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Effects of Combined Endothelin A Receptor and Renin-Angiotensin System Blockade on the Course of End-Organ Damage in 5/6 Nephrectomized Ren-2 Hypertensive Rats

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Key Words
5/6 nephrectomy · Endothelin receptor type A · AT_1 receptor blocker · End-organ damage · Hypertension

Abstract
Our previous studies in rats with ablation nephrectomy have shown similar cardiorenal protective effects of renin-angiotensin system (RAS)-dependent treatment (combination of angiotensin-converting enzyme inhibitor and angiotensin II receptor blocker) and RAS-independent treatment (combination of α- and β-adrenoreceptor antagonist and diuretics). Moreover, selective blockade of endothelin (ET) receptor type A (ET_A) improved survival rate and attenuated hypertension and organ damage in Ren-2 transgenic rats. Therefore, we were interested in whether ET_A receptor blockade could have additive effects to the classical blockade of the RAS. Transgenic rats underwent 5/6 renal ablation at the age of 2 months and were treated for 20 weeks with RAS blockers alone (angiotensin II receptor blocker – losartan, and angiotensin-converting enzyme inhibitor – trandolapril), ET_A receptor blocker alone (atrasentan) or with the combination of RAS and ET_A receptor blockade. RAS blockade normalized blood pressure and improved survival. It decreased cardiac hypertrophy and proteinuria as well as tissue angiotensin II and ET-1 levels. In contrast, ET_A receptor blockade only partially improved survival rate, reduced blood pressure, attenuated the development of cardiac hypertrophy and transiently reduced proteinuria. However, no additive cardio- and renoprotective effects of ET_A and RAS blockade were noted at the end of the study.

Introduction

It is well recognized that the renin-angiotensin (RAS) and endothelin (ET) systems are important vasoconstrictor systems in the pathogenesis of hypertension. It has also been demonstrated that concomitantly enhanced activities of the RAS and ET systems critically contribute to the development of hypertension-induced

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end-organ damage [1–4]. In addition, it is now well known that the detrimental effects of the ET system in the development of end-organ damage are mediated via the activation of ET receptors type A (ET_A) [4]. Treatment of hypertension is now generally recognized as a critical strategy to slow progression of chronic kidney disease (CKD) to end-stage renal disease (ESRD) [5–7]. Even though pharmacological blockade of the RAS using classical agents such as angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II (ANG II) receptor blockers (ARB) has significantly contributed to the prevention of progression of CKD, there still remains a significant group of patients with overt proteinuria and progression of CKD to ESRD. Therefore, there is an ongoing search for new pharmacological means of protection from progressive CKD [7–10].

The aforementioned role of the interaction of the RAS and ET systems in the development of end-organ damage serves as a rationale for the evaluation of the combined RAS and ET_A blockade on the progression of CKD and development of ESRD. However, conflicting results regarding the effects of combined RAS and ET_A receptor blockade as compared to isolated RAS blockade were obtained. Recent comprehensive studies performed by Benigni's group have, on one hand, shown that combined RAS and ET_A blockade offered greater renoprotection than isolated RAS inhibition in rats with advanced type 1 diabetes [11]. On the other hand, the combined RAS and ET_A blockade did not exhibit better renoprotective actions than isolated RAS blockade in a model of rats with type 2 diabetes [12]. Similarly, a protective effect of combined RAS and ET_A blockade as compared to monotherapy with ACEi on the progression of CKD was observed in a low-renin model of renal damage [13], but no additive renoprotection was found in a model of chronic transplant nephropathy [14]. Since we have found in our recent studies that selective ET_A receptor blockade in Ren-2 transgenic rats (TGR), a model of hypertension (produced by the insertion of a murine renin gene into its genome) [15] with inappropriately activated RAS especially at the kidney tissue level, had antihypertensive and cardio- and nephroprotective effects [16–19], we have assumed that this model would be suitable for a comparison of the effects of isolated RAS blockade with combined RAS and ET_A receptor blockade on the progression of CKD.

Therefore, in the present study we first evaluated whether selective ET_A receptor blockade had antihypertensive and cardio- and renoprotective effects in rats with ablation nephrectomy, a model of CKD, and, second, whether combined treatment with ETA receptor and RAS blockade may have an additional beneficial effect in this model of CKD.

Materials and Methods

The present study was performed in accordance with guidelines and practices established by the Institute for Clinical and Experimental Medicine Animal Care and Use Committee, which are in accordance with national law and European union policy (EEC Council Directive 86/609, OJL 358-1, December 1987). All animals used in the study were housed in facilities accredited by the Czech Association of Laboratory Animal Care.

Animals

Male heterozygous TGR [strain name TGR(mRen2)27] and their normotensive transgene-negative Hannover Sprague-Dawley (HanSD) rats as controls were housed at 25°C under a 12-hour light/dark cycle and had free access to chow (normal rat chow – 0.45% NaCl) and water. All animals used in this study were bred at the Department for Experimental Medicine of the Institute for Clinical and Experimental Medicine from stock animals supplied from Max Delbrück Center for Molecular Medicine of Berlin, Germany.

Experimental Design

Studies were started in 2-month-old male TGR and their transgene-negative controls (HanSD), in which 5/6 nephrectomy (NX) was carried out under anesthesia (tiletamine + zolazepam, Virbac SA, Carros Cedex, France, 8 mg/kg, and xylazine, Spofa, Czech Republic, 4 mg/kg intramuscularly), as described previously [20]. Animals were treated either with a combination of the ACEi trandolapril (Gopten, Abbott, Prague, Czech Republic, 6 mg/l in drinking water) and the ARB losartan (Lozap, Zentiva, Prague, Czech Republic, 100 mg/l in drinking water), or with the ET_A receptor blocker atrasentan (5 mg kg^{-1} day^{-1}, Abbott, USA in drinking water), or with the combination of ACEi + ARB + ET_A blocker for 20 weeks. The dose of atrasentan was adjusted weekly according to actual water intake. The doses of ET_A receptor antagonist, ACEi and ARB were chosen on the basis of previous studies that have demonstrated that this dose of atrasentan effectively blocked ET_A receptors and this combination of trandolapril and losartan elicited maximal inhibition of RAS [16, 20]. Systolic BP (SBP) was measured with automated tail cuff system (Hatteras Instruments, Cary, N.C., USA). In accordance with recommendations for BP measurements in experimental animals [21], this method is adequate for detecting intergroup differences in SBP over time, and therefore is optimal for long-term studies. This method is regularly used in our laboratory [16–20] and was previously validated; a close correlation was found between measurements by tail-plethysmography and direct BP measurements using indwelling catheter in conscious rats. It is important to emphasize that this method does not allow accurate measurements of diastolic BP and mean arterial pressure. To obtain all BP parameters in experimental animals, employment of a radiotelemetry system for direct BP measurements would be required; however, this approach is not suitable for long-term studies in large groups of 5/6 NX animals. We are aware that this is a drawback of

all studies employing the tail-cuff method for BP measurements. However, despite this limitation we have demonstrated in pilot experiments with the tail-cuff method that the direct BP measurement by radiotelemetry in 5/6 NX TGR provide similar results [20].

At the end of the experiment, animals were decapitated and plasma and tissue ANG II and tissue ET-1 levels were measured as described and validated previously [16–20, 22]. This approach was used because we previously showed that plasma and tissue ANG II concentrations in anesthetized animals are higher than those obtained from decapitated conscious rats, and that normotensive animals exhibit a greater increase in renin secretion in response to anesthesia and surgery than ANG II-induced hypertensive intrarenal renin-depleted animals [23]. In addition, this standardized approach allowed us to compare the present results with those obtained in our previous studies employing TGR rats; this helped to evaluate the role of the RAS and of ET in the pathophysiology of hypertension and hypertension-induced end-organ damage on the basis of our previous work [16–20, 22, 24]. Since previous studies have shown that ET-1 acts predominantly as a paracrine agent and that plasma ET-1 levels do not reflect the local activity of the ET system, we decided to use tissue ET-1 concentration as marker of the activity of the ET system; therefore, plasma ET-1 levels were not measured in the present study [3, 4, 16]. Body weight was assessed every week and at weeks 4, 12 and 20 after 5/6 NX or sham operation, the animals were placed in individual metabolic cages, and after appropriate habituation training, their 24-hour urine was collected for determination of daily proteinuria and urinary creatinine excretion, as described previously [20, 22]. Blood samples were taken and plasma creatinine concentrations were measured by the picric acid colorimetric method using a commercially available kit (Lachema, Brno, Czech Republic). Weights of kidney and heart were assessed at the end of experiment. The ratio of left ventricle weight to tibial length (LVW/TL) was used to evaluate the degree of cardiac hypertrophy.

The following experimental groups were investigated in the present study: (1) sham-operated HanSD rats + water (initial n = 20); (2) sham-operated TGR + water (initial n = 20); (3) 5/6 NX TGR + water (initial n = 80); (4) 5/6 NX TGR + ET_A blockade (initial n = 33); (5) 5/6 NX TGR + ACEi + ARB (initial n = 40); (6) 5/6 NX TGR + ACEi + ARB + ET_A blockade (initial n = 40).

Histological Examination

At the end of the experiments, the left kidney was quickly removed, fixed in 4% buffered formaldehyde, dehydrated and embedded. Paraffin sections were stained with hematoxylin/eosin and periodic acid-Schiff reaction and were examined using a Nikon Eclipse E 600 light microscope. Slides were evaluated in a blind fashion. As described previously [20], 100 glomeruli per section were randomly selected, and the degree of glomerular damage was evaluated using a semiquantitative scoring method: grade 0: normal glomeruli, grade 1: sclerotic area up to 25% or distinct adhesion present between capillary tuft and Bowman's capsule, grade 2: sclerotic area 25–50% glomeruli, grade 3: sclerotic area 50–75%, grade 4: sclerotic area 75–100%. The glomerulosclerosis index (GSI) was calculated using the following formula: $GSI = (1 \times n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4)/(n_1 + n_2 + n_3 + n_4)$ – where n_x is the number of glomeruli in each grade of glomerulosclerosis.

Renal cortical and tubulointerstitial injury was evaluated according to Nakano et al. [25] for inflammatory cell infiltration, tubular dilatation and/or atrophy, or interstitial fibrosis and was graded semiquantitatively using the following scale of lesions: grade 0, no abnormal findings; (1) mild (<25% of cortex); (2) moderate (25–50% of cortex); (3) severe (>50% of cortex). The lesions were assessed in at least 30 random and nonoverlapping fields in the renal cortex.

Morphometric evaluation of the glomerular volume was made in the same kidney sections that were examined for morphological changes, using the method validated by Lane et al. [26] and employed in our recent study [24] using Nikon NIS-Elements AR 3.1 morphometric program (Nikon, Tokyo, Japan). Briefly, this method consists of determination of mean glomerular profile area and calculation of mean glomerular volume from the following formula: glomerular volume = $area^{1.5} \times 1.38/1.01$, where 1.38 is β, the shape coefficient for a sphere, and 1.01 is the size distribution coefficient assuming a 10% coefficient of variation.

Statistical Analysis

Statistical analysis of data was performed using Graph-Pad Prism software (Graph Pad Software, San Diego, Calif., USA). ANOVA for repeated measurements, followed by Student-Newman-Keuls test, was performed for the BP analysis within the groups. Statistical comparisons of other results were made by Student's t test or one-way ANOVA. Unless noted, values are expressed as mean ± SEM and n represents the number of animals. A p value less than 0.05 was considered statistically significant.

Results

All sham-operated HanSD and TGR survived until the end of the experiment. As shown in figure 1a, untreated 5/6 NX TGR exhibited a final survival rate of 21% with the steepest fall in survival after the second month of renal ablation. In contrast, all three treatments, i.e. isolated ET_A blockade, ACEi + ARB and ACEi + ARB + ET_A blockade, substantially improved survival rates after 5/6 NX to 73, 90 and 95%, respectively. Until week 18 after 5/6 NX, all three regimes improved the survival rates to a similar level, but after week 18, the regimes that included blockade of the RAS were more efficient than isolated ET_A receptor blockade (fig. 1a).

As shown in figure 1b, SBP in sham-operated HanSD remained in the normotensive range throughout the course of the experiment. In contrast, sham-operated TGR had severe hypertension from the beginning of the study and 5/6 NX caused a further substantial increase in SBP from 190 ± 4 to 245 ± 4 mm Hg ($p < 0.05$). Isolated ET_A blockade prevented this worsening and SBP in this group remained at the same level as observed in sham-operated TGR. In contrast, treatment with ACEi + ARB or ACEi + ARB + ET_A blockade rapidly decreased SBP in

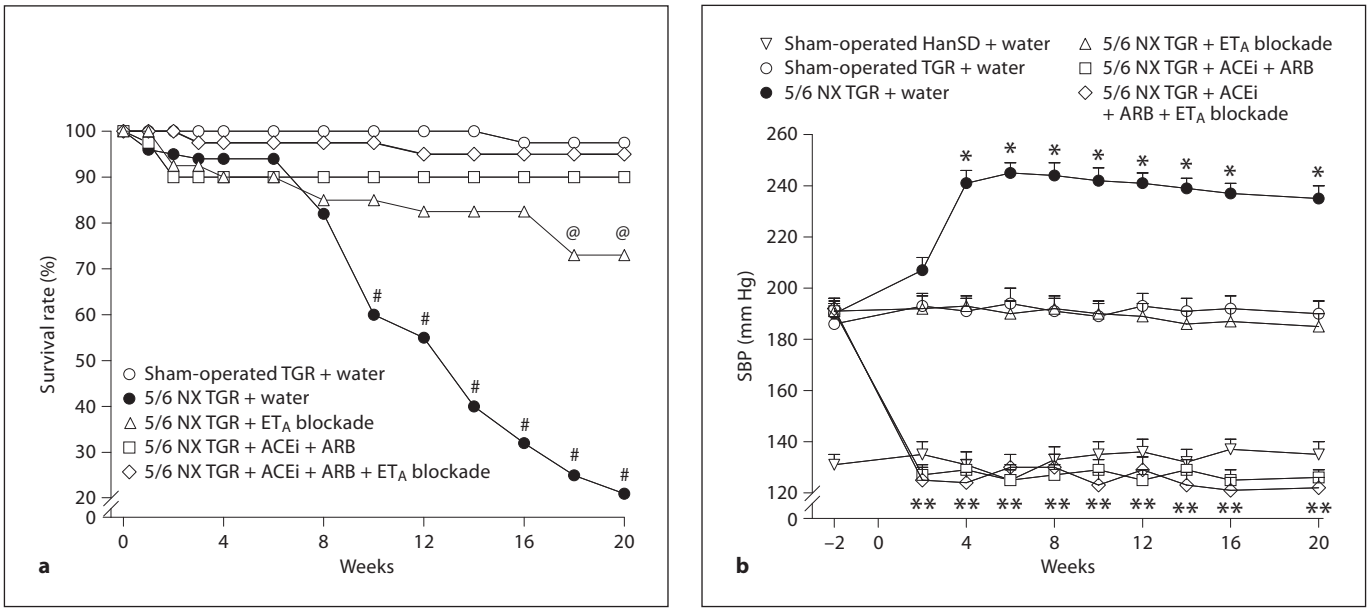

Fig. 1. Survival rates (**a**) and SBP (**b**) in sham-operated HanSD rats, in heterozygous Ren-2 TGR, and in 5/6 nephrectomized (5/6 NX) TGR, untreated (water) or receiving either a combination of trandolapril, an ACEi, and losartan, an ARB, or a combination of ACEi + ARB and ET_A blockade. * $p < 0.05$ compared with basal values. # $p < 0.05$ compared with treated groups at the same time point. @ $p < 0.05$ compared with groups receiving ACEi + ARB.

5/6 NX TGR to levels that were not significantly different from those in sham-operated HanSD rats.

Sham-operated TGR clearly showed cardiac hypertrophy (fig. 2a; evaluated as LVW/TL) when compared with sham-operated HanSD (28.1 ± 0.4 vs. 23.8 ± 0.3, $p < 0.05$). 5/6 NX induced a marked increase in LVW/TL when compared with sham-operated TGR (35.5 ± 1.3 vs. 28.1 ± 0.4, $p < 0.05$). Treatment with ET_A receptor blockade prevented this increase in LVW/TL after 5/6 NX. Both ACEi + ARB as well as ACEi + ARB + ET_A blockade not only prevented the development of cardiac hypertrophy in 5/6 NX TGR, but the LVW/TL ratio was even lower than observed in sham-operated HanSD (18.5 ± 0.3 and 19.3 vs. 23.8 ± 0.3, $p < 0.05$ in both cases).

As shown in figure 2b, sham-operated HanSD showed minimal proteinuria with a small rise throughout the experiment (from 11 ± 2 to 16 ± 3 mg/24 h), which was significantly lower than in sham-operated TGR (from 25 ± 2 to 34 ± 5 mg/24 h, $p < 0.05$). Untreated 5/6 NX TGR exhibited already at week 4 after 5/6 NX a dramatic increase in proteinuria as compared with sham-operated TGR (149 ± 8 vs. 25 ± 2 mg/24 h, $p < 0.05$) which progressively increased at week 20 after 5/6 NX to 245 ± 15 mg/24 h. Treatment with ET_A receptor blocker temporarily attenuated the increase in proteinuria at week 4 after 5/6 NX; subsequently, proteinuria reached similar values as observed in untreated 5/6 NX TGR. Remarkably, treatment with ACEi + ARB as well as with ACEi + ARB + ET_A blockade not only prevented the increase in proteinuria after 5/6 NX, but reduced it even to levels observed in sham-operated HanSD.

There were no significant differences in creatinine clearance between sham-operated HanSD rats and sham-operated TGR throughout the experimental period (fig. 2c), indicating that despite marked hypertension and associated proteinuria and renal glomerular damage (see below) at the age of 7 months sham-operated TGR did not exhibit a significant impairment of renal function. These findings are in accordance with previous studies showing that heterozygous TGR exhibit a high resistance to the development of hypertension-induced renal damage [17–20]. However, untreated 5/6 NX TGR exhibited already at week 4 after 5/6 NX a significantly lower creatinine clearance as compared with sham-operated TGR. All three treatment regimens prevented decreases in creatinine clearance in 5/6 NX TGR.

Plasma ANG II levels in sham-operated TGR were significantly higher than in sham-operated HanSD rats (12 ± 1 vs. 6 ± 1 fmol/ml, $p < 0.05$) and were not affected by 5/6 NX or by treatment with any of the regimes (fig. 3a).

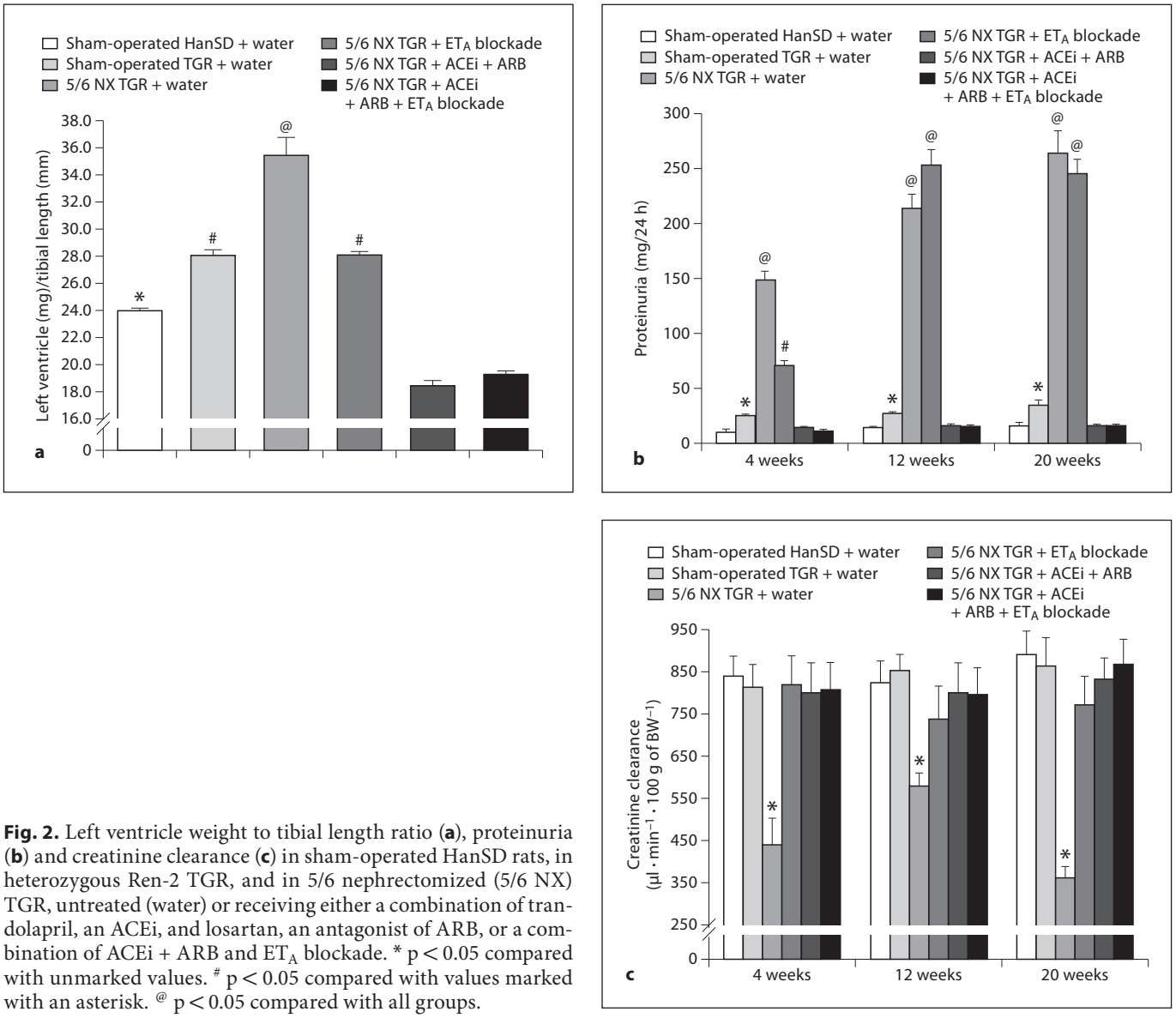

Fig. 2. Left ventricle weight to tibial length ratio (**a**), proteinuria (**b**) and creatinine clearance (**c**) in sham-operated HanSD rats, in heterozygous Ren-2 TGR, and in 5/6 nephrectomized (5/6 NX) TGR, untreated (water) or receiving either a combination of trandolapril, an ACEi, and losartan, an antagonist of ARB, or a combination of ACEi + ARB and ET_A blockade. * $p < 0.05$ compared with unmarked values. # $p < 0.05$ compared with values marked with an asterisk. @ $p < 0.05$ compared with all groups.

As shown in figure 3b, ANG II concentrations in kidney cortex were again significantly higher in sham-operated TGR than in sham-operated HanSD rats (90 ± 9 vs. 56 ± 8 fmol/g, $p < 0.05$). After 5/6 NX, the kidney cortex ANG II levels further increased (to 156 ± 36 fmol/g) and were not altered by the treatment with ET_A receptor blockade. In contrast, under both ACEi + ARB as well as ACEi + ARB + ET_A blockade therapies, 5/6 NX was followed by a decrease in kidney ANG II concentrations even below values observed in sham-operated HanSD rats (12 ± 3 and 13 ± 3 vs. 56 ± 8 fmol/g, respectively; $p < 0.05$ in both cases).

These findings are in accordance with our current knowledge that kidney tissue concentrations of ANG II are much higher than can be explained by concentrations delivered by arterial blood flow and that there is substantial evidence that the majority of intrarenal ANG II is generated locally in the kidney tissue [1]. Our findings that combined RAS blockade not only prevented the rise in kidney ANG II levels in 5/6 NX animals but also decreased ANG II below the values observed in sham-operated HanSD rats indicate that the remnant kidney, besides the ability to produce ANG II from endogenous intrarenal components by ACE-dependent pathway, has

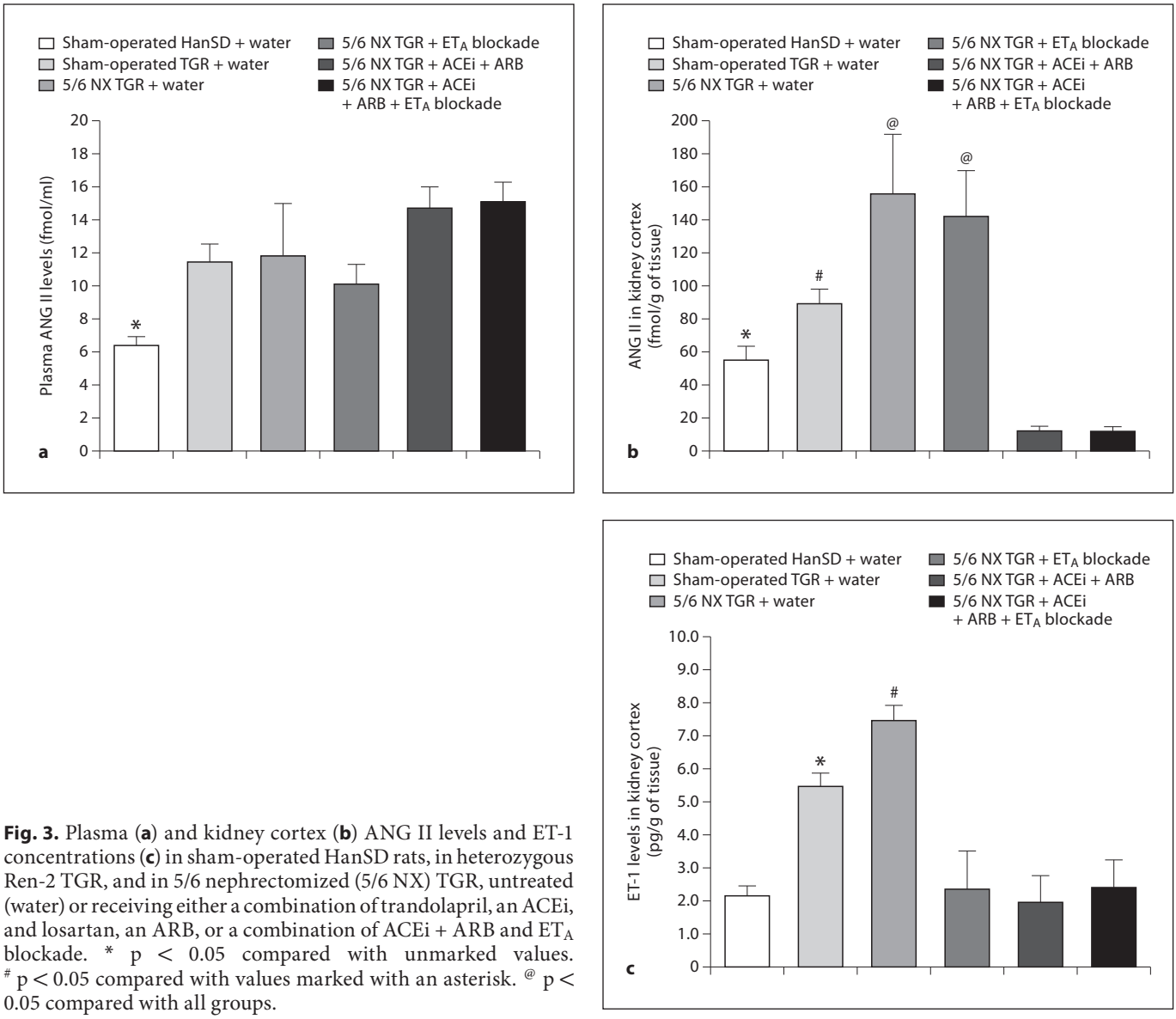

Fig. 3. Plasma (**a**) and kidney cortex (**b**) ANG II levels and ET-1 concentrations (**c**) in sham-operated HanSD rats, in heterozygous Ren-2 TGR, and in 5/6 nephrectomized (5/6 NX) TGR, untreated (water) or receiving either a combination of trandolapril, an ACEi, and losartan, an ARB, or a combination of ACEi + ARB and ET_A blockade. * $p < 0.05$ compared with unmarked values. # $p < 0.05$ compared with values marked with an asterisk. @ $p < 0.05$ compared with all groups.

also the capacity to uptake ANG II from the circulation by intrarenal ANG II receptors type 1 (AT_1). These findings are in good agreement with the results of our recent study in 5/6 NX TGR [20] and with previous studies aimed at evaluating the role of intrarenal RAS in the pathophysiology of hypertension and hypertension-associated end-organ damage [1, 16–19, 22, 24]. The finding that plasma ANG II levels in 5/6 NX animals treated by ACEi + ARB were not decreased is in good agreement with previous studies and is consistent with the concept that interruption of the short-loop negative feedback effect of AT_1 results in increased renin secretion and plasma ANG II levels which offset the effect of ACEi treatment on circulating ANG II concentrations [1, 2, 27].

ET-1 concentrations in kidney cortex (fig. 3c) were significantly higher in sham-operated TGR than in sham-operated HanSD (5.4 ± 0.4 vs. 2.2 ± 0.3 pg/g, $p < 0.05$; 5/6 NX resulted in a significant rise in ET-1 levels in kidney cortex to 7.5 ± 0.4 pg/g). All three treatment regimens markedly reduced ET-1 concentrations in 5/6 NX TGR to levels observed in sham-operated HanSD.

As shown in figure 4a, sham-operated HanSD exhibited a minimal degree of GSI that was significantly lower than in sham-operated TGR (0.02 ± 0.01 vs. 0.09 ± 0.01,

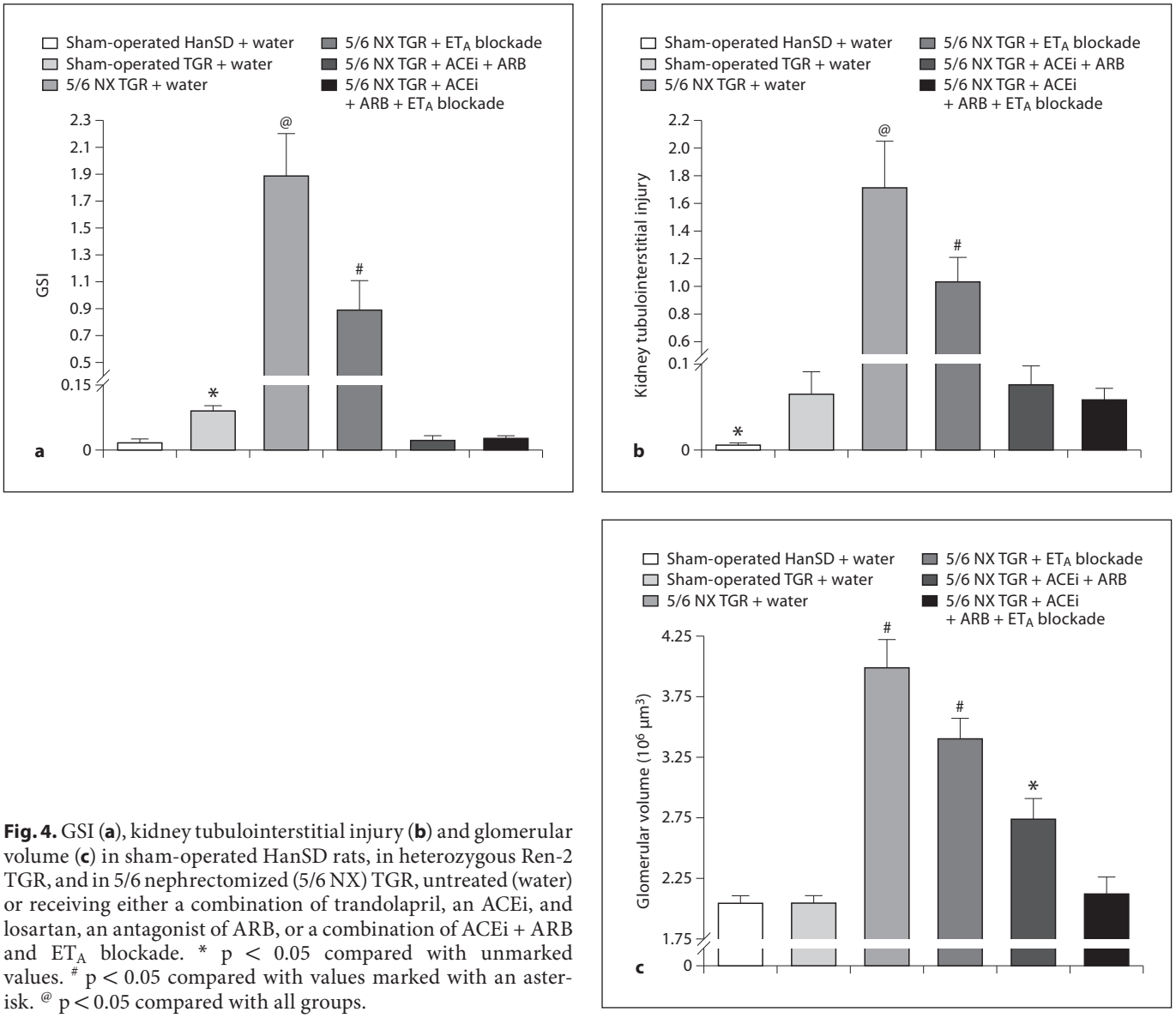

Fig. 4. GSI (**a**), kidney tubulointerstitial injury (**b**) and glomerular volume (**c**) in sham-operated HanSD rats, in heterozygous Ren-2 TGR, and in 5/6 nephrectomized (5/6 NX) TGR, untreated (water) or receiving either a combination of trandolapril, an ACEi, and losartan, an antagonist of ARB, or a combination of ACEi + ARB and ET_A blockade. * $p < 0.05$ compared with unmarked values. # $p < 0.05$ compared with values marked with an asterisk. @ $p < 0.05$ compared with all groups.

$p < 0.05$). 5/6 NX resulted in a dramatic increase in GSI. Treatment with the ET_A receptor blocker significantly attenuated the increase in GSI as compared with untreated 5/6 NX TGR (0.88 ± 0.22 vs. 1.89 ± 0.31, $p < 0.05$). Treatment with ACEi + ARB or with ACEi + ARB + ET_A blockade completely prevented the increase in GSI after 5/6 NX and even reduced it to levels observed in sham-operated HanSD.

Renal tubulointerstitial injury (fig. 4b) in sham-operated HanSD and TGR revealed the same pattern of distribution as observed with GSI, but in 5/6 NX treatment with ACEi + ARB or ACEi + ARB + ET_A blockade only reduced the degree of renal tubulointerstitial injury to values observed in TGR, and did not normalize it to levels found in sham-operated HanSD.

As shown in figure 4c, there was no significant difference between the glomerular volume in sham-operated HanSD and sham-operated TGR. 5/6 NX elicited a marked increase in the glomerular volume in TGR as compared with sham-operated TGR (3.99 ± 0.21 vs. $2.05 \pm 0.06 \times 10^6$ μm^3, $p < 0.05$). Treatment with the ET_A receptor blocker significantly reduced glomerular volume in 5/6 NX TGR. In addition, in 5/6 NX TGR treatment with ACEi + ARB was more efficient in reducing

glomerular volume than ET_A receptor blockade which, however, still remained significantly larger than that observed in sham-operated TGR (2.74 ± 0.16 vs. $2.05 \pm 0.06 \times 10^6$ μm^3, $p < 0.05$). In contrast, treatment with ACEi + ARB + ET_A blockade fully normalized glomerular volume after 5/6 NX to the levels observed in sham-operated TGR and sham-operated HanSD.

Discussion

To our knowledge, this is the first study that evaluates the role of the ET system in the progression of CKD and specifically tries to answer the question whether addition of the selective ET_A receptor blockade to the standard RAS blockade will exhibit additional beneficial effects on the progression of CKD in a model of hypertension with an inappropriately activated endogenous RAS. The issue whether combined RAS and ET_A receptor blockade offers better renoprotection than isolated RAS inhibition has been recently studied in experimental models of diabetes type 1 and type 2, but conflicting results were obtained [11, 12]. Since it is well recognized that the RAS plays an important role in the progression of CKD to ESRD [4, 6, 8–10], employing TGR appears to be an innovative and appropriate approach for evaluating whether the combined RAS and ET_A receptor blockade has additional renoprotective effects when compared with isolated RAS blockade.

The first new finding of our present study is that 5/6 NX in TGR elicited additional increases in intrarenal ET-1 concentrations to more than 3-fold higher levels than those in sham-operated normotensive HanSD rats. In addition, treatment with the ET_A receptor antagonist substantially improved the survival rate, prevented the increase in BP and the worsening of cardiac hypertrophy after 5/6 NX in TGR. Moreover, chronic ET_A receptor blockade prevented the decreases in creatinine clearance, temporarily attenuated the increase in proteinuria, ameliorated the degree of renal glomerular and tubulointerstitial injury and reduced glomerular volume after 5/6 NX in TGR. These organ-protective changes were associated with normalization of intrarenal ET-1 concentrations to levels observed in sham-operated HanSD. With regard to the only temporary antiproteinuric effect of ET_A receptor blockade, it is important to emphasize that chronic treatment with the ET_A receptor antagonist markedly improved survival rate as compared with untreated 5/6 NX TGR and, since we have observed that animals with the highest proteinuria and BP died first, one might consider that the increase in proteinuria in 5/6 NX TGR treated with the ET_A receptor antagonist during the experiment is a 'negative' consequence or side effect of the improved survival rate in these animals. Taken together, our present findings strongly support the notion that activation of the ET system and of the ET_A receptor-mediated augmentation of intrarenal ET-1 concentrations contributes to the rate of progression of CKD and development of ESRD and hypertension-associated end-organ damage in 5/6 NX TGR.

This view is in accordance with previous studies demonstrating renoprotective effects of treatment with an ET antagonist on the course of end-organ damage in models of diabetic nephropathy and in rats with renal mass reduction, a model of hypertensive nephropathy [11, 13]. In addition, our previous studies in homozygous TGR, a model of ANG II-dependent malignant form of hypertension, have also shown cardio- and renoprotective effects of ET blockade [16, 17]. It is important to mention that in our previous studies evaluating the effects of ET blockade on the development of hypertension-induced end-organ damage in either heterozygous TGR or homozygous TGR [16–19], animals were not exposed to the additional experimental manipulations such as surgical removal of renal cortical tissue. It is therefore conceivable that the combination of hypertension with enhanced systemic and tissue RAS activity with marked reduction of renal tissue is an extremely damaging combination that cannot be fully prevented by pharmacological blockade of the ET system alone. Collectively, our current data implicate an important role of the ET system in the pathophysiology of end-organ damage and provide the rationale for targeting the ET system in the treatment of CKD progression.

As a second major finding of the present study, we observed that RAS-dependent antihypertensive therapy applied in TGR subjected to 5/6 NX markedly improved the survival rate, normalized BP, prevented the development of proteinuria, cardiac hypertrophy and renal glomerular and tubulointerstitial injury. This was associated with suppression of enhanced intrarenal activity of the RAS even below the level observed in normotensive sham-operated HanSD and normalization of kidney ET-1 concentrations. These findings are in good agreement with results of our recent study showing that in this model of hypertension with increased intrarenal RAS activity the combined inhibition of RAS at more than one target site exhibits marked cardio- and renoprotection after 5/6 NX [20]. However, it is important to emphasize that despite all these marked cardio- and renoprotective effects, the

RAS blockade did not fully normalize survival rate and glomerular volume to values observed in sham-operated normotensive HanSD. It is conceivable that with the extended time after 5/6 NX the renoprotective effects of RAS blockade may become progressively less effective. This possibility requires the need of further search for a multi-drug strategy in the treatment of progression of CKD and prevention of ESRD.

The third major finding of our present study is that the addition of ET_A receptor blockade to the inhibition of the RAS, i.e. a combined RAS and ET_A receptor blockade, did not improve the survival rate and did not exhibit additive cardio- and renoprotective effects as compared with isolated RAS inhibition. Nevertheless, it is noteworthy to emphasize that the combined RAS and ET_A receptor blockade exhibited a tendency to greater survival rate in 5/6 NX TGR as compared with isolated RAS inhibition (95 vs. 90%), which, however, did not reach statistical significance.

We cannot offer a fully satisfactory explanation for this surprising lack of additional cardio- and renoprotective actions of the combined blockade of the RAS and ET system; however, some issues or aspects should be considered.

With regard to cardiac hypertrophy, recent studies have demonstrated that the severity of cardiac hypertrophy in ANG II-dependent models of hypertension is exclusively dependent on BP level [20, 28, 29]. Because the RAS-dependent antihypertensive therapy in our present study decreased SBP even below levels observed in sham-operated normotensive HanSD rats, the addition of the ET_A receptor antagonist could not exhibit further BP reduction and, therefore, additional cardioprotective effects cannot be expected.

Concerning the absence of renoprotective effects, it is now well recognized that the degree of proteinuria, development of kidney tubulointerstitial injury and glomerulosclerosis directly correlate with intrarenal ET-1 concentrations, which is an accepted marker of intrarenal ET system activity [10, 30, 31]. In addition, it is also well known that ANG II itself activates renal ET-1 production [32], and it has been demonstrated that in ANG II-dependent model of hypertension a direct correlation between intrarenal activity of RAS and ET systems exists [32, 33]. In line with this evidence, we observed that intrarenal ET-1 levels in 5/6 NX TGR treated with combination of ACEi and ARB were already lowered to levels observed in sham-operated normotensive HanSD rats. In view of this knowledge and our current data, it is reasonable to assume that the addition of ET_A receptor blockade to the combined RAS inhibition could not exhibit additional renoprotective actions in 5/6 NX TGR because the intrarenal activity of the ET system was already suppressed to the degree detected in normotensive control animals. Nevertheless, one beneficial effect of combined RAS and ET_A receptor blockade has been noticed and specifically, only this antihypertensive regime normalized glomerular volume in 5/6 NX TGR to values observed in sham-operated normotensive HanSD rats. It has been reported that the nephroprotection of antihypertensive therapy is also associated with a reduction of the size of the glomerulus [34, 35], an observation which we have recently confirmed in TGR [24]. One could assume that in the long-term prospect the combined RAS and ET_A receptor blockade as antihypertensive regime should exhibit further amelioration of end-organ damage within the kidney as compared with RAS-dependent antihypertensive therapy. Therefore, to address this issue, future studies are required with longer duration than the current one which then may lead to more conclusive results.

In summary, our present findings indicate, first, that the progression of CKD after renal mass reduction in hypertensive TGR is associated with marked intrarenal activation of the RAS and ET system. Second, treatment with an ET_A receptor antagonist improved survival rate and attenuated the progression of CKD and development of ESRD in 5/6 NX TGR, indicating that increased ET activity mediated through ET_A receptors plays an important role in the pathophysiology of progression of CKD after renal mass reduction. Third, the combined RAS and ET_A receptor blockade did not provide superior cardio- or renoprotective effects in 5/6 NX TGR as compared with RAS-dependent antihypertensive therapy, even though there was a tendency towards an improved survival rate.

All these findings should be considered in attempts to develop new therapeutic approaches for the treatment of progression of CKD and reduction of the incidence of ESRD.

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