

# Abolition of Hypertension-Induced End-Organ Damage by Androgen Receptor Blockade in Transgenic Rats Harboring the Mouse Ren-2 Gene

OVIDIU BALTATU,\* CÉCILE CAYLA,\* RADU ILIESCU,\*<sup>†</sup> DMITRII ANDREEV,\*<sup>‡</sup> CYNTHIA JORDAN,<sup>§</sup> and MICHAEL BADER\*

\*Max-Delbrück-Center for Molecular Medicine (MDC), Berlin-Buch, Germany; <sup>†</sup>Department of Pharmacology at the University of Medicine and Pharmacy “Gr. T. Popa,” Iasi, Romania; <sup>‡</sup>Medical Faculty of Moscow Medical Sechenov Academy, Moscow, Russia; and <sup>§</sup>University of California, Department of Psychology, Berkeley, California.

**Abstract.** A sexual dimorphism in hypertension has been observed in both human and laboratory animal studies. The mechanisms by which male sex hormones regulate cardiovascular homeostasis are still not yet fully understood and represent the subject of this study. The possible involvement of androgen receptors in the development of hypertension and end-organ damage in transgenic rats harboring the mouse Ren-2 renin gene [TGR(mREN2)27] was studied. Male TGR(mREN2)27 rats were treated with the androgen receptor antagonist Flutamide starting at 4 wk of age. Also, an androgen receptor mutation (testicular feminization mutation [tfm]) was introduced in these rats by crossbreeding male TGR(mREN2)27 rats with tfm rats. The resulting offspring male rats that contain the tfm mutation are insensitive to androgens.

Flutamide treatment or tfm mutation produced a significant attenuation of the development of hypertension. Besides a reduction in cardiac hypertrophy, urinary albumin excretion was blunted and no histologic characteristics of end-organ damage were observed in the kidney after Flutamide treatment. Testosterone levels increased 15-fold after Flutamide treatment and 2.7-fold by the tfm mutation. Also, plasma estrogens and luteinizing and follicle-stimulating hormones were significantly increased. Plasma renin concentrations and activity but not plasma angiotensinogen were reduced. Our results indicate that androgens contribute not only to the development of hypertension, but even more importantly to end-organ damage in TGR(mREN2)27 rats.

Men are predisposed to hypertension and cardiovascular diseases more than age-matched, premenopausal women (1). A sexual dimorphism in hypertension has been observed both in human and laboratory animal studies (2). The mechanisms responsible for the gender differences in BP control are not yet clear and continue to be subject of active investigation (3). Evidence is accumulating that androgens may play an important role in gender-associated differences in BP regulation. Several studies have indicated that androgens may mediate hypertension and renal injury (4–8). However, the mechanisms by which male sex hormones regulate cardiovascular homeostasis are not yet fully understood (3) and represent the subject of this study. There are indications for an interrelation between androgens and the renin-angiotensin system (RAS) (3); therefore, we studied the consequences of androgen receptor blockade on the development of malignant hypertension in

transgenic TGR(mREN2)27 rats with overactive RAS (9,10). Androgen receptor inhibition was achieved by treatment with the antagonist Flutamide or by introducing a testicular feminization mutation (tfm) mutation, and BP as well as end-organ damage and RAS and pituitary-gonadal hormones were evaluated.

## Materials and Methods

### Rat Strains

Male transgenic heterozygous rats [TGR(mREN2)27] ( $n = 24$  rats) were obtained from the animal facilities of the Max-Delbrück-Center for Molecular Medicine, Berlin, Germany. Female Long-Evans rats carrying the X-linked recessive mutation (tfm) were provided by the Department of Psychology, University of California, Berkeley, California. The rats were housed individually, synchronized to a 12-h light-dark cycle, at ambient temperature  $23 \pm 2^\circ\text{C}$ . A standard rat diet (ssniff R-ZUCHT) and tap water were supplied ad libitum.

### Study Design

All experimental protocols were performed in accordance with the guidelines for the human use of laboratory animals by the Max-Delbrück-Center for Molecular Medicine and approved by an ethical committee.

To study the involvement of androgen receptors in the development of hypertension and end-organ damage in male TGR(mREN2)27 rats, two experimental strategies were used:

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Correspondence to Dr. Michael Bader, Max-Delbrück-Center for Molecular Medicine (MDC), Robert-Rössle-Str. 10, Berlin-Buch, D-13092, Germany. Phone: 49-30-9406-2193; Fax: 49-30-9406-2110; E-mail: mbader@mdc-berlin.de

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1. Treatment with Flutamide (specific nonsteroidal competitive antagonist of the androgen receptor, 30 mg/kg per d subcutaneously (11) starting at 4 wk of age ( $n = 12$ ) before the development of hypertension. A group of 12 rats received subcutaneous injections solely of the Flutamide solvent and represented the reference group.
2. Male TGR(mREN2)27 rats were crossbred with female Long-Evans carrying the X-linked recessive mutation in the rat androgen receptor (*tfm*). The resulting offspring male rats that contained the *tfm* mutation are insensitive to androgens. The male littermates lacking the *tfm* mutation represented the control group.

BP development was followed telemetrically, as described previously (12). The rats were implanted at the age of 7 to 8 wk with radiofrequency transmitters (TA11PA-C40; Data Sciences, St. Paul, MN). For the implantation of the transmitter, rats were anesthetized with 10 mg/100 g body wt Ketamin (Ketavet Parke-Davis, Berlin, Germany) plus 0.02 mg/100 g body wt xylazine (Rompun; Bayer, Leverkusen, Germany). The catheter of the transducer was implanted into the abdominal aorta just below the bifurcation of renal arteries, and the sensor itself was fixed to the peritoneum.

At the age of 12 wk, when the hypertension levels became stable, the 24-h urine was collected. The rats were sacrificed by decapitation under light ether anesthesia. Plasma was collected for hormone analysis. Cardiovascular organs were excised for histology and gene expression analysis.

### Genotyping of the *tfm* Mutation

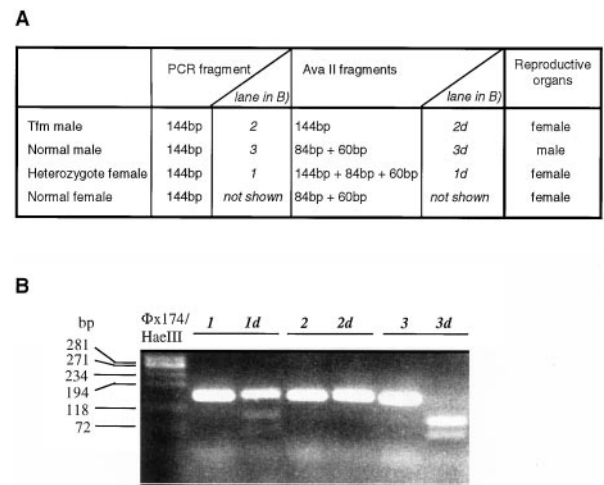
The offspring of female Long-Evans rats carrying the *tfm* mutation in the androgen receptor gene on the X chromosome and transgenic males harboring the mouse Ren-2 renin gene [TGR(mREN2)27] was analyzed to identify animals carrying the mutation. The mutation consists of a single base change from G in wild-type DNA to A in the *tfm* DNA in the coding region (13). The presence of this mutation erases the recognition site for the restriction enzyme *Ava*II (GGTCC). For genotyping, oligonucleotides were designed to amplify by PCR a fragment flanking the *tfm* mutation (forward primer: CTTCCG-CAACTTGCATGTGG; reverse primer: TCATTGAAAACCAGGT-CAGG). The amplified fragment has a length of 144 bp and is digested by *Ava*II in two fragments of 84 and 60 bp only in the case of the presence of the wild-type allele. Together with the morphology of the reproductive organs, this PCR allows to distinguish all possible genotypes of the rats (Figure 1).

### Kidney Damage Evaluation

Urine was collected by placing the rats into metabolic cages for 24 h. Rat urinary albumin (index of kidney damage) was determined by Immundiagnostics (Bensheim, Germany) using a specific ELISA. For histologic analysis, kidneys were excised, decapsulated, and fixed with 10% formalin in 0.01 M phosphate-buffered saline (PBS), pH 7.4, and dehydrated by immersing them stepwise into various concentrations of ethyl alcohol from low to high. The tissues were then embedded in paraffin and sectioned into 4- $\mu$ m-thick slices, and the sections were stained with Goldner trichrome. Cytoplasm, muscle tissue, and erythrocytes are stained in red; collagen is stained in green. At least five randomly selected areas per sample were observed.

### Heart Hypertrophy Evaluation

The hearts were excised, washed in ice-cold saline, blotted dry, and weighed. The left ventricles were separated and weighed.



**Figure 1.** Genotyping of rats. Rats were genotyped by assessment of the morphology of the reproductive organs and a PCR for the androgen receptor gene followed by an *Ava*II digestion of the product. (A) Predicted *Ava*II fragments and morphology of reproductive organs for each genotype. (B) Example of PCR result for different genotypes;  $\Phi$ X174 DNA digested with *Hae*III was used as a size marker.

### Hormone Measurements

Plasma was obtained from trunk blood collected on EDTA (6.25 mmol/L) after centrifugation at 4000 rpm. On the basis of a published report (14), plasma renin concentration and activity were determined using an indirect enzyme-kinetic assay based on the generation of angiotensin I with modification of the pH optimum to measure rat and mouse plasma renin activity (PRA) and plasma renin concentration (PRC). Angiotensinogen, testosterone, estrogens, and luteinizing and follicle-stimulating hormones were measured by RIA.

### Gene Expression Studies

Total RNA was isolated from the kidney using the TRIzol Reagent (Life Technologies, Eggenstein, Germany) followed by chloroform-isopropanol extraction, according to the protocol of the manufacturer. Specific mRNAs for rat or mouse renin were determined by ribonuclease protection assay (RPA), using the Ambion RPA II kit (AMS Biotechnology, Witney, UK) as described previously (15). For semi-quantitative determination of mRNA levels, band intensities were normalized to the housekeeping gene  $\beta$ -actin.

### Statistical Analyses

Data were analyzed by independent-samples *t* test between two-group comparisons or by GLM (general linear model)—general factorial or repeated measures procedure (SPSS 8.0) for multi-group and multifactorial analysis. Criterion for significant differences between groups of study was a *P* value less than 0.05. Results are expressed as the mean  $\pm$  SEM.

## Results

### Effect of Flutamide Treatment or *tfm* Mutation on Body Weight

The body weight was not significantly altered by the Flutamide treatment (Flutamide: 377.9  $\pm$  5.2 g versus control: 362.4  $\pm$  18.1 g at the end of experiment). The *tfm* rats weighed

significantly less than the wild-type controls ( $tfm^+$ :  $366.6 \pm 6.9$  g versus  $tfm^-$ :  $427.0 \pm 24.1$  g at the end of experiment).

*Effect of Flutamide Treatment or tfm Mutation on BP*

Untreated TGR(mREN2)27 rats developed fulminant hypertension starting at 10 wk of age as measured by telemetry (Figure 2). Flutamide treatment or the presence of the *tfm* mutation decreased significantly the levels of systolic BP (Figure 2). Furthermore, Flutamide increased the survival rate from approximately 76% at the age of 12 wk to 100%.

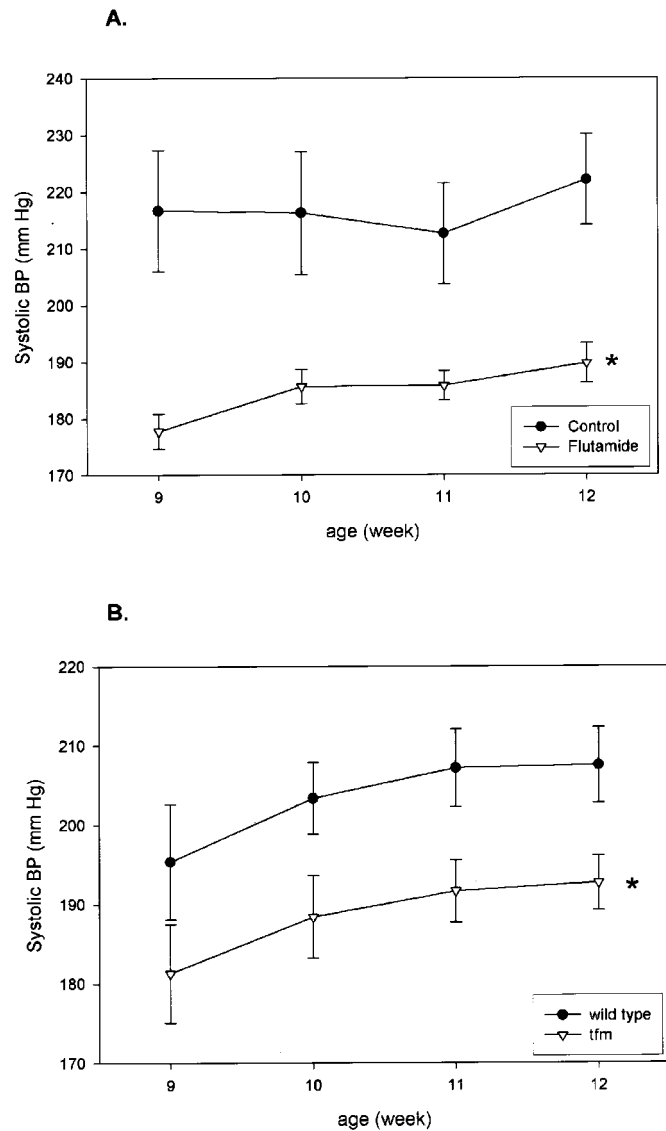


Figure 2. Effect of androgen receptor antagonism or insensitivity on the development of hypertension. (A) Untreated TGR(mREN2)27 rats (Control,  $n = 5$ ) compared with TGR(mREN2)27 rats treated with Flutamide (Flutamide,  $n = 6$ ). (B) Normal TGR(mREN2)27 *tfm*-negative (wild-type,  $n = 7$ ) compared with TGR(mREN2)27 littermates harboring the *tfm* mutation, (*tfm*,  $n = 7$ ). \* significantly different ( $P < 0.05$ ).

*Effect of Flutamide Treatment or tfm Mutation on End-Organ Damage*

Untreated hypertensive TGR(mREN2)27 rats developed signs of malignant hypertension with end-organ damage, including renal and cardiac pathology. The urinary albumin (as index of kidney damage) was drastically reduced by either Flutamide treatment or the presence of *tfm* mutation (Figure 3A). Furthermore, the kidney morphologic signs characteristic for the malignant hypertension (fibrinoid necrosis of arterioles and onion-shaped proliferative lesions) disappeared after Flutamide treatment (Figure 4). The TGR(mREN2)27 rats carrying the *tfm* mutation and their control littermates had normal kidney morphology at light microscopy. However, the urinary albumin, which is a marker that provides evidence of kidney damage before the development of lesions detectable by light microscopy (16–18), reached high levels in the control male littermates lacking the *tfm* mutation (Figure 3B). The lack of

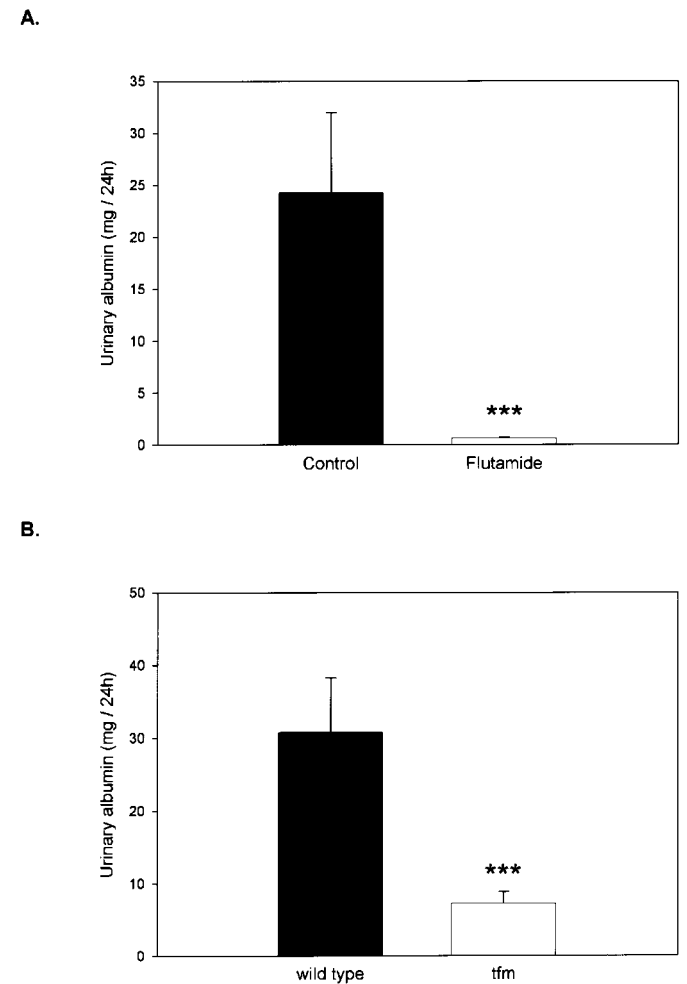
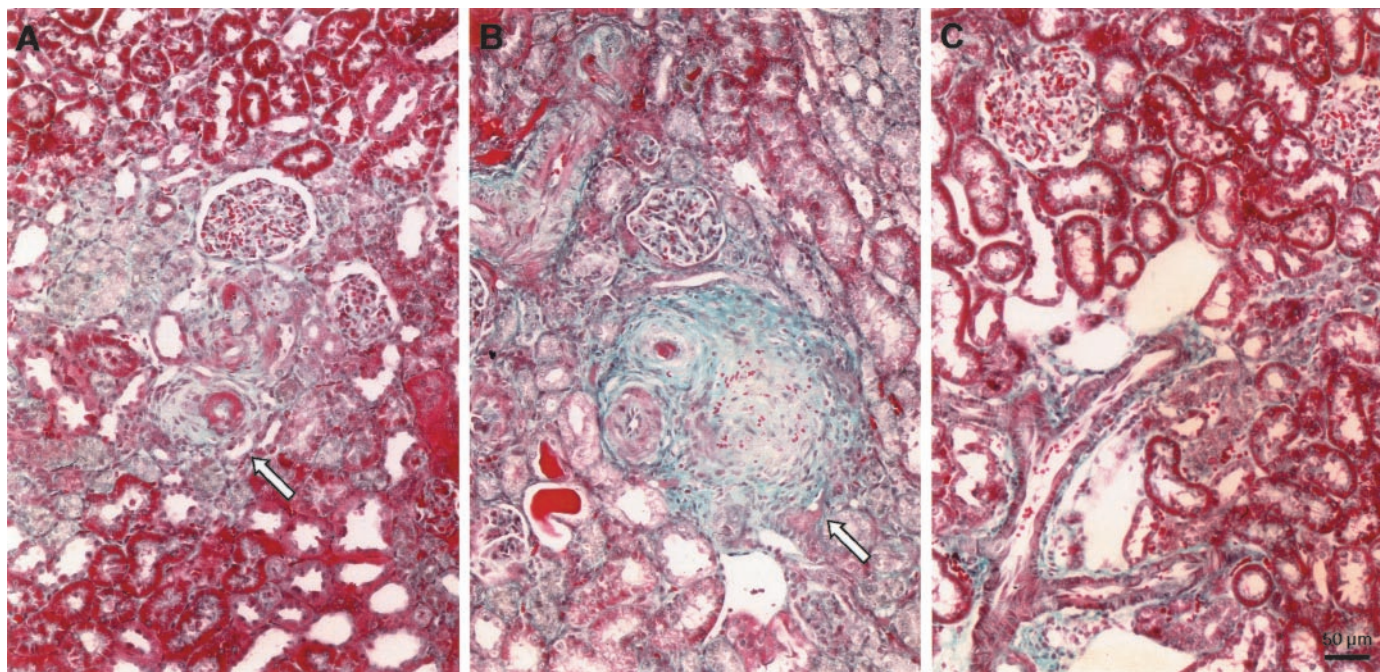


Figure 3. Effect of androgen receptor antagonism or insensitivity on urinary albumin excretion. (A) Untreated TGR(mREN2)27 rats (Control,  $n = 8$ ) compared with TGR(mREN2)27 rats treated with Flutamide (Flutamide,  $n = 6$ ). (B) Normal TGR(mREN2)27 *tfm*-negative (wild-type,  $n = 7$ ) compared with TGR(mREN2)27 littermates harboring the *tfm* mutation, (*tfm*,  $n = 7$ ). \*\*\* significantly different ( $P < 0.005$ ).





**Figure 4.** Abolition of hypertension-induced kidney histopathology by Flutamide treatment. While in the kidney of untreated TGR(mREN2)27 fibrinoid necrosis, concentric wall thickening of arterioles (A and B) and hyperplastic arteriosclerosis is observed; Flutamide-treated TGR(mREN2)27 exhibit a completely normal histologic aspect of the kidney (C).

morphologic signs characteristic for the malignant hypertension in the control male littermates lacking the *tfm* mutation could be due to lower BP levels compared with Flutamide controls and/or to the different genetic background of the two strains [TGR(mREN2)27 rats, having Sprague-Dawley background, crossbred with female Long-Evans carrying the *tfm* mutation results in a mixed Sprague-Dawley–Long-Evans background]. Although cardiac and left ventricular hypertrophies were reduced by both androgen receptor-targeting strategies, this effect only reached statistical significance after Flutamide treatment (Figure 5).

#### *Effect of Flutamide Treatment or tfm Mutation on RAS*

Plasma renin concentrations and activities were reduced after Flutamide treatment or by the presence of the *tfm* mutation (Table 1). RPA for rat or mouse renin mRNAs in kidney revealed significantly decreased levels after Flutamide treatment (Table 1). However, the levels of renin mRNAs were not altered significantly by the presence of the *tfm* mutation. Plasma angiotensinogen was also not altered.

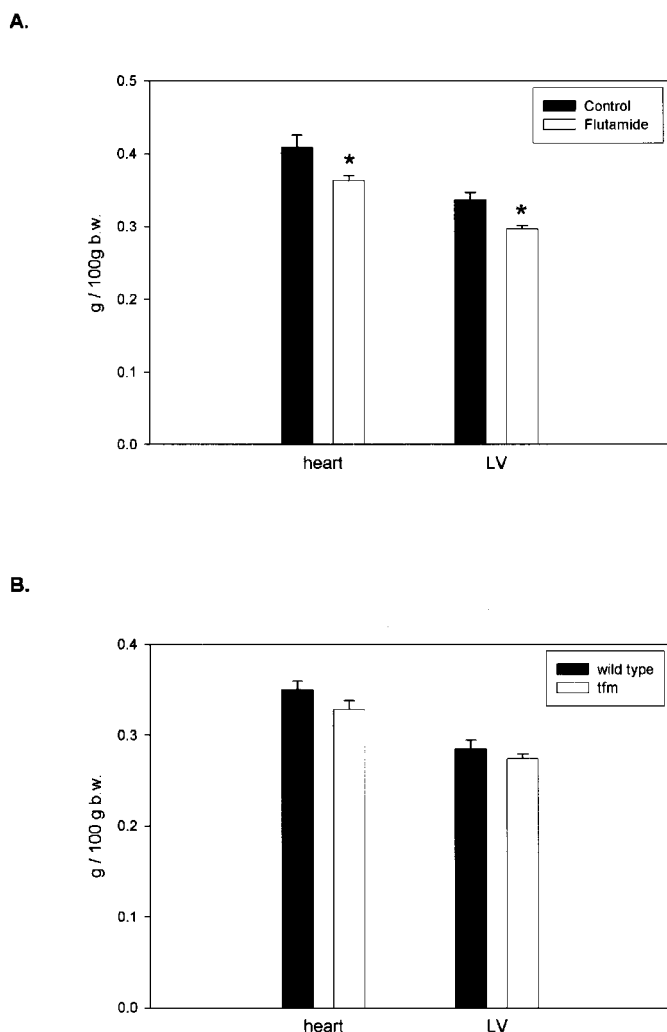
#### *Effect of Flutamide Treatment or tfm Mutation on Pituitary-Gonadal Axis*

Testosterone levels increased 15-fold after Flutamide treatment and 2.7-fold by the *tfm* mutation (Table 2). Plasma estrogens and luteinizing and follicle-stimulating hormones were significantly increased to a similar degree by both Flutamide treatment or *tfm* mutation (Table 2).

## Discussion

Gender differences have been observed in various hypertensive animal models. Male rats have higher BP than females in the spontaneously hypertensive strain (SHR) (8), and the Dahl salt-sensitive strain (19) in deoxycorticosterone-salt (20,21), and sodium-sensitive renal-wrap hypertension (22). Moreover, several reports have indicated a role of androgens and androgen receptors in hypertension (reviewed in reference 3). In the present study, we investigated the involvement of androgen receptors in the end-organ damage in TGR(mREN2)27 rats, which represent a model of hypertension with a defined genetic cause. They develop fulminant hypertension at an early age and end-organ damage similarly to human malignant phase hypertension (23). Using a double experimental strategy, we demonstrated that androgen receptors contribute to the development of malignant hypertension induced by an overactive RAS. Androgen receptor blockade by either Flutamide or introduction of a *tfm* mutation significantly attenuated the development of hypertension in TGR(mREN2)27. Moreover, kidney damage was significantly attenuated by the *tfm* mutation. Notably, Flutamide treatment totally prevented kidney damage, as evidenced by the normal histologic picture and the absence of albuminuria. Furthermore, a significant decrease of cardiac and left ventricular hypertrophy was observed only after Flutamide treatment. These observations are particularly remarkable because the Flutamide treated and the *tfm* rats still develop equally severe hypertension with systolic BP of 185 to 190 mmHg.

It has been shown that renin-angiotensin inhibitors may



**Figure 5.** Effect of androgen receptor antagonism or insensitivity on heart and left ventricular (LV) hypertrophy. (A) Untreated TGR(mREN2)27 rats (Control,  $n = 12$ ) compared with TGR(mREN2)27 rats treated with Flutamide (Flutamide,  $n = 12$ ). (B) Normal TGR(mREN2)27 tfm-negative (wild-type,  $n = 7$ ) compared with TGR(mREN2)27 littermates harboring the tfm mutation (tfm,  $n = 7$ ). \* significantly different ( $P < 0.05$ ).

prevent end-organ damage independent of the BP reduction in TGR(mREN2)27 (24,25). Also, renin gene expression and activity can be regulated by androgens (26,27). Therefore, the prevention of end-organ damage by Flutamide treatment could be independent of BP levels, possibly due to the inhibiting effect on the RAS. Indeed, in the present study, both Flutamide and the presence of the tfm mutation reduced plasma renin concentrations and activity but not plasma angiotensinogen. In Flutamide-treated rats, the kidney mRNA levels for rat and mouse renin were reduced in correlation to lower plasma concentrations and activities. However, renin gene expression levels in the kidney were unaffected by the tfm mutation. This may be explained by the presence of a residual androgen receptor activity in the tfm rats (13). The higher levels of renin mRNA in the kidney of the tfm rats compared with the flutamide-treated animals may elicit a higher RAS activity locally in the kidney. Local angiotensin generation in the kidney, however, is a major determinant of hypertension-induced damage in this organ (10).

Androgens can promote hypertension in models characterized by high salt intake and presumably a suppressed renin/angiotensin/aldosterone axis (28) as well as in renin-dependent models (27,29). This implies that mechanisms other than renin-suppression may also be involved even in the TGR(mREN2)27 model. Flutamide is a nonsteroidal androgen receptor antagonist with no agonist activity and is used in the treatment of advanced prostate cancer (30). Its active metabolite, 2-hydroxyflutamide, inhibits the uptake and/or binding of androgens to the androgen receptor (31). Flutamide treatment induces a feedback increase of testosterone associated with a significant augmentation of plasma gonadotropin concentrations (32). Indeed, in our study Flutamide increased plasma testosterone levels 15-fold. This tremendous increase of testosterone may be involved in the lowering of BP. Besides its genomic effects, it has been reported that testosterone can induce direct vascular relaxation (33–35). On the other hand, it has also been reported that testosterone increases vascular resistance (36) by inducing the release of thromboxane A<sub>2</sub> (37).

Males possessing the tfm mutation are entirely androgen-insensitive and externally appear to be female, despite abdom-

**Table 1.** Effect of androgen receptor antagonism or insensitivity on plasma renin concentrations (PRC) and activities (PRA), angiotensinogen, and kidney renin mRNA levels

	Control $n = 7$	Flutamide $n = 6$	tfm-Negative $n = 7$	tfm-Positive $n = 7$
Rat PRC (ng AngI/ml per h)	33.8 ± 9.6	27.6 ± 2.7	18.1 ± 1.6	12.7 ± 1.8 <sup>a</sup>
Rat PRA (ng AngI/ml per h)	19.7 ± 5.9	10.1 ± 1.5	12.1 ± 1.1	7.7 ± 0.6 <sup>b</sup>
Rat renin mRNA (% of control)	100 ± 14.8	55.6 ± 6.2 <sup>a</sup>	100 ± 51.9	137.2 ± 18.9
Mouse PRC (ng AngI/ml per h)	44.8 ± 6.4	30.2 ± 1.5 <sup>a</sup>	35.3 ± 3.6	24.9 ± 1.7 <sup>a</sup>
Mouse PRA (ng AngI/ml per h)	30.5 ± 3.7	16.9 ± 1.0 <sup>b</sup>	18.4 ± 5.2	20.9 ± 4.4
Mouse renin mRNA (% of control)	100 ± 11.8	44.1 ± 2.9 <sup>b</sup>	100 ± 23.0	133.5 ± 12.9
Plasma angiotensinogen (μg/ml)	1.3 ± 0.2	1.6 ± 0.1	1.2 ± 0.1	1.0 ± 0.04

<sup>a</sup>  $P < 0.05$ , <sup>b</sup>  $P < 0.01$ , significantly different Flutamide group versus control group, or tfm-positive group versus tfm-negative group.



**Table 2.** Effect of androgen receptor antagonism or insensitivity on plasma levels of testosterone, estrogens, luteinizing (LH), and follicle-stimulating (FSH) hormones

	Control <i>n</i> = 7	Flutamide <i>n</i> = 6	tfm-Negative <i>n</i> = 7	tfm-Positive <i>n</i> = 7
Testosterone (nmol/L)	7.5 ± 1.5	117.1 ± 14.7 <sup>c</sup>	5.0 ± 1.6	13.7 ± 2.4 <sup>a</sup>
Estrogen (pmol/L)	28.3 ± 2.1	50.7 ± 1.9 <sup>c</sup>	35.8 ± 4.9	66.3 ± 8.2 <sup>c</sup>
Rat LH (μg/L)	0.5 ± 0.1	7.1 ± 0.8 <sup>c</sup>	0.8 ± 0.1	6.4 ± 0.3 <sup>c</sup>
Rat FSH (μg/L)	7.8 ± 0.8	17.2 ± 1.9 <sup>c</sup>	7.7 ± 1.2	19.5 ± 1.3 <sup>b</sup>

<sup>a</sup> *P* < 0.05, <sup>b</sup> *P* < 0.01, <sup>c</sup> *P* < 0.001, significantly different Flutamide group *versus* control group, or tfm-positive group *versus* tfm-negative group.

inal testes and normal-to-elevated circulating levels of testosterone (38,39). As after Flutamide treatment, the tfm mutation caused alteration of plasma gonadotropin and sex hormones. However, while LH, FSH, and estrogen levels increased similarly when compared with Flutamide treatment, testosterone levels increased only 2.7-fold by the presence of the tfm mutation. The differences in these hormone levels after Flutamide treatment and in the rats with the tfm mutation may also explain the more pronounced beneficial effects of Flutamide on cardiac hypertrophy and renal damage. However, direct effects of Flutamide independent of its antiandrogenic actions cannot be ruled out for the explanation of this discrepancy.

Flutamide as well as the tfm mutation significantly increased not only the plasma levels of testosterone but also of estrogens. This effect on estrogens could also represent an antihypertensive and cardioprotective as well as renoprotective mechanism. Epidemiologic studies are indicating that estrogen replacement therapy has a protective effect on the cardiovascular system in postmenopausal women (40). The protective action of estrogen is mediated by intracellular receptors and, thus, by alterations of gene expression. In addition to the genomic effect and like testosterone, estrogens can have a direct vasorelaxing action without altering gene expression (41). Recently, it has been found that estrogens can directly activate a membrane potassium ion (Maxi-K) channel, as one mechanism for their rapid modulation of vascular tone (42,43). Estrogen is also known to lower renin levels (44) and to inhibit the sympathetic outflow (45,46), which contributes to its renin downregulation effect (46,47). As TGR(mREN)27 rats have increased plasma concentration of norepinephrine (25) and flutamide treatment has been shown to decrease catecholamine levels in SHR (11), sympathetic inhibition may also contribute to the attenuation of hypertension after androgen receptor blockade.

To summarize, the major findings of this study are: (1) androgen receptors are contributing to the hypertension and end-organ damage caused by an overactive RAS; (2) androgen receptor blockade inhibits renin expression and activity; (3) pituitary-gonadal hormone levels are increased by both Flutamide treatment or tfm mutation; and (4) Flutamide had a stronger inhibiting effect on the RAS and a more pronounced beneficial action on cardiovascular organs than the tfm mutation.

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