Abstract—Rodents studies suggest that androgens are involved in sex-specific differences in blood pressure. In humans, there is no difference in blood pressure between boys and girls, but after puberty, blood pressure increases more in men than in women. We investigated androgen-dependent regulation of the α-subunit of the epithelial sodium channel (αENaC) in human kidney and in the human renal cell line immortalized human renal proximal tubular cell line (HKC-8). We used microarray technique to analyze androgen-dependent gene regulation and performed quantitative RT-PCR for verification. Promoter constructs for human αENaC were used in transfection studies to analyze the regulation by testosterone. We investigated the in vivo effect of testosterone on αENaC in a rat model and used the mouse collecting duct cell line M-1 for transepithelial electrophysiological measurements. The androgen receptor (AR) was expressed in male kidney and HKC-8 cells. αENaC mRNA expression increased 2- to 3-fold after treatment with testosterone in HKC-8 cells. The induction by testosterone was completely blocked by adding the AR antagonist flutamide. Analysis of the αENaC promoter sequence identified a putative AR response element (ARE) located 140 nucleotides upstream from the transcription start site. HKC-8 cell transfection studies showed that testosterone directly upregulated gene expression via this ARE. In vivo, testosterone treatment of orchietomized rats resulted in an increased renal αENaC mRNA expression. In testosterone-treated mouse M-1 cells, amiloride caused a significant stronger decrease in short circuit current than in control cells. These data show that αENaC expression is directly regulated by androgens in vitro and in vivo and highlight a potential mechanism explaining the reported gender differences in blood pressure. (Hypertension. 2005;46:787-798.)

Key Words: blood pressure ■ gender ■ kidney ■ sodium channels

Androgens are known to play an important role in renal tubular epithelial cell growth, hypertrophy, and erythropoetin production, and may be important determinants of sex-specific differences in blood pressure.1 In spontaneously hypertensive rats, males have higher blood pressures than females,2 an effect that is reversed by male castration. Furthermore, increases in blood pressure were observed in castrated male and female rats after the administration of testosterone,3,4 whereas administration of the androgen receptor (AR) antagonist flutamide attenuated hypertension.4 These studies strongly implicate androgens in the regulation of blood pressure in the rat, but the mechanisms for this are still unknown.

In humans, there is no difference in blood pressure between boys and girls, but during and after puberty, boys show higher blood pressure than age-matched girls,5,6 and men have a higher overall mean arterial pressure than women,7 regardless of ethnic origin.8 The higher blood pressure and stronger progression of hypertension in men is associated with a higher risk and mortality for cardiovascular diseases than in women.9,10

Although androgens appear to play an important role in the pathogenesis of sex-specific differences in hypertension, it is clear that other sex steroids are also involved in the control of blood pressure. For example, estrogens are known to modulate vascular endothelial function, resulting in vasodilatation and lowering of blood pressure of women.11,12 The role of androgens in blood pressure regulation and the pathogenesis of hypertension have not been extensively investigated. Because the kidneys play a major role in the regulation of blood pressure,13 we used a human renal cell line and an in vivo rat model to investigate androgen effects on key target genes associated with renal sodium and water reabsorption.

Materials and Methods

Cell Culture

Cells from the simian virus 40–infected human proximal tubule cell line (HKC-8)14 were cultured in standard culture medium (470 mL...
DMEM/Ham F12, 5 mL 2 mmol/L glutamine [all from Sigma Chemical Co], and 25 mL of FCS [Gibco Invitrogen Ltd] final concentration 5%) in 75 cm² flasks. They were incubated at 37°C (95% O₂ and 5% CO₂) until they were confluent. HKC-8 cells were transferred to 12- or 24-well plates so that each well contained 1 mL androstenedione (all from Sigma Chemical Co), respectively. These cells, we added 50 nmol/L of unlabeled DHEA, testosterone, or estradiol. Preliminary experiments were done for the incubation time (4 hours). For incubations with 150 000 cpm of 3H-DHEA, 1 male and 1 female kidney were cultured using established methodology.16 Specific PCRs were set up as described previously.16 Samples were amplified using initial denaturation of 94°C for 5 minutes followed by 34 cycles (38 cycles for kidney) and one final elongation step of 72°C for 7 minutes. Amplification products were run on a thin-layer chromatography (TLC) plate (20 × 20 cm). TLC aluminum plates coated with silica gel 60F₂₅₄ (Fluka Chemika) along with unlabeled control steroids and detected as described previously.16 Briefly, the plates were run in mixture of dichloromethane:acetone (92.5:7.5) for 75 minutes. After drying at 80°C for 10 minutes, they were run for an additional 45 minutes in the same mixture and the same direction. After the run, the plates were scanned by a Bio Scan 3000 image analyzer (Bioscan Inc.) then sprayed with Liebermann-Burchard reagent and heated for detection of unlabeled control steroids under UV light (360 nm). The running distance of each control steroid was compared with the running distance of the radiolabeled steroids for identification of the metabolites in the sample. Percentage conversions were calculated using radiolabeled spots that had been detected. The Bio-Rad protein assay (Bio-Rad) was performed to determine the concentration of total protein.

**Western Immunoblot Analysis**

Proteins were prepared from whole cell lysates, 1 male and 1 female kidney, as well as 2 primary kidney cell lines from male donors. Briefly, 40 µg of total protein for each sample was subjected to SDS-PAGE, transferred electrophoretically onto polyvinylidene fluoride membrane (Immobilon-P; Millipore), and blocked with TBS-Tween containing 5% milk powder for 1 hour. For detection of ARs, rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc) were diluted 1:500 and membranes incubated overnight at 4°C. After a

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**TABLE 1. Primer Sequences and Product Size of Target Genes**

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Product Size (bp)</th>
<th>Primer Sequence (Forward Primer, Reverse Primer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSD11B2</td>
<td>419</td>
<td>5'-ACGTTATGGTGAAGCTAGGCA-5' 5'-AGGAGTTCAAGCTGATG-5'</td>
</tr>
<tr>
<td>MR</td>
<td>336</td>
<td>5'-TCCCTATGCTGAAAATCG-5' 5'-AGAGTGGCGAACAGCTGT-5'</td>
</tr>
<tr>
<td>sgk</td>
<td>699</td>
<td>5'-AGGCGATTTGGGAAAGGT-5' 5'-GCCAAGAGCGAGCAAAAGC-5'</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>601</td>
<td>5'-CCACAGTACAGCTCTCTG-5' 5'-TTTTCACAAGGTGGGA-5'</td>
</tr>
<tr>
<td>kNBC-1</td>
<td>400</td>
<td>5'-CAC ACT GCG CCC AGA GTA TT-5' 5'-GTTCCTCAAAAGCTGG-5'</td>
</tr>
<tr>
<td>NHE2</td>
<td>398</td>
<td>5'-GGGCTCAACTTTGGAAGA-5' 5'-GCTGCTGTCTCTCAATAGC-5'</td>
</tr>
<tr>
<td>NHE3</td>
<td>398</td>
<td>5'-GGAGTCAAAGCTGAGATGG-5' 5'-AGCCTGTCAGCTGAAAGGA-5'</td>
</tr>
<tr>
<td>SRD5A1</td>
<td>239</td>
<td>5'-CAGATCCCGGTTTCTAAATGGA-5' 5'-AACAGCTGAAGAAAGCAGAAGC-5'</td>
</tr>
<tr>
<td>SRD5A2</td>
<td>226</td>
<td>5'-CTGGAAGAATACCATCAGAAG-5' 5'-GCTTCCGAACTATTGGG-5'</td>
</tr>
<tr>
<td>17β-HSD 1</td>
<td>219</td>
<td>5'-TTTCAATGAGCTTATGATCGCCAAGCA-5' 5'-TTGCCGTGGAAGATTTGGA-5'</td>
</tr>
<tr>
<td>17β-HSD 2</td>
<td>181</td>
<td>5'-CTGTGACAGTGGTGTAGTGGT-5' 5'-TTATGTCAGCTGCGGCTG-5'</td>
</tr>
<tr>
<td>17β-HSD 4</td>
<td>527</td>
<td>5'-AGTTCTCTCTCTCTTGTTGCTCGAAG-5'-GGGTCTCTTCTTCTTCTTCCT-5'</td>
</tr>
<tr>
<td>17β-HSD 5</td>
<td>476</td>
<td>5'-ACTCTATGCGTCATGGAGATTG-5'-CTGCGCTGCGTGAGATTTGGA-5'</td>
</tr>
<tr>
<td>AR</td>
<td>585</td>
<td>5'-CGGAAGCTGAAAGGACTTG-5' 5'-ATTTCAGCCAGCTACACTCT-5'</td>
</tr>
</tbody>
</table>

HSD11B2 indicates 11β-hydroxysteroid dehydrogenase type 2; MR, mineralocorticoid receptor; AR, androgen receptor; SRD5A, 5α-reductase; NHE, sodium/proton exchanger (SLC9A); kNBC-1, kidney sodium bicarbonate cotransporter 1 (SLC4A4); sgk1, serum glucocorticoid-regulated kinase 1.

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**Enzyme Assays**

We investigated the metabolism of DHEA, androstenedione, and testosterone in HKC-8 cells. Before incubation, cells were washed with 0.5 mL of FCS-free DMEM/Ham F12, then 1 mL of serum-free incubation medium was added. All incubations were done in triplicates. Preliminary experiments were done for the incubation time (4 to 24 hours). For incubations with 150 000 cpm of 3H-DHEA, 17β-hydroxyprogesterone, or 17α-androstenedione, respectively. These were incubated for 10 hours at 37°C.
series of washes, the membranes were exposed to secondary anti-goat rabbit antibody (horseradish peroxidase conjugate; DAKO Ltd) with a dilution of 1:1000. Proteins were detected using enhanced chemiluminescence (ECL; Amersham) and autoradiography. AR blocking peptide (Santa Cruz Biotechnology Inc) was used to block the AR-specific band.

**Microarray Analysis**
HKC-8 cells were treated either with 500 nmol/L testosterone or with the corresponding amount of ethanol (solvent) for 48 hours. RNAs from 4 treated cultures were pooled as well as RNAs from 4 control cultures. Biotin-labeled cRNA was generated from 10 μg of total RNA according to Affymetrix technical protocol (GeneChip Expression Analysis Manual). A total of 15 μg of cRNA was hybridized to Human Genome U133 sets (Affymetrix). Affymetrix software (Data Mining Tool; version 3.0) was used for analyzing microarray data. Actin and GAPDH were used to assess RNA samples and assay quality. Accordingly, results are presented as qualitative (detection) and quantitative (signal) measures of expression level, which represent arbitrary data, and comparisons between testosterone-treated and control cultures were expressed as fold change. The interassay variability between microarrays was <5%.

**Quantitative RT-PCR**
HKC-8 cells were treated for different times (0, 6, 12, 24, and 48 hours) with testosterone (500 nmol/L) as well as with different testosterone concentrations (0, 0.01, 0.05, 0.1, 0.5, 1, 10, 50, 100, 500, and 1000 nmol/L) for 48 hours in triplicates versus ethanol-treated control cultures. In addition, HKC-8 cells were treated with different concentrations (0.001, 0.1, 10, and 1000 μmol/L) of the AR antagonist flutamide (Sigma-Aldrich Company Ltd). In further experiments, HKC-8 cell cultures were treated in triplicates with different concentrations (0, 1, 10, and 100 nmol/L) of testosterone, 5α-dihydrotestosterone, or dexamethasone for 48 hours. All incubations were performed in FCS-free incubation media and repeated 3 times. RNA was extracted as mentioned above. The α-subunit of the epithelial sodium channel (αENaC) and serum and glucocorticoid-regulated kinase 1 (skg1) mRNA expression levels were analyzed using an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems) as described previously. All reactions were performed with the housekeeping gene (18S). Oligonucleotide primers and a Taqman probe for αENaC were as follows: forward, CCACGCTTCTCGTGTACTCA; reverse, TGGCGATAG- CATTGGGAGA; and probe, TGGCGCTTCGTCATCCCGAG. The primers and probe used for skg1 real-time PCR were a commercially available Assay on Demand (Applied Biosystems). According to manufacturer guidelines, data were expressed as threshold cycle value (CT) in construct 2 using the Quick-change Site-Directed Mutagenesis Kit (Stratagene) and primers 5′-CAACAGTGTAAAAAGAACAGAACACTCGGCGCTAGGCC and 5′-GGGCTAGCGGCGGCTAGTTCTCTGTCTTTTATACGTGTTG. The mutated construct 2 was transfected into HKC-8 cells and incubated with testosterone as described above.

**Animal Experiments**
Male Wistar rats aged 8 to 10 weeks with a body weight (bw) of 180 to 200 g were obtained from Charles River Breeding Laboratories (Kisslegg, Germany). All animal experimentation described was conducted in accordance with accepted standards of humane animal care such as the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Animals received water and normal rat chow ad libitum. They were orchietomized and treated with a long-lasting testosterone preparation (testosterone undecanoate; 100 or 500 mg/kg bw), with a 5α-dihydrotestosterone preparation (75 mg per pellet per 21-day release), or with placebo. Each group consisted of 4 animals. After 14 days, the animals were killed and the kidneys removed. RNA was extracted and quantitative real-time PCR performed as described above.

**Electrophysiological Transepithelial Measurements**
The mouse cell line M-1 was grown on inserts with a semipermeable membrane. After incubating the confluent M-1 cells, the semipermeable membrane was mounted in a 4-electrode Ussing chamber separating the apical and basolateral compartment. The 2 compartments were filled with a solution containing 140 mmol/L sodium, 123.8 mmol/L chloride, 5.4 mmol/L potassium, 1.2 mmol/L calcium, 1.2 mmol/L magnesium, 2.4 mmol/L HPO₄²⁻, 0.6 mmol/L H₂PO₄⁻, 21 mmol/L HCO₃⁻, 10 mmol/L D(+)-glucose, 0.5 mmol/L β-hydroxybutyrate, 2.5 mmol/L glucose, 10 mmol/L D(+)-mannose, and antibiotics (10 mg/L imipenem/cilastatin and 50 mg/L azlocillin) and continuously bubbled with 95% O₂ and 5% CO₂ at 37°C. After a period of equilibration (1 to 2 hours), periodic measurements of transepithelial resistance (Rₑ), voltage (Vₑ), and short circuit current (Iₑ) were obtained once every 30 s using a computer-controlled voltage-clamp device (CVC 6; Fiebig). Iₑ values were corrected for bath resistance. Once baseline readings had stabilized, 10 μmol/L amiloride (Sigma Chemical Co) was added to the apical compartment. Iₑ values after a period of 10 minutes before and after amiloride were averaged and compared for change in Iₑ attributable to amiloride. Control of electrophysiological function of M-1 cells was performed by evaluating the response to theophylline (Sigma Chemical Co).

**Statistical Analysis**
Data are expressed as mean±SD unless otherwise stated. Statistical analysis on real-time PCR data were performed on mean dct values (and not on fold changes) to exclude potential bias attributable to averaging data that had been transformed through the equation 2⁻Δct. Statistical analysis of comparisons between groups was undertaken using paired and unpaired t tests where appropriate; otherwise, the Mann–Whitney rank sum test or ANOVA Bonferroni was used.
Results

Renal AR Expression
RT-PCR analysis showed strong expression of mRNA for the ARs in HKC-8 cells as well as in T47D (breast epithelial) cells (Figure 1A). AR mRNA was also detected in kidney and 2 primary kidney cell cultures from male donors; whereas in a single “female” kidney, the signal was minimal. Western blot analysis confirmed the strong expression of AR in HKC-8 cells as well as in T47D cells (Figure 1B). Kidneys from 3 males showed positive protein detection, whereas no AR protein was found in the kidney from a female (Figure 1B).

Enzyme Assay and Enzyme Expression
Previous studies have reported androgen synthesis and metabolism in human kidney tissue. Therefore, we investigated androgen metabolism in the human renal cell line HKC-8. There was no significant metabolism of DHEA or testosterone in HKC-8 cells (data not shown). HKC-8 cells showed expression of 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) and 17β-HSD4 mRNAs but not 17β-HSD1 and 17β-HSD5 (Figure 2). Androstenedione was metabolized to the single metabolite 5α-androstane-1 (data not shown) by 5α-reductase 1, which was highly expressed in all kidney cell lines.
tissues (Figure 2). Surprisingly, testosterone was not converted to 5α-dihydrotestosterone in HKC-8 cells, probably because first, the micromolar Km value of 5α-reductase 1 for testosterone, and second, the lack of 5α-reductase 2 expression. Subsequently, in functional studies, we incubated HKC-8 cells with testosterone. The HKC-8 cells derive originally from a proximal tubule cell line, and therefore, we tested their enzyme, ion transporter, and receptor expression. These cells showed features of polarization such as the formation of domes indicative of transepithelial transport. HKC-8 cells typically expressed channels of the proximal tubule, such as the basolateral electrogenic Na+HCO3 cotransporter kNBC-1 and the luminal Na+/H+ exchangers NHE-2 and NHE-3 (Figure 3), which have both been implicated in apical sodium absorption. However, the cell line also showed features characteristic of distal tubule cells, including expression of mRNA for mineralocorticoid receptor (MR), sgk1, αENaC, and 11β-HSD2 (Figure 3), and abundant 11β-HSD2 activity (conversion of cortisol to cortisone [4 pmol/mg protein per hour]). Although there was abundant αENaC expression (Figure 3), βENaC and γENaC expression was not identifiable in HKC-8 cells under basal conditions (data not shown).

**Microarray Analysis**

To assess the effects of testosterone on gene expression in HKC-8 cells, DNA microarray analysis was performed using mRNA isolated from vehicle- and testosterone-treated (500 nmol/L) cells. Data from primary and comparison files (testosterone-treated versus control) on HKC-8 cells were analyzed, and candidate genes were selected using Microarray Suite 5.0 (Affymetrix). A total of 22 283 (U133A) entries in comparison analysis were sorted out according to the criteria: (1) genes with absent signal in control and treated cells arrays were deleted; (2) genes with difference signal of no change were deleted; (3) genes with signal log ratio 1 for increase or above 1.0 for decrease were deleted (arbitrary cutoff point of 2-fold change); and (4) expressed sequence tag entries were not analyzed. This filtering procedure resulted in a single data set testosterone-treated versus control with changes in gene expression of 2-fold.

Incubation of HKC-8 cells with testosterone for 48 hours resulted in significant changes (≥2-fold) in 9 genes on U133A microarrays, downregulation of 6, and upregulation of 3 genes (Table 2). αENaC was significantly upregulated (2-fold) by testosterone in HKC-8 cells. The microarray analysis confirmed that βENaC and γENaC are not expressed under basal conditions in HKC-8 cells and were not regulated by testosterone (Table 2). The AR and sgk were present under basal conditions but showed no change after testosterone treatment (Table 2).

**Quantitative PCR**

To confirm that the Affymetrix DNA microarrays accurately identified αENaC gene expression changes, we performed

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**Table 2. Expression of Genes According to cDNA Arrays With RNA Extracted From Control HKC-8 Cells and Testosterone (500 nmol/L)-Treated HKC-8 Cells**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>Testosterone</th>
<th>Fold Change Ctr vs Testo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Detection</td>
<td>Signal</td>
<td>Detection</td>
</tr>
<tr>
<td>Serum amyloid A2</td>
<td>2.8</td>
<td>A</td>
<td>27.7</td>
</tr>
<tr>
<td>TATA box binding protein</td>
<td>8</td>
<td>M</td>
<td>23.7</td>
</tr>
<tr>
<td>αENaC</td>
<td>77.3</td>
<td>P</td>
<td>174.6</td>
</tr>
<tr>
<td>Vit D 24-hydroxylase</td>
<td>15.7</td>
<td>P</td>
<td>6.4</td>
</tr>
<tr>
<td>Cytochrome b245</td>
<td>21.8</td>
<td>P</td>
<td>7.6</td>
</tr>
<tr>
<td>Asparagine synthetase</td>
<td>290.8</td>
<td>P</td>
<td>64.3</td>
</tr>
<tr>
<td>Cystine glutamate exchanger</td>
<td>110.1</td>
<td>P</td>
<td>36.9</td>
</tr>
<tr>
<td>Histidine-rich glycoprotein</td>
<td>23.9</td>
<td>P</td>
<td>10.2</td>
</tr>
<tr>
<td>Long-chain acyl-CoA synthetase 5</td>
<td>15.6</td>
<td>P</td>
<td>5.2</td>
</tr>
<tr>
<td>Androgen receptor</td>
<td>97.8</td>
<td>P</td>
<td>108.7</td>
</tr>
<tr>
<td>Sgk</td>
<td>56.5</td>
<td>P</td>
<td>67.6</td>
</tr>
<tr>
<td>NBC-4</td>
<td>63.3</td>
<td>P</td>
<td>48.2</td>
</tr>
<tr>
<td>βENaC</td>
<td>11.3</td>
<td>A</td>
<td>12.4</td>
</tr>
<tr>
<td>γENaC</td>
<td>21</td>
<td>A</td>
<td>29.2</td>
</tr>
<tr>
<td>NHE-2</td>
<td>19.1</td>
<td>A</td>
<td>2.6</td>
</tr>
<tr>
<td>NHE-3</td>
<td>3.2</td>
<td>A</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Detection indicates qualitative measure indicating whether the transcript is reliably detected (Present, P) marginally detected (Marginal, M), or not detected (Absent, A); Signal, quantitative measure of the relative abundance of a transcript; Fold change, change in expression level for a transcript between control and testosterone treatment. 
separate quantitative RT-PCR assays. Expression of αENaC mRNA increased significantly after 6-hour treatment with testosterone and reached a highly significant level (2.5-fold induction) at 48 hours (Figure 4A). Dose dependency of testosterone response was evident (Figure 4B), with IC50 = 10 nmol/L. The highest increase in αENaC expression (3.5-fold) was observed using testosterone concentrations between 50 and 100 nmol/L (Figure 4B). In comparison, 100 nmol/L of dexamethasone resulted in a 4.5-fold increase in αENaC expression in HKC-8 cells (Figure 4C). Surprisingly, 5α-dihydrotestosterone concentrations up to 100 nmol/L did not cause a change in αENaC expression in HKC-8 cells (Figure 4D). Treatment with the AR antagonist flutamide resulted in an inhibition of the testosterone effect in a dose-dependent fashion compatible with an interaction with the AR. A total of 10 μmol/L flutamide completely abolished the testosterone effect on αENaC expression (Figure 5).

We then investigated the regulation of sgk1 in HKC-8 cells after treatment with testosterone, 5α-dihydrotestosterone, or dexamethasone. There was no effect of testosterone or 5α-dihydrotestosterone on sgk1 mRNA expression, but dexamethasone significantly induced sgk1 mRNA expression (mean ΔCT ± SD 8.2 ± 0.8 control to 6.8 ± 0.8 100 nmol/L dexamethasone; P < 0.05).

**αENaC Promoter Region**

On analyzing the sequence of the αENaC promoter, we found a nuclear factor κB binding side, AP-1 and AP-4 sides, octamer transcription factor-1 (Oct-1), E12, several Sp1 motifs, and a retinoid X receptor-α/retinoic acid receptor-α binding side within the exon 1A sequence.20 Upstream of the transcription start site of exon 1A, several Sp1 sites, AP-1, AP-2α, and AP-4 sites, GATA-1 and NF-1 sites, as well as 2 CCAAT/enhancer-binding protein α and 1 Oct-1 sites were found. At nucleotide position 155-140 upstream of the exon 1A transcription start site, an ARE was found (AGAACA-GAATGCTCT). This demonstrates >90% identity with the reported glucocorticoid receptor hormone response element (GRE).21

**Figure 4.** Testosterone stimulates αENaC expression. Time course of the fold change in αENaC mRNA expression between control HKC 8 cells and testosterone treated HKC-8 cells (500 nmol/L testosterone used; A) and differing testosterone concentrations for 48 hours (B). Dexamethasone (C) but not 5α-dihydrotestosterone (D) increased αENaC mRNA in HKC-8 cells. Zero hours and 0 nmol/L were set as 1. Statistical calculations are comparing different time points/concentrations with start at 0 hour or 0 nmol/L. For each time point/concentration, 3 flasks of control cells and 3 flasks of treated cells were grown. cDNA from each flask was assessed at least in duplicate using singleplex quantitative TaqMan real-time PCR with 18S as housekeeping gene. All incubations were repeated 3 times. *P < 0.05; **P < 0.01; ***P < 0.001.
Testosterone increases αENaC mRNA expression in the kidney (Figure 9).

Effects of Testosterone and Other Steroids on αENaC Promoter–Reporter Constructs

Transient transfection of HKC-8 cells was performed using either an empty pGL3 vector or the pGL3 vector with construct 1 (no ARE sequence), 2, 3, or 4 (all containing the ARE) into HKC-8 cells (Figure 6A). The empty pGL3 vector and construct 1 were not influenced by increasing testosterone concentrations (Figure 6B). However, with increasing testosterone concentrations, luciferase activity of construct 2, containing the putative ARE, showed a dose dependency and reached a >3-fold increase (Figure 6B). Constructs 3 and 4 (containing the ARE) showed a 2-fold increase in luciferase activity by testosterone treatment (Figure 6B). At 100 nmol/L testosterone, construct 2 had a significantly higher luciferase signal (P<0.005) than constructs 3 and 4 (Figure 6B). Construct 2 showed a small but not significant induction in luciferase activity by 100 nmol/L 5α-dihydrotestosterone (Figure 6C). The induction of luciferase activity by 100 nmol/L testosterone of construct 2 could be blocked by treatment with the AR antagonist flutamide, whereas construct 1 (no ARE sequence) showed no induction by testosterone or flutamide treatment (Figure 7A). In addition, mutation of the putative ARE abolished the induction by testosterone (Figure 7B).

In an additional experiment, we compared the effect of physiological concentrations of aldosterone, cortisol, and testosterone on αENaC promoter–reporter constructs. Physiological testosterone concentrations (10 nmol/L) showed a similar fold induction of construct 2 (containing the ARE) as 0.5 nmol/L aldosterone (Figure 8). Physiological concentrations of cortisol (100 nmol/L) showed a significantly higher fold induction of construct 4 (Figure 8).

Animal Experiments

Testosterone treatment in rats resulted in significantly increased αENaC mRNA expression in the kidney (Figure 9). This was found for both testosterone concentrations tested. Treatment with the more potent androgen 5α-dihydrotestosterone also resulted in an increase of αENaC mRNA expression in the kidney, but this effect was less pronounced than for testosterone (Figure 9).

Electrophysiological Transepithelial Measurements

The Rₑ of the M-1 cells ranged from 50 to 200 Ωcm². The Iₑ measured in these studies is clearly mediated predominantly by epithelial Na⁺ channels (ENaCs). Addition of 10 μmol/L amiloride, an inhibitor of ENaC, to the apical compartment rapidly decreased Iₑ. The vehicle control group (ethanol) showed a significantly lower response to amiloride than M-1 cells treated with testosterone (Figure 10).

Discussion

Gender differences in the kidney were investigated in previous studies using cultured mesangial cells showing that estradiol had a modest proliferative effect and increased matrix metalloproteinase activity, whereas testosterone was not effective. This was regarded as “protective” effect of female gender on the progression of renal disease.22–24 Recently, it was shown that physiological testosterone levels induced an increase in apoptosis in human tubule cell line (HK-2) cells and human primary cultures of proximal tubular epithelial cells.25 However, this is the first report of the effects of testosterone on epithelial sodium transport in a human-derived renal cell line.

The human renal cell line HKC-8 expressed typical features of distal tubular cells such as 11β-HSD2, MR, and sgk1 and was chosen as a human-derived model to analyze the effects of androgens on genes associated with sodium homeostasis. It is necessary to emphasize that the HKC-8 cells are a transformed cell line, and therefore, the data obtained should be interpreted with caution.

Interestingly, HKC-8 cells converted androstenedione to 5α-androstenedione because of a high 5α-reductase 1 expression (Figure 2), which is a similar finding to cultured rat inner medullary collecting duct cells.26 Testosterone was neither activated to 5α-dihydrotestosterone nor inactivated to androstenedione and was not converted to estradiol, and therefore, we used testosterone for our experiments. The AR was strongly expressed in HKC-8 cells but also in human primary cultures of proximal tubular epithelial cells.27 This was found for both testosterone concentrations tested. Treatment with the more potent androgen 5α-dihydrotestosterone also resulted in an increase of αENaC mRNA expression in the kidney, but this effect was less pronounced than for testosterone (Figure 9).
Recently, it was suggested that genetic variations (A(2139)G polymorphism) in the sodium channel nonvoltage-gated 2 subunit gene encoding for ENaC could lead to an increased risk of hypertension. The 3 subunits of ENaC appear to be differentially regulated in specific tissues. In kidney, aldosterone causes an increase in ENaC mRNA expression but does not alter mRNA expression of the other 2 subunits, whereas in colon, aldosterone mainly induces β- and γ-subunit expression. Glucocorticoids induce αENaC mRNA expression in lung, kidney, and colon but β- and γ-subunit mRNA expression only in lung and colon and not in kidney. We identified a similar expression pattern of ENaC subunits under basal conditions in the human renal cell line HKC-8: αENaC was abundantly expressed, whereas βENaC and γENaC were not detectable. Testosterone upregulated only mRNA expression for αENaC (Table 2; Figure 4A and 4B), an effect that was mediated by the AR in that it was antagonized completely by the AR antagonist flutamide (Figure 5). Serum testosterone levels in healthy men range between 13 and 30 nmol/L and 0.5 to 2 nmol/L in

![Figure 6. Direct effects of testosterone on αENaC gene promoter activity.](image-url)
healthy women. Testosterone may also be formed through intrarenal synthesis as reported previously. Extrapolating from our dose-response studies (Figure 4B), physiological testosterone concentrations and possible intrarenal androgen synthesis may be high enough to cause a 2- to 3-fold increase in αENaC mRNA expression in men. Changes in sodium transport by increasing αENaC expression without affecting β- and γ-subunit expression may be subtle (2- to 3-fold), as shown by Sayegh et al and in this study, but it is an interesting finding and a possible explanation for the differences in blood pressure in normal subjects occurring with the onset of puberty.

We performed electrophysiological experiments and showed that testosterone treatment of M-1 cells resulted in a significantly larger response to amiloride than control cells. This indicates that testosterone enhances the amiloride-sensitive sodium transport across the renal epithelia (Figure 10). The relative incongruity between the large upregulatory effect of testosterone on αENaC message in HKC-8 cells and the small functional response obtained in M-1 cells may be attributable to species (human versus mouse) and/or compartmental differences (proximal versus distal origin of the cell lines). In our studies, we used defined media without FCS to avoid nonspecific effects that may be induced by serum or other undefined factors. Although the difference between testosterone treatment and control was relatively small, this difference was consistent and significant. However, we were able to demonstrate that this effect of testosterone is not only an in vitro phenomenon in renal cell lines, but that this effect is also present in an in vivo rat model (Figure 9). In vivo testosterone treatment of rats resulted in a significant increase in αENaC expression in the rat kidney.

Surprisingly, 5α-dihydrotestosterone did not change αENaC mRNA expression in HKC-8 cells, although a small but not significant induction of αENaC promoter construct 2 by 5α-dihydrotestosterone was observed (Figure 6C). According to the in vitro data, we found a significant lower increase in renal αENaC mRNA expression with the more potent androgen 5α-dihydrotestosterone in our in vivo rat model (Figure 9). Recent evidence suggests that different AREs could respond differentially to testosterone versus 5α-dihydrotestosterone, indicating that target gene DNA sequences may be important factors regulating testosterone versus 5α-dihydrotestosterone differential transactivation.

In addition, it is possible that the testosterone–AR-ARE complex recruits different AR coregulators compared with 5α-dihydrotestosterone–AR-ARE complex. Another possible explanation for this finding may be the expression of

Figure 7. A, Constructs 1 and 2 were transiently transfected into HKC-8 cells and treated with 100 nmol/L testosterone (T) in the presence or absence of AR antagonist flutamide (48-hour incubation). Luciferase activity levels (normalized by cotransfection with a renilla luciferase construct) were reported as fold increase compared with the corresponding construct (ctr) without steroid, which was set as 1. Means of quadruplicate incubations and duplicate measurements±SD. ***Significant (P<0.001) to ctr. B, Changes (percent) in luciferase activity of construct 2 (gray bars) and ARE mutant construct 2 (open bars) by increasing concentrations of testosterone. Means of quadruplicate incubations and duplicate measurements±SEM. *P<0.05 compared with the corresponding construct without steroid.

Figure 8. Comparison of physiological aldosterone, cortisol, and testosterone concentrations on αENaC gene promoter activity. Constructs were transiently transfected into HKC-8 cells in the presence or absence of 0.5 nmol/L aldosterone (A), 100 nmol/L cortisol (F), or 10 nmol/L testosterone (T; 48-hour incubation). Luciferase activity levels (normalized by cotransfection with a renilla luciferase construct) were reported as fold increase compared with the corresponding construct (ctr) without steroid, which was set as 1. Means of quadruplicate incubations and duplicate measurements±SD. ***Significant (P<0.001) to ctr.
17β-HSD2 and 17β-HSD4 (Figure 2), inactivating 5α-dihydrotestosterone to 5α-androstanedione at a “prereceptor” level in HKC-8 cells.

Dexamethasone increased αENaC mRNA expression in HKC-8 cells, which is in agreement with studies by others using human lung cell lines. This has been explained by a GRE in the 5'-flanking region of the gene encoding αENaC (SCNN1A). This response element is in fact an ARE with 93% similarity to GRE, which may explain transcriptional regulation of αENaC by testosterone, glucocorticoids, and mineralocorticoids. It is located 155-140 nucleotides upstream of the transcription start side of exon 1A in the SCNN1A gene and seems to regulate transcription start sites of exons 1A and 1B. We located putative transcription factor binding sites of Oct-1 and NF-1 proximal of the AR binding site, which may potentiate the relative responsiveness of the AR-regulated promoter. Our promoter–reporter studies indicated that this ARE is required for the direct transcriptional upregulation of αENaC mRNA by testosterone. Promoter construct 2 containing the ARE showed a significantly higher luciferase activity than construct 1, which does not contain the ARE sequence (Figure 6B), and this testosterone-dependent increase of construct 2 was blocked by flutamide (Figure 7A). In addition, testosterone-mediated transcription was abolished by mutation of the ARE in construct 2 (Figure 7B). Without testosterone, construct 2 showed a 2-fold higher luciferase activity than construct 1 (data not shown), which may be attributable to several Sp1 consensus sites in the construct 2 sequence. The activity of TATA-less promoters, such as the human αENaC promoter, is frequently dependent on Sp1 sites in the proximal promoter region. Interestingly, construct 3, which is 200-bp longer at the 5'-end than construct 2, and construct 4, which spans >1.5 kb, also showed a significant increase in luciferase activity by testosterone, but not as high as construct 2 at high testosterone concentrations. This suggests the existence of possible repressor sites in this region: 1 AP-2α, 2 NF-1, 2 Sp1, and 1 polyomavirus enhancer activator 3 (PEA3) consensus sites were found in this region of the promoter. In corneal epithelial cells, AP-2 functions as a repressor of K3 keratin expression, opposing the effects of Sp1. Competition has been proposed between NF-1 proteins and Sp1 for binding at adjacent sites as a means for NF-1 to repress Sp1 activation of the promoter. PEA3 belongs to the PEA/external tran-

Figure 9. Fold increase in kidney αENaC mRNA expression in orchiectomized rats with testosterone or 5α-dihydrotestosterone treatment for 14 days compared with controls. Each group consisted of 4 animals. Each kidney was investigated at least in triplicate. Controls were set as 1. Means±SD. *P<0.05 and **P<0.001 compared with control.

Figure 10. Ieq (in μA/cm²) in response to addition of 10 μmol/L amiloride to the apical compartment in untreated (control) and testosterone-treated M-1 mouse cortical collecting duct cells. A, Time course. B, Change of Ieq attributable to amiloride; means±SEM (n=7).

Figure 6A. Short circuit current Ieq (in μA/cm²) in response to addition of 10 μmol/L amiloride to the apical compartment in untreated (control) and testosterone-treated M-1 mouse cortical collecting duct cells. A, Time course. B, Change of Ieq attributable to amiloride; means±SEM (n=7).
scribed spacer family, which is able to interact directly with the AR and modulate androgen-dependent gene expression. Physiological testosterone concentrations (10 nmol/L) had the same effect as physiological aldosterone concentrations (0.5 nmol/L) on the promoter activity (Figure 8), and 100 nmol/L cortisol showed a significantly higher increase of luciferase activity for construct 4 (Figure 8). Because of the artificial system, it is difficult to draw conclusions for clinical or pathophysiological situations from these differences for these hormones.

In addition to direct transcriptional regulation, there are several additional mechanisms that may alter ENaC activity (eg, post-translational modification by sgk1). Sgk1 is a mineralocorticoid-inducible gene and regulates ENaC distribution at the luminal side of the epithelial cell through phosphorylation of Nedd4-2. Phosphorylated Nedd4-2 is not able to mediate ubiquination and degradation of ENaC from the cell surface. We found an increase in sgk1 mRNA expression with dexamethasone but not after incubation with androgens in HKC-8 cells (Table 2).

In conclusion, we described the direct upregulation of αENaC mRNA expression by testosterone via the AR in vitro in the human renal cell line HKC-8 and in vivo in a rat model.

Perspectives

In men, androgens either from circulation or from intrarenal synthesis can bind to the AR in distal tubular cells and may increase αENaC mRNA expression and epithelial sodium transport. This mechanism provides a possible explanation for the higher normal blood pressure observed in men compared with women. Future studies should explore whether this may be also an important target for management of the increased susceptibility to hypertension in men.

Acknowledgments

This work was supported by a Deutsche Forschungsgemeinschaft postdoctoral research fellowship grant (QU142/1-1) to M.Q., and the Medical Research Council (cooperative core group grant). This work received the Schoeller-Junkmann Award 2005 of the German Endocrine Society. We would like to thank David M. Smith, Carl Montague, and Kay Garnett (AstraZeneca, UK) for help with microarray analysis. Thanks to Kevin Eardley, Department of Nephrology, Queen Elizabeth Hospital, Birmingham, UK, for the primary kidney cell lines, and to Beverly Hughes for T47D cells. We also thank Wiebe Arlt, Nicole Draper, and Elizabeth A. Walker, all of Birmingham, UK, for help with the promoter analysis and transfection studies.

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Hypertension. 2005;46:787-798; originally published online September 19, 2005; doi: 10.1161/01.HYP.0000184362.61744.c1

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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