

# Molecular Basis of Human Salt Sensitivity: The Role of the 11 $\beta$ -Hydroxysteroid Dehydrogenase Type 2\*

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## ABSTRACT

Salt-sensitive subjects (SS) increase their blood pressure with increasing salt intake. Because steroid hormones modulate renal sodium retention, we hypothesize that the activity of the 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ HSD2) enzyme is impaired in SS subjects as compared with salt-resistant (SR) subjects. The 11 $\beta$ HSD2 enzyme inactivates 11-hydroxy steroids in the kidney, thus protecting the nonselective mineralocorticoid receptor from occupation by glucocorticoids. We performed an association study using a recently identified single *AluI* polymorphism in exon 3 and a polymorphic microsatellite marker of the HSD11B2 gene in 149 normotensive white males (37 SS and 112 SR). The activity of the enzyme 11 $\beta$ HSD2 was assessed by determining the urinary ratio of cortisol (THF+5 $\alpha$ THF) to cortisone (THE) metabolites by gas chromatography in all the 37 SS subjects and in 37 age- and body habitus-matched SR volunteers. Mean (THF+5 $\alpha$ THF)/THE ratio was markedly elevated in SS subjects compared with SR subjects (1.51  $\pm$  0.34 vs. 1.08  $\pm$  0.26,  $P$  < 0.0001), indicating enhanced access of glucocorticoids to the mineralocorticoid receptor in SS subjects. In 58% of SS subjects this ratio was higher than the maximum levels in SR subjects. The

salt-induced elevation in arterial pressure increased with increasing (THF+5 $\alpha$ THF)/THE ratio ( $r^2$  = 0.51,  $P$  < 0.0001). A total of 12 alleles of the polymorphic microsatellite marker were detected. Homozygosity for the allele A7 was higher in SS subjects than in SR subjects (41 vs. 28%,  $P$  < 0.005), whereas the occurrence of the allele A7 with allele A8 was lower in SS subjects than in SR subjects (8 vs. 15%,  $P$  < 0.03). The prevalence of salt sensitivity was 35% in subjects with allele A7/A7, whereas salt sensitivity was present in only 9% of the subjects with allele A7/A8. The (THF+5 $\alpha$ THF)/THE ratio was higher in subjects homozygous for the A7 microsatellite allele as compared with the corresponding control subjects. The prevalence of the *AluI* allele was 8.0% in SR subjects and 5.4% in SS subjects and did not correlate with blood pressure. The decreased activity of the 11 $\beta$ HSD2 in SS subjects indicates that this enzyme is involved in salt-sensitive blood pressure response in humans. The association of a polymorphic microsatellite marker of the gene with a reduced 11 $\beta$ HSD2 activity suggests that variants of the HSD11B2 gene contribute to enhanced blood pressure response to salt in humans. (*J Clin Endocrinol Metab* 84: 3745–3749, 1999)

**I**N mineralocorticoid target organs the 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ HSD2) converts cortisol to the receptor-inactive cortisone, thus protecting the nonselective mineralocorticoid receptor from occupation by cortisol (1–3). Mutations in the HSD11B2 gene, as occur in the syndrome of apparent mineralocorticoid excess (4–6) or inhibition by licorice (7), result in a compromised 11 $\beta$ HSD2 enzyme activity, which in turn lead to overstimulation of the mineralocorticoid receptor by cortisol and sodium retention, hypokalemia, and hypertension (8).

Patients with essential hypertension do not have overt signs of mineralocorticoid excess, however, more subtle changes such as a positive correlation between blood pressure and serum sodium levels or a negative correlation with potassium levels may suggest a corticosteroid influence (9).

Recent studies have shown that the half-life of cortisol is significantly prolonged and the excretion of urinary cortisol metabolites increased in some patients with essential hypertension (10, 11). Moreover, a genetic association of a HSD11B2 flanking microsatellite and hypertension was also reported (12). In the “four-corner study,” an impaired conversion of cortisol to inactive metabolites has also been reported in young men with higher blood pressure whose parents also had high blood pressure (13). Together, these studies suggest that the 11 $\beta$ HSD2 enzyme may play a role in essential hypertension and in the sensitivity of blood pressure to dietary salt. A salt-sensitive (SS) response of blood pressure has not only been observed in patients with hypertension, but has also been well documented in young normotensive individuals (14). These subjects also display a number of traits, including increased pressor response to vasoactive substances, or mental stress, suppression of the renin-angiotensin system, and insulin resistance, features that can also be found in patients with essential hypertension (15). It has, therefore, been suggested that SS normotensive individuals may be genetically predisposed to the development of hypertension (16).

Thus, the main hypotheses addressed in this project were: 1) whether the recently described *AluI* [Glu<sup>178</sup>/Glu (G534A)] polymorphisms within (17) or the polymorphic microsatel-

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lite marker flanking (GenBank AF071493) (18) the HSD11B2 gene are related to salt-sensitivity in young Caucasian normotensive subject; and 2) whether changes in blood pressure due to salt-sensitivity are associated with changes in 11 $\beta$ HSD2 activity as assessed by the urinary excretion ratio of the cortisol (THF+5 $\alpha$ THF) to cortisone (THE) metabolites.

## Subjects and Methods

### Subjects

The 149 study subjects were unrelated young Caucasian male volunteers recruited among medical students from the Free University of Berlin. All subjects underwent routine clinical and laboratory evaluations to ensure that none had hypertension, hyperlipidemia, diabetes mellitus, or hepatic or renal disease. Only subjects with a blood pressure <140/85 mm Hg were included in the study. All participants gave their informed consent to participate in the study.

As described previously (19, 20), subjects were given a standardized low-salt diet containing 20 mmol sodium, 20 mmol chloride, 60 mmol potassium, and 20 mmol calcium per day for 7 days. Thereafter, the same diet with a daily supplement of 20 tablets of sodium (10 mmol NaCl per tablet; a gift of Ciba-Geigy, Horsham, UK) or placebo was administered in a randomized single-blind crossover fashion for 7 days, respectively. Throughout the dietary period, compliance was assessed by measuring the daily 24-h urinary sodium excretion by standard laboratory methods. At the end of each week, following a 30-min resting period, blood pressure was measured for assessment of salt sensitivity in the recumbent subject over 1 h at 5-min intervals with an automatic oscillometric device (DINAMAP 1846 SX; Critikon, Tampa, FL). As in previous studies (19–21), salt sensitivity was defined as a significant drop in mean arterial pressure >3 mm Hg under the low-salt diet calculated as the difference between the average of the 60 readings under the high- and low-salt periods ( $P < 0.05$ , paired  $t$  test for independent samples). The SEM for a single 60-min period ranged between 0.35 and 0.65 mm Hg. Salt sensitivity defined according to this protocol is a well-reproducible phenomenon in normotensive individuals (21).

### PCR analysis

PCR amplification of exon 3 was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer Corp., Oak Brook, IL) as described previously (17). Primers were derived from the intronic sequences flanking exon 3 published elsewhere (4). PCR analysis of the polymorphic microsatellite marker (18) was performed by the automated fluorescent genotyping method using an unlabeled forward primer (5'-CCAGC-CAGGTTGGAAGTGTG-3') and a fluorochrome-labeled reverse primer (5'-CAGTACGGTCTCCCCATCT-3'). PCR reactions were performed by using 100 ng template DNA, 20 pmol of each primer, and 5 nmol of each dNTP in supplied PCR buffer with 2.5 U Ampli-Taq Gold (Perkin Elmer Corp.) in a total volume of 50  $\mu$ L. Initial denaturation was at 95°C for 10 min, followed by 30 cycles of 95°C for 15 sec, 58°C for 15 sec, and 72°C for 45 sec. Final extension was at 72°C for 7 min.

### Gel Analysis and sequencing

PCR products of exon 3 were analyzed on 12% acrylamide gels containing 7.25% glycerol using a two-buffer system, 4  $\mu$ L of the PCR sample were loaded and DNA was visualized by silver staining (22). Sequence changes were detected by double band shifts on the gel. Identified variants were further analyzed by restriction digest of PCR products with the *AluI* enzyme cutter, according to standard methods.

The fluorescently labeled polymorphic marker fragments were analyzed using an ABI GENESCAN (Version 2.0.1 fc2) software on the Model ABI PRISM 377 (Version 1.1) automated sequencer (PE Applied Biosystems, Foster City, CA). Genotype data were generated using ABI GENOTYPER (Version 1.1r8) DNA fragment analysis software (PE Applied Biosystems).

### Urinary steroid profile analysis

Urine samples were analyzed by gas chromatography according to the method of Shackleton (23) [for the determination of tetrahydrocortisol (THF), 5 $\alpha$ -tetrahydrocortisol (5 $\alpha$ THF), and tetrahydrocortisone (THE)]. The analytical procedure consisted of hydrolysis, solid phase extraction, derivatization, and purification of the samples by gel filtration. A total of 2.5 mL urine mixed with 0.5 mL acetate buffer 0.5 M were hydrolyzed (for 3 h at 55°C) with Sigma Chemical Co. (Buchs, Switzerland) Type I powdered Helix promatia enzyme (12 mg) and 12.5  $\mu$ L Boehringer Mannheim (Rat-kreuz, Switzerland)  $\beta$ -glucuronide/aryl sulfatase liquid enzyme. The resulting free steroids were extracted with a Sep-Pak cartridge and taken to a final solution of 4 mL in methanol. To this extract an internal standard mixture (5 $\alpha$ -androstane-3 $\alpha$ , 17 $\alpha$ -diol, stigmaterol, and cholesteryl butyrate, 2.5  $\mu$ g each) was added, and the sample was derivatized to form the methyloxime-trimethylsilyl ether. Derivatization mixtures were purified by gel filtration on Lipidex-5000 columns. Samples were analyzed on a Carlo Erba (Milan, Italy) Gaschromatograph 2100 equipped with a Merck and Co., Inc. (Darmstadt, Germany) Hitachi D-2500 Chromato-Integrator. The derivatized steroid samples were analyzed during a temperature-programmed run (210–270°C) over a 40-min period. Several steroid mixtures containing known amounts of all metabolites to be measured were analyzed as calibration standards, and the values obtained in the urine steroid analysis were determined relative to this calibration. In each case, peak areas were quantified against the ones of androstane and stigmaterol internal standards, and the mean values were taken as final results.

### Other biochemical variables

Plasma and urine potassium, sodium, and creatinine were measured by standard laboratory techniques. Plasma renin activity (PRA) and plasma aldosterone concentrations (PAC) were assayed by radioimmunoassay as previously described (24).

### Statistics

Values are expressed as mean  $\pm$  SD. Median values and 95% confidence intervals (CIs) were used when appropriate.

Statistical differences between means were assessed by non-parametric analysis, and the 2x2 contingency tables by  $\chi^2$  test.

## Results

The physical data, the blood pressure values on low-salt diet, the blood pressure increases on high-salt diet, and the urinary excretion of sodium and creatinine on low-sodium diet of the SS and salt-resistant (SR) subjects are outlined in Table 1. Age, height, and weight did not differ in SS subjects as compared with SR subjects. In the two groups, the mean blood pressure was identical on low-salt diet. When the diet was changed from a low to a high salt intake, blood pressure increased in SS subjects and decreased slightly in SR subjects (Table 1). The mean ( $\pm$ SD) values on a high-salt diet were 116/61  $\pm$  10/10 and 108/56  $\pm$  7/7 mm Hg, ( $P < 0.0001$ ) in SS and SR subjects, respectively.

### Genotyping

Exon 3 of the HSD11B2 gene was amplified by PCR in all 149 subjects. Gel analysis of the PCR products revealed a total of 11 migration variants (7.4%). Migration variants were further analyzed after restriction digest with the *AluI* cutter demonstrating the presence of the *AluI* restriction polymorphism in all variants. All 11 subjects were heterozygous for the polymorphic marker. The *AluI* marker was positive in 2 of 37 SS subjects (5.4%) and 9 of 112 SR subjects (8.0%).

A total of 12 different alleles of the polymorphic microsatellite marker were detected among the 298 alleles analyzed. The length of PCR products varied from 356 nucleotides for allele A1 to 378 nucleotides for allele A12. Heterozygosity in this population reached 68%. Homozygosity for the allele A7 was higher in SS subjects than in SR subjects (41 vs. 28%,  $P < 0.005$ ), whereas the occurrence of the allele A7 with allele A8 was lower in SS subjects than in SR subjects (8 vs. 15%,  $P < 0.03$ ). The prevalence of salt sensitivity was 35% in subjects with allele A7/A7, whereas salt sensitivity was present in only 9% of the subjects with allele A7/A8.

**TABLE 1.** Physical findings, blood pressure, and urinary data in SS and SR normotensives (mean  $\pm$  SD).

	SS	SR
Number	37	112
Age (yr)	24.7 $\pm$ 2.3	24.9 $\pm$ 2.3
Height (cm)	183 $\pm$ 6	181 $\pm$ 7
Weight (kg)	77 $\pm$ 9	76 $\pm$ 9
Body mass index (kg/m <sup>2</sup> )	22.9 $\pm$ 1.9	22.9 $\pm$ 2.0
Blood pressure under low salt		
Systolic (mm Hg)	111 $\pm$ 9	110 $\pm$ 8
Diastolic (mm Hg)	58 $\pm$ 9	58 $\pm$ 8
Mean (mm Hg)	78 $\pm$ 8	79 $\pm$ 6
Changes in blood pressure during high salt		
Systolic (mm Hg)	5.1 $\pm$ 4.1	-1.8 $\pm$ 5.1 <sup>a</sup>
Diastolic (mm Hg)	4.0 $\pm$ 2.5	-2.5 $\pm$ 3.5 <sup>a</sup>
Mean (mm Hg)	5.0 $\pm$ 1.6	-2.9 $\pm$ 3.7 <sup>a</sup>
Urine sodium excretion		
Low-salt diet (mmol/day)	28 $\pm$ 12	28 $\pm$ 14
High-salt diet (mmol/day)	230 $\pm$ 51	225 $\pm$ 52

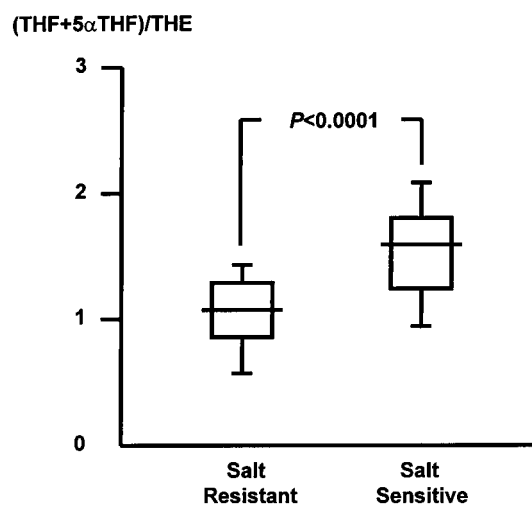
<sup>a</sup>  $P < 0.0001$  SS vs. SR volunteers.

### Phenotyping

The mean (THF+5 $\alpha$ THF)/THE ratio in urines collected on low-salt diet was markedly elevated in SS subjects as compared with SR subjects (1.51  $\pm$  0.34 vs. 1.08  $\pm$  0.26,  $P < 0.0001$ ) (Fig. 1). Median values (and 95% CIs) for these ratios were 1.56 (1.35–1.61) in SS subjects and 1.10 (0.92–1.15) in SR patients; in 58% of SS subjects the ratio was higher than the maximum level observed in SR subjects. The urinary (THF+5 $\alpha$ THF)/THE ratio was  $>1.5$  in 21 SS subjects but only in 1 SR subject, and in 8 SS patients this ratio was  $>1.8$ . The increased ratio of (THF+5 $\alpha$ THF)/THE in SS subjects was due to a decreased production of THE (3280  $\pm$  1890 vs. 4530  $\pm$  2380  $\mu$ g/day,  $P < 0.01$ ), whereas THF+5 $\alpha$ THF [4840  $\pm$  2670 vs. 4730  $\pm$  2440  $\mu$ g/day,  $P =$  not significant (NS)] did not differ significantly in SS and SR subjects in 24-hour urines. There was a positive correlation between the salt-induced increase in mean arterial pressure and the urinary ratio of (THF+5 $\alpha$ THF)/THE ( $r^2 = 0.51$ ,  $P < 0.0001$ ) (Fig. 2). Subjects with the *AluI* polymorphic marker showed lower (THF+5 $\alpha$ THF)/THE ratios than *AluI*-negative volunteers (1.05  $\pm$  0.21 vs. 1.33  $\pm$  0.38,  $P < 0.01$ ). The urinary ratio of (THF+5 $\alpha$ THF)/THE was significantly higher in subjects with the A7/A7 microsatellite allele homozygosity (1.45  $\pm$  0.29) than in subjects with other alleles (1.24  $\pm$  0.39,  $P < 0.05$ ) or with the allele pair A7/A8 (1.15  $\pm$  0.25,  $P < 0.01$ ).

### Other biochemical variables

We analyzed the relationship between urinary steroid metabolites and PRA, PAC, and potassium in the subjects whose urinary (THF+5 $\alpha$ THF)/THE ratios were measured. PRA was significantly lower in SS subjects as compared with SR patients (0.97  $\pm$  0.79 vs. 1.12  $\pm$  0.97 ng/mL/h,  $P < 0.05$ ), as was PAC (280  $\pm$  20 vs. 370  $\pm$  19 pmol/L,  $P < 0.05$ ), whereas plasma potassium did not differ significantly in the two groups (4.2  $\pm$  0.3 vs. 4.4  $\pm$  0.3 mmol/L,  $P =$  NS) although there was a tendency for lower potassium levels in SS subjects than in SR subjects. PRA was positively associated with PAC ( $r^2 = 0.57$ ,  $P < 0.0001$ ). There was a weak correlation



**FIG. 1.** Activity of the 11 $\beta$ HSD2 as assessed by the urinary (THF+5 $\alpha$ THF)/THE excretion ratio in 37 SS and 37 age- and body-habitat-matched SR subjects (median and 95% CI).



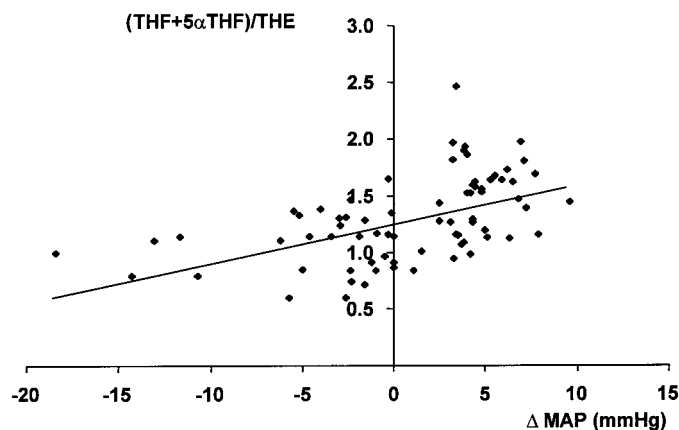


FIG. 2. Relationship between salt-induced changes in mean arterial pressure ( $\Delta$ MAP) and the urinary (THF+5 $\alpha$ THF)/THE ratio as a measure of 11 $\beta$ HSD2 activity ( $r^2 = 0.51$ ,  $P < 0.0001$ ).

between urinary (THF+5 $\alpha$ THF)/THE ratio and PAC ( $r^2 = -0.23$ ,  $P < 0.05$ ) and a tendency for a negative correlation between urinary (THF+5 $\alpha$ THF)/THE ratio and PRA ( $r^2 = -0.18$ ,  $P = \text{NS}$ ).

### Discussion

The present study demonstrates that a salt-induced blood pressure increase is associated with impaired 11 $\beta$ HSD2 activity, as measured by the urinary excretion ratio of (THF+5 $\alpha$ THF)/THE in young Caucasian SS men. Moreover, allele analysis of a polymorphic microsatellite flanking the HSD11B2 gene reveals a correlation with salt-induced blood pressure elevation and impaired 11 $\beta$ HSD2 activity, indicating that some individuals with SS blood pressure have subtle genetic abnormalities of the 11 $\beta$ HSD2 enzyme, causing a decrease in renal cortisol inactivation and an increased blood pressure susceptibility to salt.

The prevalence of salt sensitivity in this group was 25%, in accordance with an approximate 30% frequency observed in the normotensive white population (15). The activity of the 11 $\beta$ HSD2 was decreased in some, but not all, SS subjects (Fig. 1). Although normal values of the urinary (THF+5 $\alpha$ THF)/THE ratio for children are on average  $1.1 \pm 0.3$  (25, 26), for young adults these values range from 0.6–1.3, with an average of  $1.21 \pm 0.06$  (27). Moreover, these values are  $1.30 \pm 0.07$  in normal adult males and  $1.15 \pm 0.11$  in normal adult females (27). In 57% of SS subjects the urinary (THF+5 $\alpha$ THF)/THE was  $>1.5$  (1.52–2.47), whereas in the SR group this ratio was  $<1.4$  in all but three subjects, and none of them had a ratio  $>1.65$ . Therefore, considering the urinary (THF+5 $\alpha$ THF)/THE excretion ratio as an intermediate phenotype, it seems that approximately half of the subjects with blood pressure susceptibility to salt display a decreased 11 $\beta$ HSD2 activity.

Our data are in apparent contrast with a recent observation that subjects with highest urinary-free cortisol show the least sensitivity of blood pressure to dietary sodium (28). There are two explanations for this discrepancy. First, in the study by Litchfield *et al.* (28) only urinary-free cortisol but not cortisone or (THF+5 $\alpha$ THF)/THE ratio were measured, thereby not allowing a direct evaluation of 11 $\beta$ HSD2 activity; second,

a reduced 11 $\beta$ HSD2 activity correlates with a decreased urinary excretion of free cortisone rather than an increased urinary-free cortisol excretion (27). Thus, measuring cortisone and its metabolites or (THF+5 $\alpha$ THF)/THE ratio in the urine seems to be the most appropriate assay of renal 11 $\beta$ HSD2 activity, although it should be emphasized that the most specific test for 11 $\beta$ HSD2 activity is provided by the metabolism of 11 $\alpha$ -[ $^3\text{H}$ ] cortisol, as described by Ulick *et al.* (8).

There are two possible mechanisms for a reduced 11 $\beta$ HSD2 activity. On one hand, it is possible that circulating factors may inhibit the renal 11 $\beta$ HSD2 enzyme in the SS population (29). In a recent study, such inhibitors were described in some essential hypertensive patients (29). A low-salt diet increased these inhibitors in high/normal-renin but not in low-renin hypertensives (29). Considering that most investigators reported low levels of plasma renin in SS subjects (15), it seems unlikely that such factors play a key role in the reduced 11 $\beta$ HSD2 activity of the SS subjects investigated. On the other hand, the presence of a genetic variant of the enzyme, with only slightly reduced activity, deserves consideration. This aspect has been recently predicted by the description of a young girl with low-renin hypertension but without the characteristic features of apparent mineralocorticoid excess (30). Analysis of the urinary excretion of steroid metabolites indicated a mild form of the syndrome, which was confirmed by genetic analysis. The identified mutant showed only a slightly reduced enzymatic activity *in vitro* (30). Based on this observation, we analyzed two known polymorphic markers of the HSD11B2 gene (17, 18) and their linkage to the intermediate phenotype of decreased 11 $\beta$ HSD2 activity or the distant phenotype of salt-induced elevation in blood pressure.

The lack of correlation of the *AluI* (G534A) variant with either salt sensitivity or impaired 11 $\beta$ HSD2 activity indicates that this polymorphism has no functional significance, at least in the heterozygous state. This observation is in line with the recently reported absence of a positive association between the *AluI* (G534A) marker and severe essential hypertension in sibships with multiple hypertensive subjects (18).

To analyze whether some Caucasian individuals with SS blood pressure may have more subtle genetic abnormalities of the 11 $\beta$ HSD2 enzyme, responsible for an increased blood pressure susceptibility to salt, the microsatellite marker described by Brand *et al.* (18) was used. The analysis revealed a positive association with the allele A7 homozygosity and a negative correlation with allele pair A7/A8 of the microsatellite marker with salt sensitivity in this study suggest that the activity of the 11 $\beta$ HSD2 enzyme may be genetically determined by variants in the HSD11B2 promoter or by the presence of undetected mutations in the HSD11B2 gene itself, an issue deserving further investigation.

The CA-repeat allele polymorphism was recently analyzed in a large series of families with essential hypertension (18), and no correlation was found between this marker and blood pressure in this group (18). The apparent divergent findings of the latter investigation as compared with the present study is explained by the lack of selection for the blood pressure response to salt load in the patients studied

by Brand *et al.* (18). In fact, an association with the HSD11B2 locus and essential hypertension was suggested by Watson *et al.* (12) using a different microsatellite, which was shown to be significantly linked to essential hypertension in blacks. Blacks are more prone to develop low-renin hypertension, a form of salt-dependent hypertension (15). Considering the relationship between genotype and phenotype, in all subjects with the allele pair A7/A7 (the (THF+5 $\alpha$ THF)/THE ratio was higher than in the other patients. Even though some subjects homozygous for the A7 allele were SR, a risk remains that some individuals may develop a salt susceptibility later in life. In fact, SR patients with this allele pair had, on average, higher (THF+5 $\alpha$ THF)/THE ratios than the rest of the SR subjects.

In line with the findings in subjects with mutations in the HSD11B2 gene whose plasma renin and aldosterone levels are suppressed (4, 5, 30), we found lower PRA and PAC in the SS patients as compared with the SR group, and these values tended to be inversely correlated with the urinary (THF+5 $\alpha$ THF)/THE ratio. Thus, the relatively lower plasma renin and aldosterone levels, along with the decreased 11 $\beta$ HSD2 activity in SS patients, strongly suggest a role of this enzyme in the salt-induced blood pressure increase in this population.

The present findings indicate that in some young normotensive white male subjects the blood pressure response to salt load is related to a genetically determined reduction in the activity of the enzyme 11 $\beta$ HSD2. This might predispose to the development of hypertension with increasing age, along with excessive salt consumption. Biochemical analysis and targeted genotyping of the 11 $\beta$ HSD2 enzyme may help to detect subjects at risk to develop hypertension or hypertensive patients, whose condition is mediated by renal sodium retention via this mechanism. This would help to tailor dietary salt restriction (31) and to choose antihypertensive drugs that modulate mineralocorticoid effects in selected patients.

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