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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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Abstract

Objective: 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-VDCC) and attenuated angiotensin II-dependent vasoconstriction. We were interested whether bosentan (nonselective ET_A/ET_B receptor antagonist) would have similar effects.

Methods: Young 4-week-old (preventive study) and adult 8-week-old (regression 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).

Results: 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.

Conclusion: Although bosentan did not result in any blood pressure lowering effects, it substantially influenced NO-dependent vasodilation and calcium influx through L-VDCC 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.

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, Hypertension Res, 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, Life Sci, 2016]. A similar reduction of Ca 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; Vaneckova 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 non-selective 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.

MATERIALS AND 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 (regression 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) started 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 (5 mg/kg) was administered following NO-donor nitroprusside (5 mg/kg). 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 regression studies (Fig. 1). The BP difference was more pronounced in young animals than in adults (181 ± 5 mmHg vs. 156 ± 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 regression 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 observed.

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 regression 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 regression studies (Fig. 4A and 4B).

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 vasoactive 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; Vaneckova et al, KBPR, 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 regression study, suggesting that young heterozygous TGR animals are not more sensitive to the increased sodium intake.

We confirmed our previous findings (Vaněčková et al., *Hypertens Res*, 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) [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 diets 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 regression 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 which 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.

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Conflict of interest

There are no conflicts of interest.

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FIGURE LEGENDS

Fig. 1. The blood pressure of young (preventive study)(A) and adult (regression 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 ± SEM. * p<0.05, vs. control HanSD rats.

Fig. 2. The participation of major vasoactive systems contributing to BP maintenance – angiotensin II-dependent vasoconstriction (RAS blockade)(A), pentolinium-induced vasoconstriction (SNS blockade)(B) and nitric oxide synthase-dependent vasodilation (NOS blockade)(C). MAP changes were recorded at the end of the experiment in conscious young (preventive study)(A) and adult (regression 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 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-sensitive BP reduction elicited by the inhibition of calcium influx through the voltage-dependent calcium channels (L-VDCC) in young (preventive study)(A) and adult (regression 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 ± SEM. * p<0.05, vs. control HanSD rats, @ p<0.05 vs. TGR on normal salt diet.

Fig. 4. The correlation of mean arterial pressure after NOS inhibition with L-NAME with MAP response to nifedipine-induced inhibition of calcium influx through the voltage-dependent calcium channels (L-VDCC) in young (preventive study)(top panel) and adult (regression study) (bottom panel) rats.

Fig. 1

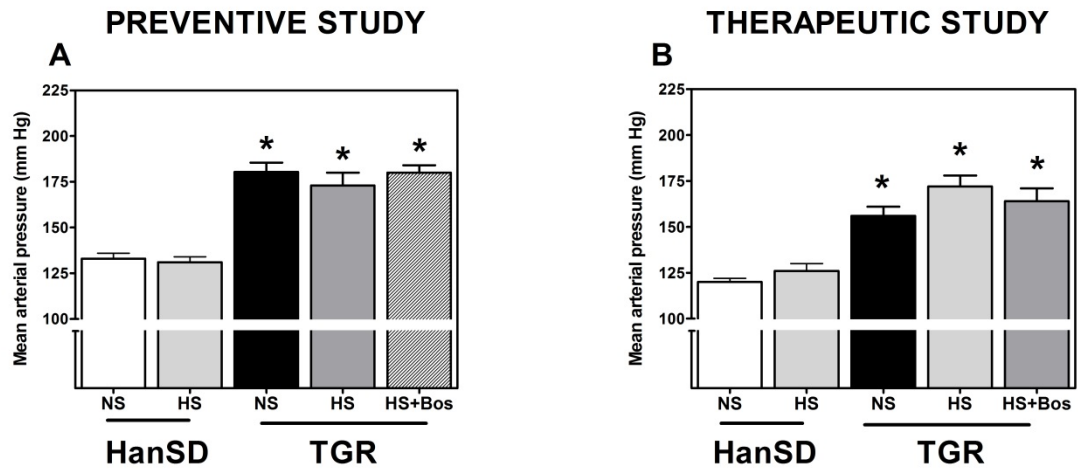


Fig. 2

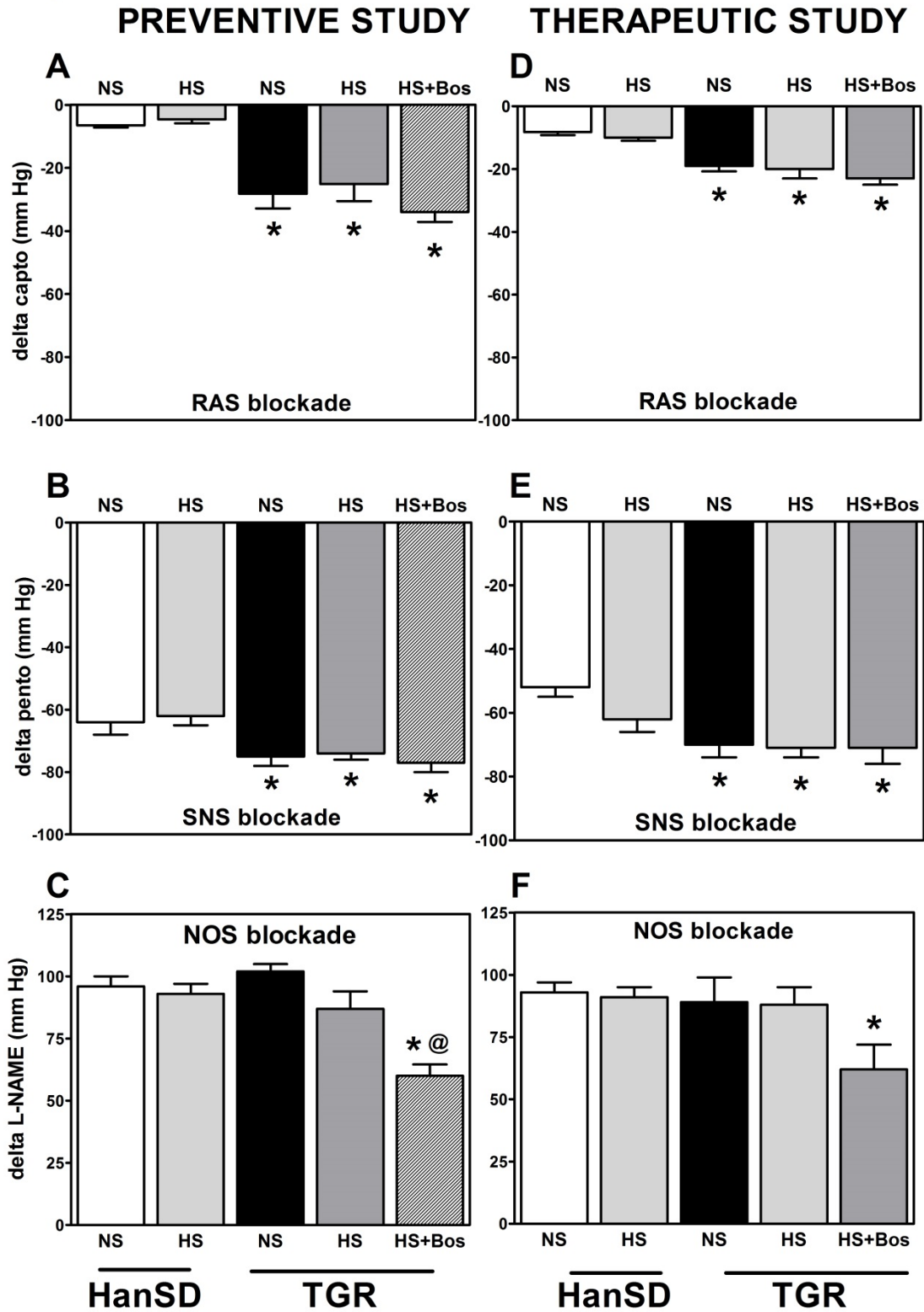


Fig. 3

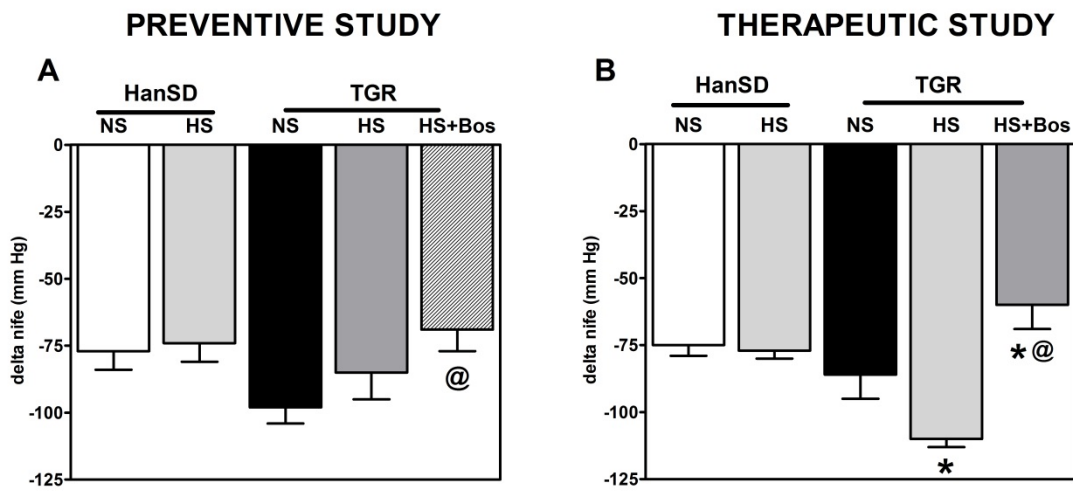


Fig. 4

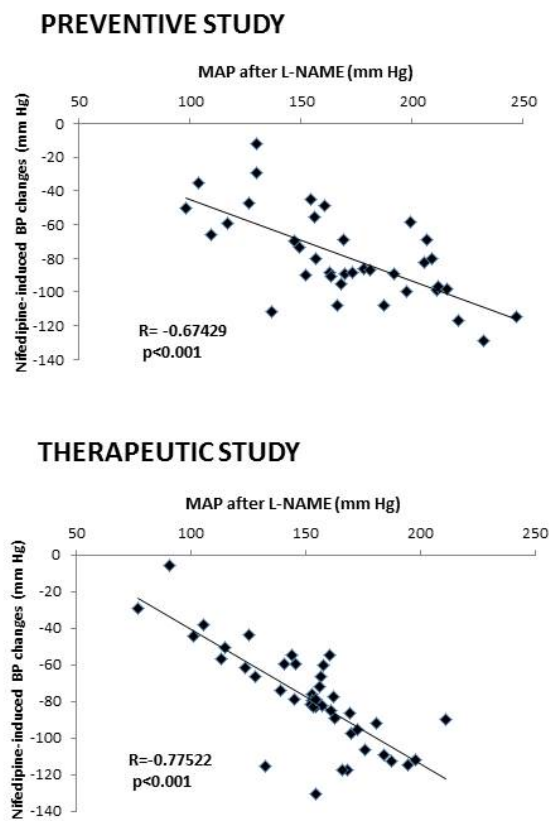


Table 1.

PREVENTIVE STUDY

	HanSD-NS (7)	HanSD-HS (7)	TGR-NS (10)	TGR-HS (12)	TGR-HS-bosentan (9)
Body weight (g)	408±8	432±9	389±14	387±15	403±16
Heart weight (mg)	1165±30	1243±80	1406±41*	1533±57*	1488±55*
Heart weight/ Body weight (mg/g)	285±7	286±15	363±5*	397±8*®	372±16*
Kidney weight (mg)	3010±80	3487±145	2971±118	3408±110	3496±137®
Kidney weight/ Body weight (mg/g)	738±18	806±25	765±13	885±20*®	869±22*®

REGRESSION STUDY

	HanSD-NS (11)	HanSD-HS (10)	TGR-NS (10)	TGR-HS (10)	TGR-HS-bosentan (7)
Body weight (g)	523±12	497±8	552±12	527±12	549±20
Heart weight (mg)	1268±27	1242±35	1770±32*	1821±54*	1886±61*
Heart weight/Body weight (mg/g)	242±2	249±4	322±9*	346±10*	346±13*
Kidney weight (mg)	3709±107	3531±92	3755±98	3989±142	4232±129*
Kidney weight/Body weight (mg/g)	708±12	710±16	680±12	758±21®	774±23®

* against HanSD

® against TGR-NS