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Chronic Endothelin Receptor Blockade Reduces End-Organ Damage Independently of Blood Pressure Effects in Salt-Loaded Heterozygous Ren-2 Transgenic Rats

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Summary

The present study was performed to evaluate the role of an interaction between the endothelin (ET) and the renin-angiotensin systems (RAS) in the development and maintenance of hypertension and in hypertension-associated end-organ damage in heterozygous male and female transgenic rats harboring the mouse Ren-2 renin gene (TGR). Twenty-eight days old heterozygous TGR and age-matched transgene-negative normotensive Hannover Sprague-Dawley rats (HanSD) were randomly assigned to groups with normal-salt (NS) or high-salt (HS) intake. Nonselective ET_A/ET_B receptor blockade was achieved with bosentan (100 $mg.kg^{-1}.day^{-1}$). All male and female HanSD as well as heterozygous TGR on NS exhibited 100 % survival rate until 180 days of age (end of experiment). HS diet in heterozygous TGR induced a transition from benign to malignant phase hypertension. The survival rates in male and in female heterozygous TGR on the HS diet were 46 % and 80 %, respectively, and were significantly improved by administration of bosentan to 76 % and 97 %, respectively. Treatment with bosentan did not influence either the course of hypertension (measured by plethysmography in conscious animals) or the final levels of blood pressure (measured by a direct method in anesthetized rats) in any of the experimental groups of HanSD or TGR. Administration of bosentan in heterozygous TGR fed the HS diet markedly reduced proteinuria, glomerulosclerosis and attenuated the development of cardiac hypertrophy compared with untreated TGR. Our data show that the ET receptor blockade markedly improves the survival rate and ameliorates end-organ damage in heterozygous TGR exposed to HS diet. These findings indicate that the interaction between the RAS and ET systems plays an important role in the development of hypertension-associated end-organ damage in TGR exposed to salt-loading.

Key words
Hypertension • Endothelin system • Renin-angiotensin system • Bosentan • End-organ damage

Introduction

Growing body of evidence indicates that the endothelin (ET) system plays an important role in the pathogenesis of hypertension and associated end-organ damage in angiotensin II (ANG II)-dependent models induced by exogenously administrated ANG II, but not in the models with enhanced endogenous production of ANG II (d´Uscio *et al.* 1997, Ehmke *et al.* 1999, Müller *et al.* 2000, Alexander *et al.* 2001, Ficai *et al.* 2001, Müller *et al.* 2002, Sasser *et al.* 2002, Saam *et al.* 2003, for review see Moreau and Schiffrin 2003). The reasons for this discrepancy regarding the role of ET system in the ANG II-dependent model of hypertension remain unclear.

To determine the contribution of an interaction between endothelin-1 (ET-1) and ANG II to the development of hypertension and related end-organ damage in a model of hypertension dependent on the endogenous activation of the renin-angiotensin system (RAS), we utilized the rat strain transgenic for mouse Ren-2 renin gene [TGR; strain name TGR (mRen2)27]. The development of hypertension in this strain is a result of insertion of the mouse Ren-2 renin gene into the rat genome (Mullins *et al.* 1990). Thus, TGR represents a model of hypertension with a well-defined genetic background, in which the development of hypertension can be attributed to a single gene alteration and is clearly ANG II-dependent (for review see Langheinrich *et al.* 1996). Being characterized by normal plasma ANG II and by overexpression of the Ren-2 transgene in the adrenal cortex and in the arterial wall, the TGR seems to be an optimal model of ANG II-dependent hypertension with endogenous activation of the RAS (Hilgers *et al.* 1992, Peters *et al.* 1993, Jacinto *et al.* 1999, for review see Langheinrich *et al.* 1996).

However, only limited and conflicting data regarding the role of the ET system in TGR are available at present. On one hand, it has been reported that ET blockade resulted in hypotensive effects in male and female heterozygous TGR pointing to an important ET-dependent component in this model of hypertension (Gardiner *et al.* 1995, 2000, Kelly *et al.* 2000). In addition, we have recently showed that nonselective ET_A/ET_B receptor blockade markedly improves the survival rate and ameliorates end-organ damage in homozygous male TGR without a significant lowering of blood pressure (BP) (Dvořák *et al.* 2002a,b). On the other hand, it has been demonstrated that ET receptor blockade did not lower BP and did not prevent end-organ damage in heterozygous male TGR despite increased preproendothelin mRNA expression and ET-1 concentration in renal and cardiac tissues suggesting that an ET-dependent component is negligible in this model of hypertension (Whitworth *et al.* 1995, Andreis *et al.* 2000, Rossi *et al.* 2000, Seccia *et al.* 2003). Because the variance of these findings may have resulted from different time protocols and from a different ET blocker used, the rationale of the present study was to elucidate the role of ET system in the development of hypertension in heterozygous TGR under precisely defined experimental conditions (both sexes and the long-term follow-up).

Accordingly, the first aim of the present study was to examine the effects of nonselective ET_A/ET_B receptor blockade by bosentan on the course of hypertension in heterozygous male TGR. Since it has been reported that TGR also show a salt-sensitive component of hypertension (Callahan *et al.* 1996), and we have demonstrated that high-salt diet augmented the onset of the malignant phase of hypertension in homozygous male TGR (Dvořák *et al.* 2002a,b), the second aim of this study was to delineate whether a high-salt diet would accelerate the course of hypertension and end-organ damage in heterozygous TGR. Finally, since an emerging body of evidence indicates that TGR exhibit a marked sexual dimorphism with respect to the course of hypertension (Lee *et al.* 1996, Cargnelli *et al.* 1998) and that the ET system also reveals noticeable gender differences in the intrarenal activity (Taylor *et al.* 2003), the third aim of the present study was to compare the effects of salt intake and pharmacological manipulation on the cardiovascular phenotypes in heterozygous male and female TGR.

Methods

Protocols in the present study were designed according to the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society and were approved by the Czech Animal Care and Use Committee (protocol 79#2001).

Animals

All animals used in the present study were bred at the Center for Experimental Cardiovascular Research of the Institute for Clinical and Experimental Medicine from stock animals supplied from the Max Delbrück

Center for Molecular Medicine in Berlin, Germany. Animals were kept on a 12-hour/12-hour light/dark cycle.

Diets

All diets used in the present study were produced by SEMED (Prague, Czech Republic). Rats were fed either a normal-salt diet (NS, 0.45 % NaCl, 19-21 % protein) or a high-salt diet (HS, 2 % NaCl, 19-21 % protein).

Experimental design and functional examination

Twenty-eight days old heterozygous male and female TGR and age-matched transgene-negative Hannover Sprague-Dawley (HanSD) male and female rats from several litters were randomly assigned to experimental groups with care that animals from a single litter did not prevail in any of the groups. Food intake was monitored once a week and the concentration of bosentan (Actelion, Switzerland) was added to the NS and HS diets in a concentration depending on the food intake so that the final consumption of bosentan was 100 $mg.kg^{-1}$ of body weight per day. For this purpose, new diets were prepared every week. The experimental groups with initial number of animals are shown in Table 1.

Table 1. Mean arterial pressure, heart and kidney weights, and glomerulosclerosis index (GI) in experimental groups at 180 days of age.

Group	n	MAP (mm Hg)	HW/BW (mg/g)	KW/BW (mg/g)	GI
Male HanSD + NS	13	117±4	3.12±0.19	3.29±0.17	0.33±0.21
Male HanSD + NS + Bosentan	14	119±4	3.11±0.14	3.33±0.14	0.43±0.20
Male HanSD + HS	14	116±5	3.08±0.11	3.22±0.15	0.21±0.20
Male HanSD + HS + Bosentan	14	121±6	3.09±0.10	3.31±0.16	0.40±0.25
Female HanSD + NS	16	109±6	3.12±0.14	3.27±0.19	0.28±0.19
Female HanSD + NS + Bosentan	15	112±6	3.14±0.17	3.22±0.12	0.33±0.24
Female HanSD + HS	14	113±5	3.11±0.09	3.41±0.20	0.41±0.22
Female HanSD + HS + Bosentan	15	114±4	3.08±0.12	3.21±0.17	0.37±0.26
Male TGR + NS	24	199±7*#	3.82±0.27*	3.36±0.19	1.44±0.18*
Male TGR + NS + Bosentan	26	192±6*#	3.84±0.22*	3.23±0.21	1.68±0.13*
Male TGR + HS	22	191±9*#	4.49±0.28*+	3.29±0.19	2.24±0.12*+
Male TGR + HS + Bosentan	25	193±8*#	3.78±0.25*	3.35±0.18	1.60±0.16*
Female TGR + NS	32	158±6*	3.68±0.19*	3.32±0.16	1.51±0.15*
Female TGR + NS + Bosentan	24	159±5*	3.63±0.21*	3.31±0.21	1.43±0.16*
Female TGR + HS	35	157±8*	4.27±0.23*+	3.20±0.13	2.33±0.14*+
Female TGR + HS + Bosentan	34	158±9*	3.67±0.20*	3.25±0.19	1.39±0.13*

HanSD, transgene-negative rats; TGR, heterozygous transgenic rats (mRen2)27; NS, normal salt diet; HS, high salt diet; MAP, mean arterial pressure; HW, heart weight; BW, body weight; KW, kidney weight. * $P<0.05$ vs. HanSD rats; # $P<0.05$ male TGR vs. female TGR; + $P<0.05$ untreated TGR fed HS vs. TGR fed NS or HS and treated with bosentan

Systolic blood pressure (SBP) was measured in conscious animals by tail plethysmography from 29 to 40 days of age every two days, from 41 to 60 days of age every three days and thereafter every week until the end of the experiment (180 days of age). At each measurement, BP was determined as the mean of four measurements. This method was previously validated in our laboratory by Heller and Hellerová (1998). At 40, 80, 120 and 170 days of age, rats were placed into individual metabolic cages and their 24-hour urine was collected (after at least 3 days of training period) for protein determination and calculation of clearance of endogenous creatinine (a blood sample for determination of plasma concentration of creatinine and electrolytes was taken on the second day in the morning). Body weights were obtained during SBP measurements and from day 60 until

the end of the experiment rats were weighed additionally twice a week. At the end of experiments, animals were anesthetized with thiopental sodium (50 mg.kg^{-1} of body weight), and the right carotid artery was cannulated with PE-50 tubing for direct measurement of mean arterial pressure (MAP).

Morphological examination

The ratios of heart weight/body weight (mg/g) and right kidney weight/body weight (mg/g) were used as indices of cardiac hypertrophy (HW/BW) and kidney hypertrophy (KW/BW), respectively. The left kidney was quickly removed, fixed in 4 % formaldehyde, dehydrated and embedded. Paraffin sections were stained with hematoxylin-eosin and periodic acid-Schiff reaction (PAS). Slides were evaluated in a blind fashion. Fifty glomeruli in each kidney were examined on a semi-quantitative scale as described previously (Yagil *et al.* 2002): *grade 0*, all glomeruli normal; *grade 1*, one to two glomeruli were affected; *grade 2*, more than 2 but less than 17 glomeruli were affected; *grade 3*, 17 or more glomeruli were affected.

Tissue ET-1 evaluation

In separate groups of HanSD and heterozygous TGR fed either NS or HS diet (n = 8 in each group) from 28 to 80 days, ET-1 concentrations were examined in kidney cortex and left ventricle. The purpose of this protocol was to evaluate whether tissue ET-1 levels are increased before transition to the malignant phase of hypertension in this model. This time protocol was chosen because at this time point (80 days of age) each group still exhibited 100 % survival rate. ET-1 levels were assessed by the ELISA system from Amersham (Braunschweig, Germany) as described and validated previously (Bäcker *et al.* 2001).

Statistical analysis

All values are expressed as mean ± S.E.M. Two-way repeated-measures ANOVA were used to detect differences within each experimental group. For comparison between heterozygous TGR and HanSD rats, repeated-measures ANOVA was used with a test of interaction to determine whether the average change after experimental manipulation (diet manipulation and pharmacological treatment) was different between TGR and HanSD rats. One-way ANOVA was used for the evaluation of heart and kidney weights, ET-1 concentrations and glomerulosclerosis data. Statistical significance was defined as $p<0.05$.

Results

Survival rate

All male and female HanSD fed either the NS or HS diet with or without bosentan treatment survived until the end of the experiments (180 days of age). Likewise all heterozygous male and female TGR fed NS diet with or without bosentan administration exhibited 100 % survival rate. In contrast, only 46 % (10 of 22 rats) heterozygous male TGR exposed to HS intake survived until the end of the experiment (Fig. 1A). The administration of bosentan significantly increased the survival rate in this group to 76 % (19 of 25 rats). Likewise, HS diet in heterozygous female TGR resulted in the survival rate of 80 % (28 of 35 rats) which was significantly higher than that of heterozygous male TGR ($p<0.05$). This was improved by treatment with bosentan to 97 % (33 of 34 rats) ($p<0.05$) (Fig. 1B).

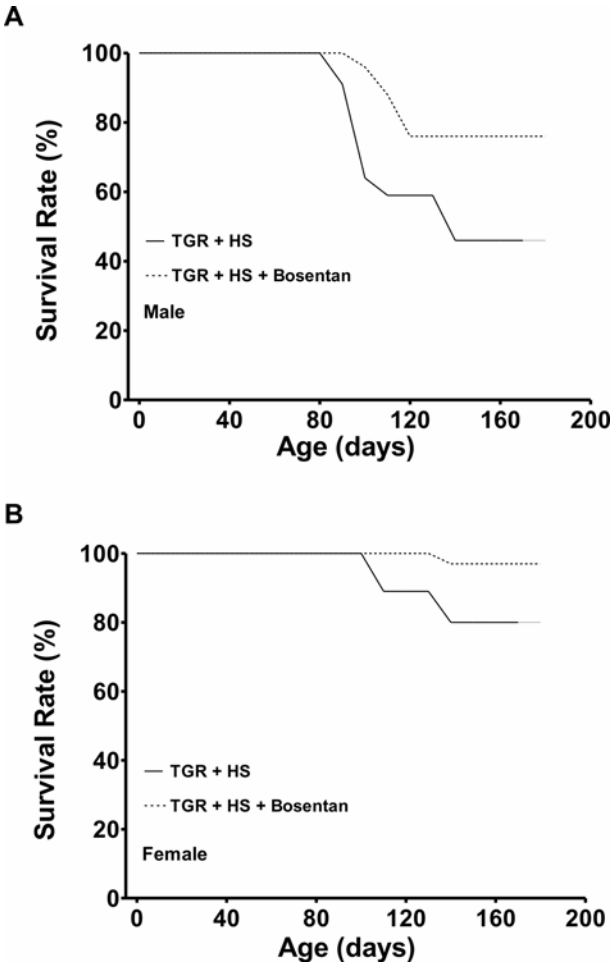

Fig. 1. Survival rate of male (A) and female (B) heterozygous Ren-2 transgenic rats (TGR) fed high salt (HS) diet.

Blood pressure

SBP determined by tail plethysmography in conscious male and female HanSD on NS diet remained within the normotensive range throughout the whole experiment and the final SBP was not different between male and female rats (138±5 and 128±6 mm Hg, respectively). HS diet did not significantly increase SBP at any time point throughout the experiment either in male or female HanSD and the final SBP was not significantly higher compared with animals fed the NS diet (139±6 vs. 138±5 in males and 137±5 vs. 128±6 mm Hg in females, respectively). Treatment with bosentan did not significantly alter SBP either in male or female HanSD fed HS or NS diet (data not shown). There were also no significant differences in MAP measured intraarterially in anesthetized rats on day 180 (Table 1).

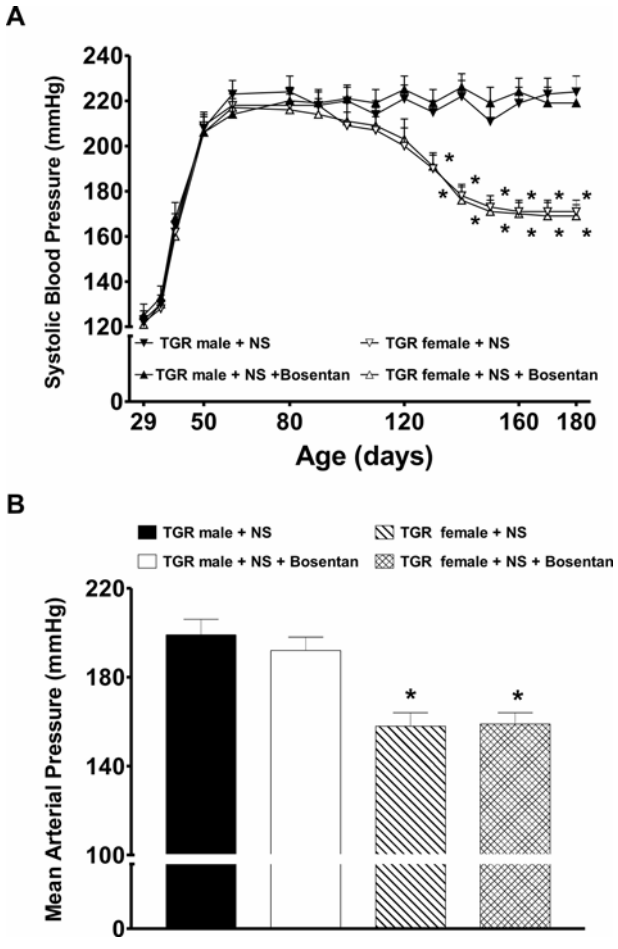

Fig. 2. Changes in systolic blood pressure in male and female heterozygous Ren-2 transgenic rats (TGR) fed normal (NS) diet (**A**). Mean arterial pressure measured at the end of experiment (180 days of age) (**B**).

As shown in Figure 2A, both heterozygous male and female TGR fed the NS diet developed hypertension at day 40 of age and reached the peak of BP at day 65 of age. In heterozygous male TGR fed the NS diet, SBP was stable until the end of the experiment. In contrast, in heterozygous female TGR fed the NS diet, SBP after reaching its peak (between 65 to 80 days of age), started to decrease spontaneously (at the age of 130 days) and the final SBP was significantly lower compared with male heterozygous TGR fed the NS diet. Treatment with bosentan did not alter this pattern of SBP in heterozygous male or female TGR fed the NS diet. Heterozygous female TGR fed the NS diet either untreated or treated with bosentan, had significantly lower MAP at the end of experiment than heterozygous male TGR fed the NS diet without or with bosentan (158±6 vs. 199±7 and 159±5 vs. 192±6 mm Hg, respectively, $p<0.05$ in both cases) (Fig. 2B).

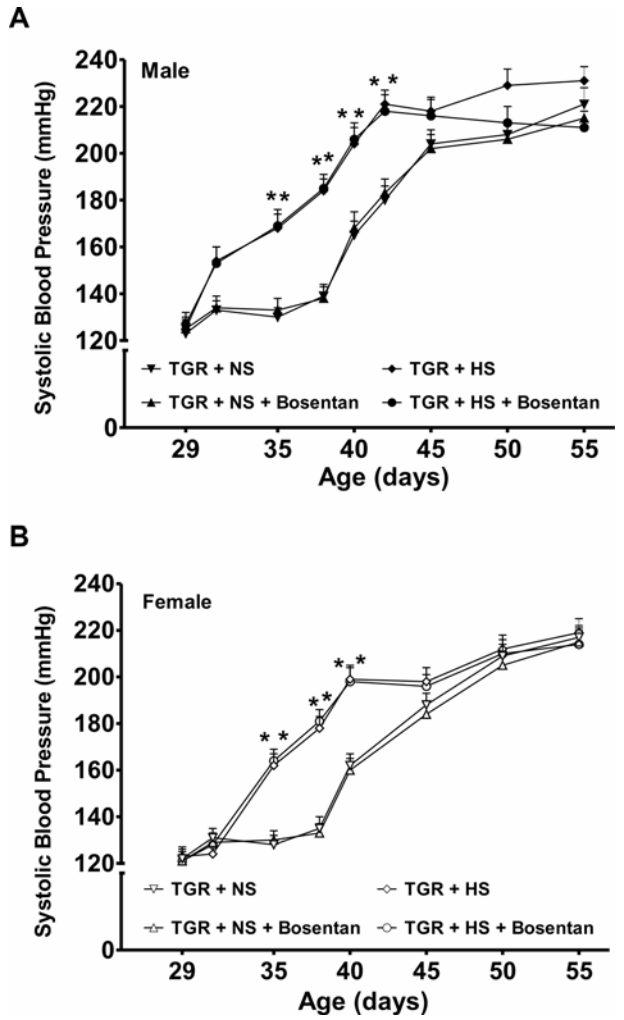

Fig. 3. Changes in systolic blood pressure in male TGR fed either NS or high salt (HS) diets (**A**). Changes in systolic blood pressure in female TGR fed either NS or HS diets (**B**). ***** $P<0.05$ vs. values at the same time point.

As shown in Figure 3A, the HS diet caused a significant acceleration of hypertension development in heterozygous male TGR between days 35 to 42 of age as compared with animals fed the NS diet and bosentan treatment did not prevent this acceleration. Likewise, between 35 to 40 days of age heterozygous female TGR fed the HS diet exhibited significantly higher SBP compared with animals fed the NS diet. Similarly, the administration of bosentan did not attenuate this BP increase (Fig. 3B).

Body and organ weights and glomerulosclerosis index

The course of body weight (BW) gain in male and female HanSD was not altered either by dietary manipulation or by the treatment with bosentan. The course of BW gain in heterozygous male and female TGR fed either the NS diet alone or the NS diet with bosentan was almost identical to that observed in HanSD animals. However, heterozygous male and female TGR exposed to HS diet exhibited a profound loss of BW just before they started to die (Figs 3A and 3B). In contrast, heterozygous male and female TGR fed the HS diet with bosentan showed the same BW gain as NS treated rats (data not shown)

Data on HW/BW and KW/BW are summarized in Table 1. As expected, heterozygous male as well as female TGR on NS diet exhibited a higher index of HW/BW compared with male and female HanSD fed the NS diet and treatment with bosentan did not alter this pattern. Heterozygous male as well as female TGR exposed to HS diet had a significantly higher HW/BW compared with NS fed littermates. Administration of bosentan in these groups of TGR lowered their HW/BW to levels observed in the animals fed NS diet. Neither diet manipulations nor treatment with bosentan did significantly change the HW/BW in male or female HanSD. There were no significant differences in the KW/BW index among the groups (Table 1).

As shown in Table 1, heterozygous male and female TGR fed the NS diet exhibited markedly higher values of glomerulosclerosis index compared with male and female HanSD. HS diet further increased the glomerulosclerosis index in these groups of TGR. Administration of bosentan in heterozygous male and female TGR fed the HS diet substantially reduced the glomerulosclerosis index to the levels observed in NS fed animals.

Table 2. Proteinuria, clearance of endogenous creatinine at different time points in male and female experimental groups**.**

Group	Day 40		Day 80		Day 120		Day 170	
	Proteinuria (mg/24 h)	C_{Cr} ($\mu l.min^{-1}.g^{-1}$)	Proteinuria (mg/24 h)	C_{Cr} ($\mu l.min^{-1}.g^{-1}$)	Proteinuria (mg/24 h)	C_{Cr} ($\mu l.min^{-1}.g^{-1}$)	Proteinuria (mg/24h)	C_{Cr} ($\mu l.min^{-1}.g^{-1}$)
Male HanSD + NS	3.1±1.1	7.7±0.6	4.1±1.2	7.6±0.5	5.2±1.1	7.2±0.4	5.4±1.3	7.8±1.4
Male HanSD + NS + B	2.9±0.9	7.6±0.5	3.6±1.1	7.8±0.7	4.9±1.2	7.3±0.5	5.3±1.2	7.6±1.1
Male HanSD + HS	6.4±1.3	6.8±0.7	7.7±1.5	7.1±0.4	6.2±1.1	7.1±0.6	7.8±1.1	7.0±0.9
Male HanSD + HS + B	5.4±1.2	7.2±0.6	54±1.3	6.6±0.5	5.7±0.8	7.6±0.5	7.7±1.2	7.1±0.8
Male TGR + NS	4.8±1.1	6.7±0.7	39.8±3.7*	6.7±0.6	36.5±2.9*	8.1±0.9	41.5±2.7*	6.9±1.2
Male TGR + NS + B	5.3±0.9	7.4±0.5	8.8±2.8	6.4±0.6	9.1±2.9	7.7±0.6	9.3±2.4	7.4±1.3
Male TGR + HS	35.8±3.7*	7.0±0.5	75.9±3.9*$^{+}$	6.7±0.7	75.1±2.7*$^{+}$	6.6±0.6	75.9±3.8*$^{+}$	6.8±1.1
Male TGR + HS + B	6.5±1.3	7.3±0.6	31.5±3.3*	6.4±0.8	37.2±4.1*	6.4±0.7	36.4±3.9*	7.7±1.1
Female HanSD + NS	4.6±1.2	7.4±0.8	4.1±0.6	7.1±0.9	4.2±0.9	7.2±0.4	4.7±1.2	6.8±0.9
Female HanSD + NS+ B	4.8±1.1	7.0±0.7	5.9±0.9	7.2±0.6	4.4±0.7	7.1±0.7	4.9±1.1	7.3±0.7
Female HanSD + HS	5.4±1.3	7.1± 0.9	5.1±0.7	7.2±0.5	5.7±0.9	7.2±0.5	5.8±1.2	7.1±0.6
Female HanSD + HS + B	5.2±0.9	7.2±0.8	4.7±0.9	7.3±0.6	5.1±0.8	7.0±0.5	5.3±1.1	6.9±0.8
Female TGR + NS	5.1± 0.8	6.9±0.8	5.1±1.3	7.0±0.8	4.5±0.7	7.2±0.6	4.4±0.7	7.2±0.6
Female TGR + NS+ B	4.7±0.9	7.1±0.6	4.6±1.2	7.4±0.9	4.3±0.8	7.3±0.7	4.7±0.8	7.2±0.5
Female TGR + HS	5.4±1.1	7.3±0.7	37.5±3.4*	6.1±0.3	20.8±2.3*	7.1±0.5	19.5±2.1*	6.8±0.6
Female TGR + HS + B	5.3±1.2	7.2±0.8	5.3±1.1	6.3±0.7	5.9±1.1	7.2±0.6	6.3±1.4	7.1±0.5

HanSD, transgene-negative rats; TGR, heterozygous transgenic rats (mRen2)27; NS, normal salt diet; HS, high salt diet; B – bosentan, C_{Cr}, clearance of endogenous creatinine (expressed per gram of body weight). * $P<0.05$ vs. HanSD rats; $^{+}$ $P<0.05$ untreated TGR fed HS vs. TGR fed NS or HS and treated with bosentan. Data shown in this table at each time point are from animals that survived until the end of the experiment (180 days of age).

Proteinuria, clearance of endogenous creatinine and urinary electrolyte excretion

All male and female HanSD fed either the NS or the HS diet exhibited minimal proteinuria and stable renal function (measured as clearance of endogenous creatinine) throughout the entire experimental period. Bosentan treatment did not affect these parameters in any of the experimental groups (Table 2)

As shown in Table 2, heterozygous male TGR fed the NS diet exhibited a progressive increase in proteinuria throughout the experimental period reaching final values that were eight times higher than those in male HanSD rats ($p<0.05$). Proteinuria was significantly reduced in rats treated with bosentan ($p<0.05$). The HS diet caused a significant increase in proteinuria in untreated heterozygous male TGR compared with NS fed animals ($p<0.05$). Bosentan treatment again significantly reduced the proteinuria in heterozygous male TGR fed the HS diet. In contrast to males, heterozygous female TGR fed the NS did not show any significant increase in proteinuria throughout the entire experiment compared with female HanSD rats. HS diet increased proteinuria in heterozygous female TGR compared with NS fed littermates ($p<0.05$), but the onset of this change was delayed and also the degree of proteinuria was lower compared with male TGR ($p<0.05$) (Table 2). Bosentan administration normalized proteinuria in heterozygous female TGR fed the HS diet to levels observed in HanSD animals.

Data on proteinuria and endogenous creatinine clearance obtained from all experimental groups throughout the study are shown in Table 2. As expected, animals fed the HS diet exhibited significantly higher daily urinary sodium excretion compared with rats fed the NS diet (between 1780 to 1980 vs. 920 to 980 µmol/24 h, $p<0.05$).

ET-1 tissue concentrations

As shown in Figure 4A, kidney cortex ET-1 concentrations in male HanSD rats were not significantly different from those in heterozygous male TGR fed the NS diet (0.20±0.04 vs. 0.24±0.05). The HS diet did not significantly change kidney cortex ET-1 levels in HanSD, but elicited a significant increase in TGR (0.23±0.03 vs. 0.48±0.04, $p<0.05$). Likewise, left ventricular ET-1 concentrations were not significantly different between HanSD and TGR rats fed the NS diet (0.42±0.06 vs. 0.56±0.09), but the HS diet caused a significant increase in TGR animals (0.49±0.05 vs. 0.78±0.06, $p<0.05$) (Fig. 4B).

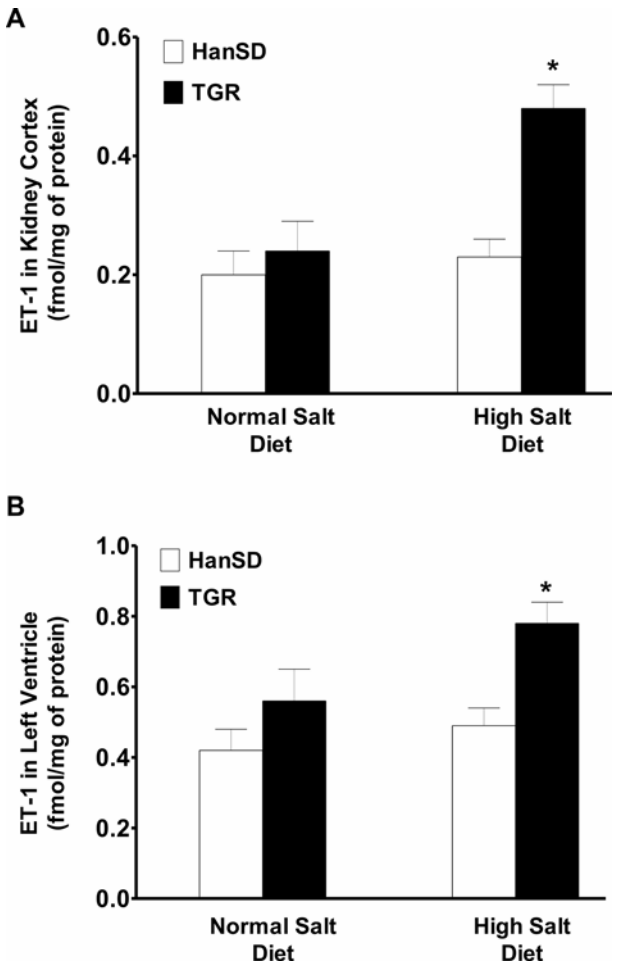

Fig. 4. Kidney cortex (**A**) and left ventricle (**B**) ET-1 levels in transgene-negative (HanSD) and homozygous male Ren-2 transgenic rats (TGR). ***** P<0.05 vs. all other values.

Discussion

The first aim of the present study was to evaluate the effects of nonselective ET_A/ET_B receptor blockade on the course of hypertension in heterozygous TGR. We found that treatment with bosentan did not alter the course of hypertension either in heterozygous male or female TGR implying a minor ET-dependent component in this model of hypertension. Our findings confirm the results obtained by Rossi´s group (Rossi *et al.* 2000, Seccia *et al.* 2003), but do not support previous findings that suggest a major contribution of the ET system to the maintenance of hypertension in TGR (Gardiner *et al.* 1995, 2000, Kelly *et al.* 2000). We cannot offer a fully satisfactory explanation for this discrepancy, but one possibility should be considered. In the aforementioned studies TGR of various ages were used, i.e. young rats were used in studies with no effect of bosentan, whereas old ones were employed in studies reporting a hypotensive effect of ET blockade. In addition, it has

been recently reported that the ET_A receptor blockade attenuated the development of hypertension in adult Dahl salt-sensitive rats but not in young animals (Dobešová *et al.* 2003). Therefore, it is pertinent to assume that ET system contributes to the pathogenesis of hypertension in mature but not in immature TGR. However, the newest study of Rothermund *et al.* (2003) does not support this concept. They demonstrated that ET receptor blockade did not decrease BP in heterozygous TGR with already established hypertension. The claim that the role of ET system in the pathogenesis of hypertension in TGR is more pronounced in adult than in young animals is in agreement with the fact that pressor systems such as vasopressin or angiotensin are less important in salt-dependent hypertension of immature rats compared to that of adult rats (for review see Zicha and Kuneš 1999). It is clear that more detailed studies are needed to address this issue.

However, with respect to the lack of BP lowering effect of bosentan treatment in TGR, the nonselective nature of this compound should be considered. Emerging body of evidence indicates that hypertensinogenic and target-organ damaging ET-1 actions are produced *via* ET_A receptor activation especially in the models of malignant and salt-loaded hypertension (d´Uscio *et al.* 1997, Orth *et al.* 1998, Rothermund *et al.* 2001, for review see Moreau and Schiffrin 2003). In addition, Vassileva *et al.* (2003) have recently demonstrated that activation of ET_B receptors participates in the pressure-natriuresis mechanism and it has been proposed that ET may participate *via* this mechanism in the long-term regulation of BP. Therefore, it is logical to assume that the selective ET_A blockade would have a BP lowering effect in TGR. However, the recent results from Rossi´s and Rothermund´s groups do not support this notion. They found that neither bosentan nor a selective ET_A receptor antagonist prevented the development of hypertension or did not lower BP in TGR (Seccia *et al.* 2003, Rothermund *et al.* 2003). Moreover, the expectation that ET_A receptor activation leads to vasoconstriction and ET_B receptor stimulation causes vasodilatation may be oversimplified, because it is known that two distinct subtypes of ET_B receptors exist (ET_{B1} and ET_{B2}). ET_{B1} receptors are present on the vascular endothelium and cause vasodilatation through the release of nitric oxide and prostaglandins (De Nucci *et al.* 1998), whereas ET_{B2} receptors are found on vascular smooth muscle cells and mediate non-ET_A vasoconstriction (Rasmussen *et al.* 1998). Moreover, ET_B receptors have been reported to stimulate aldosterone production (for review see Nussdorfer *et al.* 1999) and it has been recently demonstrated that aldosterone plays an important role in the development of cardiac and renal vascular fibrosis (Park and Schiffrin 2002, Pu *et al.* 2003, Seccia *et al.* 2003). Therefore, it seems that the nonselective ET_A/ET_B receptors blockade is a suitable experimental approach for the evaluation of the overall role the ET system in the development of ANG II-dependent hypertension. It is obvious, however, that more detailed studies employing selective ET receptor antagonist are needed to address the role of ET system and its specific ET receptor subtypes in the development of hypertension in TGR.

The second aim of this study was to assess the effects of HS diet on the course of hypertension and associated end-organ damage in heterozygous TGR. With respect to BP we found that HS intake accelerated the development of hypertension in heterozygous male and female TGR. In addition, our present study shows that in heterozygous TGR the HS diet caused a marked increase in mortality and deteriorated the cardiac and renal injury. Bosentan administration elicited a considerable improvement of survival rate and a substantial attenuation of end-organ damage in heterozygous male and female TGR fed the HS diet without influencing BP levels. These results are in good agreement with our recent observation made in homozygous TGR showing that nonselective ET receptor blockade resulted in a considerable improvement of survival rate and a substantial attenuation of end-organ damage in homozygous TGR fed the HS diet without altering BP levels (Dvořák *et al.* 2002a). Taken together, our data indicate that the ET-dependent component plays an important role in the development of end-organ damage in heterozygous male as well as female TGR fed HS diet. Our findings further support the notion that the interaction of ET-1 and ANG II contributes to the development of hypertension-induced end-organ damage (d´Uscio *et al.* 1997, Breu *et al.* 2000, Müller *et al.* 2000, Ficai *et al.* 2001, Müller *et al.* 2002, for review see Moreau and Schiffrin 2003).

In addition, our results suggest that heterozygous male as well as female TGR carry a salt-sensitive component of hypertension. These observations are in contrast with a report by Chung *et al.* (1993) who found no BP changes in TGR when given 8 % NaCl diet for 10 days. However, this difference might be explained by their use of adult animals (3 to 5 months old), while we

used young TGR. This explanation agrees with our findings that acceleration of hypertension occurred only between 35 to 40 days of age, whereas we did not observe any effects of HS diet on BP in elderly animals. Moreover, it is also important to mention that we used only 2 % NaCl and the differences in the salt-loading protocol might therefore account for different BP responses. Nevertheless, a previous study of Callahan *et al.* (1996) which showed that 2 % NaCl diet exacerbated the progression of hypertension in heterozygous male TGR, supports our findings.

The third aim of the present study was to evaluate if gender differences influence the course of hypertension and end-organ damage in TGR and if they would modify responses to dietary manipulation and pharmacological treatment. We found that heterozygous male and female TGR fed the HS diet exhibited similar pattern of the course of hypertension, survival rate and associated hypertension-induced end-organ damage. In addition, the response to bosentan treatment has a lot in common. However, the onset of the malignant phase of hypertension (characterized by enhanced mortality rate, increased proteinuria and body weight loss) was significantly delayed in heterozygous female TGR fed the HS diet compared with males. This is in good agreement with previous observations in various hypertensive models showing that males have higher BP and more serious hypertension-induced end-organ damage than age-matched females (for review see Reckelhoff 2001). Even if the mechanisms responsible for the gender differences in the severity of hypertension and end-organ damage are not yet fully understood, several reports suggest that the interaction between the RAS and androgen receptors accounts for these gender differences (for review see Reckelhoff 2001). This notion is further supported by recent findings that androgen receptor blockade significantly attenuated the development of hypertension and, even more important, completely prevented end-organ damage in male TGR (Baltatu *et al.* 2002).

Of special interest is our observation that BP in heterozygous female TGR fed the NS diet reached its peak between 65 to 80 days of age, then started to decrease spontaneously at the age of 130 days. At the end of the experiment it reached values which were significantly lower than those in heterozygous male TGR. Such a marked sexual dimorphism of BP changes in TGR was already reported by Cargnelli *et al.* (1998) who found that BP in heterozygous female TGR spontaneously returned to normotensive levels by the age of 250 days. It remains unclear whether the diminished expression of the Ren-2 gene throughout the lifetime in females is responsible for this BP decline, but the available data on the Ren-2 gene expression in females would rather argue against this possibility (for review see Langheinrich *et al.* 1996 and Lee *et al.* 1996). Therefore, the reasons for this sexual dimorphism of BP phenotype in heterozygous TGR are not known and will require more comprehensive studies.

The critical issue of the present study is related to the problem which are the underlying mechanism(s) responsible for the beneficial effects of bosentan treatment on survival rate and end-organ damage in heterozygous TGR fed the HS diet.

Multiple independent evidence indicate that the beneficial effects of ET blockade may be BP independent. It has been shown that antihypertensive treatment with hydralazine, reserpine and hydrochlorothiazide which decreased BP to normotensive levels, barely delayed end-organ damage in ANG II-dependent model of hypertension, whereas treatment with bosentan had markedly beneficial effect (Müller *et al.* 2000). Furthermore, it has been demonstrated in two-kidney, one-clip Goldblatt hypertensive rats that chronic ET_A receptor blockade attenuated cardiac hypertrophy without a reduction of BP (Ehmke *et al.* 1999). Moreover, two studies reported that mice overexpressing ET-1 developed pronounced end-organ damage independently of the increase in systemic BP (Hoecher *et al.* 1997, Shindo *et al.* 2002). With respect to the underlying mechanism(s), it has been found in these rats that treatment with bosentan inhibited the activation of nuclear factor-kappa B (NF-κB) and transcription factor activator protein (AP-1) in the kidney and in the heart independently of BP (Müller *et al.* 2000). Since ET-1 *via* ET_A receptor activation stimulates mitogen-activated protein kinase (MAPK) pathways which are important regulators of cell proliferation and play an important role in the inflammatory response and consequently in the fibrosis process, the alternative mechanism for the beneficial effects of ET blockade might be the inhibition of MAPK pathways (for review see Guijarro and Egido 2001, Luft 2002).

Alternatively, the beneficial effects of ET blockade might be related to the blockade of aldosterone-mediated deleterious actions on organ structure. It has recently been shown that ET-1 plays a critical role in the development of end-organ damage in aldosterone-infused

rats exposed to HS intake and that ET blockade prevented cardiac and aortic fibrosis independently of BP changes (Park and Schiffrin 2002). In addition, it has been reported that ET-1 exerts an important permissive action on basal aldosterone secretion and that treatment with bosentan decreased basal aldosterone secretion in heterozygous TGR (Andreis *et al.* 2000). This notion is further supported by recent findings that ET receptor blockade in aldosterone-infused rats decreased oxidative stress (Pu *et al.* 2003). Moreover, ET-1 markedly stimulates tissue superoxide production (Li *et al.* 2003). As heterozygous TGR exposed to salt-loading exhibit enhanced tissue ET-1 concentrations, it is likely that they also increased tissue superoxide levels. Since it is well known that oxidative stress increases the activity of NF-κB (Alexander 1995) and that oxidative stress plays an important role in ANG II-dependent models of hypertension (for review see Reckelhoff and Romero 2003), it seems conceivable that the beneficial effects of the ET blockade may be attributed to the blockade of the deleterious effects of aldosterone and oxidative stress on organ structure. Further studies are needed to elucidate the exact underlying mechanism(s) of our experimental observation regarding the beneficial effects of bosentan treatment.

In conclusion, we demonstrated in the present study that the chronic treatment with bosentan substantially improved the survival rate in heterozygous TGR given HS diet in the absence of BP reduction. Administration of bosentan in these groups of TGR also markedly decreased cardiac hypertrophy and renal damage, thus providing considerable protection from end-organ damage. Our data suggest that the interaction of the RAS and ET system plays an important role in the development of end-organ damage in heterozygous TGR exposed to salt-loading.

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