

Distinct Effects of Bosentan on NO-Dependent Vasodilation and Calcium Influx in Heterozygous Ren-2 Transgenic Rats on High-Salt Diet

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Summary

Our studies in hypertensive Ren-2 transgenic rats (TGR) demonstrated that chronic administration of atrasentan (ET_A receptor antagonist) decreased blood pressure by reduced Ca²⁺ influx through L-type voltage-dependent calcium channels (L-VDC) and attenuated angiotensin II-dependent vasoconstriction. We were interested whether bosentan (nonselective ET_A/ET_B receptor antagonist) would have similar effects. Young 4-week-old (preventive study) and adult 8-week-old (therapeutic study) heterozygous TGR and their normotensive Hannover Sprague-Dawley (HanSD) controls were fed normal-salt (NS, 0.6 % NaCl) or high-salt (HS, 2 % NaCl) diet for 8 weeks. An additional group of TGR fed HS was treated with bosentan (100 mg/kg/day). Bosentan had no effect on BP of TGR fed high-salt diet in both the preventive and therapeutic studies. There was no difference in the contribution of angiotensin II-dependent and sympathetic vasoconstriction in bosentan-treated TGR compared to untreated TGR under the condition of high-salt intake. However, bosentan significantly reduced NO-dependent vasodilation and nifedipine-sensitive BP component in TGR on HS diet. A highly important correlation of nifedipine-induced BP change and the BP after L-NAME administration was demonstrated. Although bosentan did not result in any blood pressure lowering effects, it substantially influenced NO-dependent vasodilation and calcium influx through L-VDC in the heterozygous TGR fed HS diet. A significant correlation of nifedipine-induced BP change and the BP after L-NAME administration suggests an important role of nitric oxide in the closure of L-type voltage dependent calcium channels.

Key words

Hypertension • Bosentan • Vasoactive systems • Blood pressure regulation

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Introduction

Ren-2 transgenic rat (TGR) is a typical model of angiotensin II-dependent hypertension in which the insertion of the mouse renin-2 gene into the genome of Hannover Sprague Dawley (HanSD) rats is associated with high blood pressure (Mullins *et al.* 1990). Our previous study demonstrated that the contribution of distinct vasoactive systems regulating blood pressure is age- and strain-dependent. Thus, RAS-dependent vasoconstriction is higher in homozygous rats and its role declines with the age of animals in both strains. By contrast, the sympathetic component of BP is more pronounced in adult rats (Vaněčková *et al.* 2012).

Our interest was focused on the mechanisms by which different antihypertensive drugs affected blood pressure in this experimental model of hypertension. We have demonstrated that the chronic treatment of TGR animals with three different types of RAS blockers

(angiotensin receptor type 1 blockers, angiotensin converting enzyme inhibitors or direct renin inhibitors) similarly normalized their blood pressure; this effect being mediated mainly by the substantially attenuated RAS-dependent and partly by moderately reduced sympathetic vasoconstriction. When atrasentan (ET_A receptor antagonist) was added to these RAS blockers, it did not influence the BP and hence the major vasoactive systems. However, it substantially decreased nifedipine-sensitive calcium influx through voltage-dependent calcium channels (Vaněčková *et al.* 2016). A similar reduction of calcium influx was also demonstrated in a study evaluating the effects of ET_A receptor blockade alone showing a four-fold decrease of this parameter in heterozygous TGR on a high-salt diet. Moreover, the blood pressure lowering effect of atrasentan was also associated with a substantial attenuation of angiotensin II-dependent vasoconstriction. Of note was the attenuation of nitric oxide-dependent vasodilation which was not accompanied by a compensatory activation of other vasodilator systems (BK_{Ca} channels or vasodilator prostanoids) (Vaněčková *et al.* 2015).

There is some controversy on the superiority of the selective ET_A over the nonselective (dual) ET_A/ET_B receptor blockade. Some authors favor the selective ET_A blockers due to their antihypertensive, anti-inflammatory and antiproliferative effects, while others prefer the use of dual ET_A/ET_B endothelin receptor blockers (Vaněčková *et al.* 2018). Our studies in Ren-2 transgenic rats have shown that atrasentan decreased blood pressure and reduced cardiac hypertrophy in both young and adult heterozygous as well as homozygous TGR. Similarly, the positive effects on blood pressure and organ protection of selective ET_A blockers were found in salt-loaded stroke-prone SHR (Okada *et al.* 1995), Dahl salt-sensitive rats (Barton *et al.* 1998, Okada *et al.* 2000), DOCA-salt rats (Allcock *et al.* 1998, Matsumura *et al.* 1999), and Sabra rats (Rothermund *et al.* 2003). By contrast, none of our studies with mixed ET_A/ET_B endothelin receptor blockade in TGR demonstrated the lowering of blood pressure, although bosentan had positive effects on their survival and exerted slight renoprotective effects in some studies (Dvořák *et al.* 2004, Opočenský *et al.* 2004, Vaněčková *et al.* 2005, Vaněčková *et al.* 2006, Opočenský *et al.* 2006, Vernerová *et al.* 2008). The beneficial organoprotective effects of the nonselective ET receptor blockade were also found in rats with diabetic

nephropathy (Ding *et al.* 2003) and in double transgenic rats (Muller *et al.* 2000). Moreover, the hypotensive effects of the nonselective ET receptor blockade were demonstrated in the Goldblatt 2K1C model (Li *et al.* 1996), DOCA-salt hypertensive rats (Schiffrin *et al.* 1996), double transgenic rats (Muller *et al.* 2000) and young TGR (Rossi *et al.* 2000).

Many experimental studies support the idea that young animals are more sensitive to various pharmacological interventions (Zicha *et al.* 1986, Zicha and Kuneš 1999). Not surprisingly, the therapies applied at a younger age (preventive treatment) are usually more effective than those in animals with established hypertension (therapeutic treatment). Interestingly, one of the exceptions from this general rule is the study of Zicha *et al.* (2012) which demonstrated that ambrisentan (ET_A receptor antagonist) was more effective in reducing the blood pressure of adult young salt-sensitive Dahl rats compared to young ones (Zicha *et al.* 2012).

Endothelin-1 production is enhanced during increased salt intake; this occurs in the renal collecting duct, specifically the inner medullary collecting duct (IMCD), which upon stimulation by high salt intake produces ET-1 to promote the elimination of salt load by autocrine inhibition of Na⁺ reabsorption (Kohan *et al.* 2011). Moreover, Herrera *et al.* (2005) reported that with a high salt intake, medullary tonicity increased – this process being associated with the activation of ET_B receptors, which leads to enhanced eNOS activity, NO production and inhibition of Na⁺ transport in renal thick ascending limb *in vitro*.

Therefore, we were interested (i) whether bosentan could have some effects on the major vasoactive systems contributing to BP maintenance, and (ii) whether nonselective endothelin receptor blockers might, similar to selective ET_A receptor blockade, affect the NO-dependent vasodilation and calcium influx through the voltage-dependent calcium channels.

Methods

Ethics statement

All the procedures and experimental protocols were approved by the Ethical Committee of the Institute of Physiology, Czech Academy of Sciences (Protocol Nr. 139/2013), and conform to the European Convention on Animal Protection and Guidelines on Research Animal Use (Directive 2010/63/EU).

Animals and in vivo experimental strategies

Four-week-old young (prevention study) or 8-week-old adult (therapeutic study) male heterozygous (mRen-2) 27 transgenic rats (TGR) and their transgene-negative control Hannover Sprague Dawley (HanSD) rats were housed at 23 °C under a 12 h light/dark cycle, given a normal salt diet (0.6 % NaCl) or high-salt diet (2.0 % NaCl) and tap water *ad libitum* for 8 weeks. The additional group of TGR fed a high-salt diet was treated with bosentan, which was mixed to the diet (100 mg/kg/day).

All rats used in this study were bred at the Institute of Clinical and Experimental Medicine from stock animals supplied by the Max Delbrück Center for Molecular Medicine in Berlin, Germany.

After 8 weeks of treatment, the involvement of major vasoactive systems (RAS, SNS and NO) contributing to blood pressure regulation was determined. Additionally, the calcium influx through the L-type voltage-dependent calcium channel (L-VDCC) was evaluated after the complete blockade of major vasoactive systems. One day prior to the experiment, the cannulation of the carotid artery (PE 50) and the jugular vein (PE 10) was performed under 2.5 % isoflurane anesthesia to enable BP measurement and the infusion of drugs, respectively. The cannulae were exteriorized in the interscapular region. Blood pressure and heart rate (HR) were recorded using a pressure transducer and a multichannel recorder (ADInstruments, Bella Vista, Australia) employing the modified protocol of Minami *et al.* (1995). Briefly, rats were adapted in transparent plastic cages for 30 min. Then, the RAS blockade with captopril (10 mg/kg) was followed by the ganglionic blockade of SNS with pentolinium (5 mg/kg) 15 min later. After NO synthase inhibition with L-NAME (30 mg/kg), the BP was monitored for a further 20 min. Finally, the L-type voltage-dependent calcium channel blocker nifedipine (0.4 mg/kg) was administered. The BP levels before and after particular blockades were determined and the respective BP changes were calculated. All drugs were dissolved in saline and administered as intravenous bolus injections in a volume of 1 ml/kg of body weight. At the end of the experiment the rats were euthanized by anesthetic overdosage and their hearts and kidney were weighed.

Drugs

All drugs were purchased from Sigma (St. Louis, Missouri, USA) except for bosentan (a contribution from

Martine Clozel, Actellion).

Statistical analysis

The results are expressed as the mean \pm SEM. The statistical differences were evaluated by one-way and two-way analysis of variance (ANOVA) (Instat, La Jolla, California, USA) followed by the Fisher LSD *post hoc* test. $P < 0.05$ values were considered to be statistically significant.

Results

The blood pressure of TGR animals on a normal salt diet was significantly elevated compared to normotensive HanSD rats in both the prevention and therapeutic studies (Fig. 1). The BP difference was more pronounced in young animals than in adults (181 \pm 5 mm Hg vs. 156 \pm 5 mm Hg). The feeding of a high-salt diet had no effect on the BP in either HanSD rats or TGR in both studies. However, it increased the relative kidney weights in TGR rats in both studies and cardiac hypertrophy in TGR in the prevention study (Table 1). Bosentan did not exert any effect on BP or body parameters in TGR.

The contribution of Ang II-dependent vasoconstriction was proportional to basal BP level – being four-fold higher in young TGR in the prevention study while only two-fold higher in adult TGR in the therapeutic study (Fig. 2A, D). By contrast, although the sympathetic vasoconstriction substantially contributed to the increased BP in both age groups of TGR, its contribution was greater in adult TGR (Fig. 2B, E). No effects of a high-salt diet or bosentan on angiotensin II-dependent or sympathetic vasoconstriction were found.

NO-dependent vasodilation was not different between TGR and control HanSD animals in either study (Fig. 2C, F). In addition, the high-salt diet had no effect on this parameter in hypertensive or normotensive rats. On the other hand, bosentan significantly lowered NO-dependent vasodilation in both the prevention and therapeutic studies. This was also true for nifedipine-sensitive BP reduction elicited by the inhibition of calcium influx through the voltage-dependent calcium channels (Fig. 3). Moreover, we analyzed the relationship between the MAP measured after NO synthase inhibition with L-NAME and nifedipine-sensitive BP change and we found a highly significant correlation of these parameters in both the prevention and therapeutic studies (Fig. 4A, B).

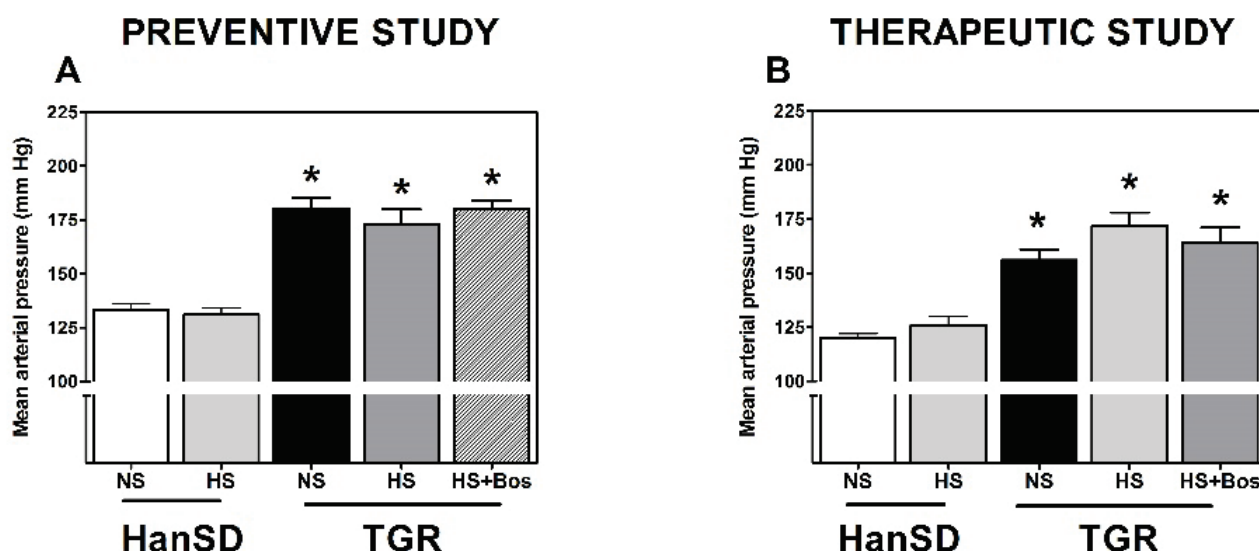


Fig. 1. The blood pressure of young (preventive study)(A) and adult (therapeutic study)(B) normotensive control Hannover Sprague Dawley (HanSD) and hypertensive heterozygous Ren-2 transgenic rats (TGR) on normal salt (NS) diet, high-salt (HS) diet, or HS treated with nonselective ET_A/ET_B receptor antagonist bosentan (Bos) measured at the end of the study. The data are the means \pm SEM. * $p < 0.05$, vs. control HanSD rats.

Table 1. Body and organ weights in young (preventive study) and adult (therapeutic study) normotensive control Hannover Sprague Dawley (HanSD) and hypertensive heterozygous Ren-2 transgenic rats (TGR) on normal salt (NS) diet, high-salt (HS) diet, or HS treated with nonselective ET_A/ET_B receptor antagonist bosentan.

| PREVENTIVE STUDY | | | | | |
|--------------------------------------|----------------|----------------|----------------|----------------------------|-----------------------------|
| | HanSD-NS | HanSD-HS | TGR-NS | TGR-HS | TGR-HS-bosentan |
| Number of rats | 7 | 7 | 10 | 12 | 9 |
| Body weight (g) | 408 \pm 8 | 432 \pm 9 | 389 \pm 14 | 387 \pm 15 | 403 \pm 16 |
| Heart weight (mg) | 1165 \pm 30 | 1243 \pm 80 | 1406 \pm 41* | 1533 \pm 57* | 1488 \pm 55* |
| Relative heart weight (mg/100 g BW) | 285 \pm 7 | 286 \pm 15 | 363 \pm 5* | 397 \pm 8* [@] | 372 \pm 16* |
| Kidney weight (mg) | 3010 \pm 80 | 3487 \pm 145 | 2971 \pm 118 | 3408 \pm 110 | 3496 \pm 137 [@] |
| Relative kidney weight (mg/100 g BW) | 738 \pm 18 | 806 \pm 25 | 765 \pm 13 | 885 \pm 20* [@] | 869 \pm 22* [@] |
| THERAPEUTIC STUDY | | | | | |
| | HanSD-NS | HanSD-HS | TGR-NS | TGR-HS | TGR-HS-bosentan |
| Number of rats | 11 | 10 | 10 | 10 | 7 |
| Body weight (g) | 523 \pm 12 | 497 \pm 8 | 552 \pm 12 | 527 \pm 12 | 549 \pm 20 |
| Heart weight (mg) | 1268 \pm 27 | 1242 \pm 35 | 1770 \pm 32* | 1821 \pm 54* | 1886 \pm 61* |
| Relative heart weight (mg/100 g BW) | 242 \pm 2 | 249 \pm 4 | 322 \pm 9* | 346 \pm 10* | 346 \pm 13* |
| Kidney weight (mg) | 3709 \pm 107 | 3531 \pm 92 | 3755 \pm 98 | 3989 \pm 142 | 4232 \pm 129* |
| Relative kidney weight (mg/100 g BW) | 708 \pm 12 | 710 \pm 16 | 680 \pm 12 | 758 \pm 21 [@] | 774 \pm 23 [@] |

Data are means \pm SEM. Significantly different: * $p < 0.05$ vs. HanSD, [@] $p < 0.05$ vs. TGR on normal salt diet.

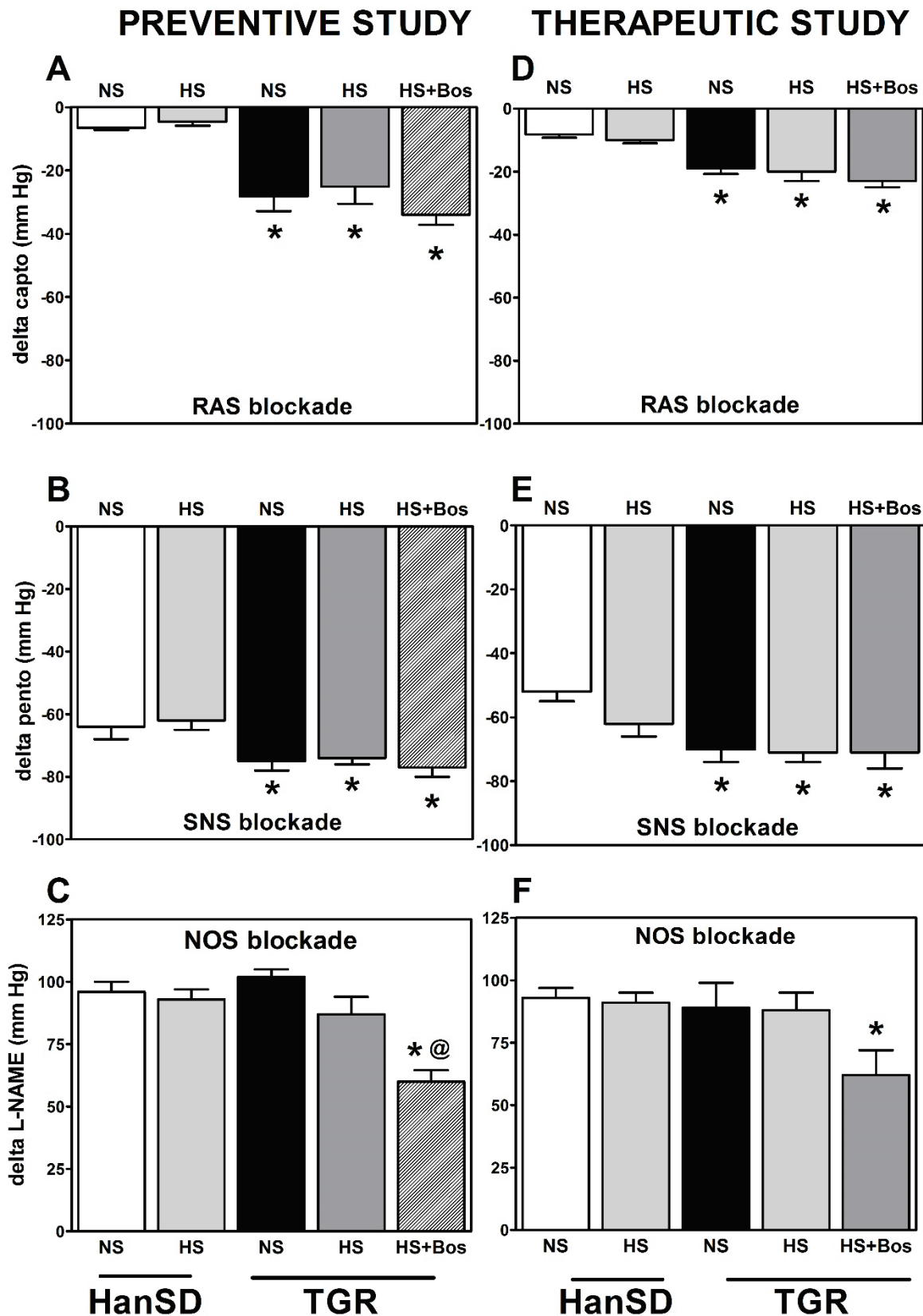


Fig. 2. The participation of major vasoactive systems contributing to BP maintenance – angiotensin II-dependent vasoconstriction (RAS blockade) (**A, D**), sympathetic vasoconstriction (SNS blockade) (**B, E**) and nitric oxide-dependent vasodilation (NOS blockade) (**C, F**). MAP changes were recorded at the end of the experiment in conscious young (preventive study) (**A-C**) and adult (therapeutic study) (**D-F**) normotensive control Hannover Sprague Dawley (HanSD) and hypertensive heterozygous Ren-2 transgenic rats (TGR) on normal salt (NS) diet, high-salt (HS) diet, or HS diet treated with nonselective ET_A/ET_B receptor antagonist bosentan (Bos). The data are the means ± SEM. * p<0.05 vs. control HanSD rats, @ p<0.05 vs. TGR on normal salt diet.

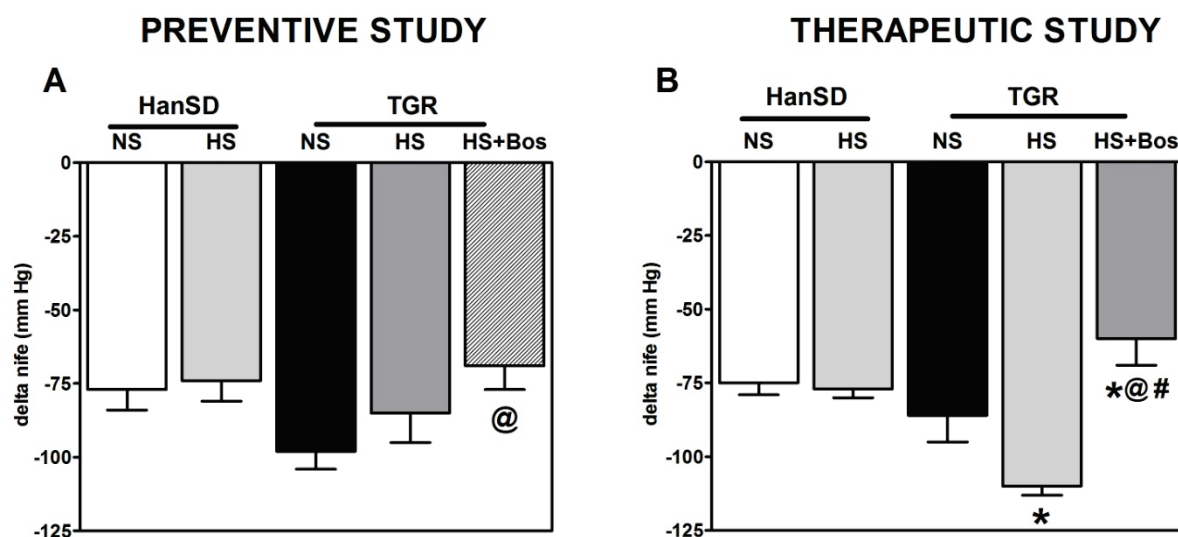


Fig. 3. Nifedipine-induced BP reduction elicited by the inhibition of calcium influx through the voltage-dependent calcium channels (L-VDCC) in young (preventive study) (A) and adult (therapeutic study) (B) normotensive control Hannover Sprague Dawley (HanSD) and hypertensive heterozygous Ren-2 transgenic rats (TGR) on normal salt (NS) diet, high-salt (HS) diet, or HS treated with nonselective ET_A/ET_B receptor antagonist bosentan (Bos). The data are the means \pm SEM. * $p < 0.05$, vs. control HanSD rats, @ $p < 0.05$ vs. TGR on normal salt diet, # $p < 0.05$ vs. TGR on high-salt diet.

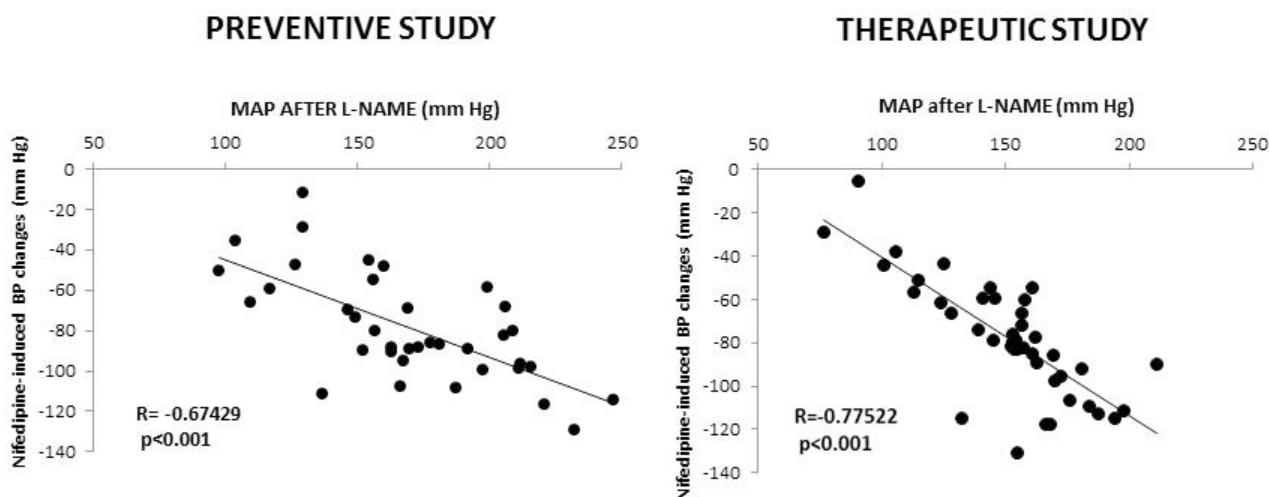


Fig. 4. The correlation of mean arterial pressure after complete vasoactive blockade (captopril, pentolinium and L-NAME) with MAP response to nifedipine-induced inhibition of calcium influx through the voltage-dependent calcium channels (L-VDCC) in young (preventive study) (left panel) and adult (therapeutic study) (right panel) rats.

Discussion

Our study demonstrated that i) high-salt intake did not aggravate hypertension development in TGR rats, ii) their high BP is dependent on different vasoconstrictor systems in young and adult hypertensive rats, iii) although bosentan had no effect on blood pressure and hence on the major vasoconstrictor systems contributing to BP maintenance, it significantly decreased NO-dependent vasodilation and nifedipine-sensitive calcium influx through the voltage-dependent calcium channels.

The blood pressure of hypertensive heterozygous male TGR was significantly higher in young rats than in the adults. This finding is quite intriguing since this phenomenon – decreasing BP with advancing age – was typically described for female TGR (Cargnelli *et al.* 1998, Vaněčková *et al.* 2011) but only rarely for aged male TGR (Kasper *et al.* 2005). A high salt intake did not influence the blood pressure level either in the preventive or in the therapeutic study, suggesting that young heterozygous TGR animals are not more sensitive to the increased sodium intake.

We confirmed our previous findings (Vaněčková

et al. 2012) that the contribution of major vasoconstrictor systems to high blood pressure in heterozygous TGR is age-dependent – RAS-dependent vasoconstriction being the leading system in young animals while the sympathetic-vasoconstriction prevailed with advancing age.

The fact that we did not observe any blood pressure lowering effect of nonselective endothelin receptor antagonist bosentan in Ren-2 transgenic rats is not unexpected since both our study and others did not show any substantial blood pressure decrease either in heterozygous (Andreis *et al.* 2000, Vaněčková *et al.* 2006, Vernerová *et al.* 2008) or in homozygous rats (Vaněčková *et al.* 2005, Opočenský *et al.* 2006). In this context, it is also not surprising that no changes in the contribution of major vasoconstrictor systems following bosentan treatment were observed in the present study.

By contrast, a substantial decrease of L-NAME-induced NO-dependent vasorelaxation was discerned in bosentan-treated salt-loaded TGR, which is a finding similar to that seen with a selective endothelin receptor A antagonist atrasentan in our previous study (Vaněčková *et al.* 2015). While the reduction of NO-dependent vasodilation is understandable in the present study with bosentan, having in mind that ET_B receptors mediate the endothelin effects on nitric oxide and cyclooxygenase production, the same finding with a selective ET_A blockade was rather surprising. We cannot offer a satisfactory explanation for this finding but one should consider a possible interaction of both receptors, which has been recently described by Vercauteren *et al.* (2017). These authors demonstrated that for fluid retention encountered during an endothelin ET_A receptor blockade, the increased vascular permeability resulting from the concurrent activation of ET_B receptors is responsible.

There was a significantly attenuated nifedipine-sensitive calcium influx through L-VDCC in TGR on high-salt diet treated with bosentan. A similar decrease,

although more prominent was reported in salt-loaded TGR rats subjected to atrasentan treatment (Vaněčková *et al.* 2015). Our previous assumption that this is associated with a higher activation of endothelin-induced calcium influx due to high salt intake could be excluded since this was true only in the therapeutic study but not in the prevention study. Our correlation analysis revealed that a nifedipine-induced BP change correlates strongly with the basal BP, a phenomenon that has been reported for other hypertensive strains (Zicha *et al.* 2014). However, when the salt-loaded TGR subjected to bosentan treatment was added, the correlation was attenuated. In addition, a highly important correlation of nifedipine-induced BP change was also observed for the BP after L-NAME administration, thus suggesting a major role of nitric oxide in the closure of L-type voltage dependent calcium channels.

In conclusion, the selective or nonselective endothelin receptor antagonists share important similarities which are independent of blood pressure, both groups affecting NO-dependent vasodilation and calcium influx through L-type voltage-dependent calcium channels.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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