Expression of Adrenal Cytochromes P-450 in Testosterone-Induced Hypertension

Samuel Gallant, Josephine Alfano, Maud Charpin, and Alexander C. Brownie

Chronic treatment of rats with the naturally occurring androgen, testosterone, leads to hypertension and cardiovascular disease. This effect is believed to be mediated through the adrenal gland and in particular by action on the steroid 11β-hydroxylase enzyme system. To study the possible mechanism of this effect, the enzyme system was examined at several time periods up to the time that hypertension develops. Rats were treated with testosterone (10 mg/day) for 3, 7, 21, and 42 days. Levels of cytochrome P-450 enzyme and messenger RNA (mRNA) were determined as well as 11β-hydroxylase enzyme activity. A significant decrease in enzyme activity was observed after 3 days of treatment. This correlates with a profound decrease in the level of cytochrome P-450 enzyme as determined by Western blot analysis. A large decrease in cytochrome P-450 mRNA was also observed after 3 days of treatment. All three parameters remained low throughout the treatment period. The decrease in 11β-hydroxylase enzyme activity appears to result from a lower enzyme level brought about by decreased concentrations of mRNA transcripts. (Hypertension 1991;18:523-528)

It has long been recognized that chronic treatment of rats with androgens results in hypertensive cardiovascular disease.1 The primacy of the adrenal gland was suggested by Salgado and Selye,2 who showed that hypertensive disease failed to occur in adrenalectomized rats. These investigators also postulated that androgens induced adrenal dysfunction by interfering with the corticosteroid biosynthetic pathway in a way that resulted in excessive production of mineralocorticoid. Subsequent in vivo and in vitro studies supported this hypothesis.3-5 The major defect in androgen-treated animals is impaired 11β-hydroxylation of 11-deoxycorticosterone (DOC) to corticosterone.4-5 This results in the accumulation of DOC, a mineralocorticoid that is known to produce hypertension in rats.6,7

The mechanism for this defect in corticosteroidogenesis is unclear. While early studies demonstrated that androgens are competitive inhibitors of 11β-hydroxylase,8 it is unlikely that this is their sole mechanism of action. Consistent with this idea are spectral data by Fink et al9 that suggested a decrease in adrenal mitochondrial cytochrome P-450 and an increase in cytochrome P-450 levels in androgen-treated rats. Also, we have recently shown10 by Western blotting techniques that there is a profound decrease in adrenal cytochrome P-450 enzyme levels in rats treated with the synthetic androgen, 17α-methyltestosterone (MAD). A possible explanation for these observations was offered by Hornsby,11 who suggested that androgens by acting as pseudosubstrates bind to cytochrome P-450 enzyme and, since they are unable to be hydroxylated, generate free radicals that lead to a loss of 11β-hydroxylase activity. This is a mechanism that apparently applies in bovine adrenocortical cells in culture. Alternatively, the loss in enzyme activity may result from effects of androgens on the gene expression of cytochrome P-450.

To investigate this possibility, we have in the present study examined the consequence of androgen administration on a number of parameters involved in adrenal gland function. Adrenal levels of cytochromes P-450 and P-450 and their corresponding messenger RNA (mRNA) transcripts were determined in rats given either placebo or testosterone during a 6-week period, and these were correlated with mitochondrial 11β- and 18-hydroxylation of DOC.

Methods

Experimental Animals

Female Holtzman Sprague-Dawley rats, 35 days old, were obtained from Harlan Sprague Dawley, Indianapolis, Ind., and maintained in a controlled environment of 12-hour light/dark cycles with constant temperature
of 22°C. After 1 week, all rats underwent right nephrectomy under Ketaset-Rompun anesthesia. After a 2-day recovery period, animals received daily subcutaneous injections of either vehicle (0.2 ml corn oil) or 10 mg testosterone (Sigma Chemical Co., St. Louis, Mo.) suspended in vehicle. Animals were fed Purina Lab Chow and were given 1% saline ad libitum as drinking solution. Systolic blood pressures were determined indirectly by the tail-cuff method in unanesthetized warmed animals (Narco BioSystems, Houston, Tex.). Three consecutive readings were recorded and averaged for each animal.

Preparation of Adrenal Mitochondria

Animals were killed by decapitation, and the adrenal glands were exposed and enucleated in situ to separate fasciculata-reticularis from glomerulosa zones. For each animal, one adrenal gland was taken for preparing mitochondria for enzyme assays and Western blotting, and the contralateral adrenal was taken for RNA preparation. The inner zones were homogenized in Tris-buffered sucrose, pH 7.4, and mitochondria were prepared by differential centrifugation as previously described.15 There were five to six animals in each group at each time period investigated. Although the data presented are from one experiment, similar findings were obtained in several experiments.

11β-Hydroxylase and 18-Hydroxylase Enzyme Assays

 Mitochondria were diluted to a protein concentration of 0.6–1.0 mg/ml in (mM) sucrose 50, NaCl 50, MgCl₂ 5, KCl 5, and Tris 100, pH 7.4. 11-Deoxycorticosterone was added as substrate to give a final concentration of 90 μM. After 5 minutes preincubation at 37°C, isocitrate (10 mM) was added as a source of reducing equivalents. Samples were incubated for 3 minutes, and the reaction was stopped by freezing in liquid nitrogen.

11-Deoxycortisol was added as an internal standard, and samples were extracted into methylene chloride; the organic phase was washed with 0.1N NaOH and water and then dried under a stream of nitrogen. The extracted steroids were redissolved in 0.3 M sodium acetate, pH 6.0, precipitated in 2.5 volumes of absolute ethanol, and suspended in water before Northern blot analysis. The RNA concentration was estimated spectrophotometrically by measuring the absorbance at 260 nm.

For Northern blot analysis, 10 μg total adrenal RNA was fractionated on a 1% (wt/vol) agarose-formaldehyde gel and transferred to GeneScreen nylon membrane (New England Nuclear Research Products, Boston, Mass.) in sodium phosphate (25 mM, pH 7.0) buffer.18 To confirm that approximately equal amounts of RNA were loaded in each lane, the 28S and 18S ribosomal RNA bands were visualized under UV illumination after the addition of ethidium bromide. Before transfer the gel was incubated with 50 mM NaOH and then neutralized with sodium phosphate buffer, pH 7.0. The membrane was baked at 80°C to fix the RNA and then prehybridized and hybridized with the various radiolabeled probes at 42°C. After washing and drying, the membrane was exposed to x-ray film with intensifying screens (Lightning Plus, Du Pont, Wilmington, Del.) at −70°C. The relative intensities of the bands were estimated using a scanning laser densitometer (Pharmacia-LKB, Piscataway, N.J.) with integration of peak heights. The size of transcripts was determined by comparison with an RNA ladder (GIBCO-BRL, Grand Island, N.Y.) after staining with ethidium bromide.

Western Blotting

 Polyclonal antibodies to cytochrome P-450₁₁β were raised in rabbits by immunization against a peptide conjugated to keyhole limpet hemocyanin (Calbiochem, San Diego, Calif.) with m-maleimidobenzoic acid N-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, Mo.) according to Sambrook et al.19 The sequence of the peptide used was C I P Q Y S R

RNA Preparation and Northern Blot Analysis

The plasmids containing the complementary DNAs (cDNAs) encoding for bovine cytochrome P-450₁₁β and bovine adrenodoxin were kindly provided by Dr. Michael Waterman, University of Texas Health Sciences Center, Dallas, Tex. The plasmid containing the cDNA encoding for mouse cytochrome P-450₁₁β was a generous gift from Dr. Keith Parker, Duke University, Durham, N.C. The actin cDNA was purchased from ONCOR, Gaithersburg, Md. Each plasmid was subjected to digestion with the appropriate restriction enzyme (BamH1 for adrenodoxin, EcoR1 for cytochrome P-450₁₁β, and EcoR1 for cytochrome P-450₁₁α) to excise the cDNA of interest and then subjected to agarose gel electrophoresis. The separated fragments were cut from the gel and extracted and isolated with GeneClean (BIO 101, La Jolla, Calif.) according to the manufacturer's directions. All cDNAs were radiolabeled by random priming16 using [α-32P]dCTP (ICN Biomedicals, Costa Mesa, Calif., 1–2x10⁶ cpm/μg DNA) and an oligolabeling kit (Pharmacia Molecular Biology Products, Piscataway, N.J.).

Total adrenal RNA was prepared by homogenization in 4 M guanidinium isothiocyanate and centrifugation through a 5.7 M cesium chloride cushion as described by Maniatis et al.17 The RNA pellet was dissolved in 0.3 M sodium acetate, pH 6.0, precipitated in 2.5 volumes of absolute ethanol, and suspended in water before Northern blot analysis. The RNA concentration was estimated spectrophotometrically by measuring the absorbance at 260 nm.

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TABLE 1. Steroid 11β- and 18-Hydroxylase Activities of Control and Testosterone-Treated Rats

<table>
<thead>
<tr>
<th>Length of treatment</th>
<th>Controls</th>
<th>Testosterone-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18-Hydroxylase</td>
<td>11β-Hydroxylase</td>
</tr>
<tr>
<td>3 days</td>
<td>6.43±0.297</td>
<td>12.72±0.60</td>
</tr>
<tr>
<td>7 days</td>
<td>3.97±0.251</td>
<td>8.18±0.537</td>
</tr>
<tr>
<td>21 days</td>
<td>3.67±0.178</td>
<td>9.33±0.455</td>
</tr>
<tr>
<td>42 days</td>
<td>2.79±0.118</td>
<td>7.15±0.26</td>
</tr>
</tbody>
</table>

Enzyme activities are in nanomoles product/minute/milligram protein. Values are mean±SEM. At all time intervals studied, both enzyme activities have a p<0.01 for testosterone vs. control.

N K W L and was synthesized by Multiple Peptide Systems, San Diego, Calif. With the exception of cysteine, which was added as a convenient coupling point, the peptide sequence was derived from the published nucleotide sequence for rat cytochrome P-450(11β).20 Rabbits were immunized at 15–20 sites along the back with an initial 200-μg total dose of conjugate in Freund's complete adjuvant. Thereafter, rabbits were given 100 μg of conjugate in Freund's incomplete adjuvant at biweekly intervals. Specific antibodies to cytochrome P-450(11β) were obtained in two of three rabbits after 10 weeks of immunization. Specificity was assumed after observing only one band on Western blots of a rat mitochondrial extract. Also, Western blot analysis showed a lack of reactivity of the antibodies with the bovine cytochrome P-450 standard (a gift from Dr. David Lambeth, Emory University, Atlanta, Ga.) and with a Leydig cell tumor extract (which contains cytochrome P-450(11β) but no cytochrome P-450(11β)).

Adrenal mitochondrial samples (30 μg protein) were treated with an extraction buffer consisting of 0.1% sodium dodecyl sulfate (SDS) and 1% cholate in phosphate-buffered saline, pH 7.4,10 and were subjected to discontinuous SDS-polyacrylamide gel electrophoresis using a 5% stacking gel and a 7.5% separating gel.21 After overnight electrophoresis, proteins on the gel were transferred onto nitrocellulose sheets and immunoblotted according to Towbin et al22 but using non-fat powdered milk as blocking agent. After transfer, the blots were incubated overnight with primary antibody to cytochrome P-450(11β) and then for 2 hours with secondary antibody (anti-rabbit iodine-125-labeled immunoglobulin G, 1×10⁶ cpml/ml, New England Nuclear). After autoradiography the blots were scanned by laser densitometry, and the peak heights determined. Autoradiograms were also used as templates to cut out bands from immunoblots. The bands were then counted in a Packard Multi-PriAs 1 gamma counter (Packard, Downers Grove, Ill.) to determine relative amounts of radioactivity present. Protein was estimated by the method of Bradford.23

Results

Blood Pressures and Body Weights

The blood pressures of rats given testosterone for 6 weeks were significantly different from those of control rats injected with vehicle alone (129±2.2

mm Hg in controls versus 147±6.1 mm Hg in the testosterone-treated group). Three of 13 androgen-treated rats had blood pressures above 170 mm Hg, and five were above 140 mm Hg. In the control group (n=12) no blood pressures were above 150 mm Hg, and only one was above 140 mm Hg. The body weights of both groups increased during the course of the experiment, and the final body weights were similar (270±7.5 g in controls versus 266±4.1 g in the testosterone-treated group).

11β-Hydroxylase and 18-Hydroxylase Activity

The effect of testosterone treatment on adrenal mitochondrial metabolism in vitro is presented in Table 1. At all time periods tested, testosterone-treated animals showed a marked reduction in their ability to form corticosterone and 18-hydroxy-DOC from added DOC. Corticosterone formation in the testosterone-treated rats was approximately two thirds of control values after 3 days of treatment and was suppressed to 45% of control values by the end of the 42-day treatment period.

Cytochrome P-450(11β) Levels

Analysis of the enzyme activity data supports previous findings that adrenal mitochondria isolated from androgen-treated animals have an impaired ability to convert added DOC to the natural end products, corticosterone and 18-hydroxy-DOC. It was important to determine the cause of this impairment. To this end we generated antibodies to cytochrome P-450(11β) to be used for immunoblot analysis. As is clearly seen in Figure 1, testosterone treatment resulted in a marked decline in the level of immunoreactive cytochrome P-450(11β) measured in mitochondria. Testosterone decreased the levels of this enzyme to 33% of control values after only 3 days of treatment. By 42 days, the levels were down to 18% when compared with controls.

Expression of Cytochromes P-450(11β) and P-450(11α) Messenger RNA

To determine whether the decline in cytochrome P-450(11β) levels associated with androgen treatment could be attributed to the steady-state levels of mRNA transcripts for the protein, we isolated adrenal RNA from control and testosterone-treated animals. Northern blot analysis revealed that in both groups, the cytochrome P-450(11β) radiolabeled probe

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FIGURE 1. Autoradiogram of cytochrome P-450\textsubscript{11\beta} isolated from adrenal mitochondria of control and testosterone-treated rats. Samples were subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis, and the level of enzyme was determined by immunoblotting with specific antibodies directed against cytochrome P-450\textsubscript{11\beta} as described in "Methods." Each lane is equivalent to 30 μg of mitochondrial protein. C, control rats treated with vehicle; T, rats treated with 10 mg testosterone/day; S, crude mitochondrial cytochrome P-450\textsubscript{11\beta} standard.

hybridized to an RNA species of 3.6 kb (Figure 2). The level of cytochrome P-450\textsubscript{11\beta} mRNA decreased dramatically in the testosterone-treated group after only 3 days of treatment. After 1 week of androgen treatment, the level of cytochrome P-450\textsubscript{11\beta} mRNA declined further but then appeared to rebound slightly after 3 and 6 weeks of treatment. To assess the selectivity of the effects of testosterone, we also probed for cytochrome P-450\textsubscript{18\alpha} mRNA. In both groups the radiolabeled probes hybridized to an RNA species of 2.3 kb. In contrast to the effect on cytochrome P-450\textsubscript{11\beta} mRNA, the steady-state levels of cytochrome P-450\textsubscript{11\beta} mRNA in the testosterone group remained equal to or exceeded the control group for the first 3 weeks of treatment. There was a slight decrease in cytochrome P-450\textsubscript{11\beta} mRNA after 42 days of treatment with testosterone. Actin mRNA levels were also determined and did not appear to change during the treatment period (Figure 3.) The mRNA for adrenodoxin, the non-heme iron protein involved in the steroid hydroxylation electron transport chain of adrenal mitochondria, also appeared to decline in response to androgens (Figure 3). The signal, however, was too weak to permit quantitative assessment.

Discussion

The results obtained in the present study are consistent with our hypothesis that androgen administration produces profound and selective alterations in adrenal cytochrome P-450\textsubscript{11\beta}. The mRNA transcript for this enzyme is markedly reduced after only 3 days of treatment, and this reduction is reflected in a decrease in cytochrome P-450\textsubscript{11\beta} levels as determined by immunoblot analysis. Furthermore, as would be predicted, the ability of mitochondria to hydroxylate DOC to corticosterone and 18-hydroxy-DOC is greatly diminished. In contrast, cytochrome P-450\textsubscript{18\alpha} is relatively resistant to androgens.\textsuperscript{10} In another study\textsuperscript{9} we have shown that there is an increase in cholesterol side-chain cleavage and increased

TABLE 2. Relative Amounts of Cytochromes P-450\textsubscript{11\beta}, P-450\textsubscript{18\alpha}, and Actin Messenger RNAs and Cytochrome P-450\textsubscript{11\beta} Enzyme Levels in Testosterone-Treated Rats

<table>
<thead>
<tr>
<th>Length of treatment</th>
<th>Actin mRNA</th>
<th>P450\textsubscript{11\beta} mRNA</th>
<th>P-450\textsubscript{11\beta} enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>125</td>
<td>142</td>
<td>20</td>
</tr>
<tr>
<td>7 days</td>
<td>103</td>
<td>134</td>
<td>12</td>
</tr>
<tr>
<td>21 days</td>
<td>104</td>
<td>114</td>
<td>16</td>
</tr>
<tr>
<td>42 days</td>
<td>92</td>
<td>78</td>
<td>27</td>
</tr>
</tbody>
</table>

Values are expressed as percent of control.
binding of cholesterol to cytochrome P-450_{18} in adrenal mitochondria isolated from MAD-treated rats. Those data are consistent with the present data showing that the level of the cytochrome P-450_{18} mRNA transcripts was at or slightly above control levels during the entire treatment period.

The role of androgens in regulating adrenal cortical function is poorly characterized. Although it is known that adrenal androgen receptors do exist, their function is unclear. Much of the evidence thus far suggests that androgens have an inhibitory role. Thus, it has been demonstrated that androgen administration results in adrenal atrophy, inhibition of adrenal protein synthesis, and also inhibition of enzyme activity. The latter includes 5α reductase and cytochrome P-450_{11B,4,5,9,29-31}. The mechanisms involved in these actions are not known. Adrenal atrophy has been attributed to inhibition of adrenocorticotropic hormone (ACTH) secretion. The results of the present study do not support this contention. ACTH is known to regulate the transcription of cytochrome P-450_{18} enzyme. The present data show that cytochrome P-450_{18} mRNA levels in testosterone-treated rats are similar to those in non-treated controls. This suggests adequate secretion of ACTH. Furthermore, Mazzocchi et al found increased circulating ACTH levels in male rats chronically treated with testosterone.

The mechanism involved in the selective decrease in cytochrome P-450_{11B} mRNA is also unknown. Two obvious possibilities that need to be investigated are the effect of testosterone on the repression of transcription of cytochrome P-450_{11B} and its effect on the rate of message degradation. What seems less important, although not precluded, as a mechanism for the loss of cytochrome P-450_{11B} activity is oxidative damage due to the interaction of testosterone with cytochrome P-450_{11B} in a pseudosubstrate mode. The primary effect appears to be on mRNA. The relatively good correlation among the decreases in cytochrome P-450_{18} mRNA, 11β-hydroxylase activity, and cytochrome P-450_{11B} levels suggest that testosterone is primarily affecting transcription and not translation of the message. Similar actions of testosterone on ornithine decarboxylase mRNA have recently been described in rat Sertoli cells in culture.

The present study clearly shows that testosterone administration to rats causes a profound and selective reduction in mRNA transcripts coding for cytochrome P-450_{11B}. This decrease leads to a deficiency in cytochrome P-450_{11B}, which then results in excessive production of DOC and presumably the development of hypertension. The possibility, however, that extra-adrenal effects of androgens on the vasculature contribute to this experimental model of hypertension cannot be excluded. The mechanism by which testosterone affects transcription remains to be determined.

Acknowledgments

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KEY WORDS: adrenal cortex • testosterone • cytochrome P450 • steroid hydroxylases • steroid-induced hypertension
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