrophotometer by the method of Omura and Sato.

Cholesterol side-chain cleavage (CSCC) activity was determined by measuring the pregnenolone formed from endogenous cholesterol in adrenal mitochondria incubated at 37°C under air in the presence of cyanoketone, an inhibitor of sterol 3B-ol-dehydrogenase, and isocitrate as a source of reducing equivalents. The reaction medium contained 50 mM sucrose, 50 mM NaCl, 5 mM MgCl2, 5 mM KCl, and 100 mM MOPS (Morpholinopropan Sulphonic Acid, pH = 7.4; Calbiochem). The pregnenolone formed was measured by RIA.

Mitochondrial 11β- and 18-hydroxylase assays were performed in the same reaction medium described above; 60 μM DOC substrate as well as a source of reducing equivalents were added. The method of assay employed high-pressure liquid chromatography for simultaneous assay of 11β- and 18-hydroxylase activity.

Spectral Analyses

Adrenal cortical mitochondrial cytochrome P450 systems, consisting of P45011β and P45018, were examined by light absorption spectrophotometry. The extent of cholesterol association with P45018 was determined by the heat-generated Type I absorbance change (HGI) which develops as the mitochondria are warmed from 1°C to 37°C. An indirect measure of cholesterol association with P45018, the pregnenolone-induced Type II (reverse Type I) absorbance change (P11), was determined by adding saturating concentrations of pregnenolone to warmed adrenal mitochondrial samples. The extent of DOC binding to P45011β was measured by adding saturating amounts of DOC to warmed adrenal mitochondria and measuring the observed Type I absorbance change (DOC).

Results

Body Weights and Blood Pressures

Figure 1 shows the weekly mean systolic blood pressures and body weights of representative control and MAD-treated rats. Body weight increased more rapidly in the control rats. By 4 weeks, the blood pressures of the MAD-treated group were significantly greater than those of the controls (p < 0.05). Eight of 14 (57%) MAD-treated rats and only one of 13 (7%) controls became hypertensive by 7 weeks. Rats were considered hypertensive when systolic blood pressures were in excess of 150 mm Hg.

Peripheral Serum Steroid Determinations

Figure 2 shows the peripheral serum concentrations of DOC, 18-hydroxy-DOC, and B from quiescent control and MAD-treated male rats which were killed at 0600, 1200, 1800 or 2400 hours after 2 or 4 weeks of treatment. A circadian rhythm for B, 18-hydroxy-DOC, and DOC in the control rats at both 2 and 4
weeks is evident when the concentrations of these steroids at 1800 hours (lights off) are compared with those at 0600 hours (lights on). The low basal concentrations of all three steroids at 0600 hours in these control rats indicate that they were quiescent. The apparent increase of serum B concentrations after 4 weeks of treatment, above that observed after 2 weeks in both control and MAD-treated rats, may be the result of an age-related maturation of the circadian rhythm of plasma corticosterone concentrations.80

The MAD-treated rats also showed a circadian rhythm of B, 18-hydroxy-DOC, and DOC similar to that seen in the controls, with a high point at 1800 hours and a low point at 0600 hours. However, the absolute concentrations determined in the MAD-treated rats varied from those achieved by the controls. At 0600 hours, concentrations of DOC, B, and 18-hydroxy-DOC were higher in the MAD-treated rats after both 2 and 4 weeks. However at 1800 hours, B and 18-hydroxy-DOC concentrations were lower in the MAD-treated animals, but DOC concentrations remained higher than those of the control group. Even when the effects of stress are minimized, as they have been during these experiments, DOC concentrations were higher in the MAD-treated rats at all times studied, and were maximal at 1800 hours. The data indicate that these differences in serum steroid concentrations were magnified at 1800 hours.

Adrenal Mitochondrial Incubations

Mitochondrial 11β- and 18-hydroxylase and CSCC activities were compared in the quiescent control and MAD-treated rats which were killed at 0600, 1200, 1800 and 2400 hours after either 2 or 4 weeks (table 1). These data indicate that MAD treatment decreases by approximately the same proportion both 11β and 18-hydroxylase activities. When the CSCC activities were examined, a circadian rhythm was evident in both control and MAD-treated rats. Activities measured at 1800 hours were higher than those measured at 0600 hours in both groups at 2 and 4 weeks. Moreover, the MAD-treated rats showed a greatly increased rate of CSCC activity above control activity at 0600 and 1200 hours after 2 and 4 weeks. Although these data are expressed per nmole of cytochrome P450, similar differences between control and MAD groups are observed if CSCC activity is expressed as pregnenolone formed per mg protein.

Spectral Data

Cholesterol and DOC association with cytochrome P450 of adrenal mitochondria isolated from the various groups is shown in table 2. Cholesterol

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**Table 1. Effect of MAD Treatment on Adrenal Mitochondrial 11β- and 18-Hydroxylase and Cholesterol Side-Chain Cleavage Activities**

<table>
<thead>
<tr>
<th>Activity*</th>
<th>Rat</th>
<th>After 2 wks of treatment</th>
<th>After 4 wks of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0600</td>
<td>1200</td>
</tr>
<tr>
<td>11β-hydroxylase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>6.83</td>
<td>9.00</td>
</tr>
<tr>
<td>MAD</td>
<td></td>
<td>2.90</td>
<td>3.22</td>
</tr>
<tr>
<td>18-hydroxylase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>2.66</td>
<td>5.03</td>
</tr>
<tr>
<td>MAD</td>
<td></td>
<td>1.58</td>
<td>1.65</td>
</tr>
<tr>
<td>Cholesterol side chain cleavage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>0.08</td>
<td>0.18</td>
</tr>
<tr>
<td>MAD</td>
<td></td>
<td>0.28</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*Enzyme activities are expressed in nmole of product formed/minute/nmole P450. Each value represents the mean of duplicate assays; taken at 6-hour intervals during a 24-hour period.
FIGURE 2. Mean peripheral serum steroid concentrations of MAD-treated and control rats quiescently killed at the hours indicated either 2 or 4 weeks after the initiation of treatment. Data are expressed in μg/dl or ng/ml. Vertical lines indicate ± SE. Significance was determined by Student's t test. Asterisk (*) indicates p < 0.05; dagger (†) indicates p = 0.067.

TABLE 2. Cholesterol and DOC Association with Cytochrome P450 of Adrenal Mitochondria Isolated from Quiescent Control and MAD-treated Rats at Various Times of the Day*

<table>
<thead>
<tr>
<th>Time of Day (hrs)</th>
<th>HG I Control</th>
<th>HG I MAD</th>
<th>P II Control</th>
<th>P II MAD</th>
<th>DOC I Control</th>
<th>DOC I MAD</th>
<th>Cytochrome P450 (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 2 wks of treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0600</td>
<td>19.7</td>
<td>36.0</td>
<td>10.3</td>
<td>30.0</td>
<td>50.9</td>
<td>22.7</td>
<td>1.84</td>
</tr>
<tr>
<td>1200</td>
<td>21.2</td>
<td>48.1</td>
<td>13.7</td>
<td>41.2</td>
<td>41.6</td>
<td>20.6</td>
<td>1.75</td>
</tr>
<tr>
<td>1800</td>
<td>26.6</td>
<td>44.7</td>
<td>22.3</td>
<td>38.2</td>
<td>42.8</td>
<td>17.5</td>
<td>2.19</td>
</tr>
<tr>
<td>2400</td>
<td>36.2</td>
<td>55.3</td>
<td>29.8</td>
<td>50.0</td>
<td>44.7</td>
<td>27.6</td>
<td>2.01</td>
</tr>
<tr>
<td>After 4 wks of treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0600</td>
<td>24.0</td>
<td>47.9</td>
<td>15.3</td>
<td>42.7</td>
<td>50.0</td>
<td>24.0</td>
<td>2.06</td>
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<td>1200</td>
<td>21.6</td>
<td>34.9</td>
<td>10.8</td>
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<td>27.4</td>
<td>1.55</td>
</tr>
<tr>
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<td>48.0</td>
<td>22.7</td>
<td>46.0</td>
<td>45.5</td>
<td>24.0</td>
<td>1.83</td>
</tr>
<tr>
<td>2400</td>
<td>30.5</td>
<td>51.6</td>
<td>22.6</td>
<td>43.6</td>
<td>42.5</td>
<td>16.4</td>
<td>1.88</td>
</tr>
</tbody>
</table>

*HGI and DOCI are expressed as ΔA 390-420/nmole P450 × 10. PII is expressed as ΔA 420-390/nmole P450 × 10.
association with P450<sub>aceto</sub>, measured by HGI and PII assays on adrenal mitochondria, was increased in the MAD-treated groups above control values at all time periods studied. DOC binding to P450<sub>11β</sub>, measured by D OCI assays on adrenal mitochondria, on the other hand, was decreased in the MAD-treated groups at all time periods. Total adrenal mitochondrial P450 concentrations were reduced by about 40% after treatment with MAD.

Discussion

Previous studies suggest that following the injection into a rat of MAD, a Δ<sup>5</sup>-3β-ol-steroid, the corresponding Δ<sup>5</sup>-3-ketone, MT, is produced in sufficient quantities to inhibit steroid 11β-hydroxylation. This results in an increased secretion of DOC and, eventually, hypertensive cardiovascular disease. Another effect of chronic MAD treatment is a significant decrease in total adrenal mitochondrial cytochrome P450.<sup>31, 32</sup> This decrease has been related to the reduced activity of steroid 11β-hydroxylation. However, cytochrome P450<sub>aceto</sub> which is involved in the rate-limiting step of corticosteroidogenesis, CSCC, is also localized to the mitochondria of the adrenal cortical cell.<sup>30</sup> Our studies show that, in contrast to steroid 11β- and 18-hydroxylation, the activity of CSCC is not reduced by chronic treatment with MAD. Thus, we can now propose that the androgen treatment selectively decreases 11β- and 18-hydroxylation in adrenal cortical mitochondria and leads to an increase in the secretion of DOC.

In the present study we have shown for the first time that, compared to controls, MAD-treated rats have elevated plasma DOC concentrations. Furthermore, we have shown the advantage of carrying out measurements at the high point of the circadian rhythm when the concentrations of DOC achieved are much higher than those seen in quiescent rats killed in the morning at the start of the light period. At the high point of the circadian rhythm one can readily see the reduced ability of MAD-treated rats to maintain normal concentrations of corticosterone and 18-hydroxy-DOC whereas there are increased concentrations of DOC. These studies also show that despite alterations in corticosteroidogenesis brought about by treatment with MAD there is a circadian rhythm in DOC, 18-hydroxy-DOC, and B, with the highest values occurring near the start of the dark period. Apparently, these three corticosteroids are under similar control, with their presumed source being the zona fasciculata- reticularis.

There is some evidence that treatment of rats with androgen reduces ACTH secretion.<sup>44</sup> However, despite that, our studies show that a circadian rhythm of corticosteroids is retained in androgen-treated rats. In control rats there is an apparent circadian rhythm in CSCC activity with a maximum around 1800 hours and a minimum around 0600 hours. These results are similar to those in another report from our laboratory.<sup>31</sup> There is a less well-defined circadian rhythm in CSCC activity in the MAD-treated groups. This is related to a higher activity at 0600 and 1200 hours compared to controls. This higher rate of cholesterol side-chain cleavage in MAD-treated rats at 0600 and 1200 hours correlates with higher plasma B and 18-hydroxy-DOC concentrations in MAD-treated groups. At these times, when control animals are showing very low B and 18-hydroxy-DOC concentrations, DOC/B ratios are not significantly higher in MAD-treated rats at 2 weeks. However, when the 1800-hour blood samples are analyzed, it is clear that the DOC/B ratio is much higher in the MAD-treated rats than in controls. The advantage of measuring corticosteroid concentrations on blood samples withdrawn at or near the high point of the circadian rhythm has been shown by us for adrenal regeneration hypertension<sup>18</sup> and now this is also applicable to androgen-induced hypertension.

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