Early Endothelin-A Receptor Blockade Decreases Blood Pressure and Ameliorates End-Organ Damage in Homozygous Ren-2 Rats

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Abstract—We have recently found that nonselective endothelin ET₄/ET₆ receptor blockade markedly improves survival rate and ameliorates end-organ damage in male homozygous rats transgenic (TGR) for the mouse Ren-2 renin gene without lowering blood pressure. Because activation of the ET₆ receptor may be responsible for the detrimental effects of ET in the development of hypertension, this study was performed to determine whether ET₆ or ET₄/ET₆ receptor blockade exerts these beneficial effects. TGR and age-matched normotensive Hannover Sprague-Dawley rats fed a high-salt diet received either vehicle or bosentan and atrasentan (ABT-627) as nonselective ET₄/ET₆ and selective ET₆ receptor blockers, respectively, from 29 until 90 days of age. The survival rate of 48% in untreated TGR was significantly (P<0.01) improved to 79% by bosentan and to 92% by ABT-627 (ABT-627 versus bosentan P<0.05). Proteinuria, glomerulosclerosis, and cardiac hypertrophy, as well as ET-1 content in left ventricular tissue, were significantly reduced by bosentan and to a greater degree by ABT-627, which also significantly attenuated the rise in blood pressure (P<0.05). Our data indicate that the ET system, especially via ET₆ receptors, plays an important role in the development of hypertensive end-organ damage and confirm the concept that the predominant role of ET₆ receptors within the peripheral vasculature is to mediate the vasorelaxant actions of ET-1. They also demonstrate that selective blockade of ET₆ receptors is superior to nonselective ET₄/ET₆ in attenuating hypertension, hypertensive organ damage, and survival rate. (Hypertension. 2005;46[part 2]:969-974.)

Key Words: hypertension ■ kidney ■ antihypertensive drugs ■ end-organ damage ■ systolic ■ experimental models

Endothelin-1 (ET-1) has been described as one of the most powerful vasoconstrictors 1,2 that also plays a role in the regulation of renal hemodynamics and salt and water excretion. 3 Moreover, numerous studies have shown that the ET system plays an important role in the pathogenesis of salt-sensitive models of hypertension and in associated end-organ damage. 4 The nephroprotective effect of ET receptor blockade in these models is comparable with blockade of the renin-angiotensin system. 5 The beneficial effects of ET receptor blockers in modulating target-organ damage arise from their antiproliferative actions. 6 There is, however, a large discrepancy in the effect of ET between various models of hypertension. Animal models with exogenously administered angiotensin II (ANG II) exhibit an ET-dependent component, whereas hypertensive models with endogenously enhanced production of ANG II do not. 7

In our previous study, we showed that bosentan treatment substantially improved the survival rate in homozygous rats transgenic (TGR) for the mouse Ren-2 renin gene fed either normal-salt or high-salt diet without blood pressure (BP)-lowering effect. 8 Whereas the protective action of nonselective ET blockade has attracted much attention in homozygous TGR, 5,9 there is no information available concerning the effect of selective ET₆ blockade on the course of hypertension and end-organ damage in homozygous TGR and little information is available in heterozygous animals. 10,11 It is well-known that young animals are more susceptible to various hypertensive stimuli 12 and also that interventions made in these early periods are more effective than when adopted lately in the life. 13 However, in Dahl-sensitive rats it has been shown that ET₆ receptor blockade was efficient in adult but not in young animals. 14

The transgenic hypertensive TGR(mRen2)27 rat model 15 is a valuable monogenic model of renin-dependent (and thus ANG II-dependent) hypertension, which exhibits functional and structural changes of the kidney usually found in hyper-
tension, ie, reduced glomerular filtration rate and proteinuria associated with glomerulosclerosis. Because it is generally accepted that ET is involved in elevating blood pressure (BP) principally in experimental models of salt-sensitive hypertension and in TGR who exhibit a salt-sensitive component of hypertension, we evaluated the effects of either nonselective ET receptor blockade or selective ET<sub>A</sub> receptor blockade in TGR on survival and BP on a high-salt regimen.

Materials and Methods

The protocols in the present study were designed according to the Guiding Principles in the Care and Use of Laboratory Animals as published by the Council of the American Physiological Society and were in adherence to the Guide for the Care and Use of Laboratory Animals. The protocols in the present study were designed according to the Guiding Principles in the Care and Use of Laboratory Animals approved by the Czech Animal Care and Use Committee (protocols 79/2001 and 923/2003).

Animals

Homozygous TGR (strain name TGR(mRen2)27) and their normotensive HanSD (HanSD) control rats were kept at room temperature (25°C) with 12-hour light–dark cycle. All animals used in this study were bred at the Center 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. The animals were switched to a high-salt diet (HS) (2% NaCl) immediately after weaning (29 days) and received tap water ad libitum. At the same time, either nonselective ET<sub>A</sub>/ET<sub>B</sub> blockade by bosentan or selective ET<sub>A</sub> receptor blockade by atramentan (ABT-627, A-147627) was started. Bosentan (Actelion, Alschwil, Switzerland) was mixed to the high-salt diet depending on the real food intake to achieve a final consumption of 100 mg·kg<sup>-1</sup>·day<sup>-1</sup>.<sup>19</sup> ABT-627 was given with the drinking fluid, adjusted weekly to maintain proper dosage of 5 mg·kg<sup>-1</sup>·day<sup>-1</sup>.<sup>18</sup>

The following experimental groups were investigated:
- Male HanSD + HS (n=24)
- Male TGR + HS (n=21)
- Male TGR + HS + bosentan (n=16)
- Male TGR + HS + ABT-627 (n=16)

Experimental Design and Functional Examination

Twice per week, rats were weighed and systolic blood pressure (SBP) was measured by the tail-cuff method previously validated in our laboratory.<sup>20</sup> At the age of 50 and 80 days, animals were individually housed in metabolic cages and measurements of fluid consumption, urine excretion, as well as proteinuria, were monitored over 24 hours.

On termination of the experiment (day 90), animals were weighed, anesthetized with thiopental sodium (50 mg·kg<sup>-1</sup>) and mean arterial pressure (MAP) was monitored directly in the carotid artery using data acquisition system PowerLab (ADInstruments, Mountain View, Calif). Kidneys and hearts were weighed. Ratios of kidney weight/ body weight (KW/BW) and heart weight/body weight (HW/BW) were used as indices of organ hypertrophy. Right kidney cortex and left ventricles were frozen in liquid nitrogen (LN<sub>2</sub>) for ET-1 determination using enzyme-linked immunosorbent assay test (Amersham, Braunschweig, Germany). Left kidneys were fixed in 4% buffered formaldehyde, embedded, and taken for morphological examination. Paraffin sections were stained with hematoxylin eosin and periodic acid-Schiff reaction (PAS). Slides were evaluated in a blind manner. As described previously,<sup>20</sup> 50 glomeruli were examined on a semi quantitative scale: grade 0=all glomeruli normal; grade 1=1 to 2 glomeruli affected; grade 2=2 to 17 glomeruli affected; and grade 3=17 or more glomeruli affected.

Statistical Analysis

Statistical analysis of data was performed using Graph-Pad Prism software (Graph Pad Software, San Diego, Calif). Group comparisons were determined by 2-way ANOVA. Statistical comparisons of the results obtained for heart and kidney weights and for ET-1 concentration were made by 1-way ANOVA. Unless noted, values are expressed as mean±SEM and n represents the number of animals. P<0.05 was considered significant.

Results

BP

SBP data are shown at a time point in which the survival rate of rats was still 100%. After weaning (day 29), SBP started to rise gradually in all 3 homozygous TGR groups on high-salt diet (Figure 1A). This increase was similarly steep in male TGR on HS diet without or with bosentan treatment reaching 236±5 and 228±5 mm Hg on day 50, respectively. Treatment with the selective ET<sub>A</sub> receptor blocker ABT-627 caused a strong attenuation of SBP increments, starting already at the age of 32 days. By day 50, SBP was substantially lower in ABT-627-treated TGR (190±5 mm Hg) when compared with male TGR without (236±5 mm Hg, P<0.01) or with bosentan treatment (228±5 mm Hg, P<0.01). No significant difference in MAP was found on termination of the experiment (day 90) between male TGR on high-salt diet without or with bosentan treatment reaching 129±4 mm Hg.

Survival Rate

All male HanSD on high-salt diet survived until the end of experiment. Starting from day 50, high-salt regimen caused a
substantial and progressive increase in mortality in untreated animals, with survival rate being only 48% by day 90 (Figure 2A). Both bosentan and ABT-627 treatment decreased mortality in homozygous animals fed the high-salt diet with survival rates of 79% and 92%, respectively \((P<0.01)\), with a significantly greater effect of ABT-627 \((P<0.05)\).

**Proteinuria**

Because there were no statistically significant differences in proteinuria between groups at the age of 50 days, results are not shown. At the age of 80 days, male HanSD on high-salt diet exhibited a significantly smaller proteinuria than all homozygous TGR groups (Figure 2B). Untreated TGR fed HS exhibited a 4-fold higher proteinuria in comparison with control HanSD animals \((78.4\pm2.3 \text{ mg/24 hours; } P<0.01)\). Bosentan treatment partly decreased proteinuria, whereas ABT-627 markedly ameliorated proteinuria to \(36.8\pm1.4 \text{ mg/24 hours, respectively; both } P<0.01\).

**Body and Kidney Weight**

All survivors of male homozygous groups of TGR gained weight until 6 to 7 weeks of age; thereafter, body weight remained stable until the end of the experiments. In HanSD, body weight increased gradually and was significantly higher when compared with homozygous TGR. There was no difference in kidney/body weight ratio between all 4 groups (data not shown).

**Left Cardiac Ventricular Hypertrophy**

The ratio of left heart ventricular weight to body weight was significantly increased in all homozygous TGR on high-salt diet when compared with control HanSD rats on the same diet (HS) (Figure 3A). In untreated TGR, it rose to \(4.07\pm0.07\), ie, to a level that was 63% higher than that of HanSD. Bosentan treatment caused a substantial decrease in LW/BW ratio \((3.71\pm0.06); \) With ABT-627, the development of left ventricular hypertrophy was significantly attenuated \((3.39\pm0.06; P<0.01)\).

**Glomerulosclerosis**

According to renal histological changes, glomerulosclerosis indices were calculated and are shown in Figure 3B. Figure 4A to 4D shows morphological changes of renal injury. HanSD on a HS diet showed no signs of renal damage, ie, there was only focal segmental pattern of glomerular sclerosis. On the contrary, after HS diet, glomeruli of homozygous TGR showed advanced sclerotic changes and widespread vascular sclerosis. Thus, in TGR on HS, glomerulosclerosis index was strongly elevated when compared with kidneys from HanSD. Glomerulosclerosis indices in homozygous TGR without or with bosentan treatment were almost the same; vascular changes were slightly milder in TGR with bosentan treatment. This renal injury, however, was substantially attenuated by ABT-627, showing no significant abnormalities of vessels and glomeruli, thus histological changes resembled those of HanSD.

**ET-1 Tissue Concentration**

As shown in Figure 5A, kidney cortex ET-1 levels in male homozygous TGR on HS diet exceeded almost 4-fold values...
of HanSD on the same diet (0.68±0.11 versus 0.22±0.03 fmol/mg protein, \( P<0.05 \)). After bosentan treatment, ET-1 content in TGR decreased to levels seen in HanSD (0.20±0.02 fmol/mg protein), whereas after ABT-627 treatment, ET-1 content was only moderately below values seen in untreated TGR (0.56±0.02 fmol/mg protein, not significant).

Left ventricular ET-1 content in untreated homozygous TGR fed a HS diet was almost 4-fold that of HanSD (2.15±0.21 versus 0.52±0.06, \( P<0.05 \)) (Figure 5D). Both bosentan and ABT-627 treatment substantially decreased these levels to those of HanSD (0.89±0.12 and 0.42±0.03, respectively, not significant).

**Discussion**

To our knowledge this is the first study addressing the effects of early treatment with selective ET\( _\alpha \) receptor blockade versus nonselective ET\( _\alpha/ET_\beta \) receptor blockade in homozygous TGR on HS. We have recently found that the nonselective ET\( _\alpha/ET_\beta \) receptor blocker bosentan markedly improved the survival rate and ameliorated end-organ damage in male homozygous TGR without lowering BP.\(^5\) The current study extends these findings to investigate the role of ET\( _\alpha \) receptors in the pathogenesis and treatment of end-organ damage in conditions of HS intake. Both bosentan and ABT-627 markedly improved survival rates of homozygous TGRs. Only treatment with ABT-627 but not with bosentan induced a significant decrease in BP. Moreover, whereas nonselective ET receptor blockade was moderately effective in heart and kidney protection, selective ET\( _\alpha \) receptor blockade was found to have substantial nephroprotective and cardioprotective effects, ie, reduce proteinuria, glomerulosclerosis index, and also left ventricular hypertrophy. The difference between the efficacy of ABT-627 and bosentan may be questionable because it could be a matter of potency. It has been found that ABT-627 as ET receptor antagonist is more potent than bosentan and their potency is greatly influenced by binding to serum albumin.\(^{21,22}\) Although we did not evaluate the efficacy of ABT-627 in TGR, the dose used in our experiments is generally accepted and confirmed by other authors,\(^{23,24}\) although both higher doses (35 and 70 mg per kg per day),\(^13\) and even the lower dose (2 mg per kg per day\(^25\)) were used. However, higher doses may have unspecific side effects; therefore, a greater benefit may be obtained with an intermediate dose.\(^13\) Concerning bosentan, we have previously performed the experiments in homozygous male TGR and HanSD in which a dose of bosentan (100 mg per kg per day) completely blocked BP responses to an intravenous bolus dose of ET-1 (250 ng).\(^8\) Also, this dose resembles that used by other authors.\(^{26,11,27}\)

Our present results are in agreement with our previous findings that nonselective ET receptor blockade with bosentan improved survival rate but had no effect on the course of hypertension in homozygous\(^8\) or heterozygous TGR.\(^11,10\) Similarly, Karam et al\(^28\) also found that the renoprotective actions of bosentan were independent of any BP-lowering effect. However, regarding the role of selective ET\( _\alpha \) receptor blockade conflicting data have been reported. Whereas some authors reported that ET\( _\alpha \) blockade lowers BP in various hypertensive models, ie, in Sabra salt-sensitive rats,\(^{29}\) DOCA-salt rats,\(^30\) salt-loaded SHR-SP,\(^31\) or rats transgenic for human angiotensinogen and renin genes.\(^32\) Rothermund et al\(^10\) did not find a protective effect of ET\( _\alpha \) blockade in heterozygous Ren-2 animals. Although quite opposite to our findings, their results could be explained by different timing of the experiments, ie, different age of animals, and by using heterozygous instead of homozygous animals. First, although we have started treatment immediately after weaning, their results were obtained in animals between 10 to 30 weeks of age. Moreover, Blezer et al\(^13\) found in stroke-prone SHR that early-onset but not late-onset of ET\( _\alpha \) receptor blockade prevented the development of cerebral edema, decreased BP and proteinuria, and increased the survival rate. Thus it is
possible that only early ET_a receptor blockade is effective in reducing end-organ damage. The implication that ET plays a role in the pathogenesis of hypertension and proteinuria but not in the maintenance of established target-organ damage, however, needs further examination. Second, the difference between heterozygous and homozygous animals is probably related to the severity of hypertension, which is accompanied by higher ET-1 production in homozygous animals.\textsuperscript{9} Whitworth et al\textsuperscript{35} found that only heterozygous animals with severe hypertension have significantly higher preproET-1 mRNA expressions in kidney tissue when compared with rats with benign hypertension. Moreover, only in those models of malignant hypertension that have been shown to have elevated plasma levels of ETA, ETA receptor blockade exhibited a hypotensive effect suggesting a role for ET in this condition.\textsuperscript{34} Third, although not very likely, the possibility cannot be fully excluded that various ET_a receptor blockers may produce different effects.

The role of ET in end-organ damage appears to be quite clear, because both bosentan, and especially ABT-627, significantly reduced overall mortality rates. There exist, however, conflicting data regarding the nephroprotective and cardioprotective effects of ET_a receptor blockade. Beneficial effects were found in rats with congestive heart failure,\textsuperscript{35} in Sabra salt-sensitive hypertension,\textsuperscript{29} or stroke-prone SHR,\textsuperscript{36} whereas Rothermund et al\textsuperscript{10} or Rossi et al\textsuperscript{11} observed no beneficial effect in the Ren2 model. We cannot offer a satisfying explanation for these discrepant findings except for the use of heterozygous instead of homozygous animals since Rossi et al\textsuperscript{11} used animals of the same age as we did.

The beneficial effects of ET_a receptor blockade can be clearly demonstrated by our histological findings. The most striking changes were found in the vasculature. They are characterized by hyaline sclerosis of afferent arterioles and fibrotic intimal thickening of preglomerular arteries caused by hypertrophy and hyperplasia of smooth muscle cells. The extent of glomerular involvement was directly proportional to the severity of vascular changes. Glomerular structural changes ranged from slight basement membrane wrinkling and mild mesangial matrix expansion to total glomerular collapse and sclerosis. Treatment with bosentan slightly attenuated the vascular damage but did not substantially reduce the glomerulosclerosis index, whereas ABT-627 fully prevented kidney parenchyma from hypertensive damage.

There still remains the crucial question of why this difference exists between the efficacy of selective versus nonselective ET receptor blockade in lowering BP and what is the underlying mechanism(s). A possible explanation could be the different actions of ET-1 via ETA and ETB receptors and their respective blockers at the molecular level. Whereas binding of both ABT-627 and ET-1 results in partial receptor internalization, only ET-1 is capable of triggering intracellular functional responses.\textsuperscript{37} Moreover, in rat aortic rings, ABT-627 is even able to reverse an ET-1 induced contraction. However, whether these observations offer a correct explanation for our findings remains speculative. With special emphasis on HS intake, one should keep in mind an additional role of ET_b receptors, which bind and remove ET-1 from the circulation and thus reduce ET_a receptor activation. Thus, in Sprague-Dawley rats Pollock and Pollock\textsuperscript{38} found that ET_a receptor blockade produced an increase in mean arterial pressure that was significantly higher in rats on the high-salt diet. These authors hypothesized that in response to salt loading ET-1 participates in BP regulation through ET_b receptors. Also, it is possible to assume that nonselective blockade not only reduces deleterious effects of ET_a receptor blockade but is detrimental due to blockade of beneficial vasorelaxant actions of ET_b receptors, which is mediated through the release of nitric oxide and prostaglandins.\textsuperscript{39} The question is even more complicated because, first, 2 distinct ET_b receptors were identified\textsuperscript{40,41} (ET_b1 present on vascular endothelium cause vasodilation whereas ET_b2 on vascular smooth muscle cells mediate vasoconstriction) and, second, different actions of these receptors are reported in various species\textsuperscript{40,42,43} (in contrary to other species, Brooks found no ET_b-mediated vasoconstriction in dogs). Moreover, Just et al\textsuperscript{41} speculated that not only dual (constrictor and dilator) actions of ET_b receptors have to be considered but also interactions with ETA receptors evaluating their physiological function. This topic therefore needs further examination.

Taken together, the aforementioned findings imply that the ET system may be activated secondary to an activation of the renin-angiotensin system. But only in conditions of severe hypertension or exogenously increased ANG II levels, when ET-1 expression is stimulated to a significantly elevated tissue level, ET-1 appears to play a major role in cardiovascular and renal damage. Moreover, our data clearly show that selective blockade of ETA receptors is superior to nonselective ETA/B receptor blockade in attenuating hypertension, hypertensive organ damage, and survival rate.

Perspectives

It is not yet well-understood whether the beneficial effect of early selective ET_a blockade on BP and end-organ damage observed in homozygous Ren-2 transgenic rats on HS diet will also be effective in animals with already-established hypertension. This question together with the participation of ET_B receptors on BP regulation needs further evaluation. However, when considering the therapeutic implication of our findings, our data clearly support the idea that selective ETA blockade may be preferable to nonselective one in protecting from hypertension and hypertension-associated organ damage.

Acknowledgments

This study was supported by grant NR/7826-3 awarded to I.V. by the Ministry of Health of the Czech Republic. H.J.K. is supported by the German Research Foundation (Kra 433/14). We thank to Drs Detlev Ganten and Michael Bader for providing us with breeders of our Ren-2 transgenic colony. We also greatly appreciate the help of Abbot Laboratories (North Chicago, Ill) for generously providing us with ABT-627.

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Hypertension. 2005;46:969-974; originally published online September 12, 2005; doi: 10.1161/01.HYP.0000173426.06832.b5

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