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Addition of ET_A receptor blockade increases renoprotection provided by renin–angiotensin system blockade in 5/6 nephrectomized Ren-2 transgenic rats

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ABSTRACT

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_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_A receptor blocker (atrasentan, 5 $mg \cdot kg^{-1} \cdot day^{-1}$ 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_A receptor blockade. SBP was not affected by the addition of ET_A blockade while proteinuria and renal glomerular damage were further reduced.
Significance: Our data show that a combined RAS and ET_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.

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_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

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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; Vernerová 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 ET_A receptor blockade alone retarded the progression of CKD and development of ESRD in 5/6 NX Ren-2 transgenic rats (TGR) (Vaněčková et al., 2012), a model of ANG II-dependent hypertension with endogenous activation of RAS (Mullins et al., 1990). This prompted us recently to examine whether the addition of the selective ET_A 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; Vaněčková et al., 2005; Kang et al., 2009; Sasser et al., 2002; Rossi et al., 1999; Cao et al., 2000; Vernerová et al., 2009; Kohan, 2010; Briet and Burns, 2012; Vaněčková et al., 2012). However, combined RAS and ET_A receptor blockade did not exhibit greater renoprotection as compared with RAS blockade alone (Vaněčková et al., 2012). In earlier studies, the treatment with ET_A receptor antagonist did not yield consistent results: some groups reported important renoprotection (Vaněčková et al., 2005; Kang et al., 2009; Cao et al., 2000; Vernerová et al., 2009; Brochu et al., 1999; Benigni et al., 1993; Potter et al., 1997) while other workers found no significant effect of ET_A 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 ET_A receptor blockade compared with rats after RAS inhibition alone. Moreover, only the combined treatment did normalize the glomerular volume to control HanSD levels (Vaněčková 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 ET_A 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 (Gopten; Abbot, 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 (Vaněčková et al., 2012; Kujal et al., 2010). ET_A receptor blockade was achieved with atrasentan (Abbott, Illinois, USA), 5 $mg \cdot kg^{-1} \cdot day^{-1}$ in drinking water. The dose of atrasentan was adjusted weekly to actual water intake; such dosage was previously found to effectively block ET_A receptors (Vaněčková et al., 2005; Vaněčková et al., 2012). The treatment either with RAS alone or with a combination of RAS and ET_A blockade was started at the age of 9 weeks.

Experimental protocols

Series 1: effects of RAS blockade alone and combined RAS and ET_A 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; Hatteras Instruments Co. and RTBP 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, Virbac SA, Carros Cedex, France, 8 mg/kg; and xylasine, Spofa, Czech Republic, 4 mg/kg intramuscularly), as described previously (Vaněčková 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 + ET_A 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 (Vaněčková 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 (Vaněčková et al., 2012; Kujal et al., 2010; Huskova et al., 2006; Červenka et al., 2008; Husková et al., 2010; Honetschlagerová et al., 2013; Vaňourková et al., 2010). Tissue concentration of endothelin-1 (ET-1) in kidney cortex was measured as described in our previous studies (Vaněčková et al., 2005; Vaňourková 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: $GSI = [(1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)]/ (n_0 + n_1 + n_2 + n_3 + n_4)$, where n_x 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

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