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Heterozygous knockout of transforming growth factor-β1 protects Dahl S rats against high salt-induced renal injury

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Chen CC, Geurts AM, Jacob HJ, Fan F, Roman RJ. Heterozygous knockout of transforming growth factor-β1 protects Dahl S rats against high salt-induced renal injury. Physiol Genomics 45: 110–118, 2013. First published December 18, 2012; doi:10.1152/physiolgenomics.00119.2012.—The present study employed a zinc-finger nuclease strategy to create heterozygous knockout (KO) rats for the transforming growth factor-β1 (Tgfβ1) gene on the Dahl SS/Jr genetic background (Tgfβ11/-/H9252 Dahl S). Intercrossing Tgfβ11/-/H9252 rats did not produce any homozygous KO rats (66.4% +/-, 33.6% +/-), indicating that the mutation is embryonic lethal. Six-week-old wild-type (WT) littersmates and Tgfβ11/-/H9252 Dahl S rats were fed a 0.4% (low salt, LS) or 8% NaCl (high salt, HS) diet for 5 wk. Renal cortical expression of TGF-β1, urinary TGF-β1 excretion, proteinuria, glomerular injury and tubulointerstitial fibrosis, and systemic blood pressure were similar in WT and Tgfβ11/-/H9252 Dahl S rats maintained on the LS diet. The expression and urinary excretion of TGF-β1 increased to a greater extent in WT than in Tgfβ11/-/H9252 Dahl S rats fed an HS diet for 1 wk. Systolic blood pressure rose by the same extent to 235 ± 2 mmHg in WT and 239 ± 4 mmHg in TGF-β11/-/H9252 Dahl S rats fed a HS diet for 5 wk. However, urinary protein excretion was significantly lower in Tgfβ11/-/H9252 than in the WT animals. The degree of glomerular injury and renal cortical and outer medullary fibrosis was markedly less in TGF-β11/-/H9252 than in the WT animals. The degree of glomerular injury and renal cortical and outer medullary fibrosis was markedly less in TGF-β11/-/H9252 than in the WT animals. The degree of glomerular injury and renal cortical and outer medullary fibrosis was markedly less in TGF-β11/-/H9252 than in the WT animals. The degree of glomerular injury and renal cortical and outer medullary fibrosis was markedly less in TGF-β11/-/H9252 than in the WT animals. The degree of glomerular injury and renal cortical and outer medullary fibrosis was markedly less in TGF-β11/-/H9252 than in the WT animals.

hypertension; glomerular injury; renal fibrosis; kidney disease; transforming growth factor beta 1

TRANSFORMING GROWTH FACTOR-β1 (TGF-β1) is a multifunctional profibrotic cytokine that has been implicated in the cardiac, renal, and vascular pathology associated with hypertension and diabetes (6, 20, 24, 28, 29, 37). Indeed, renal and vascular TGF-β1 levels have been reported to be elevated in diabetic nephropathy (5, 8, 25, 34), salt-sensitive hypertension (21, 27, 35, 36), glomerulonephritis (3, 4, 23), and angiotensin II-induced hypertension (22). Elevated levels of TGF-β1 are thought to increase collagen deposition and cause thickening of the glomerular basement membrane (GBM). Excessive expression of TGF-β1 has also been shown to promote podocyte apoptosis and detachment from the GBM, leading to breakdown of the glomerular permeability barrier, proteinuria, and renal interstitial fibrosis (18).

Anti-TGF-β therapy has been reported to have a renoprotective effect in many experimental models of renal disease. For example, lowering TGF-β levels with decorin attenuates the development of proteinuria in models of glomerulonephritis (2, 3). Chronic administration of an antibody to the TGF-β type II receptor reduced extracellular matrix (ECM) accumulation in the Thy-1 rat model of proliferative glomerulonephritis (16). Reducing the expression of TGF-β1 using antisense oligodeoxynucleotides or a neutralizing TGF-β antibody protected against expansion of the mesangial matrix (8) and reduced proteinuria in several animal models of renal disease (1, 7, 14, 37). Our laboratory has reported that chronic administration of an anti-TGF-β antibody to inactivate Tgfβ1, 2, and 3 isoforms reduced mean arterial pressure, proteinuria, and renal and cardiac fibrosis in Dahl S rats (10, 21). However, the exact isoform involved and the mechanism of the renoprotection remains to be determined.

One way to better assess the role of TGF-β1 in the pathogenesis of hypertension-induced renal injury would be to perform studies in knockout (KO) animals. However, during embryogenesis, TGF-β1 has proven to be essential in development prior to implantation, and for yolk sac endothelial cell differentiation and hematopoiesis (11, 15). As a consequence, a global TGF-β1 KO mouse model has not been created although it should be possible to develop a cell-specific conditional animal (9). The lack of embryonic stem cells has also hampered attempts to generate KO rats. However, the recent development of zinc finger nuclease (ZFN) technology has allowed for the creation of gene KOs in rats (12). In the present study we generated a TGF-β1 KO rat on Dahl S background to determine its role in the development of salt-sensitive hypertension induced renal injury.

MATERIALS AND METHODS

The Tgfβ11/-/H9252 Dahl S strain (SS-Tgfβ1em3Mcwi) was initially produced under protocols approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin. Rats were transferred and housed in the Laboratory Animal Care facility at the University of Mississippi Medical Center that is approved by the American Association for Accreditation. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center.

ZFN-mediated KO of Tgfβ1 in the Dahl S rat. The Tgfβ11/-/H9252 Dahl S rats were created as previously described (12). In brief, ZFN constructs specific to the rat Tgfβ1 gene were designed, assembled,
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and validated by Sigma Aldrich to target the exon 3 sequence
CTGTCCTCCACTCCGCTGGCTCTAGTGCCTGAGCCTCCG. The
ZFN monomers bind to each underlined sequence on opposite strands of the gene. SS/MCiHsd (Dahl S) 1-cell rat embryos were injected with messenger RNAs encoding the Tgfβ1/ZFNs at a concentration of 10 ng/μl and transferred to pseudopregnant recipients. DNA extracted from ear punch biopsy samples from founder-generation animals were screened with a PCR (TGF-β1, sense: 5'-AATGCCAGTCATTTG-GATGC-3' and antisense: 5'-TAAAGCTTAACTCGTCGGC-3') and CEL-I mutation assay for nonhomologous end joining mis-repair events as previously described (13).

Generation of TGF-β1−/− KO line. Several heterozygous founder rats were tested for ZFN cleavage using Surveyor Nuclease Assay as previously described (12), identified, and backcrossed to Dahl S rats to produce a heterozygous line and the colony was maintained at the University of Mississippi Medical Center by brother-sister mating. Each animal was genotyped by PCR analysis by using genomic DNA previously described (12), identified, and backcrossed to Dahl S rats. From ear punch biopsy samples from founder-generation animals were tested for ZFN cleavage using Surveyor Nuclease Assay as previously described (13).

Comparison of the development of hypertension and proteinuria in wild-type (WT) and TGF-β1−/− Dahl S rats. These experiments were performed on WT and TGF-β1−/− Dahl S littermates maintained from weaning on a 0.4% NaCl diet (113755; Dyets, Bethlehem, PA) diet. When the rats were 6 wk of age they were switched to 8% NaCl diet (100078, Dyets). Systolic blood pressure (SBP) was measured by tail-cuff (MC4000 Blood Pressure Analysis System; Hatteras Instruments, Cory, NC), and 24-h urine samples were collected once a week for 5 wk. At the end of the experiment, the right kidney was preserved in 10% neutral-buffered formalin for histological analysis, and the left kidney was snap-frozen in liquid nitrogen and used for Western blot and measurements of TGF-β1 levels. Urinary concentration of protein was measured using the Bradford method, albumin was measured using Albumin Blue 580 fluorescence assay (17), and TGF-β1 levels were measured using a TGF-β1 Emax Immunoassay System ELISA kit (Promega, Madison, WI). Since TGF-β1 levels may be affected by renal damage caused by prolonged exposure to high salt (HS), additional experiments were performed on a second group of 6 wk old WT and TGF-β1−/− littermates that were fed an HS diet for 1 wk.

Comparison of the expression of TGF-β1, TGF-β2, TGF-β3, and markers of renal injury in WT and TGF-β1−/− Dahl S rats. The renal cortex was separated from the medulla and homogenized in lysis buffer containing 20 mM HEPES, 10 mM sodium chloride, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM EDTA, and protease inhibitor cocktail. The homogenates were centrifuged at 5,000 g for 5 min and 9,000 g for 15 min. The supernatant was collected, and Western blots were incubated with primary antibodies against TGF-β1 (sc-146; Santa Cruz Biotechnology, Santa Cruz, CA), TGF-β2 (sc-90, Santa Cruz Biotechnology), TGF-β3 (ab15537; Abcam, Cambridge, MA), COL4A1 (ab19808, Abcam), α-smooth muscle actin (SMA; sc-32251, Santa Cruz Biotechnology), podocin (sc-111KO OF TGF-β1 Protects Against Renal Injury

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Fig. 1. A: a representative gel for genotyping rats. Lanes 1 and 2, wild type (WT); lanes 3 and 4, transforming growth factor (TGF)-β1−/−; lane 5, negative control; lane 6, 50 bp DNA ladder. B: the nuclease target site and location of the deletion in TGF-β1 gene. The predicted size of the PCR product is 381 bp for WT and 359 bp for knockout (KO). Square bracket denotes the sequence of the 22 bp deletion. Capital letters indicate Zinc-finger recognition sites separated by the spacer cttct; boldface indicates predicted start and stop codons of the mutant transcript. ZFN, zinc finger nuclease.
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RESULTS

Genotyping and sequencing. A representative gel is presented in Fig. 1A. PCR amplification of DNA from Dahl S rats produced a single band of 381 bp. Amplification of DNA samples from the TGF-β1+/− produced the WT band and a second band of 359 bp. Our sequence analysis confirmed that all of TGF-β1+/− animals have a 22 bp deletion in exon 3 of the TGF-β1 gene between bp 191 and 212 of the rat Tgfb1 cDNA (Fig. 1B) that is predicted to produce a frame-shift mutation in the resulting nascent transcript that results in the introduction of a premature stop codon at bp 267 and again at 315 to produce a predicted 33 amino acid-truncated protein. It is expected that this heterozygous deletion could lead to reduced expression of the intact TGF-β1 protein and the expression of a truncated inactive form of TGF-β1.

Backcross breeding of heterozygous TGF-β1+/− rats to Dahl S rats generated pups with the expected 1:1 ratio across

21009), and nephrin (ab58968, Abcam) overnight followed by 1:10,000 dilution of a horseradish peroxidase-coupled secondary antibody (sc-2004 or sc-2005) for 1 h. The blots were exposed to SuperSignal West Dura Substrate (Thermo Scientific, Rockford, IL) and imaged using a ChemiDoc photo documentation system (Bio-Rad). Equal loading of homogenate protein was confirmed by stripping and reprobing with a GAPDH antibody (sc-166574) overnight followed by a 1:20,000 dilution of horseradish peroxidase-coupled secondary antibody (sc-2005) for 1 h or by imaging total protein on the membrane with Ponceau S.

Measurement of TGF-β1 levels. Free TGF-β1 levels in renal tissue were measured after homogenization of the renal cortical samples in the homogenization buffer used for Western blots. Total TGF-β1 levels were measured after the tissue was acid activated in 1 M acetic acid for 30 min to release the latent TGF-β1. The homogenates were centrifuged at 5,000 g for 5 min and 9,000 g for 15 min, and the supernatant was collected and neutralized with 1 M sodium hydrox-ide. TGF-β1 levels in the samples were measured by ELISA (Pro-mega) according to manufacturer’s instructions, and the values were normalized to the protein concentration of the samples.

Histology. Formalin-fixed kidneys were hemisected and embedded in paraffin. Sections (3 μm) were cut and stained with Masson trichrome to evaluate degree of renal injury. The degree of glomerular injury was assessed on 100 glomeruli/section and graded from 0–4 as follows: grade 0, normal glomeruli with no overt morphological damage; grade 1, <25% glomerulus injured; grade 2, 25–49% glomerulus injured; grade 3, 50–74% glomerulus injured; grade 4, >75% glomerulus injured. The degree of tubulointerstitial fibrosis was evaluated by measuring the percentage of blue staining (collagen) seen in trichrome-stained slides. Images were captured with a Nikon Eclipse 55i microscope equipped with a Nikon DS-Fi1 color camera (Nikon, Melville, NY), and the percentage of image stained blue was quantified with NIS-Elements D 3.0 software. At least 15 cortical and 15 medullary fields were analyzed per rat for tubulointerstitial fibrosis.

Statistical analysis. Data are presented as mean values ± SE. The significance of differences in mean values between the groups were performed by two-way ANOVA for repeated measures or a one-way ANOVA followed by Tukey’s multiple comparisons test. A P value <0.05 was considered to be statistically significant.
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Fig. 4. Comparison of the expression of TGF-β1, TGF-β2, and TGF-β3 protein in the renal cortex of WT and TGF-β1+/− rats fed a 0.4% or 8% NaCl diet for 1 wk. A: representative blots. B, C, D: a comparison of the relative expression of TGF-β1, 2, and 3. *Significant difference (P < 0.05) in corresponding LS (0.4% NaCl) values within a strain; †significant difference (P < 0.05) in corresponding values between strains.

nine litters totaling 38 WTs and 38 TGF-β1+/− rats, and the males were used in our phenotyping experiments. We also tried to produce a TGF-β1 homozygous KO line. However, breeding heterozygous pairs of TGF-β1+/− animals failed to produce a single TGF-β1 homozygous KO pup. Instead, these pairs produced 39 WT and 77 TGF-β1+/− heterozygous pups in a ratio of 1:2, consistent with the view that homozygous TGF-β1 mutation is embryonic lethal.

Comparison of the development of hypertension and proteinuria in WT and TGF-β1+/− Dahl S rats. The results of these experiments are presented in Fig. 2. SBP (Fig. 2A), proteinuria (Fig. 2B), and albuminuria (Fig. 2C) were similar in WT and TGF-β1+/− maintained on a low-salt (LS) diet containing 0.4% NaCl. Blood pressure increased similarly in WT and TGF-β1+/− Dahl S rats fed an HS diet containing 8% NaCl. Urinary protein excretion (UPE) also increased progressively in both WT and TGF-β1+/− rats over time. However, the level of UPE was 48% higher in the WT than in the TGF-β1+/− rats (463 vs. 313 mg/day, respectively) 5 wk after rats were fed an HS diet. Urine albumin excretion increased in both WT and TGF-β1+/− rats fed an HS diet for 1 wk. However, the level of urine albumin was higher in WT than in the TGF-β1+/− rats 5 wk after rats were fed an HS diet.

Fig. 5. Comparison of the expression of TGF-β1 (A), TGF-β2, and TGF-β3 (B) protein in the renal cortex of WT and TGF-β1+/− rats fed a 0.4% or 8% NaCl diet for 5 wk. *Significant difference (P < 0.05) in corresponding LS (0.4% NaCl) values within a strain; †significant difference (P < 0.05) in corresponding values between strains.
Comparison of the renal expression of TGF-β1, TGF-β2, TGF-β3, podocin, and nephrin in WT and TGF-β1−/− Dahl S rats. Free renal cortical TGF-β1 levels were very low and not significantly different in WT and TGF-β1−/− rats fed LS or HS diet for only 1 wk (Fig. 3A). Total renal TGF-β1 protein levels were also similar in WT and TGF-β1−/− rats fed a 0.4% salt diet for 1 wk (Fig. 3B). However, total TGF-β1 rose in WT rats fed an HS diet for 1 wk and was significantly higher than the value in TGF-β1−/− rats. Free and total TGF-β1 protein levels were also similar in WT and TGF-β1−/− fed an LS (0.4% NaCl) diet for 5 wk (Fig. 3, A and B). Total TGF-β1 levels increased to the same extent in both WT and TGF-β1−/− rats fed an HS diet for 5 wk (Fig. 3B), but the free form of TGF-β1 was significantly higher in the WT than TGF-β1−/− rats fed an HS diet for 5 wk (Fig. 3A). The results of the Western blot experiments for rats fed a 0.4% NaCl or HS diet for 1 wk are presented in Fig. 4. These experiments confirm that TGF-β1 expression increases in WT but not in TGF-β1−/− rats following 1 wk of an HS diet. TGF-β2 levels were not significantly altered in either WT or TGF-β1−/− rats. Baseline expression of TGF-β3 was significantly lower in TGF-β1−/− rats than in WT rats fed an LS diet for 1 wk, but levels were not significantly different after the rats were fed an HS diet for 1 wk. The comparison of the effects of exposure to HS diet for 5 wk on the expression of TGF-β is presented in Fig. 5. Baseline expression of TGF-β1 was similar in WT and TGF-β1−/− rats fed a 0.4% NaCl diet for 5 wk. The expression of TGF-β1 increased in both WT and TGF-β1−/− rats fed an HS diet, but the levels were significantly lower in TGF-β1−/− rats than WT rats (Fig. 5A). TGF-β2 protein was reduced in WT fed an HS diet for 5 wk but was not changed in TGF-β1−/− rats (Fig. 5B, left). The expression of TGF-β3 was unchanged in response to HS diet for 5 wk in either group (Fig. 5B, right).

Baseline free and total urinary TGF-β1 excretion was similar in WT and TGF-β1−/− fed 0.4% NaCl diet and did not change in either group over the course of the study (Fig. 6, A and B). Free and total urinary TGF-β1 excretion increased significantly in both WT and TGF-β1−/− rats fed an HS diet for 1 wk, but the rise in TGF-β1 levels were higher in WT compared with TGF-β1−/− rats. The rise in TGF-β1 excretion was transient and returned toward control after 5 wk on HS diet in both groups. However, TGF-β1 levels remain significantly elevated in WT but not in TGF-β1−/− rats fed an HS diet for 5 wk. The expressions of biomarkers of glomerular injury are presented in Fig. 6. Expression of podocin, an index of podocyte number and integrity, was similar in the renal cortex of WT and TGF-β1−/− rats fed 0.4% NaCl diet (Fig. 7A). The expression of podocin fell to a similar extent in both WT and TGF-β1−/− rats fed an HS diet for 5 wk. Renal cortical nephrin protein was similar in WT and TGF-β1−/− fed a 0.4% NaCl diet as shown by Fig. 7B but was significantly lower in WT than in TGF-β1−/− rats fed a HS diet for 5 wk. Renal cortical podocin and nephrin levels were not significantly different in WT and TGF-β1−/− rats that were fed an HS diet for only 1 wk (data not shown).

Histology and comparison of the renal expression of biomarkers of renal fibrosis in WT and TGF-β1−/− Dahl S rats. Representative histology for renal cortex of WT and TGF-β1−/− rats fed a 0.4% or 8% NaCl diet for 1 wk and 5 wk are presented in Fig. 8A, and comparisons of the glomerular injury scores and the degree of renal interstitial fibrosis are presented in Fig. 8, B and C. The degree of glomerular injury was similar in WT and TGF-β1−/− rats fed an HS diet for 1 wk. However, the percentage of renal interstitial fibrosis was higher in WT than in TGF-β1−/− rats. The glomerular injury scores and percentage of renal interstitial fibrosis were similar in WT and TGF-β1−/− rats fed a 0.4% NaCl diet for 5 wk. However, the degree of glomerular injury and renal interstitial fibrosis was markedly increased in both WT and TGF-β1−/− rats fed an HS diet for 5 wk, but the increase in injury was significantly less in TGF-β1−/− animals than the WT rat. The representative appearance of the renal medulla in WT and TGF-β1−/− rats fed a 8% NaCl diet for 1 and 5 wk is presented in Fig. 9A, and the percentage of medullary fibrosis is presented in Fig. 9B. TGF-β1−/− and WT rats fed an HS diet for only 1 wk exhibited very little renal medullary interstitial fibrosis, and there was no difference between the groups. TGF-β1−/− rats fed 0.4% NaCl diet for 5 wk exhibited less renal medullary interstitial fibrosis compared with WT (Fig. 9B). The fibrosis of the vasa recta capillaries and tubular necrosis increased in both WT and TGF-β1−/− animals fed an HS diet for 5 wk, but the increase was significantly less in TGF-β1−/− than in the WT rats.

COL4A1 and α-SMA levels were measured as confirmatory biomarkers of renal fibrosis. Renal cortical COL4A1 was
similar in WT and TGF-β1+/− rats fed 0.4% NaCl diet for 5 wk. Renal cortical COL4A1 expression increased fourfold in WT rats fed an HS diet for 5 wk but did increase significantly in the TGF-β1+/− rats (Fig. 10A). Renal cortical α-SMA was similar in rats fed 0.4% NaCl diet for 5 wk, but it increased in WT rats fed an HS diet but not in the TGF-β1+/− rats (Fig. 10B).

The expression of COL4A1 (Fig. 10C) and α-SMA (Fig. 10D) in the outer medulla was similar in WT and TGF-β1+/− rats fed 0.4% NaCl diet for 5 wk. Renal medullary COL4A1 and α-SMA increased in both WT and TGF-β1+/− rats fed an HS diet for 5 wk, but α-SMA levels were increased to a greater extent in WT rats than in the TGF-β1+/− rats.

**DISCUSSION**

The present study characterized the development of hypertension, proteinuria, and renal injury in WT and heterozygous TGF-β1 KO strain of Dahl S rats. The results indicate that homozygous KO of TGF-β1 is embryonic lethal, and phenotyping of the heterozygous TGF-β1+/− KO rats revealed some interesting differences in the development of hypertension-induced proteinuria and renal injury.

Both WT and TGF-β1+/− Dahl S rats developed the same degree of hypertension in response to exposure to an HS diet. SBP rose <10 mmHg in both groups in the first week on HS diet but increased by 60–70 mmHg over the next 4 wk. Exposure to an HS diet increased the expression of TGF-β1 in the urine of the WT Dahl S rats. However, knocking out one copy of the TGF-β1 gene attenuated the increase in the expression of TGF-β1 in the renal cortex and the urine during the first week the rats were fed an HS diet. After 5 wk on an HS diet, the free and activated form of TGF-β1 remained elevated in the renal cortex and urine of WT compared with TGF-β1+/− rats. This transient

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**Fig. 7.** Comparison of the expression of podocin (A) and nephrin (B) protein in the renal cortex of WT and TGF-β1+/− rats fed a 0.4% or 8% NaCl diet for 5 wk. *Significant difference (P < 0.05) in corresponding LS (0.4% NaCl) values within a strain; †significant difference (P < 0.05) in corresponding values between strains.

**Fig. 8.** Comparison of renal cortical histology (A) in WT and TGF-β1+/− rats fed a 0.4% or 8% NaCl diet for 1 and 5 wk. Glomerular injury index (B) and renal cortical interstitial fibrosis (C) were assessed. *Significant difference (P < 0.05) in corresponding values between strains.
increase in local renal TGF-β1 production in the urine is consistent with our previous findings that TGF-β1 increases following challenge with HS in Dahl S rats in the first few days but then returns toward baseline following the development of hypertension and progressive renal injury (10, 21, 30). Interestingly, the excretion of TGF-β1 excretion reached a maximum during the first week on an HS diet and then decreased after 5 wk, but renal cortical tissue TGF-β1 levels continued to increase from week 1 to 5 on an HS diet. It is unclear what mechanisms underlie this phenomenon. One possible explana-

Fig. 9. Comparison of renal medullary histology (A) in WT and TGF-β1−/− rats fed a 0.4% or 8% NaCl diet for 1 and 5 wk. Renal medullary interstitial fibrosis (B) was assessed. *Significant difference (P < 0.05) in corresponding LS (0.4% NaCl) values within a strain; †significant difference (P < 0.05) in corresponding values between strains.

Fig. 10. Comparison of the expression of COL4A1 and α-SMA protein in the renal cortex (A and B) and outer medulla (C and D) of WT and TGF-β1−/− rats fed a 0.4% or 8% NaCl diet for 5 wk. *Significant difference (P < 0.05) in corresponding LS (0.4% NaCl) values within a strain; †significant difference (P < 0.05) in corresponding values between strains.
tion may be due to the local effects of TGF-β1. TGF-β1 production was reported to be greatly increased in the glomeruli of Dahl S rats fed an HS diet and could be detected in the urine (36), consistent with our results. TGF-β1 can also cause podocyte dysfunction and injury (33), leading to increased production of experimental glomerulonephritis by antiserum against transforming growth factor beta 1. Nature 346: 371–374, 1990.


