Angiotensin II sensitivity of afferent glomerular arterioles in endothelin-1 transgenic mice

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Abstract

Background. Although endothelin I (ET-1) is a very potent vasoconstrictor, ET-1 transgenic (ET-1 tg) mice are not hypertensive. This might be due to higher bioavailability of nitric oxide (NO) in ET-1 tg, which counteracts the effect of vasoconstrictors. We hypothesized lower angiotensin II (Ang II) sensitivity of afferent arterioles in ET-1 tg.

Methods. Afferent arterioles were manually dissected and microperfused. Changes of the luminal diameter due to application of vasoactive substances were used for assessment of the reactivity of afferent arterioles. We investigated the effect of L-NAME, an unspecific NO synthase inhibitor, on basal tone, and the sensitivity of afferent arterioles to Ang II with and without pre-treatment with L-NAME. The renin-angiotensin-system was characterized by expression analysis of angiotensin-receptors and renin at the mRNA level.

Results. L-NAME reduced afferent arterioles diameters similarly in ET-1 tg and wild-types (WT). Ang II sensitivity determined by calculation of EC50 for Ang II was less in ET-1 tg compared with WT (P < 0.05). Ang II reduced luminal diameters to a lesser extent in ET-1 tg compared to WT (P < 0.05). After pre-treatment with L-NAME, Ang II sensitivity and maximum constriction of afferent arterioles were similar in ET-1 tg and WT. The expression of renin- and Ang II-receptor-mRNA in the kidney did not differ between either group.

Conclusion. The loss of differences in the maximum constriction and Ang II sensitivity of afferent arterioles between ET-1 tg and WT in the absence of NO suggests pronounced NO effects in afferent arterioles of ET-1 tg. This might contribute to the maintenance of normal renal arteriolar tone in ET-1 tg mice.

Keywords: afferent arteriole; angiotensin II; endothelin; ET-1 transgenic mouse; nitric oxide

Introduction

Endothelin-1 (ET-1) transgenic (tg) mice have higher endothelin-1 concentrations in kidney tissue and develop a phenotype characterized by progressive fibrotic remodelling of the kidney [1]. These mice do not show hypertension or elevation of blood pressure, although ET-1 is known as a potent endothelium derived vasoconstrictor [2]. Though the finding that there is no hypertension in ET-1 tg mice was initially unexpected, a second independently generated ET-1 tg mouse model [3] confirmed the findings. Recent studies on aortic rings demonstrated an enhanced endothelium-dependent relaxation in aortic rings of ET-1 transgenic mice, which was inhibited by unspecific blockade of nitric oxide (NO) synthases [4]. Furthermore, the maximum contraction to ET-1 was reduced compared with wild-type littermates. The authors concluded that there was a higher bioavailability of NO in large blood vessels of ET-1 tg mice. NO counteracts the contractile effect of ET-1 in big arteries, which may be one of the reasons for the normotension in ET-1 transgenic animals. However, investigations in resistance vessels
have not been done until now. Renal afferent (Af) and efferent arterioles are key elements in control of renal perfusion and filtration. The tone of the glomerular arterioles results from the interplay of vasoactive substances from systemic and local sources. Among them, NO plays an important role as a vasodilator. Recent studies show that the NO release is specifically stimulated by the action of Ang II in Af [5,6]. Therefore, angiotensin II (Ang II) induced constriction was used to test the hypothesis of an increased influence of NO on Af in the present study. Comparing the Ang II-sensitivity in ET-1 transgenic mice and control animals allows estimations of whether Ang II induced NO effects are increased in vessels of ET-1 transgenic mice. An increased NO effect on Af could antagonize the vasoconstrictor effect of increased ET-1 activity in the juxtaglomerular apparatus. The maintenance of a normal tone of Af by increased bioavailability of NO may be responsible for a normal renal function and possibly, among other mechanisms, for the prevention of hypertension in ET-1 transgenic mice. To test the hypothesis of a greater influence of NO in ET-1 transgenic mice, we measured Ang II sensitivity of Af with and without pretreatment with L-N^G-nitroargininemethyl-ester (L-NAME) and investigated the effect of L-NAME on basal tone. To exclude that chronic changes in the Ang II-receptor expression in ET-1 transgenic animals play a role in our study, mRNA for Ang II-receptors type 1 and type 2 was quantitatively assessed. The renin-mRNA-expression was also measured for further characterization of the renin-angiotensin-system in ET-1 transgenic mice. Since vascular remodelling has been described in large arteries in ET-1 transgenic mice [1], the media-lumen-ratio was calculated for Af to estimate the impact of remodelling of Af contraction. It is known that glomerulosclerosis progressively develops in ET-1 transgenic mice. We therefore sought for sclerotic changes using different scores, to exclude possible influences on our functional measurements.

Material and methods

Animals

Human ET-1 transgenic mice with the genetic background of NMRI mice were generated as previously described [1]. In the present study, male homozygous and heterozygous animals were used (line 856), between 3 and 6 months old. Heterozygous animals show a similar phenotype compared with the homozygous mice [1]. Male wild-type (WT) littermates served as controls. The animals were fed with standard mouse chow and allowed free access to tap water. All animal procedures conformed to the guidelines for care and handling of animals established by the US Department of Health and Public Services and published by the National Institutes of Health.

Dissection and microperfusion studies

Dissection and perfusion procedures have already been described [5]. The kidneys were immediately removed after killing the animal by breaking the neck. They were sliced along the corticomедullary axis. The Af were dissected at 4°C in albumin enriched physiological salt solution (0.1%) using a stereoscopic microscope and with the help of sharpened forceps (No.5, Dumont, Switzerland). Tubuli were removed with the exception of the region of the thick ascending limb of Henle’s loop which touches the glomerulus. Only arteries, which were located in the outer part of the renal cortex, i.e. at the end of the arterial tree, were used. Time for dissection was limited to 120 min after killing the mouse. The experiment was terminated, if perfusion was not achieved within this time span.

The arteriole with its glomerulus intact was transferred into a thermoderegulated chamber (volume 1.5 ml, VETEC, Rostock, Germany) on a stage of an inverted microscope (Axiovert 100, Carl Zeiss, Oberkochen, Germany). The perfusion system allowed movement and adjustment of concentric, holding and perfusion pipettes (Luigs & Neumann, Ratingen, Germany). The holding pipette had an aperture of roughly 26 μm at the tip and a constriction of about 20 μm. The proximal end of the arteriole was aspirated into this pipette. The inner perfusion pipette with a tip diameter of 5 μm was advanced into the lumen of the arteriole. This pipette was connected to a reservoir containing the perfusion solution and to a manometer. Af were perfused at 37°C. The pressure in the pressure head amounted to 80 mmHg. Based on investigations of the contractile response of Af to increasing perfusion pressure, we estimated the experiment to be at the lower end of the physiological pressure range when using a pressure of 80 mmHg in this perfusion set-up. The perfusion rate of this perfusion set-up was measured with the help of a radioactive labelled perfusion solution. It was calculated to about 50 nl/min using perfusion pipettes with a tip diameter of 5 μm. The perfusion rate fits to the experimentally determined single nephron filtration rate of 10 nl/min in mice [8].

After starting the perfusion, the solution temperature was gradually increased and 20 min were allowed for adaptation. Only arterioles with a good remaining basal tone were used. Hypoxic or otherwise injured vessels were readily identified by pronounced vasodilatation and failure to constrict in response to K^+-solution (100 mmol/l KCl). This was used to test the viability of the arterioles at the beginning of each experiment. The arterioles were allowed to recover for 10 min after the K^+-test.

Experiments were recorded on super video home system video tapes (video recorder AG-MD 830, Panasonic, Kadoma City, Japan). The magnification results from an objective (x40, Carl Zeiss, Germany) and projection (x1) on a 0.3” chip digital camera (CB-3803S, KGB, Tai Chung, Taiwan). Video sequences were digitized using a frame grabber card (UDT 55-LC-EZ-50, data translation, Marlboro, MA). The vessel diameters were determined using customized software (Dr. H. Siegmund, Johannes-Müller-Institute of Physiology, Humboldt-University of Berlin, Germany). The equipment allowed a resolution of 0.2 μm of the vessel structures. Basal values for the luminal diameter were obtained at the end of the adaptation period (control values). In all series, the last 10 s of the treatment period were used for statistical analysis of steady state responses. For each experiment, only one type of drug or drug combination was performed in each of the perfused arterioles.
Histomorphometry of afferent arterioles

Halves of kidneys were fixed in buffered formaldehyde (pH 7.4, 4%), dehydrated, and embedded into paraffin. From the paraffin-embedded tissues 2 µm sections were cut with a Leica microtome (Leica Microsystems AG, Wetzlar, Germany) and collected onto capillary gap microscope slides (7.5 µm) for use with imperfectly TechMate-500 immuno-stainer (Biotec Solutions, St. Barbara, CA, USA). Staining with haematoxylin and eosin was performed by standard procedures. Sections were also stained with labelled antibodies against smooth muscle actin (DAKO, Glostrup, Denmark). For further analysis, the slides were mounted on an inverted microscope (Axiolicht 35, Carl Zeiss, Oberkochen, Germany) and the sections were scanned for AF using an immersion objective with a magnification x100 and oculars x12.5. The pictures were digitized using a projection (x1) on a 0.3” chip digital colour camera (CC-8703, GKB, Tai Chung, Taiwan) and a frame grabber card (all in wonder Radeon, ATI Technologies Inc., Markham, ON, Canada). The digitized pictures had a format of 480 x 640 pixel with a calibration of 8.7 pixel per µm. Stained AF were identified by their position within the cortex and their situation in relation to the glomeruli and to the interlobular arteries. The areas of the media (stained smooth muscle cells) and of the lumen were determined using customized software (Dr R. Mrowka, Johannes-Müller-Institute of Physiology, Humboldts-University of Berlin, Germany). All vessels are, more or less, imperfectly cross-sectioned on histological slides. Therefore, the part of the arteriole with the smallest media diameter was used for the computer-aided analysis. Vessels with non-regular shape, which did not allow the measurement of the outline and inline diameters of the stained media of the vessel were excluded. In the present study, immersion-fixed tissue was used because infusion fixation in mice showed higher variability in the size of the arterioles in our hands. Immersion-fixed tissue is acceptable for measuring medial thickness [9]. Since samples of both groups were treated with the same method, comparisons are also valid. Slides were analysed independently by two investigators without knowledge about the group to which the material belonged.

Renal morphology

Samples were embedded in paraffin, and submitted to haematoxylin-eosin, Sirius red and periodic acid-Schiff (PAS) staining. The degree of interstitial matrix deposition was evaluated using an imaging analysing system (Axiovert 35, Carl Zeiss, Oberkochen, Germany) and the sections were scanned for AF using an immersion objective with a magnification x100 and oculars x12.5. The pictures were digitized using a projection (x1) on a 0.3” chip digital colour camera (CC-8703, GKB, Tai Chung, Taiwan) and a frame grabber card (all in wonder Radeon, ATI Technologies Inc., Markham, ON, Canada). The digitized pictures had a format of 480 x 640 pixel with a calibration of 8.7 pixel per µm. Stained AF were identified by their position within the cortex and their situation in relation to the glomeruli and to the interlobular arteries. The areas of the media (stained smooth muscle cells) and of the lumen were determined using customized software (Dr R. Mrowka, Johannes-Müller-Institute of Physiology, Humboldts-University of Berlin, Germany). All vessels are, more or less, imperfectly cross-sectioned on histological slides. Therefore, the part of the arteriole with the smallest media diameter was used for the computer-aided analysis. Vessels with non-regular shape, which did not allow the measurement of the outline and inline diameters of the stained media of the vessel were excluded. In the present study, immersion-fixed tissue was used because infusion fixation in mice showed higher variability in the size of the arterioles in our hands. Immersion-fixed tissue is acceptable for measuring medial thickness [9]. Since samples of both groups were treated with the same method, comparisons are also valid. Slides were analysed independently by two investigators without knowledge about the group to which the material belonged.

Blood pressure

Blood pressure and heart rate were measured by the tail-cuff method (Blood Pressure Monitor BMN-1756; Föhr Medical Instruments, Seelheim, Germany) in unanaesthetized mice that underwent 4 days of extensive training to get used to this procedure. Mean values of five subsequent measurements were calculated.

RNA-isolation and renin-mRNA-expression

One kidney of each mouse was removed immediately after killing and half of the kidney was frozen in liquid nitrogen. Specimens were kept at −80°C until analysis. Extraction of total RNA took place according to the protocol of Chomczynski and Sacchi [10]. Renal prepro-renin mRNA was analysed by Northern blot technique using 15 µg of total RNA. Synthesis of a non-radioactive labelled renin probe, its hybridization to the renin mRNA on the blot and detection of the hybridization signal were performed according to the manufacturer’s protocols (Roche Diagnostic GmbH, Grenzach-Wyhlen, Germany). The results were expressed as relative units. Renal prepro-renin contents of the samples (normalized to its ribosomal RNA) were compared to an external standard included in each blot.

Angiotensin II-receptor expression

Real-time polymerase chain reaction (PCR) was performed as previously described with an ABI PRISM 7000 sequence detection system for real-time PCR [11]. Mouse 18S ribosomal RNA for real-time PCR was chosen as an endogenous control (housekeeping gene). 18S forward primer was ACC TGG TTG ATC CTG CCA GTA G, reverse primer TTA ATG AGC AGC TCG CAG TTT C, probe TGC ATG TCT AAG TAC GCA CGG CCG TT. Ang II-type 1-receptor (AT1 receptor) forward primer was CCA TTG TCC ACC CCA TGA A, reverse primer TGA CTT TGG CCA CCA GCA T, probe TCT CGC CTC CGC AC. Ang II-type 2-receptor (AT2 receptor) forward primer CCT GCC AAG CAT CTT ATG TAG TTC, reverse primer CCG GAA ATA AAA TGT TGG CAA T, probe TTT GGT GTA TGG CTT TCT TAT CCT.

mRNA-expression of endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) nitric oxide synthase

The expression of eNOS, nNOS and iNOS mRNA in the kidney of mice was investigated using real-time PCR analyses. 1 µg of the isolated RNA was reverse transcribed using SuperScriptII Reverse Transcriptase and random hexamers (Invitrogen) according to the manufacturers instructions. Quantitative PCR analysis was performed in triplicate using a GeneAmp 5700 (Applied Biosystems) according to the manufacturer’s instructions. SYBR Green was used for the fluorescent detection of DNA generated during the PCR. The PCR reaction was performed in a total volume of 25 µl with 0.2 pmol/µl of each primer, and 2x SYBR Green master mix (Applied Biosystems); 2.5 µl cDNA corresponding to 25 ng RNA was used as template. Published sequences for mouse eNOS (NM_008713), iNOS (NM_010927) and nNOS (NM_008712) were used to design primers to bridge at least one intron for PCR amplification. Primer sequences were: eNOS sense 5’-GTT TGT CTG CCG CCA TGT C-3’ and antisense 5’-CAT GCC GCT CTC TGT TG-3’, nNOS sense 5’-TCG GCT GTG CTT TGA TGG A-3’.
and antisense 5'-TTG AAT CGG ACC TTG TAG CTC TTC-3'; iNOS sense 5'-GGC AGC CTG TGA GAC CTT TG-3' and antisense 5'-CAT TGG AAG TGA AGC GTT TCG-3'; and β-actin sense 5'-CAC CCC CGA GCA CAG CTT CTT T-3' and antisense 5'-CAT TGG AAG TGA AGC GTT CTC-3'. The expression levels of eNOS, iNOS and nNOS mRNA in the kidney of mice were normalized to β-actin using the ΔCt-method.

Solutions and drugs

Physiological salt solution (PSS) was used with the following composition: NaCl 115, NaHCO3 25, K2HPO4 2.5, CaCl2 1.3, MgSO4 1.2, and glucose 5.5 (mmol/l). The K+ solution consisted of 100 mmol/l KCl, whereby 95 mmol/l NaCl were substituted by KCl. The bicarbonate buffered solution was equilibrated with 5% CO2 and 95% O2. The pH was adjusted to 7.4 after the addition of bovine serum albumine (BSA). The concentration of BSA in PSS (bath solution) was 0.1%. The perfusate consisted of PSS with a BSA concentration of 1%. The oxygen partial pressure was between 160 and 200 mmHg in the bath and perfusion solution, and the pH was stable during the experiment. Dulbecco’s modified Eagle’s medium (DMEM, 1000 ml) was used for the dissection procedure. BSA was obtained from SERVA Electrophoresis (Heidelberg, Germany); angiotensin II and DMEM from Sigma-Aldrich (Munich, Germany); and L-N^G-nitroarginine-methyl-ester (L-NAME) from Alexis Biochemicals (Grüneberg, Germany).

Calculations and statistics

Assessment of the luminal diameter of isolated, perfused arterioles: for the last 10 s of each control and treatment periods, five measurements were averaged. The Brunner test for non-parametric analysis of longitudinal data (ANOVA) was used to test for concentration-dependent changes in the arteriolar diameter (one-way ANOVA) and to check for differences in the concentration-dependent changes in diameter between the groups (two-way ANOVA). The Mann–Whitney U-test was used to test the significance of the differences in the diameters between the groups during the control situation, and of expressions of NOS-isoforms. It was applied to test the null hypothesis regarding the expression of renin-mRNA and AT-receptors in both groups. The EC50, for the Ang II-concentration-response was calculated using the programme GraphPad Prism 4 (GraphPad Software, San Diego, CA) and differences between groups were checked with the help of Mann–Whitney U-test. Data are presented as mean ± SEM. Determination of the media-lumen-ratio: kidney sections from seven animals of each group (ET-1 transgenic animals and WT) were scanned for Af. For each group, 350 Af were obtained. Outer and luminal diameters of Af were measured three times at representative sites of the vessels. In case of an oblique cut of the arterioles the smallest diameters were taken. The areas for the media as well as for the lumen, and their ratio were computed. The mean of the three measurements for all these parameters was obtained. To compare media-lumen-ratios of corresponding groups, we calculated histograms and checked for differences according to the method of Brandt-Snedecor. The confidence level P was set to 0.05 for all statistical tests.

Results

Inhibition of NOS

Unspecific blockade of NOS with L-NAME in cumulative concentrations from 10^{-6} to 10^{-3} mol/l (each applied for 3 min) increased the basal tone of the isolated, perfused Af in both groups. As shown in Figure 1, the luminal diameter decreased in a concentration-dependent manner (P<0.05). Diameters were reduced to 77.1±5.7% (n=10) and 76.8±4.0% (n=8) at 10^{-3} mol/l L-NAME in ET-1 transgenic mice and WT, respectively. The response to L-NAME was similar in Af of ET-1 transgenic and WT mice.

Contraction to Ang II

Ang II applied in cumulative concentration from 10^{-12} to 10^{-6} mol/l (2 min each concentration) reduced the luminal diameter of Af concentration-dependent

![Fig. 1](image-url). Effect of NOS inhibitor L-NAME on the basal tone of afferent arterioles in ET-1 transgenic animals (tg) and wild-type littermates (WT). Data are presented as absolute values (left panel) and as percent of the control diameter (right panel). The concentration-response curves do not differ comparing ET-1 transgenic animals and WT.
(Figures 2–3) in both groups. In WT, the luminal diameter amounted to $54.8 \pm 3.9\%$ ($n = 13$) at $10^{-8}$ mol/l Ang II. In ET-1 transgenic mice, the luminal diameter was reduced to $69.2 \pm 6.5\%$ ($n = 7$) at $10^{-8}$ mol/l Ang II. Comparing the EC$_{50}$ reveals the lower Ang II sensitivity in ET-1 transgenic animals compared with WT ($P < 0.05$; Figure 4). The lumen of Af in ET-1 tg was greater at high Ang II concentrations compared to the WT, i.e. the Ang II induced constriction was less pronounced.

In vitro pre-incubation with L-NAME ($10^{-4}$ mol/l, 15 min) significantly reduced luminal diameters (Figures 2, 5) in both groups without differences between ET-1 transgenic mice and their wild-type littermates. The following application of Ang II in increasing cumulative concentrations reduced the diameters in both groups significantly. Remarkably, the Ang II sensitivity was drastically increased ($P < 0.05$) compared with the experiment without L-NAME pretreatment. However, it did not differ between ET-1 transgenic mice and WT. Diameters were reduced to $13.2 \pm 7.0\%$ ($n = 9$) in WT and to $14.6 \pm 6.4\%$ ($n = 9$) in transgenic mice at $10^{-8}$ mol/l Ang II.

**Histomorphology of afferent arterioles**

Analysis of the morphology of the afferent arterioles showed a skewed distribution of frequencies of media-lumen-ratios of Af for both groups. The kurtosis was shifted to higher media-lumen-ratios in ET-1

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**Fig. 2.** Microperfusion of isolated afferent arterioles of wild type littermates. Parts of the glomerulus and the holding pipette are visible on the pictures at the extreme left and right side, respectively. Effect of Ang II ($10^{-8}$ mol/l, A, B, left panel) and Ang II ($10^{-8}$ mol/l) after L-NAME pretreatment ($10^{-4}$ mol/l, C, D, right panel).

**Fig. 3.** Concentration-dependent contraction in afferent arterioles of ET-1 transgenic mice (tg) and wild-type littermates (WT) due to cumulative Ang II application. Data are presented as absolute values (left panel) and as percent of the control diameter (right panel). The maximum contraction to Ang II is smaller in ET-1 transgenic mice compared with wild-type littermates (two-way ANOVA for group interaction, $P < 0.05$; (*). (*) indicates significance of the difference ($P < 0.05$).
transgenic mice (ratio 4 and 5, \( n = 350 \)) compared with WT (ratio 3 and 4, \( n = 350 \), Figure 6). The statistic test (Brandt-Snedecor) revealed significantly greater media-lumen-ratios in ET-1 tg mice (\( P < 0.05 \)). Media thickness was not changed in ET-1 tg mice.

**Kidney morphology and systemic blood pressure**

There were no significant differences with respect to glomerular matrix deposition interstitial matrix deposition between young ET-1 transgenic mice and wild-type controls (Table 1). Thus, both measures do not argue for the development of glomerulosclerosis and kidney fibrosis, respectively in ET-1 tg mice. Blood pressure likewise remained similar in ET-1 tg mice and controls (Table 1).

**Renin-angiotensin-system**

To ascertain whether the renin-angiotensin-system in ET-1 transgenic mice is chronically changed, the expression of renin (pro-renin) and Ang II-receptors was measured. Northern blot analysis of (pro)renin-mRNA showed slightly smaller values in ET-1 transgenic animals, which were not significantly different from the controls (Figure 7). Real-time-PCR of AT1 and AT2 also revealed similar expression of both receptor types in transgenic and control mice (Figure 8).

**Isoforms of the nitric oxide synthase in the kidney**

Real-time-PCR showed different expression of NOS-isoforms in the whole kidney. The strongest expression was observed for eNOS, followed by iNOS, and then nNOS (Figure 9). Chronic over-expression of ET-1 in the tg did not influence the expression of the NOS-isoforms significantly compared with wild-type controls in the whole kidney preparation.

**Discussion**

The present study shows a reduced Ang II sensitivity and a smaller constriction upon Ang II in Af of ET-1 transgenic mice. Inhibition of NOS abolishes the differences between ET-1 tg and WT. This points at an increased Ang II-induced NO release in Af of ET-1 tg, when taken into account the similar effect of L-NAME alone on basal diameter of Af in both groups. The enhanced Ang II induced influence of NO on preglomerular vessels of ET-1 tg mice might contribute to a normal arteriolar tone and kidney function in these animals.

The data show that NO is involved in the control of the basal tone in Af in ET-1 tg and controls. L-NAME, which inhibits all NOS, decreased luminal diameters in a concentration-dependent manner without significant differences between ET-1 tg and WT. The effect of L-NAME hints to a relevant NO production in the basal state, i.e. when the vessels are not under the
influence of external vasoactive substances. The data agree with the studies in isolated perfused Af in rabbits [12]. In contrast, the influence of NO on the basal tone in C57Bl/6 animals is low [5], which points to distinct strain differences in mice. In the present study, the animals had the genetic background of NMRI mice.

Af of ET-1 transgenic mice showed less Ang II sensitivity compared with the wild-type littermates. This low Ang II sensitivity is probably not due to reduced AT-receptor expression. Analysis of the mRNA-expression of AT1 and AT2 receptors in the kidney, which allows estimates of chronic influences on AT-receptor expression, did not show significant differences for AT1 and AT2 between ET-1 transgenic and WT animals. The data reveal that the mRNA-expression of AT1 compared to AT2 was similar in the kidney preparation. This does not agree with the majority of other studies in renal vessels [13]. One reason for the discrepancy could be the usage of the whole kidney preparation in the present study. The expression of AT1 and AT2 at the protein level has not been determined due to the non-specificity of available antibodies against both receptor types in mice. Further, there was no difference in the renal

Table 1. Relative kidney weight and morphometric data of 3–6 month-old ET-1 transgenic mice and corresponding controls

<table>
<thead>
<tr>
<th></th>
<th>3–6 month-old ET-1 tg mice</th>
<th>3–6 month-old littersmates</th>
</tr>
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<tbody>
<tr>
<td>KW/BW ratio (%)</td>
<td>7.2±0.2</td>
<td>6.9±0.1</td>
</tr>
<tr>
<td>Glomerular matrix (%)</td>
<td>37.0±6.9</td>
<td>39.0±4.9</td>
</tr>
<tr>
<td>Interstitial matrix score (%)</td>
<td>3.9±0.2</td>
<td>3.9±0.3</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>117.0±3.7</td>
<td>114.0±4.5</td>
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KW, kidney weight; BW, body weight. There were no significant differences with respect to vascular morphology, glomerular matrix deposition as well as interstitial matrix deposition between young ET-1 transgenic mice and wild-type controls (n = 6/group). Data are given as mean±SEM.
Fig. 9. Results of real-time polymerase chain reaction (PCR) used for determination of the expression of nitric oxide synthase (NOS) isoforms. eNOS, endothelial isoform, iNOS, inducible isoform, nNOS, neuronal isoform. The expression levels of eNOS, iNOS and nNOS mRNA in the whole kidney preparation of mice were normalized to β-actin using the ΔCt-method. There are no significant differences between ET-1 transgenic mice (tg) and wild-type controls (WT).

renin-mRNA-expression. The data suggests that the renin-angiotensin-system is not significantly changed in ET-1 transgenic mice at the mRNA level.

Remarkably, in the series of pretreatment with L-NAME, the Ang II sensitivity was drastically increased and did not differ between transgenic animals and controls. Therefore, a stronger influence of NO, which counteracts Ang II contraction is concluded in transgenic animals. The effect of L-NAME pretreatment on the contraction to Ang II is more pronounced than the effect of L-NAME on basal tone. Based on this finding, an Ang II-induced NO release must be assumed. This NO release is increased in ET-1 transgenic animals. Investigation in aortic segments of ET-1 transgenic mice supports our conclusion [4]. The authors described weaker contractions to ET-1 as well as stronger endothelium-dependent relaxation in transgenic mice [4]. Expressions of NOS-isoforms measured in the whole kidney preparation did not differ between ET-1 tg and wild-type controls at the mRNA level. This suggests that a higher expression of NOS-isoforms does not contribute to the effect of NO in ET-1 transgenic animals. However, we could not focus on renal microvessels in this analysis, because mRNA of non-structure proteins were often below the detection level in single and even in pooled, isolated afferent arterioles of mice in our hands. We cannot exclude that differences in the expression of NOS in the microvessels between ET-1 tg and controls exist. Although, the phenomenon of Ang II-induced NO release has been shown in other models [14,15], the mechanisms of Ang II-induced NO liberation in the microvasculature are not completely clear. A contraction-induced NO release [16] can be widely excluded, since L-NAME does not influence contraction of Af to norepinephrine [6]. The specific way of activation involves the angiotensin receptors. AT1 and AT2 are discussed for mediating Ang II effects on NOS [17–19]. Recent studies support the role of AT1 in the Ang II induced NO release in renal resistance vessels [20].

In a former study in ET-1 transgenic mice, the inducible NOS (iNOS) has been found to be upregulated, which is due to the pro-inflammatory action of ET-1. Immunohistochemistry showed increased staining in and around large intrarenal arteries [7]. The very first event in the cascade leading to renal damage in ET-1 tg mice is chronic sub-clinical ET-1 induced inflammation [7]. However, it is not clear, whether an upregulation of the iNOS is responsible for the observed greater influence of NO in the present study. The analysis of the mRNA-expression in the whole kidney preparation, as in the present study, did not show any differences in NOS-isoform expression, but does not exclude the possibility that arteriolar expression was different. The hypothesis has to be proven in the future.

Vascular remodelling has been described in the aorta and big renal arteries in 14 month old transgenic mice [1]. Our current data indicate that the small preglomerular resistance vessels are already affected in younger ages. Af of transgenic animals had increased media-lumen-ratios compared with controls in the same age without changes in the media thickness. These changes in arteriolar structure can influence contractions. It can be speculated that the increased media-lumen ratio facilitate the constriction, due to the reduced diameter of the Af. However, Af of transgenic mice showed weaker contractions to Ang II. This leads to the assumption that structural changes of Af in ET-1 tg mice are functionally compensated by a greater influence of NO in ET-1 transgenic mice. Our finding of vascular remodelling in transgenic animals was also recently observed in another model of ET-1 transgenic mice [3]. In the study of Shindo et al. [3], vascular casts of the renal cortex showed smaller diameter of Af and Ef, and uneven surfaces in 12-month-old mice. Taken together, ET-1 overexpression in transgenic mice induces vascular remodelling. However, the increased media-lumen-ratio did not result in a stronger constriction of Af in ET-1 tg. In contrast, the EC50 and the maximum constriction upon Ang II were smaller in ET-1 tg, which might be due to the increased influence of NO.

The analysis of the kidney morphology, i.e. glomerular matrix deposition and interstitial matrix deposition did not show differences between ET-1 tg and WT. Therefore, we can rule out that our findings of weaker constriction of Af of ET-1 tg upon Ang II were influenced by the progressive fibrotic remodelling process in the kidney, which leads to fibrosis in older (>9 months) ET-1 transgenic animals [1]. We have used 3–6-month-old mice.

One limitation of the study is the use of both heterozygous and homozygous ET-1 tg animals. This was due to the small number of resulting homozygous ET-1 transgenic mice in breeding. However, heterozygous mice were shown to exhibit a similar phenotype as compared with homozygous mice. No additional
Ang II sensitivity in ET-1 transgenic mice

Phenomena were detectable in homozygous transgenic animals [1]. We measured the concentration-response-curve of AII to cumulative L-NAME applications in homozygous and heterozygous ET-1 tg mice in the present study and found no differences in the response (data not shown). Although, we cannot completely exclude that the heterogeneity in the genotype could be responsible for some of the non-significant results in the present study.

In conclusion, using a method of micro-perfusion, we could show a reduced Ang II-sensitivity in Af. The data suggest that ET-1 over-expression in mice accompanies an increased influence of NO in afferent arterioles. An enhanced Ang II-induced NO release in pregglomerular vessels can counteract the constrictory action of ET-1 in transgenic mice. This may be a potent mechanism for compensating ET-1 effects on glomerular hemodynamics and could be important in view of the role of kidneys in the control of the systemic blood pressure.

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