Identification of Cathepsin L as a Potential Sex-Specific Biomarker for Renal Damage

Yasmina Bauer, Patrick Hess, Changbin Qiu, Axel Klenk, Bérengère Renault, Daniel Wanner, Rolf Studer, Nina Killer, Anna Katharina Stalder, Manuel Stritt, Daniel Stefan Strasser, Hervé Farine, Katalin Kauser, Martine Clozel, Walter Fischli, Oliver Nayler

Abstract—The renin-angiotensin system is a well-known regulator of blood pressure and plays an important role in the pathogenesis of cardiovascular disease and renal damage. Genetic factors, including single nucleotide polymorphisms and sex, are increasingly recognized as potential risk factors for the development of cardiovascular disease. Double transgenic rats (dTGRs), harboring human renin and angiotensinogen genes, were used in this study to investigate potential sex differences influencing renal function and renal gene expression. dTGR males and females had comparable increases in blood pressure, whereas body weight, albuminuria/proteinuria, and urine flow rate were higher in males. At 8 weeks of age, renal plasma flow and glomerular filtration rate were proportionally lower in males, and renal vascular resistance tended to be higher. Males developed more severe tubulointerstitial and vascular lesions. By the end of week 8, 40% of the males but none of the females had died. Genome expression studies were performed with RNA from kidneys of 7-week–old male and female dTGRs and control rats to further investigate the sex-related differences on a molecular level. Forty-five genes showed sex-dependent expression patterns in dTGRs that were significantly different compared to controls. Cathepsin L, one of the genes differentially expressed between the sexes, was also shown to be strongly associated with the degree of renal injury. In dTGRs, urinary cathepsin L at week 7 was higher in males (nanograms per 24 hours: male, 512±163; female, 132±70). These results reveal a potential new biomarker for the personalized diagnosis and management of chronic kidney disease. (Hypertension. 2011;57:795-801.) • Online Data Supplement

Key Words: sex difference ■ renin-angiotensin system ■ double transgenic rats ■ hypertension ■ renal disease ■ oxidative stress ■ gene expression

In developed countries, the life expectancy of men is usually shorter than that of women.1 Men tend to have higher blood pressure than do premenopausal women of similar age,2,3 and this difference is associated with a greater risk of the development of cardiovascular disease and renal damage. End-stage renal disease is indeed more frequent in men.5,6 There is ample evidence to suggest an involvement of the renin-angiotensin system (RAS) in blood pressure increase and kidney failure.7,8 An impact of sex has also been reported in experimental animal models of renal disease and hypertension, including a rat model of mild chronic inhibition of NO synthesis,9 spontaneously hypertensive rats,10 deoxycorticosterone acetate-salt rats,11 Dahl salt-sensitive rats,12 and the Goldblatt 2-kidney, 1-clip rats.13 Because, in these models, the sex-related differences in the development of hypertension and renal damage were associated with the RAS, the double transgenic rat (dTGR), expressing both human renin and angiotensinogen genes, provides an ideal model to explore sex-dependent responses to RAS dysregulation.

Several studies have demonstrated that dTGR males, if left untreated, develop hypertension, severe albuminuria, and severe vascular lesions in the kidney, resulting in ≈50% deaths at 7 to 9 weeks of age.14–17 Bohlender et al14 published previously that there was no significant blood pressure difference between male and female dTGRs; however, so far no sex comparison of the development of renal damage has been investigated in this model. We show for the first time a sex difference in the development of renal damage in dTGR and, using a whole-genome expression analysis, we identified gene expression changes that may play a role in sex-related disease development. One gene in particular, cathepsin L (Ctsl), was further characterized at the protein level and was shown to be a sensitive biomarker to assess the degree of kidney injury, possibly in a sex-specific way.
Methods

Animals
Three-week-old dTGRs and Sprague-Dawley (SD) rats were purchased from Harlan Laboratories (Füllinsdorf, Switzerland) and maintained under identical conditions in accordance with local guidelines of the Basel-Landschaft cantonal veterinary office. The protocol was approved by license No. 251. All of the rats were housed in climate-controlled conditions with a 12-hour light/dark cycle and had free access to normal rat chow and drinking water.

Cardiac and Renal Hemodynamics
After an adaptation period of 1 week, experiments were begun in 4-week-old male (n=15) and female (n=15) dTGRs. Weekly (weeks 4 to 7 after birth), blood was sampled from the sublingual vein, and 24-hour urine samples were collected. Urinary sodium, creatinine, albumin, and protein concentrations were determined using a Synchron CX5 PRO (Beckman Coulter). Serum samples were used to determine serum concentrations of sodium and creatinine. These measurements allowed us to calculate urinary albumin and protein excretion, creatinine clearance, urinary sodium excretion, fractional excretion of sodium, and net tubular reabsorption of sodium. Further details of these calculations are available elsewhere.15,19

Arterial blood pressure and heart rate were measured at weeks 4 and 6 using the tail-cuff method (Harvard Apparatus). In brief, the rats were placed in a warming cupboard at 35°C for 30 minutes. The rats were then put in restraining holders on a heating blanket at 37°C. Indirect measurements of arterial blood pressure and heart rate were obtained. Per rat, 3 to 5 measurements were made at 1- to 3-minute intervals, and a mean arterial blood pressure was calculated.

Body weight and food intake were monitored twice a week from weeks 4 to 7. Terminal renal clearance experiments were conducted at week 8.

Renal Clearance Experiments
At week 8, after 24-hour urine collection, renal clearance measurements were performed as described previously.20–24 For details, please see online Data Supplement at http://hyper.ahajournals.org. At the end of renal clearance experiments, rats were euthanized, and the kidneys and heart were obtained from all of the animals. Both kidneys were weighed, and the ratio of kidney weight:body weight was calculated. The left kidney was cut longitudinally and fixed in 10% buffered formalin. Renal histopathologic and morphometric analyses are described in the online Data Supplement.

Statistical Analysis of Physiological and Histological Data
Data are expressed as mean±SEM. Statistical analyses were performed using an ANOVA using terms for sex, time, and their interaction (Statistica, StatSoft). When a significant F-ratio was observed, the data were further analyzed with a Student-Newman-Keuls procedure.

Histological data were analyzed by Wilcoxon rank-sum analysis. Statistical significance was defined as P<0.05.

Gene Expression Analysis

Organ Sampling and Total RNA Extraction
Additional groups of 7-week-old dTGR males and females and their respective SD controls (n=8 per sex and per strain) were euthanized for microarray studies. Mean arterial pressure and proteinuria of these animals were also measured to facilitate integration of in vivo and gene expression data. The kidneys were collected in RNeater (Ambion), and ~70 mg of kidney tissue were homogenized using the Utraturrax T8. Total RNA was then prepared with the RNA minikit according to the manufacturer’s instructions (Qiagen).

Amplification, Labeling, and Microarray Hybridization
Amplification was performed with an Agilent Low RNA Input Linear Amplification Kit (Agilent) according to the manufacturer’s instructions, using 1 µg of total RNA as starting material. The 4×44 K multiplex arrays with 41 012 oligonucleotides (60-mer) directed against the rat transcriptome were purchased from Agilent; hybridizations and washes were performed following the Agilent user’s manual. The 32 samples were hybridized to 32 arrays.

Microarray Data Preprocessing
The arrays were scanned using an Agilent microarray scanner and Scan Control software 7.5; the data were then extracted with Feature Extraction software 9.5.1. Data were first background corrected and normalized using the variance-stabilizing normalization.25

Microarray Statistical Data Analysis
After preprocessing, genes with expression changes between the transgenic models and the SD controls were identified using limma.26 Limma performs per-gene statistical tests for differential expression using a moderated t statistic. The resulting P values were adjusted for multiple testing to control the false discovery rate, using the algorithm of Benjamini and Hochberg.27

We identified genes that were differentially expressed between dTGR females and SD females, between dTGR males and SD males, and the difference between these 2 contrasts, that is, dTGR females versus SD females versus dTGR males versus SD males, while controlling the false discovery rate at 0.05. All of the microarray results may be found at the GEO Web site (http://www.ncbi.nlm.nih.gov/geo/) under accession No. GSE19058.

Verification of Microarray Expression Data Using Real-Time PCR (Quantitative PCR)
Total RNA was reverse transcribed and quantified in real time using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) as described previously.28 TaqMan Assays-on-Demand (Applied Biosystems) were used for the following genes: Aprot Rn01490054_m1, Arg1 Rn00567522_m1, Cryl Rn01503058_g1, Cts1 Rn00565793_m1, Prlr Rn01525463_m1, Sod3 Rn00563570_m1, and Vegfa Rn01511605_m1. The predeveloped TaqMan assays (Applied Biosystems) B2 mol/L Rn00560856_m1, Gapdh 4352338E, and Gusb Rn00566655_m1 were examined as internal controls. Expression values were calculated as cycle threshold – geometric mean of B2m, Gapdh, and Gusb and expressed in relative expression values (1= no detectable expression, defined as cycle threshold >40 cycles), and the expression values were then translated into log10. All of the amplifications were performed in duplicate.

Ingenuity Pathway Analysis
Differentially expressed genes were uploaded into Ingenuity Pathways Analysis (Ingenuity Systems) with corresponding fold changes and P values. For filtering we used a false discovery rate of 0.05. For gene probes that were replicated on the array, their average log2 fold change was used.

Measurement and Statistical Data Analysis of Cathepsin L and Kidney Injury Molecule 1
Addional groups of 3-week-old dTGR males and females and their respective SD controls (n=6 per sex and per strain) were used to measure cathepsin L (CTSL) and kidney injury molecule (Kim1) 1 in urine. Creatinine and proteinuria were also measured in these animals to verify our initial finding.

CTSL and Kim-1 were quantified in urine using ELISA kits from USCN Life Sciences Inc and R&D Systems, respectively. After sampling, urine samples were immediately stored at −20°C until use. All of the measurements were performed according to the manufacturer instructions. Before measurement, samples were centrifuged for 10 minutes at 1200g and diluted in assay buffers 1:10 and 1:4 for CTS and Kim-1, respectively. To allow the comparison between the different groups, data were normalized to urine volume in 24 hours. To assess the difference of the measured proteins over
time, we fit a linear model using limma\textsuperscript{26} that takes into account
time, transgenic versus wild type, sex, and the interactions of these
factors.

**Immunohistochemistry**

Immunohistochemistry for the detection of CTSL was performed on
5-μm paraffin longitudinal sections of rat kidneys. Using rabbit
anti-rat CTSL (Lifespan Biosciences Inc) and biotinylated donkey
anti-rat as a secondary antibody (Jackson Immunoresearch Labora-
tories). Staining was performed with the Leica Bond automated
stainer system using a standard program (Leica, Biosystems AG).
Sections were counterstained with hematoxylin, dehydrated, and
embedded in Medimount (Biosystems).

**Quantification of CTSL Immunostaining**

The quantification of CTSL immunostaining was performed by an
in-house developed software called Orbit, which applies pattern
recognition methods to the digitalized tissue slides. In a first step an
image is subdivided into medulla, cortex, and capsula regions. In a
second step each pixel of the cortex region is classified as back-
ground, tubulus, and tissue stained positive for CTSL by a machine
learning classifier. The final measurement is the number of pixels
classified as CTSL stained relative to tubular area. A 1-sided Welch
2 sample \(t\) test was performed to compute the \(P\) values.

**Results**

Body weights and daily food intake were similar in males and
females at week 4 and progressively increased in males and
females from week 5 onward. The increases in body weight and
daily food intake in males were greater than in females
(Figure S1, available in the online Data Supplement at
http://hyper.ahajournals.org).

There were no significant differences in mean arterial
blood pressure and heart rate between males and females at
weeks 4 and 6, as measured indirectly by tail-cuff methods.
At week 8, measured directly under anesthetized conditions,
no differences in mean arterial blood pressure (female:
176±9 mm Hg; male: 180±5 mm Hg) and heart rate (female:
411±7 bpm; male: 403±7 bpm) were observed.

Albuminuria and proteinuria were slightly more severe in
males than in females already at week 4 and progressively
increased over time in males, whereas only moderate changes
were observed in females (Figure 1). The ANOVA indicates
that the effect of time on albuminuria, a proteinuria, is
different between dTGR males and dTGR females.

By week 7, urine albumin had increased \(\approx 26\)-fold, that is,
13.0±3.8 versus 0.5±0.1 mg/24 hours \((P<0.001)\), and
urinary protein had increased \(\approx 16\)-fold, that is, 67.7±15.8
versus 4.3±0.4 mg/24 hours \((P<0.001)\) in males relative to
values in their female counterparts.

Sodium concentration was higher in males than in females
and net tubular reabsorption of sodium was significantly
higher in male dTGRs at weeks 5 and 6, whereas urine flow
rate and urinary sodium excretion were higher from weeks 5
to 7 (Table S1).

Fractional excretion of sodium was also higher at weeks 6
and 7. Serum creatinine concentration in males was not
different from that in females from weeks 4 to 6. At week 7,
serum creatinine had increased by one third in males com-
pared with females \((0.71±0.07 versus 0.53±0.01 mg/dL; \(P<0.05)\).

In males, both renal plasma flow and glomerular filtration
rate were significantly and proportionally lower than in
females at week 8, leading to a significantly lower fractional
filtration. Renal vascular resistance also tended to be higher
in males (Figure S2). As shown in Table S2, the ratio of
kidney weight:body weight was higher in males.

All 15 of the females remained alive at the end of week 8,
whereas 6 of 15 males were dead; 1 rat died in week 5 and 1
in week 6. For reasons of animal welfare, 4 rats were
euthanized during week 7 because of poor physical condition.

**Histology**

The histological appearance of renal injury in the dTGRs
resembled the hemolytic-uremic syndrome vasculopathy\textsuperscript{29}
and was characterized by prominent wall thickness and
fibrinoid necrosis in small arteries and arterioles. Semiqual-
titative histological lesion scoring revealed that there were
significantly more severe tubulointerstitial lesions (male: 0.91±0.09; female: 0.60±0.06) and renal vascular lesions
(male: 1.73±0.08; female: 1.27±0.04) in males than in
females (Table S2). The glomeruli, however, had minimal
histological changes in both males and females. Comput-
erized morphometric analysis of glomerular size showed
that glomerular surface area was significantly smaller
in males than in females, 1.86±0.02 \(\mu\)m\(^2\)×10\(^3\) and
2.09±0.02 \(\mu\)m\(^2\)×10\(^3\), respectively (Table S2).

**Gene Expression Studies**

Separate groups of 7-week–old dTGR males and females and
their respective SD controls were euthanized for microarray
studies. Arterial blood pressure and proteinuria were similar
in both physiology/histology and gene expression groups (data not shown).

Gene expression levels in dTGR males and dTGR females were compared, and 5571 gene probes displayed differential expression at a false discovery rate of 0.05. A subset of 358 differentially expressed gene probes showed sex-related expression changes in dTGRs that were significantly different from the sex-related expression changes in SD rats.

These gene probes were grouped into 3 categories: (1) significant sex-related expression changes in both dTGRs and SD rats (74 gene probes); (2) significant sex differences in SD rats but not in dTGRs (180 gene probes); and (3) significant sex difference in dTGRs but not in SD rats (104 gene probes).

Approximately half of the differentially expressed gene probes could be mapped to a gene name using Ingenuity Pathways Analysis. To further minimize the number of false positives, a gene was selected only if the expression change between dTGRs and SD rats was >60% and the change in expression between males and females was ≥50% in dTGRs or SD rats. This filtering resulted in a list of 45 remaining genes that were classified into 8 groups according to their known biological function, including the following: (1) cell replication/cell growth/cell development; (2) coagulation; (3) estrogen regulation; (4) immune response; (5) kidney injury/proteinuria; (6) metabolism; (7) oxidative stress; and (8) signaling (Figure S3).

Six genes, prolactin receptor (Prlr), cathepsin L (Ctsl), vascular endothelial growth factor A (Vegfa), cryptochrome 1 (Cry1), superoxide dismutase 3 (Sod3), and arginase (Arg1), encoding proteins with reported physiological relevance to cardiovascular disease, were selected for further investigations using quantitative PCR. In these experiments, the Ctsl and Sod3 genes were confirmed to be differentially expressed in dTGR males versus SD males, and Arg1, Cry1, Ctsl, Prlr, Sod3 and Vegfa were confirmed to be differentially expressed in dTGR females versus SD females.

Compared with control animals, the expression levels of Ctsl and Sod3 genes were lower in female dTGRs (respectively, 30% and 20% decreases) but increased in dTGR males (respectively, 30% and 40% increases; Figure S4). In addition, the expression level of Vegfa was reduced by 20% in females and reduced by 50% in male dTGRs compared with control animals. Ctsl, Sod3, and Vegfa showed higher expression levels in SD females compared with SD males. We selected Ctsl for further biomarker investigation, because the encoded protein has been shown to play a role in renal damage.30,31

**Immunohistochemistry**

**Immunohistochemical** staining of kidney sections at week 7 revealed CTSL expression in the proximal tubules. Morphometric analysis showed no significant CTSL expression changes (P=0.2) in female dTGRs compared with SD females. However, the mean CTSL levels in dTGR males were increased 1.70-fold (P=0.004) when compared with SD male controls (Figure S4).

**Discussion**

Development of hypertension and renal damage is associated with the activation of the RAS, and the frequency, severity, and progression rate of renal damage are affected by sex in animals and humans. In this study, we used dTGRs harboring human renin and angiotensinogen genes to investigate poten-
tial sex differences in blood pressure, renal function, and renal gene expression.

We found that male dTGRs exhibited more severe albuminuria/proteinuria, impairment of renal function, and renal structural damage and had a higher mortality rate than female counterparts. However, consistent with the findings of Bohlender et al., we observed no sex difference in the development of hypertension. To date, most dTGR studies have been conducted in males. To the best of our knowledge, this study is the first to demonstrate a sexual dimorphism in the development of renal damage in this model.

Long-term angiotensin II infusion in normotensive rats results in moderate hypertension and marked vascular, glomerular, and tubulointerstitial injury. In dTGRs, arterial hypertension induced by the human components of the RAS causes mechanical stress to endothelial and smooth muscle cells that may in part account for the development of renal damage. Although hemodynamic forces alone are capable of inducing end-organ damage, the important contributory role of local angiotensin II formation in the kidney has been emphasized in the development of renal damage.

In the present study, higher daily intake of food (sodium) and higher net tubular reabsorption of sodium were found in males compared with females. However, both the urinary flow rate and urinary sodium excretion were higher in males, as was the fractional excretion of sodium. It is therefore conceivable that there is no sex-related difference in sodium retention in dTGRs.

We used a genome-wide expression analysis to investigate the observed sex-specific progression of kidney failure in dTGRs at a molecular level. This approach revealed 2 genes, that is, Sod3 and Ctsl, that showed sex-specific expression changes in diseased animals that coincided with worsening of kidney function in males. Both genes have been implicated previously in the development of proteinuria and renal organ damage. Sod3 encodes a member of the superoxide dismutase family. Superoxide dismutases are antioxidant enzymes that catalyze the dismutation of 2 superoxide radicals into hydrogen peroxide and oxygen and thereby protect tissue from oxidative stress. An increase of Sod3 expression in dTGR males and not in dTGR females may reflect an increased demand for antioxidants in the diseased kidney as compensation for the increased oxidative stress. In the renal wrap model, another model of hypertension-associated renal disease, markers of renal injury differ between the sexes but the increase in CTSL with disease progression is greater in males. Consequently, in our study KIM-1 performed better in renal protection in females. Before disease development, CTSL expression is higher in female than in male rats, but the increase in CTSL with disease progression is greater in males. There is evidence that CTSL is regulated by estrogen in osteoclasts. Ovariectomized mice increase cathepsin K and CTSL expression by 200% to 400% compared with control. We could, therefore, hypothesize that the expression of CTSL and its protective role in female dTGR kidneys may be under the control of estrogen. Alternatively, our finding could underscore the value of CTS1 as a novel diagnostic biomarker detecting the degree of tubulointerstitial damage in kidney disease. Increased KIM-1 levels reflect renal injury but do not necessarily correlate with the degree of tissue damage, which was less pronounced in female than in male dTGRs. Consequently, in our study KIM-1 performed as a strong prognostic factor, whereas CTSL was a diagnostic marker for the observed tubulointerstitial damage.

**Perspectives**

In conclusion, male dTGRs were more prone to develop albuminuria/proteinuria, impaired renal function, and renal structural damage and had a higher mortality rate than females, although there was no difference in the development of hypertension. This study combined biochemical, physio-

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Ctsl, the second gene identified in this screen, is involved in lysosomal degradation of filtered proteins that have been reabsorbed into the proximal tubule. The rat Ctsl gene is closely related to human CTS2. In both dTGRs and SD rats, Ctsl expression levels are lower in males than in females. Under disease development, only the male dTGRs showed an increase in the expression of Ctsl when compared with SD controls. It has been reported previously that female rats have a higher capacity for protein degradation in the proximal tubule than males and that lysosomal peptidases increase in males after castration. CTSL activity in the proximal tubule increases in proteinuric rats, possibly in response to the increased load of filtered protein. CTSL is also expressed in a cytosolic form in glomerular podocytes, where it is directly involved in destabilization of the filtration barrier. In Ctsl-deficient mice, proteinuria and foot process effacement, that is, alteration of the podocyte filtration barrier, are abolished.

With the desire to further characterize CTSL in renal injury, we investigated urine CTSL protein levels in male and female dTGR and SD control animals and performed quantitative immunohistochemistry to demonstrate expression level and localization of CTSL protein in the kidney sections from our study. The immunostaining revealed a predominant localization of CTSL protein in lysosomes in the tubulointerstitial regions of the kidney, in strong association with the prevalent interstitial damage seen in our histological sections. We found that CTSL protein expression closely reflected the difference in disease severity between dTGR males and females.

Recently a set of biomarkers has been identified and approved by the Food and Drug Administration and the European Medicines Agency to monitor acute kidney failure in rodents in the context of preclinical drug development. Among those markers, increased levels of KIM-1 in urine were shown to detect tubular kidney injury before changes in serum creatinine and serum urea nitrogen. Parallel to analyzing CTSL protein expression, we have also determined KIM-1 protein levels in our study. KIM-1 levels increased over time, but there was no difference between dTGR males and dTGR females. The reason for the different protein expression pattern of KIM-1 and CTSL in our study could be linked to the potential mechanistic role of CTSL, contributing to better renal protection in females. Before disease development, CTSL expression is higher in female than in male rats, but the increase in CTSL with disease progression is greater in males. There is evidence that CTSL is regulated by estrogen in osteoclasts. Ovariectomized mice increase cathepsin K and CTSL expression by 200% to 400% compared with control. We could, therefore, hypothesize that the expression of CTSL and its protective role in female dTGR kidneys may be under the control of estrogen. Alternatively, our finding could underscore the value of CTS1 as a novel diagnostic biomarker detecting the degree of tubulointerstitial damage in kidney disease. Increased KIM-1 levels reflect renal injury but do not necessarily correlate with the degree of tissue damage, which was less pronounced in female than in male dTGRs. Consequently, in our study KIM-1 performed as a strong prognostic factor, whereas CTSL was a diagnostic marker for the observed tubulointerstitial damage.
logical, whole genome expression analyses, and immunohistochemistry to investigate sexual dimorphism and development of renal damage on a molecular level. Several candidate genes with known associations to cardiovascular disease and kidney injury were identified as differentially expressed genes in dTGRs compared with control animals and, in addition, were shown to display sex-related expression differences. One of these genes, Ctsl, was further investigated at the protein level and was shown to be a sensitive diagnostic marker of tubular injury. Further studies, such as those including castration of males and ovariecomy of females, as well as hormone replacement treatments, will be necessary to understand whether CTSL actively contributes, possibly in a sex-specific way, to the process of renal failure. In addition, translational studies investigating CTSL in the progression of renal disease will reveal the full potential of CTSL as a biomarker.

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Disclosures

All of the authors are employees of Actelion Pharmaceuticals Ltd.

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IDENTIFICATION OF CATHEPSIN L AS A POTENTIAL SEX-SPECIFIC BIOMARKER FOR RENAL DAMAGE

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Running Title: Biomarkers for renal damage in dTGR

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EXPANDED MATERIALS AND METHODS

Renal Clearance Experiments
At week 8, after 24-hour urine collection, renal clearance measurements were performed as previously described \(^1\)\(^-\)\(^5\). Briefly, the rats were anesthetized by intraperitoneal injection of 100 mg/kg thiobutabarbital-Na (Inactin, Byk-Gulden, Konstanz, Germany) and placed on a thermostatically controlled heating table to maintain body temperature at 36–38°C. A catheter was placed into the left femoral vein for infusion of synthetic plasma, inulin, and p-aminohippurate (PAH) and the left femoral artery was prepared for arterial blood pressure monitoring and periodic blood sampling. Through a small, suprapubic incision, a flanged catheter was placed in the urinary bladder for collection of urine. After a 45-minute equilibration period, two consecutive 20-minute urine collections were performed, with midpoint arterial blood sampling. Urine volume was measured gravimetrically, and inulin concentrations in urine and plasma were determined using the anthrone method \(^6\)\(^,\)\(^7\). p-Aminohippurate concentrations in urine and plasma were measured colorimetrically \(^8\). These measurements allowed calculation of inulin clearance (equal to glomerular filtration rate, GFR), PAH clearance (equal to renal plasma flow, RPF, when adjusted for renal extraction of PAH), renal vascular resistance (RVR) and filtration fraction (FF) \(^1\)\(^-\)\(^5\). The results of the two clearance periods were averaged.

Renal Histopathologic and Morphometric Analyses
The left kidney was embedded in paraffin. Sections of 5 \(\mu\)m thickness were stained with hematoxinlin eosin and examined under light microscopy. Investigators, blinded as to experimental groups, assessed the severity of the morphological changes, i.e. the presence of glomerulosclerosis, tubulointerstitial lesions, and vascular lesions in the renal cortex. Each type of lesion was graded semiquantitatively as previously described \(^9\).

To assess glomerulosclerosis, 50 glomeruli in each kidney were observed at \(\times\) 400 magnification and graded from 0 to 4+ by a semiquantitative score, according to the following criteria: 0, normal; 1+, slight glomerular damage such as a mild increase in the mesangial matrix and/or hyalinosis with focal adhesion, involving < 25% of the glomerulus; 2+, sclerosis of 25-50%; 3+, sclerosis of 50-75%; 4+, sclerosis of > 75% of the glomerulus. A glomerular damage index was calculated by averaging the grades assigned to all glomeruli.

Tubulointerstitial lesions (interstitial inflammation and fibrosis, tubular atrophy, and dilation with casts) were assessed at \(\times\) 100 magnification in every third field of each kidney (total of 10 fields/kidney) and assigned an injury grade from 0 to 3: grade 0, normal; 1, lesions involving < 25%; 2, lesions involving 25-50%; 3, lesions involving > 50% of the field. A score for tubulointerstitial lesions for each kidney was obtained by averaging the grades given to all fields.
Vascular lesions in each kidney were attributed grades of severity from 0 to 4 in 20 fields at × 200 magnification. This grade was based on both the severity of vascular wall thickening and the extent of fibrinoid necrosis in afferent arterioles, interlobular arterioles, and small arteries: grade 0, normal vessel; 1, mild vascular wall thickening; 2, moderate thickening; 3, severe thickening (onion skin pattern); and 4, fibrinoid necrosis. The vascular lesion score was obtained using the same procedure as described above.

Quantitative morphometry was performed in order to assess total glomerular surface area, delimited by the internal edge of the Bowman’s capsule. In each kidney section, 30 consecutive glomeruli, randomly distributed over the depth of the cortex, were measured. Glomerular polar sections were not measured. The glomerular surface area was used as an indicator of glomerular hypertrophy.

REFERENCES
### Table S1. Renal handling of sodium and creatinine from weeks 4 to 7 after birth in male (n=13) and female (n=15) double transgenic rats (dTGRs).

Data are expressed as mean ± SEM. $S_{Na}$, serum sodium concentration; $S_{Cr}$, serum creatinine concentration; Ccr, creatinine clearance rate; V, urine flow rate; $F_{E_{Na}}$, fractional excretion of sodium; $T_{Na}$, net tubular reabsorption of sodium. *, $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$, vs. female dTGRs.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>Week 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{Na}$ (mmol/L)</td>
<td>144 ± 0.3</td>
<td>146 ± 0.5*</td>
<td>143 ± 0.3</td>
<td>144 ± 0.3**</td>
</tr>
<tr>
<td>$S_{Cr}$ (mg/dl)</td>
<td>0.56 ± 0.03</td>
<td>0.60 ± 0.04</td>
<td>0.55 ± 0.03</td>
<td>0.54 ± 0.01</td>
</tr>
<tr>
<td>Ccr (ml/min/100g)</td>
<td>0.31 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.35 ± 0.01</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>V (ml/24h)</td>
<td>8.3 ± 0.9</td>
<td>10.9 ± 1.2</td>
<td>10.8 ± 1.3</td>
<td>16.0 ± 1.9*</td>
</tr>
<tr>
<td>$U_{Na}V$ (mmol/24h)</td>
<td>0.44 ± 0.02</td>
<td>0.58 ± 0.03***</td>
<td>0.52 ± 0.04</td>
<td>0.72 ± 0.04***</td>
</tr>
<tr>
<td>$F_{E_{Na}}$ (%)</td>
<td>0.80 ± 0.06</td>
<td>0.97 ± 0.09</td>
<td>0.59 ± 0.03</td>
<td>0.68 ± 0.03</td>
</tr>
<tr>
<td>$T_{Na}$ (µmol/min)</td>
<td>39.6 ± 2.5</td>
<td>44.0 ± 3.3</td>
<td>63.0 ± 2.6</td>
<td>73.3 ± 2.6**</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Parameters</th>
<th>KW/BW (%)</th>
<th>Glomerular sclerosis index (score)</th>
<th>Tubulointerstitial lesions (score)</th>
<th>Renal vascular lesions (score)</th>
<th>Glomerular surface area (µm² x10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female dTGRs</td>
<td>0.80 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>0.60 ± 0.06</td>
<td>1.27 ± 0.04</td>
<td>2.09 ± 0.02</td>
</tr>
<tr>
<td>Male dTGRs</td>
<td>0.92 ± 0.03***</td>
<td>0.03 ± 0.01*</td>
<td>0.91 ± 0.09**</td>
<td>1.73 ± 0.08***</td>
<td>1.86 ± 0.02***</td>
</tr>
</tbody>
</table>

Table S2. **Body weight, relative kidney weight, and renal structural damage in male (n=13) and female (n=15) double transgenic rats (dTGRs) at week 8 after birth.** Data are expressed as mean ± SEM. BW, body weight; KW, kidney weight. *, P < 0.05; **, P < 0.01; ***, P < 0.001, vs. female dTGRs.
Supplementary Figures
Figure S1. Body weight (top panel) and daily food intake (bottom panel) from weeks 4 to 7 after birth in male and female double transgenic rats (dTGRs). *, $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$, vs. female dTGRs.
Figure S2. Glomerular filtration rate (GFR), renal plasma flow (RPF), filtration fraction (FF) and renal vascular resistance (RVR) in male (n = 8) and female (n = 15) double transgenic rats (dTGRs) at week 8 after birth. *, P < 0.05; ** P < 0.01, vs. female dTGRs.
Figure S3. Summary of the main biological responses involved in the expression dimorphism in dTGR. The genes are grouped into 3 categories (i) genes that show sex-related expression changes in both dTGR and Sprague-Dawley (SD) (yellow), (ii) genes that show sex-related expression changes in dTGR but not in SD (red) and (iii) genes that show sex-related expression changes in SD but not in dTGR (blue).
Figure S4. Relative expression levels of genes that show gender-related expression changes in dTGR (VEGFA), or in both dTGR and SD (CTSL2 and SOD3) and that were verified by qPCR. The x-axis shows the log10 of gene expression; i.e., a value of 5 corresponds to an expression value of 100,000. In all cases the differences between dTGR and SD are significant at a threshold of 0.01 using a t-test.
**Figure S5.** Boxplot presentation of morphometric analysis of immunohistochemical staining for CTSL in the rat kidney. Relative values of CTSL staining normalized to the tubular area in a longitudinal section of the kidney. Left, comparison of female dTGR versus SD controls; right, males dTGR versus SD controls. Significance was calculated with a Welch one sided t-test and reached significance for male rats, $P = 0.004$.