Addition of ET\textsubscript{A} receptor blockade increases renoprotection provided by renin–angiotensin system blockade in 5/6 nephrectomized Ren-2 transgenic rats

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\textbf{A B S T R A C T}
Aims: There is evidence that in addition to hypertension and hyperactivity of the renin–angiotensin system (RAS), enhanced intrarenal activity of endothelin (ET) system contributes to the pathophysiology and progression of chronic kidney disease (CKD). This prompted us to examine if this progression would be alleviated by addition of type A ET receptor (ET\textsubscript{A}) blockade to the standard blockade of RAS.

Main methods: Ren-2 transgenic rats (TGR) after 5/6 renal ablation (5/6 NX) served as a model of CKD. For RAS inhibition a combination of angiotensin-converting enzyme inhibitor (trandolapril, 6 mg/L drinking water) and angiotensin II type 1 receptor blocker (losartan, 100 mg/L drinking water) was used. Alternatively, ET\textsubscript{A} receptor blocker (atrasentan, 5 mg kg\textsuperscript{-1} day\textsuperscript{-1} in drinking water) was added to the combined RAS blockade. The follow-up period was 44 weeks after 5/6 NX, and the rats' survival rate, systolic blood pressure (SBP), proteinuria and indices of renal glomerular damage were evaluated.

Key findings: The survival rate was at first improved, by either therapeutic regime, however, the efficiency of RAS blockade alone considerably decreased 36 weeks after 5/6 NX: final survival rate of 65% was significantly lower than 91% achieved with combined RAS and ET\textsubscript{A} receptor blockade. SBP was not affected by the addition of ET\textsubscript{A} blockade while proteinuria and renal glomerular damage were further reduced.

Significance: Our data show that a combined RAS and ET\textsubscript{A} receptor blockade exhibits additional beneficial effects on survival rate and the progression of CKD in 5/6 NX TGR, as compared with RAS inhibition alone.

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\textbf{Introduction}

Chronic kidney disease (CKD) is a growing medical problem of current nephrology, affecting millions of people worldwide (U.S. Renal Data System: USEDS, 2012). The natural course of progression of CKD toward end-stage renal disease (ESRD) is independent of the initial insult, and also the mechanisms underlying the progression of CKD to ESRD are the same (Brenner, 1985; Zoja et al., 2006; Hostetter, 2003). After diabetes mellitus, systemic hypertension is the second most common modifiable risk factor for the progression of CKD (Upadhyay et al., 2011; Wheeler and Becker, 2013; Mancia et al., 2007), and antihypertensive treatment is crucial among strategies used to slow down this progression i.e. provide “renoprotection” (Ptinopolou et al., 2013; Turner et al., 2012). In addition, inappropriately activated renin–angiotensin system (RAS) has a critical role in the progression of CKD to ESRD, and thus antihypertensive RAS blocking agents, such as angiotensin converting enzyme (ACE) inhibitors (ACEi) and angiotensin II (ANG II) receptor blockers (ARB), exhibit very pronounced renoprotective action (Ptinopolou et al., 2013; Turner et al., 2012; Rüster and Wolf, 2006; Macconi, 2010).

However, the effectiveness of renoprotective action of RAS inhibition is limited, especially in advanced CKD and therefore more complex pharmacologic strategies targeting also control systems other than RAS are needed (Perico et al., 1994; Gordon and Kopp, 2011). This is supported by findings that vasoconstrictor endothelin-1 (ET-1) activating type A ET receptors (ET\textsubscript{A}) contribute to the pathophysiology of certain forms of hypertension (Kohan et al., 2011; Vaněčková et al., 2005; Kang et al., 2009; Sasser et al., 2002). Interestingly, it has been shown...
that concomitant enhancement of RAS and ET systems activity critically contributes to the development of end-organ damage (Rossi et al., 1999; Cao et al., 2000; Vernérová et al., 2009; Kohan, 2010; Briet and Burns, 2012). In addition, we demonstrated recently that 5/6 renal mass reduction (5/6 NX), a model of CKD, resulted in a marked activation of intrarenal RAS and ET systems, and that ETA receptor blockade alone retarded the progression of CKD and development of ESRD in 5/6 NX Ren-2 transgenic rats (TGR) (Váněková et al., 2012), a model of ANG II-dependent hypertension with endogenous activation of RAS (Muñoz et al., 1990). This prompted us recently to examine whether the addition of the selective ETA receptor blockade to the standard RAS blockade will exhibit additional beneficial effects on the progression of CKD in 5/6 NX TGR (Kohan et al., 2011; Váněková et al., 2005; Kang et al., 2009; Sasser et al., 2002; Rossi et al., 1999; Cao et al., 2000; Vernérová et al., 2009; Kohan, 2010; Briet and Burns, 2012; Váněková et al., 2012). However, combined RAS and ETA receptor blockade did not exhibit greater renoprotection as compared with RAS blockade alone (Váněková et al., 2012). In earlier studies, the treatment with ETA receptor antagonist did not yield consistent results: some groups reported important renoprotection (Váněková et al., 2005; Kang et al., 2009; Cao et al., 2000; Vernérová et al., 2009; Brochuu et al., 1999; Benigni et al., 1993; Potter et al., 1997) while other workers found no significant effect of ETA receptor blockade on the course of end-organ damage after 5/6 NX (Pollock and Polakowski, 1997). Nevertheless, at the end of our earlier experiments with TGR rats (20 weeks after 5/6 NX) the survival rate tended to be higher after combined RAS and ETA receptor blockade compared with rats after RAS inhibition alone. Moreover, only the combined treatment did normalize the glomerular volume to control HanSD levels (Váněková et al., 2012). Noteworthy, there is evidence on strict correlation of glomerular size (reflecting growth) and the degree of glomerulosclerosis, which gave rise to the view that glomerular hypertrophy is the crucial process underlying progression of CKD (Yoshida et al., 1989a). Not surprisingly, renoprotective effects of antihypertensive therapies are associated with the reduction of glomerular size (Yoshida et al., 1989b). All these data made us postulate that in the very long-term perspective the combined RAS and ETA receptor blockade should exhibit better renoprotection compared with that obtained with RAS blockade alone. In the present study we tested this hypothesis in 5/6 NX TGR rats.

Materials and methods

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

Animals

Ren-2 transgenic rats (TGR) are a monogenetically defined form of hypertension, in which murine Ren-2 gene was inserted to the genome of Hannover Sprague Dawley (HanSD) rats. Male heterozygous TGR [strain name TGR(mRen2)27] and HanSD rats were housed at 25 °C under a 12 h light/dark cycle and had free access to normal rat chow, 0.45% NaCl content, and water. All animals used in this study were bred at the Department for Experimental Medicine, Institute for Clinical and Experimental Medicine, from stock animals supplied by Max Delbrück Center for Molecular Medicine, Berlin, Germany.

Therapeutic regimes

A combination of angiotensin converting enzyme inhibitor trandolapril (Coppen; Abbott, Prague, Czech Republic), 6 mg/L drinking water and of angiotensin receptor type II blocker losartan (Lozap; Zentiva, Prague, Czech Republic), 100 mg/L drinking water, was used (Váněková et al., 2012; Kujal et al., 2010). ETA receptor blockade was achieved with atrasentan (Abbott, Illinois, USA) 5 mg/kg·day in drinking water. The dose of atrasentan was adjusted weekly to actual water intake; such dosage was previously found to effectively block ETA receptors (Váněková et al., 2005; Váněková et al., 2012). The treatment either with RAS alone or with a combination of RAS and ETA blockade was started at the age of 9 weeks.

Experimental protocols

Series 1: effects of RAS blockade alone and combined RAS and ETA receptor blockade on survival rate and signs of end-organ damage

Male HanSD rats aged seven weeks and TGR derived from several litters, were randomly assigned to experimental groups. In order to detect inter-group differences in systolic blood pressure (SBP) over time, SBP was measured by tail-plethysmography, using a tail-cuff apparatus (MC 4000; Hatters Instruments Co. and KTB 1007; Kent Scientific Co.) (Kurtz et al., 2005). Three days before the starting measurements, rats were accustomed to the procedure of indirect tail-cuff SBP measurements. Measurements of SBP were started 14 days before 5/6 NX and performed at three-day intervals until the end of the experiment. On day 0 (age 9 weeks), 5/6 NX was performed under anesthesia (tiletamine + zolazepam, Ventac SA, Carros Cedeax, France, 8 mg/kg; and xylazine, Spofa, Czech Republic, 4 mg/kg intramuscularly), as described previously (Váněková et al., 2012; Kujal et al., 2010). After 24 hours' recovery, either appropriate treatment was initiated or rats were left with no treatment. The following experimental groups were investigated:

1. Sham-operated HanSD + water (initial n = 9)
2. Sham-operated TGR + water (initial n = 12)
3. 5/6 NX TGR + water (initial n = 22)
4. 5/6 NX TGR + RAS blockade (initial n = 20)
5. 5/6 NX TGR + RAS blockade + ETA blockade (initial n = 22)

The follow-up period was 44 weeks. At weeks 4, 8, 20, 30 and 40 after day 0, after appropriate habituation training, the animals were placed in individual metabolic cages and their 24-hour urine was collected for the determination of protein. This approach was previously validated and is regularly used in our studies (Váněková et al., 2012; Kujal et al., 2010). At the end of experiments, rats were decapitated (without anesthesia), and plasma and tissue ANG II levels were measured by radioimmunoassay. This approach was used because we have demonstrated recently that the measured ANG II levels are altered by anesthesia (Váněková et al., 2012; Kujal et al., 2010; Huskova et al., 2006; Červenka et al., 2008; Huskova et al., 2010; Honetschlagérova et al., 2013; Vahorokuvá et al., 2010). Tissue concentration of endothelin-1 (ET-1) in kidney cortex was measured as described in our previous studies (Váněková et al., 2005; Vahorokuvá et al., 2010).

The second half of kidney samples was used to assess renal glomerular damage. The kidneys were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. The sections stained with hematoxylin-eosin and PAS (periodic acid, for Schiff reaction) were examined and evaluated in a blind-test fashion. Fifty glomeruli in each kidney were examined on a semi-quantitative scale as described previously (Saito et al., 1987). grade 0, all glomeruli normal; grade 1, sclerotic area up to 25% (minimal sclerosis); grade 2, sclerotic area 25 to 50% (moderate sclerosis); grade 3, sclerotic area 50 to 75% (moderate-to-severe sclerosis); and grade 4, sclerotic area 75 to 100% (severe sclerosis). The glomerulosclerosis index (GSI) was calculated using the following formula: GSI = [(1 x n1) + (2 x n2) + (3 x n3) + (4 x n4)] / (n0 + n1 + n2 + n3 + n4), where n0 is the number of glomeruli in each grade of glomerulosclerosis.

Renal cortical tubulointerstitial injury was evaluated for inflammatory cell infiltration, tubular atrophy, and interstitial fibrosis, using...
At the same time point. \( p < 0.05 \) compared with other groups at the same time point.

\[ p < 0.05 \] compared with baseline values. \( # p < 0.05 \) compared with sham-operated TGR, using atrasentan (5 mg/kg \( \cdot \) day) drinking water. Alternatively, the above two-drug RAS blockade was combined with the blockade of endothelin type A receptor (ET\(_A\)) using atrazetan (5 mg kg\(^{-1}\) \cdot day\(^{-1}\)). Significant at \( p < 0.05 \) compared with baseline values. \# \( p < 0.05 \) compared with sham-operated TGR at the same time point.

**Series 2: determinations of ANG II and ET-1 concentrations in conscious animals in the early phase after 5/6 NX**

Animals in this series were divided into analogous experimental groups and were exposed to the same protocols as in series 1. The aim of this series was to evaluate the degree of activation of RAS and ET systems in the early phase of development of CKD after renal mass reduction and to assess the effects of therapeutic regimes on the activity of these systems. Four weeks after either 5/6 NX or sham-operation, rats from each experimental group (\( n = 8 \)) were decapitated and ANG II and ET-1 concentrations were measured as in series 1.

**Statistical analysis**

Statistical analysis of the data was performed using Graph-Pad Prism software (Graph Pad Software, San Diego, California, USA). ANOVA for repeated measurements, followed by Student–Newman–Keuls test, was performed for analysis of BP changes within the groups. Statistical comparison of other results was made by Student’s t-test or one-way ANOVA. Unless noted, values are expressed as mean \( \pm \) S.E.M. and \( n \) represents the number of animals. A p-value less than 0.05 was considered statistically significant.

**Results**

All the sham-operated HanSD rats survived until the end of experiment and sham-operated TGR exhibited 92% survival rate (one TGR unexpectedly died during the experiment). As shown in Fig. 1A, untreated 5/6 NX TGR began to die at weeks 9–10 after 5/6 NX, and by week 26 no animal survived. Both therapeutic regimes dramatically improved the survival rate, however the efficiency of RAS blockade alone considerably decreased beginning from 36 weeks after 5/6 NX, the final survival rate being 65%. In contrast, the combined RAS and ET\(_A\) receptor blockade remained effective and the final survival rate was 91%, which was significantly higher compared with RAS inhibition alone.

Sham-operated HanSD rats remained normotensive and sham-operated TGR were markedly hypertensive, without any significant alterations in SBP throughout the experiment (Fig. 1B). Starting from the initial SBP of 185 ± 5 mm Hg, 5/6 NX caused a further substantial increase in SBP, which reached the maximum at week 8 after 5/6 NX (236 ± 6 mm Hg, \( p < 0.05 \)). Both RAS inhibition alone and the combined RAS and ET\(_A\) receptor blockade not only prevented an increase in SBP in TGR after 5/6 NX, but also reduced SBP levels below those observed in sham-operated HanSD rats (112 ± 5 and 116 ± 4 mm Hg vs. 135 ± 4 mm Hg, \( p < 0.05 \)).

As shown in Fig. 2A, sham-operated HanSD rats showed minimal proteinuria throughout the experiment (at the end: 5.8 ± 1.2 mg/24 h). Sham-operated TGR exhibited pronounced proteinuria, more than 4-fold higher than that observed in sham-operated HanSD rats, throughout the experiment. Untreated 5/6 NX TGR revealed a dramatic increase in proteinuria reaching the maximum 8 weeks after 5/6 NX (184 ± 16 mg/24 h, \( p < 0.05 \) vs. all other corresponding values). RAS inhibition when applied alone prevented the increase in proteinuria that occurred after 5/6 NX in TGR, and till the week 20 after 5/6 NX the proteinuria was lower than in sham-operated TGR. However, after week 30 following 5/6 NX, proteinuria began to rise and at the end of experiment it was significantly higher than in sham-operated TGR (39.8 ± 3.6 vs. 25.9 ± 2.1 mg/24 h, \( p < 0.05 \)). Remarkably, the combined RAS and ET\(_A\) receptor blockade not only prevented the increases in proteinuria after 5/6 NX, but also reduced it below values observed in sham-operated TGR over the whole duration of experiment; until 30 weeks after 5/6 NX these levels were similar as observed in sham-operated HanSD rats.

Sham-operated HanSD rats showed a minimal degree of glomerulosclerosis and kidney tubulointerstitial injury (Figs. 2B and C) at the end of the study. These parameters were significantly increased in sham-operated TGR, 5/6 NX TGR under RAS blockade.
alone exhibited markedly increased GSI and kidney tubulointerstitial injury compared with sham-operated HanSD rats and sham-operated TGR. In contrast, combined RAS and ET\(_\alpha\) receptor blockade reduced GSI and kidney tubulointerstitial injury in 5/6 NX TGR to levels observed in sham-operated TGR. As shown in Fig. 2D, there was no significant difference between the glomerular volume in sham-operated HanSD rats and sham-operated TGR, 5/6 NX TGR under RAS blockade alone showed marked increases in the glomerular volume compared with sham-operated TGR (2.84 ± 0.09 vs. 1.49 ± 0.08 \(\times 10^6\) µm\(^3\), \(p < 0.05\)). The combined RAS and ET\(_\alpha\) receptor blockade significantly reduced the glomerular volume in 5/6 NX TGR. Representative figures of renal parenchyma are presented in Fig. 3 (A to D).

Sham-operated TGR clearly showed cardiac hypertrophy (evaluated as the LVW/TL ratio) (Fig. 4), both in the early and in the late phase (four and 44 weeks, respectively after either sham-operation or 5/6 NX) when compared with sham-operated HanSD rats 23.96 ± 0.45 vs. 16.72 ± 0.36 and 24.1 ± 0.59 vs. 17.1 ± 0.41, \(p < 0.05\) in both cases). Already after 4 weeks 5/6 NX induced a marked increase in LVW/TL as compared with sham-operated TGR (27.22 ± 0.56 vs. 23.96 ± 0.45, \(p < 0.05\)). The two-drug RAS inhibition alone as well as the combined RAS and ET\(_\alpha\) receptor blockade prevented the increases not only in the LVW ratio in 5/6 NX TGR but also in the early phase almost normalized and in the late phase restored the ratio to levels observed in sham-operated HanSD rats.

Plasma ANG II levels in sham-operated TGR were significantly higher than that in sham-operated HanSD, and in the early phase 5/6 NX caused a further increase to levels that were much higher compared with those in sham-operated TGR (68 ± 5 vs. 21 ± 3 fmol/mL, \(p < 0.05\)) (Fig. 5A). Both RAS inhibition alone and the combined RAS and ET\(_\alpha\) receptor blockade prevented the increases in plasma ANG II levels in 5/6 NX TGR. As shown in Fig. 5B, in the early phase after NX the total kidney ANG II concentrations exhibited a pattern of changes similar as that for plasma ANG II levels, but both treatment protocols were even able to lower kidney ANG II in 5/6 NX TGR to levels observed in sham-operated HanSD rats. As shown in Fig. 5C, in the late post-NX phase plasma ANG II levels in sham-operated TGR were still distinctly higher than that in sham-operated HanSD rats (25 ± 3 vs. 12 ± 3 fmol/mL, \(p < 0.05\)); in 5/6 NX TGR they were not affected by RAS inhibition alone or combined RAS and ET\(_\alpha\) receptor blockade. As shown in Fig. 4D, in late post-NX phase kidney ANG II was about 3-fold higher in sham-operated TGR than in sham-operated HanSD rats. Remarkably, the combined RAS and ET\(_\alpha\) receptor blockade in 5/6 NX TGR decreased intrarenal ANG II concentrations below values observed in sham-operated HanSD rats (19 ± 3 vs. 51 ± 6 fmol/g, \(p < 0.05\)). As shown in Fig. 6A, there were no significant differences in ET-1 concentrations in kidney cortex between sham-operated HanSD rats
and sham-operated TGR (0.42 ± 0.19 vs. 0.56 ± 0.21 pg/g). 5/6 NX resulted, in the early phase, in enormous (about 45-fold) rise in ET-1 levels in kidney cortex. Both RAS inhibition alone and combined RAS and ET<sub>A</sub> receptor blockade significantly and similarly attenuated increases in ET-1 concentrations in 5/6 NX TGR. As shown in Fig. 6B, there were no significant differences in ET-1 concentrations in kidney cortex among experimental groups in the late phase of the experiment.

Discussion

This study compared the effectiveness of two antihypertensive therapeutic regimes in TGR rats, a unique monogenetic model of RAS-dependent hypertension, with a particular interest for the effects of these regimes on the progression of CKD induced by 5/6 NX. The first and most important finding is that in the very long-term perspective, addition of ET<sub>A</sub> receptor blockade to the standard anti-RAS treatment, i.e. combined RAS and endothelin system inhibition brought additional beneficial effects compared with those obtained with RAS inhibition alone. The study is unique in that the follow-up period after 5/6 NX was about three times longer than 12-20 weeks usually employed (Zoja et al., 2006; Cao et al., 2000; Vaněčková et al., 2012; Brochu et al., 1999; Benigni et al., 1993; Potter et al., 1997; Pollock and Polakowski, 1997; Kujal et al., 2010; Fujihara et al., 2005), the feature which made it possible to come up to the above conclusion. In this context, it is important to note that in our previous study in 5/6 NX we have demonstrated that short-term (20 weeks) ET<sub>A</sub> blockade alone was not effective in improving renoprotection in TGR rats as compared to RAS blockade alone and moreover, no beneficial effects were demonstrated if ET<sub>A</sub> blockade was added to RAS blockade (Vaněčková et al., 2012). Since we were interested in the additional effects of ET<sub>A</sub> blockade administered concomitantly with RAS blockade, this particular group was not included in the current study. Moreover, our finding of enormous elevation of renal cortical ET-1 levels in the early phase after 5/6 NX is in accordance with earlier evidence that inappropriately activated ET system plays an important role in the pathophysiology of end-organ damage, and indicates an important role of ET system in the rate of progression of CKD to ESRD (Cao et al., 2000; Vernerová et al., 2009; Kohan, 2010; Briet and Burns, 2012; Benigni et al., 1993; Potter et al., 1997).

The second major finding of the present study is that in TGR the antihypertensive regime consisting of RAS inhibition alone markedly improved survival rate, normalized BP and cardiac hypertrophy, and lowered proteinuria until 36 weeks after 5/6 NX. Thereafter, BP clearly remained within normotensive range whereas the renoprotective effects became less evident: reduced survival rate, increased proteinuria, pronounced renal glomerular and cortical tubulointerstitial injury and augmented glomerular volume were detected. Not surprisingly, cardiac hypertrophy was reduced to a similar extent by both treatment regimens, as similar BP lowering effect was achieved by both treatments. This is in line with the evidence that left ventricular hypertrophy correlates well with the degree of hypertension and its reduction is therefore an important goal in the treatment of CKD patients. These findings indicate that efficiency of renoprotective effect of RAS blockade alone is not extended to the late stage of CKD, and further underscore the need for a search for new pharmacologic renoprotective strategies targeting systems other than RAS (Turner et al., 2012; Macconi, 2010; Perico et al., 1994; Gordon and Kopp, 2011).

The reasons for the failure of renoprotective actions of RAS inhibition alone in the advanced phase of CKD in 5/6 NX TGR are not clear. Our current data from untreated 5/6 NX TGR are consistent with the accepted view, that hypertension and increased intrarenal RAS activity are two critical determinants of the rate of progression of CKD and if they are controlled, renoprotection would be expected to be sustained (Zoja et al., 2006; Ptinopolou et al., 2013; Turner et al., 2012; Gordon and...
Fig. 4. Cardiac hypertrophy in the early phase (i.e. 4 weeks after 5/6 nephrectomy (5/6 NX)) (A) and late phase (i.e. 44 weeks after 5/6 NX) (B) in sham-operated Hannover Sprague-Dawley (HanSD, transgene-negative) rats and in heterozygous Ren-2 transgenic rats (TGR), and in 5/6 NX TGR rats, untreated (water) or receiving a combination of angiotensin-converting inhibitor and angiotensin II receptor blocker (RAS blockade), or combination of the RAS blockade and endothelin type A receptor (ET_A) antagonist (RAS blockade + ET_A blockade).

*p < 0.05 compared with unmarked values. #p < 0.05 compared with all groups.

In this context, of special interest are our results showing that 5/6 NX TGR under RAS inhibition exhibited 2-fold higher glomerular volume than that observed in sham-operated HanSD rats. One must consider here the evidence on strict positive correlation between glomerular size and the degree of glomerulosclerosis, a finding which is the basis of the so called "hypertrophy" theory which proposes that hypertrophy is the mechanism underlying the progression of CKD to ESRD (Yoshida et al., 1989a; Yoshida et al., 1989b). Therefore, it seems reasonable to assume that the lack of prevention of the harmful compensatory glomerular growth after renal mass reduction is responsible for the failure of renoprotection by RAS inhibition alone in the late phase after 5/6 NX in TGR.

What was the mechanism(s) responsible for the additional renoprotective effects of the combined RAS and ET_A receptor blockade as compared with RAS inhibition alone? Since both antihypertensive regimes brought BP to similar levels that were even lower than those seen in HanSD rats, the reason was not better control of hypertension by the complex therapy. Therefore, BP-independent renoprotective mechanisms must be considered, also in view of recent findings showing that activation of ET_A receptors and not increased BP per se, mediates intrarenal inflammatory cell infiltration and proliferation in ANG II-infused hypertensive mice (Boesen et al., 2011). Since ET-1 levels in kidney cortex were similarly lowered by both antihypertensive regimes, down to values observed in sham-operated HanSD rats, the difference in the intrarenal ET content cannot be the explanation, either.

Then, the difference in intrarenal RAS activity should be considered as a reason for better renoprotective actions of the combined RAS and ET_A receptor blockade. Indeed, we found that while RAS inhibition alone lowered whole kidney ANG II concentrations to values observed in sham-operated HanSD rats, the combined RAS and ET_A receptor blockade further significantly reduced these concentrations. Given the critical importance of the intrarenal ANG II augmentation in the
4 weeks after 5/6 NX

44 weeks after 5/6 NX

Fig. 5. Plasma and kidney angiotensin II (ANG II) levels in the early phase [i.e. 4 weeks after 5/6 nephrectomy (5/6 NX)] (A and B) and late phase (i.e. 44 weeks after 5/6 NX) (C and D) in sham-operated Hannover Sprague-Dawley (HanSD, transgene-negative) rats and in heterozygous Ren-2 transgenic rats (TGR), and in 5/6 NX TGR rats, untreated (water) or receiving a combination of angiotensin-converting inhibitor and angiotensin II receptor blocker (RAS blockade), or combination of the RAS blockade and endothelin type A receptor (ET\textsubscript{A} blocker) antagonist (RAS blockade + ET\textsubscript{A} blockade). *p < 0.05 compared with unmarked values. #p < 0.05 compared with all groups.

pathophysiology of end-organ damage in ANG II-dependent hypertension (Husková et al., 2010; Kobori et al., 2007), we hypothesize that this further suppression of intrarenal RAS activity was the main cause for improved renoprotective action of the combined RAS and ET\textsubscript{A} receptor blockade. If this was the case, what was the mechanism underlying further suppression of kidney ANG II after addition of ET\textsubscript{A} receptor blockade? Previous studies have demonstrated that enhanced intrarenal ANG II in ANG II-dependent models of hypertension as well as in the remnant kidney after 5/6 NX is a consequence of increased production of ANG II, which occurs mainly through the ACE-dependent pathway, and the ANG II type 1 (AT\textsubscript{1}) receptor-mediated uptake of ANG II from the circulation (Červenka et al., 2008; Husková et al., 2010; Kobori et al., 2007; Gonzalez-Villalobos et al., 2013). It is unlikely that selective ET\textsubscript{A} receptor blockade could alter the uptake of ANG II from the circulation. However, considering the well-known intrarenal interactions of ET and RAS systems at multiple levels (Kohan et al., 2011; Sasser et al., 2002; Rossi et al., 1999; Briet and Burns, 2012; Vaňourková et al., 2010; Barton et al., 1997; Yao et al., 2004), it is conceivable that antihypertensive therapy combining the blockade of ACE, AT\textsubscript{1} and ET\textsubscript{A} receptors could elicit further suppression of intrarenal ANG II synthesis. Mutual interactions of RAS and ET system should be considered in light of the late effects of ET\textsubscript{A} blockade on top of RAS blockade. Whether these beneficial effects could be also achieved if ET\textsubscript{A} blockade would start later than RAS blockade needs to be clarified in future experiments.

A second possible explanation is related to our finding that combined RAS and ET\textsubscript{A} receptor blockade substantially reduced glomerular volume in 5/6 NX TGR, almost to levels observed in sham-operated TGR. In the light of the aforementioned “hypertrophy” theory of the mechanism responsible for the progression of CKD (Yoshida et al., 1989a; Yoshida et al., 1989b; Ketteler et al., 1995), it is conceivable that a reduction of glomerular volume is another critical factor responsible for the improved renoprotection of combined RAS and ET\textsubscript{A} receptor blockade as compared with RAS inhibition alone. It is noteworthy that the “hypertrophy” theory assumes a central role for ANG II in the development of hypertension and kidney damage (Macconi, 2010; Ketteler et al., 1995).

Conclusion

Taking all these observations into consideration, we suggest that the mechanism(s) responsible for the additional renoprotective effects of the combined RAS and ET\textsubscript{A} receptor blockade compared with RAS inhibition alone, as demonstrated in 5/6 NX TGR, are related to the further suppression of intrarenal RAS activity and attenuation of the compensatory increase in the glomerular volume. Collectively, the
results show that in hypertensive TGR subjected to 5/6 NX the anti-hypertensive regime consisting of the combined RAS and ETA receptor blockade provides better long term renoprotection compared with RAS inhibition alone. These findings should be considered in attempts to develop new pharmacologic strategies aimed at slowing the progression of CKD to ESRD.

Conflicts of interest statement

None.

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V.C.Ch. and Z.V. contributed to the results of the present study equally and both should be considered as the first authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jclineph.2013.12.018.

References


of arterial hypertension of the European Society of Hypertension (ESH) and of the
Mullins JJ. Peters J, Gusten D. Fulminant hypertension in transgenic rats harbouring
Perico N, Arasuachategui SC, Colosimo V, Sonzogni G, Bertani T, Remuzzi G. Evidence that an
angiotensin-converting enzyme inhibitor has a different effect on glomerular injury
according to the different phase of the disease at which the treatment is started.
Pollock DM, Polakowski JS, ET, receptor blockade prevents hypertension associated with
exogenous endothelin-1 but not renal mass reduction in the rat. J Am Soc Nephrol
1997;8:1054-60.
Potter GS, Johnson RJ, Fink GD. Role of endothelin in hypertension of experimental chronic
Putnepolu AG, Pikilidou MI, Lutaridou N. The effect of antihypertensive drugs on chronic
Rossi GP, Sacchetto A, Cesari M, Pessina AC. Interactions between endothelin-1 and
Rüster C, Wolf G. Renin-angiotensin-aldosterone system and progression of renal
Saito T, Sumitohran E, Glasgow ET, Atkins RC. The enhancement of aminonucleoside
Sasser JM, Pollock JS, Pollock DM. Renal endothelin in chronic angiotensin II hypertension.
Turner JM, Bauer C, Abramowitz MK, Melamed ML, Hostetter TH. Treatment of chronic
and end-stage renal disease in the United States. Bethesda MD: National Institutes
Upadhayay A, Early A, Haynes SM, Uhlig K. Systemic review: blood pressure target
in chronic kidney disease and proteimuria as an effect modifier. Ann Intern Med
2011;154:541-8.
endothelin-A receptor blockade decreases blood pressure and ameliorates end-
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
Vaňourková Z, Kramer H, Husková Z, Červenka L, Vaněčková I. Despite similar
reduction of blood pressure and renal ANG II and ET-1 levels aliskiren but not
losartan normalizes albuminuria in hypertensive Ren-2 rats. Physiol Res
Vannelková Z, Kujal P, Kramer H, Backer A, Červenka L, Vaněčková I. End-organ damage in
hypertensive transgenic Ren-2 rats: influence of early and late endothelin receptor
Wheeler DC, Becker GJ. Summary of KDIGO guideline. What do we really know about
management of blood pressure in patients with chronic kidney disease? Kidney Int
2013;83:377-83.
endothelin-induced hypertension and oxidative stress in rats. Hypertens Res
Yoshida Y, Fogo A, Ichikawa I. Glomerular hemodynamic changes vs. Hypertrophy in
Yoshida Y, Kawamura T, Ikoma M, Fogo A, Ichikawa I. Effects of antihypertensive drugs on
Zoja C, Abbate M, Remuzzi G. Progression of chronic kidney disease: insight from animal