REVIEW

This paper is a tribute to Michal Behuliak, PhD (1982-2023) and to Czecho-Slovak scientific cooperation

Altered Balance between Vasoconstrictor and Vasodilator Systems in Experimental Hypertension

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Received October 21, 2024
Accepted November 14, 2024

Summary

Sympathetic hyperactivity and relative NO deficiency are characteristic alterations in both genetic and salt hypertension. The contribution of these abnormalities to blood pressure (BP) maintenance can be determined in conscious rats using a consecutive blockade of particular vasoactive systems. Thus, the contribution of pressor effects of angiotensin II to the maintenance of high BP is usually small, but the role of renin-angiotensin system in the development of hypertension mediated by central and peripheral effects of angiotensin II on sympathetic activity is highly important. This is even true in angiotensin-dependent hypertension of heterozygous Ren-2 transgenic rats in which sympathetic hyperactivity is increasing with age. Central sympathoexcitation in this hypertensive model can be inhibited by lower losartan doses than peripheral angiotensin II-dependent vasoconstriction. This experimental model also yielded important knowledge on nephroprotective effects of new therapeutic drugs - endothelin receptor type A blockers. A considerable part of sympathetic vasoconstriction is dependent on the interaction of Ca^{2+} sensitization (RhoA/Rho kinase pathway) and Ca^{2+} influx (through L-VDCC). The blockade of these pathways prevents a major part of sympathetic vasoconstriction. Ca^{2+} sensitization seems to be attenuated in genetic hypertension in order to compensate increased Ca^{2+} influx. In contrast, enhanced Ca^{2+} sensitization is a hallmark of salt sensitivity in Dahl rats in which salt hypertension is dependent on increased Ca^{2+} influx. The attention should also be paid to the impairment of arterial baroreflex sensitivity which permits enhanced BP responses to pressor or depressor stimuli. Some abnormalities can be studied in blood vessels isolated from hypertensive rats but neither conduit arteries nor mesenteric resistance arteries represent the vascular beds decisive for the increased peripheral resistance and high BP.

Keywords

Sympathetic vasoconstriction • NO-dependent vasodilatation • Calcium sensitization • Calcium influx • Arterial baroreflex • Spontaneously hypertensive rats • Salt hypertensive Dahl rats • Ren-2 transgenic rats • RAS blockade • SNS blockade • NOS inhibition • Endothelin • Vascular contraction and relaxation • Isolated conduit and resistance arteries • EDCF • PGI_2 • BK_{Ca} channels

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Fig. 1. Dr. Michal Behuliak graduated at the Faculty of Natural Sciences, Comenius University (Bratislava, Slovakia) in 2006. He performed his Ph.D. Thesis at the Institute of Pathological Physiology, Faculty of Medicine, Comenius University in Bratislava (2006-2010). Since 2008 he cooperated with our laboratory as a Visiting Scientist. Thereafter he became Postdoctoral Fellow, Junior and Senior Researcher at the Institute of Physiology, Czech Academy of Sciences in Prague.

Our Laboratory of Experimental Hypertension paid considerable attention to the role of various endogenous vasoconstrictors and vasodilators, which might be involved in the increase of peripheral resistance in systemic hypertension. Since 1983 we often used the acute blockade of particular vasoactive systems to estimate their contribution to BP maintenance in hypertensive or normotensive rats. Initially, we focused on two interesting vasoactive factors participating in age-dependent DOCA-salt hypertension – digoxin-like factor and vasopressin. Our studies [1-4] revealed a more important role of digoxin-like factor in BP maintenance of immature DOCA-salt hypertensive rats as compared to adult animals. In contrast, the contribution of pressor effects of vasopressin to BP control was greater in adult DOCA-salt-treated rats [5]. Furthermore, we demonstrated a more

pronounced BP reduction in young than in adult DOCA-salt hypertensive rats after the acute blockade of α_1-adrenergic receptors by prazosine [6]. We were also interested in the alterations of arterial baroreflex in young and adult salt hypertensive Dahl SS/Jr rats as we studied this model of age-dependent salt hypertension since 1986 [7-9]. At that time we demonstrated more pronounced alterations of arterial baroreflex in young than in adult salt hypertensive animals [10,11], but we did not understand fully the importance of this finding for the interpretation of our findings on the contribution of particular vasoactive systems to BP maintenance in various hypertensive models.

Balance of vasoactive systems in normotension and hypertension

In 2000 we began to use the acute sequential blockade of major vasoconstrictor and vasodilator systems (renin-angiotensin system, RAS; sympathetic nervous system, SNS; nitric oxide, NO) [12,13] using a modification of the method described by Minami *et al.* [14].

It is well known that sympathetic vasoconstriction and NO-dependent vasodilatation are major players in BP maintenance. Both vasoactive systems operate at a reasonable balance in normotensive rats. Thus, the acute blockade of sympathetic nervous system by ganglionic blocker pentolinium causes a rapid pronounced BP reduction, which is fully restored by BP elevation elicited by the acute inhibition of NO synthase with L-NAME (Fig. 2). These BP changes seem to reflect the extent of vasoconstriction or vasodilatation exerted by the studied vasoactive systems. This method can be expanded to further vasoconstrictor (angiotensin II, vasopressin, endothelin-1, superoxide) or vasodilator and (prostacyclin, acetylcholine, potassium channels) mechanisms. Thus, we can evaluate the actual contribution of various vasoactive systems to BP maintenance also in hypertensive animals.

In intact rats the BP stability is maintained by the operation of the arterial baroreflex. The application of ganglionic blockade within our procedure enables to determine the full extent of pressor and depressor reactions without the influence of compensatory baroreflex-mediated changes in heart rate and vascular sympathetic nerve activity. Thus, the magnitude of L-NAME-induced acute BP rise is twice as high when measured after ganglionic blockade compared to BP changes recorded before ganglionic blockade. This is caused by the rapid reduction of heart rate and sympathetic vasoconstriction, which compensate L-NAME-induced BP changes in intact rats [15]. Similarly, BP reduction induced by pentolinium is twice greater in intact animals than in animals pretreated with L-NAME in which the sympathetic tone is already attenuated through baroreflex operation. On the other hand, the acute ACE inhibition by captopril does not modify BP response to ganglionic blockade and *vice versa* [16].

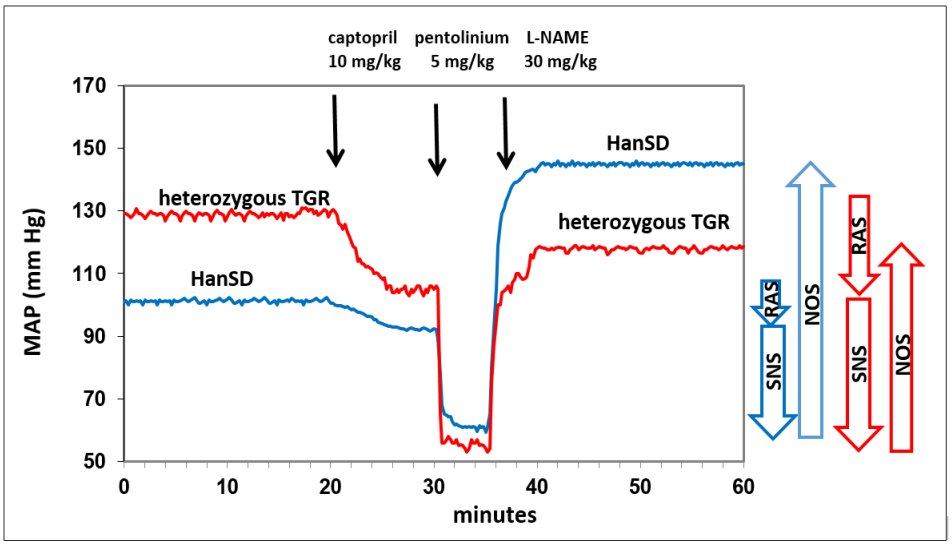

Fig. 2. The time course of BP changes achieved by acute sequential blockade of renin-angiotensin system (RAS) by captopril, sympathetic nervous system (SNS) by pentolinium and NO synthase (NOS) by L-NAME in young normotensive Han-SD rats and heterozygous TGR with angiotensin II-dependent hypertension (modified from [43]).

In order to evaluate the balance between sympathetic vasoconstriction and NO-dependent vasodilatation, we can express BP changes induced by pentolinium or L-NAME as a percentage of basal BP. This is especially important for the evaluation of NO participation in BP control. The absolute NO deficiency, i.e. the reduced L-NAME-induced BP rise, is rarely found in experimental hypertension (except that one caused by a chronic L-NAME administration). On the other hand, relative NO deficiency is often detected in various forms of experimental hypertension. In such a case, the acute L-NAME-induced BP changes are greater in animals with higher basal BP. However, this augmentation of NO-dependent vasodilatation does not fully compensate for the extent of excessive sympathetic vasoconstriction. Therefore, the absolute L-NAME-induced BP changes correlate positively with basal BP, whereas the relative BP changes correlate negatively. These relationships were disclosed in both genetic and salt hypertension, i.e. in spontaneously hypertensive rats (SHR) (Fig. 3) and in Dahl rats (Fig. 4). Similar conclusions can be made when we calculate the ratio of BP changes induced by pentolinium and L-NAME [12,17,18].

Vasoactive balance in various forms of experimental hypertension

Our initial attention was paid to the age-dependent salt hypertension in Dahl rats [12,18], genetic hypertension in hereditary hypertriglyceridemic (hHTG) rats [13], and NO-deficient hypertension of rats chronically treated with L-NAME (inhibitor of NO synthase) [19]. Later we also used this approach in recombinant inbred strains [20] which were derived from F_2 hybrids of spontaneously hypertensive rats (SHR) with normotensive Brown-Norway (BN.lx) rats by Pravenec *et al.* [21]. To our surprise, the maintenance of elevated BP was highly dependent on the enhanced contribution of sympathetic nervous system in all above mentioned models, whereas the contribution of the pressor effects of renin-angiotensin system was rather small. Another important finding was that the absolute NO deficiency was found only in L-NAME hypertension [19], whereas all other models of genetic or salt hypertension were characterized by a relative NO deficiency where augmented NO-dependent vasodilatation was insufficient to counteract enhanced sympathetic vasoconstriction [12,13,18,20,22].

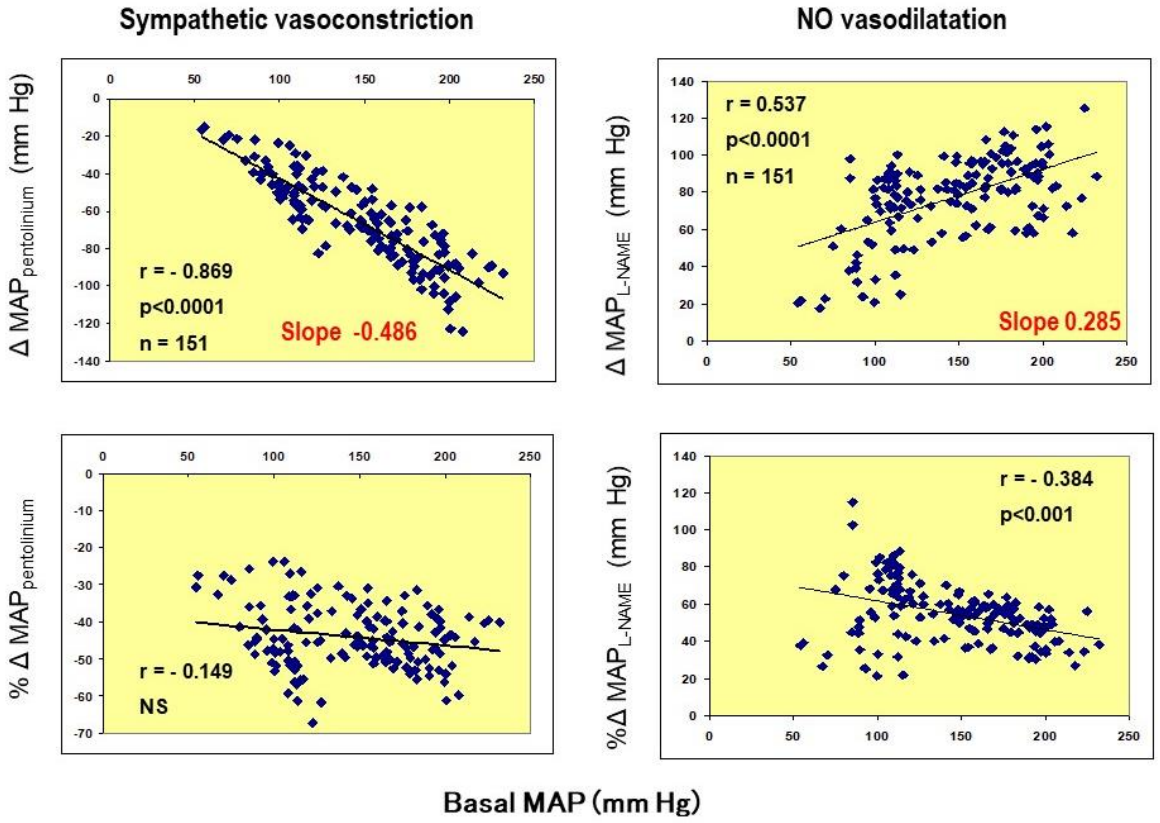

Fig. 3. The contribution of sympathetic nervous system and nitric oxide (NO) to blood pressure maintenance in spontaneously hypertensive rats (SHR) – absolute BP changes (upper panels) and relative BP changes (lower panels) (data from [22])

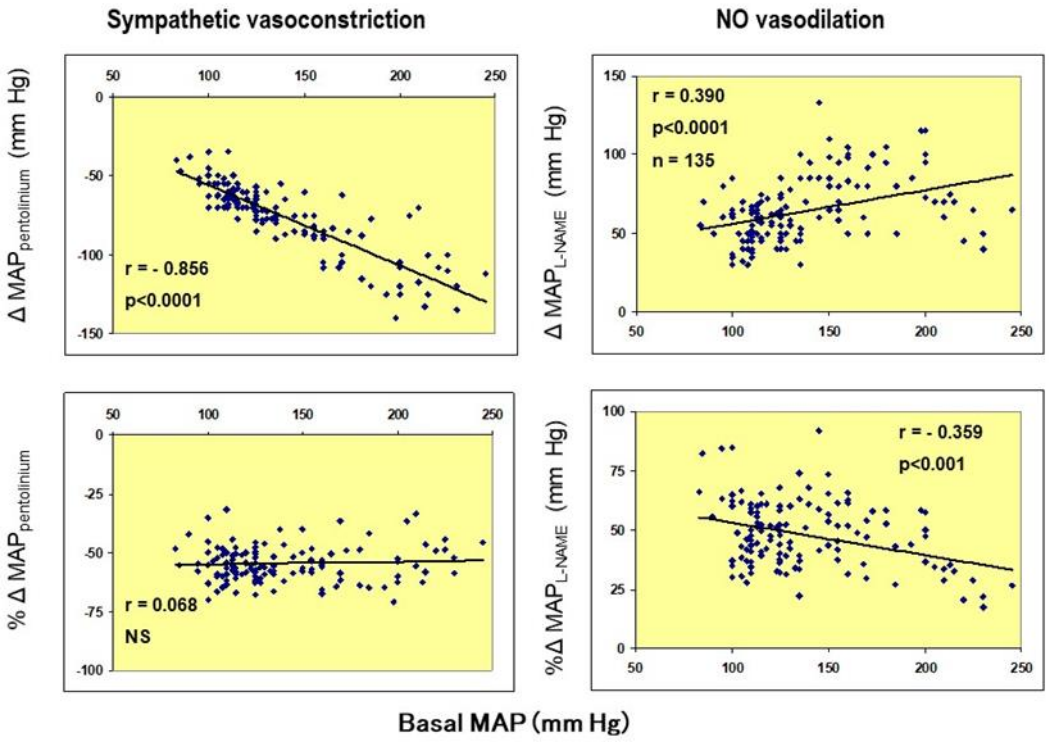

Fig. 4. The contribution of sympathetic nervous system and nitric oxide (NO) to blood pressure maintenance in Dahl rats - absolute BP changes (upper panels) and relative BP changes (lower panels) (Zicha *et al.*, unpublished data).

In Dahl rats we tried to evaluate the role of oxygen free radicals and their interaction with NO in the mechanisms maintaining blood pressure. We observed that BP reduction elicited by the acute administration of tempol (superoxide dismutase mimetic) was augmented in young salt hypertensive Dahl rats compared to either normotensive salt-resistant Dahl rats [12] or adult salt hypertensive Dahl animals [18]. The acute tempol pretreatment enhanced NO-dependent vasodilation in young salt hypertensive Dahl rats but not in other experimental groups [12]. Such changes were not observed in adult salt hypertensive Dahl rats [18]. This age-dependent difference might be ascribed to a greater impairment of baroreflex control in young Dahl rats [11], which could be responsible for the less efficient sympathetic compensation of BP reduction induced by acute tempol administration in young than in adult animals.

Behuliak *et al.* [23] tried to evaluate the contribution of various vasodilator systems in several forms of experimental hypertension, including SHR and salt hypertensive Dahl rats. He compared the vasodilator role of endogenous prostanoids, large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels and nitric oxide using their acute consecutive blockade in conscious rats. There was an enhanced vasodilation mediated by endogenous prostanoids or BK_{Ca} channels but a severe relative NO deficiency in both hypertensive models. Thus, the overall activity of vasodilator systems was not sufficient to compensate the sympathetic vasoconstriction present in hypertensive animals [23].

Our further research was focused on the changes in the balance between vasoconstrictor and vasodilator systems induced by chronic interventions in Dahl rats with salt hypertension elicited either in youth or in adulthood. The first study [24] demonstrated major protective effects of increased potassium intake against salt hypertension development in young but not in adult animals, whereas therapeutic effects of increased potassium intake in young Dahl rats with established salt hypertension were negligible. The attenuation of salt hypertension development achieved by preventive action of high K^+ intake in young Dahl rats was mediated by the reduction of sympathetic as well as nifedipine-sensitive vasoconstriction. The relative deficiency of certain vasodilator mechanisms (NO, large conductance Ca^{2+}-activated K^+ channels) was not improved by increased potassium intake [24]. The second study [25] evaluated the antihypertensive effects of chronic blockade of endothelin type A receptors by ambrisentan on the development of salt hypertension in young and adult Dahl rats, which attenuated the development of salt hypertension only in

adult but not in young rats. The BP reduction observed in adult ambrisentan-treated rats was mediated by the attenuated sympathetic BP component [25]. The third study [26], which was focused on the preventive effects of chronic antioxidant treatment in Dahl rats, indicated that chronic tempol administration attenuated salt hypertension development only in adult animals and this was again due to the reduction of sympathetic vasoconstriction. In the adult salt hypertensive rats, there was a highly significant correlation of basal BP with aortic superoxide production or renal lipoperoxidation [26].

Another series of experiments was devoted to the effects of chronic antihypertensive (captopril) or antioxidant (N-acetylcysteine) treatments in L-NAME hypertensive rats [27,28]. Both these chronic preventive interventions attenuated the development of NO-deficient hypertension but captopril treatment surprisingly decreased sympathetic vasoconstriction, whereas N-acetylcysteine administration decreased the production of reactive oxygen species and augmented NO-dependent vasodilatation [27,28]. Both preventive and therapeutic effects of chronic N-acetylcysteine administration were also demonstrated in young and adult SHR [29,30].

We performed two large F_2 hybrid studies in either Dahl rats or hereditary hypertriglyceridemic (hHTG) rats. The first study [18], which was carried out in Dahl SS/Jr x SR/Jr F_2 hybrids fed a high-salt diet, revealed a highly significant contribution of enhanced sympathetic vasoconstriction to BP maintenance and a relative NO deficiency because only half of sympathetic vasoconstriction was counteracted by the enhanced NO-dependent vasodilatation. In contrast, there was a very small contribution of angiotensin II-dependent vasoconstriction or residual BP elevation (recorded after a combined blockade of RAS and SNS) [18]. Our second study [13] disclosed in hHTG x LEW F_2 hybrids that their BP maintenance was highly dependent on sympathetic vasoconstriction and only partially dependent on the increased residual BP (structural remodeling of resistance arteries), whereas there was no significant relationship of basal BP to angiotensin II-dependent vasoconstriction or NO-dependent vasodilation. Thus, the importance of enhanced sympathetic vasoconstriction and relative NO deficiency was demonstrated even in this model [13].

Our early studies on blood pressure maintenance revealed the enhanced contribution of the sympathetic nervous system and relative NO deficiency in both genetic and salt hypertension. The experiments based upon acute ganglionic blockade followed by NO synthase inhibition demonstrated that NO-dependent vasodilatation is enhanced in hypertensive rats but not to the extent, which could effectively counteract the augmented sympathetic vasoconstriction. Increased production of reactive oxygen species might participate in the attenuation of NO-dependent vasodilatation especially in salt hypertension.

The adrenergic vasoconstriction and NO-dependent vasodilatation – the role of calcium entry through L type voltage-dependent calcium channels

There was a long-term interest of our lab in cell calcium handling [31,32], which we studied in platelets [33] or in vascular smooth muscle cells [34] of normotensive and hypertensive rats. Three young Slovak scientists – Ludovít Paulis, Silvia Líšková and Mária Pintérová – joined our lab in 2005 when we were interested in the participation of Ca^{2+} entry through voltage-dependent calcium channels of L type (L-VDCC) in adrenergic vasoconstriction which is enhanced in rats with genetic hypertension.

Our initial experiments indicated that Ca^{2+} entry plays an important role in the tonic phase of adrenergic contraction of isolated arteries, which is attenuated by the presence of endothelium. In contrast, the early rapid phase of adrenergic vasoconstriction, which is mediated by calcium mobilization from internal stores, is independent of both Ca^{2+} entry and endothelium-dependent control. This interesting phenomenon was then investigated in isolated femoral arteries of SHR and WKY rats [35]. We found that both phasic and tonic phases of adrenergic arterial contraction were enhanced in SHR arteries compared to WKY ones but nifedipine (L-VDCC blocker) acted almost entirely on the tonic contraction. Nifedipine-induced vascular relaxation was proportional to the wall tension of NE-precontracted arteries, which was greater in SHR vessels. Surprisingly, there was a highly significant correlation between basal BP level and the magnitude of nifedipine-induced BP reduction in conscious SHR, salt hypertensive Dahl rats and L-NAME hypertensive rats [17]. Our further studies [35,36] revealed that Ca^{2+} entry through L-VDCC is an important part of sympathetic vasoconstriction. Furthermore, we found that the chronic treatment of SHR with ACE inhibitor captopril lowered their BP by the decrease of sympathetic vasoconstriction, which was associated with the attenuated nifedipine-induced BP reduction to the level similar to that seen in intact WKY rats [35,37].

Our later paper [22] demonstrated a close relationship of sympathetic BP component to nifedipine-sensitive BP component in young and adult SHR, which were treated with captopril or hydralazine. This was also true in the animals in which antihypertensive treatment was withdrawn for four weeks. On the other hand, angiotensin II-dependent BP component was of minor importance in SHR. However, the relationships of pentolinium- or nifedipine-induced BP changes to basal BP in the studied SHR population were so strong that they were significant even when expressed as relative BP changes, i.e. in percentage of basal BP. The greatest surprise of our study [22] was an excellent correlation between nifedipine- and pentolinium-induced BP changes, although BP effects of pentolinium and nifedipine had to be measured in different animals because the pretreatment with the former drug substantially diminished BP effects of the latter drug and *vice versa* [36].

Another our study [38] examined the mechanisms responsible for long-term BP attenuation in SHR subjected to a transient captopril treatment in prepuberty and puberty [39]. At the age of 30-34 weeks, we compared untreated SHR with those subjected to transient ACE inhibition either in youth or in adulthood. After the drug withdrawal BP was reduced in both groups but the BP reduction was more pronounced in SHR treated in prepuberty. The reduction of sympathetic and/or nifedipine-sensitive BP components was responsible for the observed attenuation of hypertension in treated SHR [38].

A major progress in our research on the above topic was brought by Mária Pintérová, who studied the role of nifedipine-sensitive sympathetic vasoconstriction in SHR by inactivating inhibitory G proteins (Gi) using pertussis toxin (PTX) [40]. Since the overexpression of pertussis toxin (PTX)-sensitive Gi proteins, which leads to decreased cAMP levels, plays a role in the development and maintenance of high BP in SHR, she studied the involvement of Gi proteins in the pathway through which noradrenergic vasoconstriction and calcium influx through L-VDCC can be coupled. The inactivation of Gi proteins by PTX leading to enhanced cAMP production caused significantly greater BP decrease in hypertensive than in normotensive rats. Pintérová *et al.* [40] demonstrated that a pronounced reduction of BP in PTX-pretreated rats was due to a decreased sympathetic vasoconstriction, which was partially compensated by the enhancement of angiotensin II-dependent vasoconstriction. PTX pretreatment of SHR considerably attenuated not only the dose-dependent BP response to NE administration but also nifedipine-induced BP reduction. It should be pointed out that PTX pretreatment of SHR or WKY rats substantially reduced their BP sensitivity to norepinephrine (NE) compared to untreated animals. The strain differences in sympathetic vasoconstriction were almost abolished by PTX pretreatment, which also prevented the strain differences in nifedipine effects on NE-induced BP response [36,40]. It was concluded that hypotensive action of chronic Gi proteins inactivation by *in vivo* PTX treatment in SHR is associated with a major reduction of sympathetic vasoconstriction. Thus, the increased nifedipine-sensitive calcium influx through L-VDCC is the main mechanism by which the elevated sympathetic activity contributes to the maintenance of hypertension in SHR. This control of calcium entry *via* L-VDCC through augmented sympathetic activity is predominantly mediated by Gi protein-coupled pathway the effects of which are partially antagonized by the action of nitric oxide on these channels leading to endothelium-dependent vasodilatation [36].

In parallel, Silvia Líšková studied the effects of PTX pretreatment on NE-induced contraction of femoral arteries using wire myography [41]. She observed that adrenergic contraction of arteries isolated from PTX-pretreated WKY rats was attenuated in the presence as well as in the absence of endothelium. The differences in adrenergic contraction of arteries from intact or PTX-pretreated animals as well as the differences between the arteries with intact endothelium and endothelium-denuded arteries were abolished in the presence of nifedipine [41]. Surprisingly, PTX pretreatment did not abolish endothelium-dependent attenuation of NE-induced arterial contraction. The further *in vitro* studies indicated that nifedipine also abolished arterial contraction elicited by α_2-adrenergic agonist clonidine and attenuated the contraction induced by α_1-adrenergic agonist phenylephrine [36]. Michal Behuliak, who came to our lab in 2008, focused his attention on the *in vivo* vasoconstriction mediated by α_1- and α_2-adrenergic receptors. He demonstrated in conscious rats that both types of receptors participate in NE-induced BP elevation, the role of α_2-adrenergic receptors being more important in SHR than in WKY rats. In addition, his experiments indicated that in both rat strains, the α_1- and α_2-adrenergic vasoconstriction was attenuated by the pretreatment with nifedipine [36].

It should be noted that Ca^{2+} entry through L-VDCC also participate in vascular contraction and BP elevation elicited by NO synthase inhibition by L-NAME.

This was true not only *in vitro* but also *in vivo*, when nifedipine injection prevented BP rise elicited by acute L-NAME administration, whereas the injection of nifedipine after L-NAME administration lowered the increased BP [36]. Since both NO-induced cGMP and catecholamine-induced cAMP play a role in vasodilation, Pintérová *et al.* [42] studied their interaction in SHR and WKY rats. She reported that cAMP overproduction elicited by the infusion of β-adrenoreceptor agonist isoprenaline caused a more pronounced decrease of basal BP in SHR than in WKY rats. Isoprenaline infusion also prevented BP rise induced by acute NO synthase blockade and abolished the fully developed BP elevation induced by acute L-NAME administration. The cAMP-mediated prevention of L-NAME-induced BP rise was diminished by the inhibition of large-conductance Ca^{2+}-activated (BK_{Ca}) and voltage-gated (Kv) potassium channels but this was true only for SHR. In contrast, a combined blockade of BK_{Ca} and Kv potassium channels was necessary to achieve this effect in WKY rats. These results indicated that the overall contribution of K^+ channels to cAMP vasodilator mechanisms is insufficient in genetic hypertension since a concurrent activation of both types of K^+ channels is necessary to prevent BP elevation elicited by acute NO/cGMP deficiency in SHR. Thus, although the activity of K^+ channels is higher in SHR than in WKY rats, their vasodilator effects can not sufficiently match the enhanced vasoconstriction in this hypertensive strain [42].

Ca^{2+} influx through L-VDCC participates in tonic α_1- and α_2-adrenergic vasoconstriction which is also attenuated by endothelial products such as NO or PGI_2. Vasoconstrictor effects of enhanced Ca^{2+} influx are also reduced by the activation of large-conductance Ca^{2+}-activated K^+ channels in vascular smooth muscle. The acute L-VDCC blockade by nifedipine lowers blood pressure irrespective of whether it was elevated by increased adrenergic stimulation or decreased NO production. Therefore, nifedipine administration always reduced blood pressure proportionally to its initial level. The activation of BK_{Ca} channels by increased intracellular calcium or Kv channels by membrane depolarization causes vasorelaxation in both normotensive and hypertensive rats but this antihypertensive action is less efficient in SHR. The detailed comparison of BP changes elicited by the acute blockade of adrenergic vasoconstriction (pentolinium) or by the blockade of Ca^{2+} influx through L-VDCC (nifedipine) indicated that the participation of Ca^{2+} influx in BP maintenance is especially pronounced in hypertensive rats in which nifedipine-induced BP changes clearly surpassed those induced by pentolinium.

Transgenic rats with murine Ren-2 gene

A new hypertensive model, i.e. Ren-2 transgenic rats (TGR), was brought to our lab by Ivana Vaněčková who joined us in 2010. Our research on TGR started with a study that evaluated the contribution of major vasoactive systems to BP regulation in young and adult heterozygous and homozygous TGR (bearing one or two alleles of murine Ren-2 gene). The renin-angiotensin system was the major system maintaining elevated BP in young homozygous rats, while there was an important contribution of the sympathetic nervous system in heterozygous TGR which was increasing with age. Importantly, the repeated antisense therapy with oligonucleotides against angiotensin II type 1 (AT_1) receptor was most effective in young homozygous TGR, where it substantially reduced angiotensin-dependent vasoconstriction [43]. Later, we focused on the mechanism of BP lowering achieved by the selective blockade of endothelin receptor A (ET_A) with atrasentan in heterozygous TGR kept on a high-salt diet, which is a stimulus for ET system activation. Atrasentan attenuated the development of hypertension by the reduction of angiotensin-dependent vasoconstriction and reduced calcium influx through L-type voltage-dependent calcium channels (nifedipine-sensitive BP component) with no effect on sympathetic vasoconstriction. Compensatory vasodilator mechanisms (nitric oxide, endogenous prostanoids or Ca^{2+}-activated K^+ channels) were reduced in atrasentan-treated TGR, which suggests the attenuation of ET_B receptor-mediated vasodilation [44]. A combination of atrasentan with different classes of renin-angiotensin system blockers (direct renin inhibitor aliskiren, angiotensin receptor blocker losartan or angiotensin converting enzyme inhibitor trandolapril) demonstrated major effects of RAS blockers on BP level through the attenuation of both angiotensin-dependent and sympathetic vasoconstriction. Moreover, additional BP reduction achieved by their combination with atrasentan was mainly due to a reduction of calcium influx [45].

Several studies on the therapeutic potential of ET_A receptor blockade were performed in collaboration of Ivana Vaněčková with the group of Luděk Červenka from the Institute of Clinical and Experimental Medicine in Prague. These studies were performed in TGR animals in which either chronic kidney disease was induced by 5/6

nephrectomy [46-49] or heart failure was induced by aorto-caval fistula [50]. After 20 weeks of treatment of TGR with atrasentan, ET_A receptor blockade alone partially improved survival, reduced BP and transiently also decreased proteinuria. However, there was no additional effect of combination therapy with RAS inhibition (combined angiotensin receptor blockers and ACE inhibitors) at the end of the study [46]. However, the prolongation of treatment to 44 weeks led to a substantial increase of survival due to the reduction of proteinuria and glomerular damage in the group treated with a combination of atrasentan and RAS blockers [47]. Both these studies were performed in TGR animals treated early (one week) after 5/6 nephrectomy. However, the postponing of the treatment to the stage, in which chronic kidney disease is already developed (6 weeks after 5/6 nephrectomy), yielded quite opposite results. The long-term (44 weeks) treatment with ET_A receptor blockade added to combined RAS blockade did not result in any further improvement of survival, and it even worsened albuminuria, creatinine clearance, and glomerular injury [48]. The negative effects of a combination therapy with atrasentan were also demonstrated in TGR rats with 5/6 nephrectomy treated with combined RAS inhibition together with inhibition of soluble epoxide hydrolase (sEH), which resulted in a higher bioavailability of antihypertensive epoxyeicosatrienoic acids. The addition of atrasentan to the combined RAS and sEH blockade at the stage of developed chronic kidney disease did not offered any renoprotection, but even abolished the beneficial effects of adding sEH inhibitor to RAS blockade [49].

On the other hand, very promising results of ET_A blockade were achieved in two models of heart failure combined with chronic kidney damage, namely in hypertensive TGR with aorto-caval fistula [50] and normotensive HanSD rats with 5/6 nephrectomy and aorto-caval fistula [51]. In hypertensive TGR, atrasentan not only delayed the onset of decompensation phase of volume-overload heart failure, but in a combination with RAS therapy, it resulted in a reduction of albuminuria [50]. The increased renoprotection was also demonstrated in the second model, 5/6 nephrectomized normotensive HanSD with aorto-caval fistula, contributing to the lowering of their mortality.

The central mechanisms affecting BP maintenance were also studied in heterozygous Ren-2 transgenic rats. First, we compared chronic peroral and central (intracerebroventricular, icv) administration of angiotensin receptor blocker losartan and direct renin inhibitor aliskiren. Both treatments caused similar BP reduction, but their mechanisms differed – the central RAS blockade was dependent solely on the reduced sympathetic vasoconstriction, while systemic peroral administration reduced both angiotensin-dependent and sympathetic vasoconstriction, suggesting that central sympathoinhibition is more important than the attenuation of peripheral angiotensin-dependent vasoconstriction in adult TGR [52] In the developing TGR, the central sympathoinhibition is also important in their hypertension development, being susceptible to the inhibition by low doses of losartan (1 mg/kg/day), while higher doses (2 mg/kg/day) also attenuated peripheral angiotensin-dependent vasoconstriction [53]. Recently, we also paid attention to the mechanisms of α_2-adrenergic receptors agonist in both TGR and salt hypertensive Dahl rats. Both central and peroral clonidine treatment similarly reduced BP in both hypertensive rat strains. However, the sympathetic vasoconstriction was attenuated only in intracerebroventricularly treated rats but not in rats treated perorally with clonidine [54].

A model of angiotensin II-dependent hypertension yielded many important information concerning a new antihypertensive strategy, namely the blockade of endothelin receptor A. Most studies were performed in a model of chronic kidney disease induced by 5/6 nephrectomy. The effects on survival, blood pressure and renal function were dependent on whether the treatment started immediately after the nephrectomy or was postponed to the time of established chronic kidney disease. Beneficial effects were seen only when the treatment was started just after 5/6 nephrectomy. An important factor was also the length of treatment. Most positive results with ET_A receptor blockade on renal function were detected in two models of combined chronic kidney damage and heart failure (induced by aorto-caval fistula). Hypertension of TGR is usually considered an angiotensin II-dependent form of experimental hypertension. However, our data indicated the importance of angiotensin II stimulation of central mechanisms responsible for the enhanced sympathetic tone in this hypertensive model.

Calcium sensitization and calcium influx in various forms of hypertension

In 2011 we began to study the contribution of Ca^{2+} sensitization (mediated by RhoA/Rho kinase

pathway) and Ca^{2+} influx (through L-VDCC) to BP maintenance in normotensive and hypertensive rats. The interaction of these two mechanisms in BP regulation is based upon the control of phosphorylated myosin light chain (MLC), the levels of which are increased by Ca^{2+}-activated MLC kinase and decreased by Rho kinase-stimulated MLC phosphatase (Fig. 5). Our experiments started with the determination of BP reduction elicited by acute dose-dependent administration of Rho kinase inhibitor fasudil. The substantial fasudil-induced BP lowering was more pronounced in SHR than in WKY rats, suggesting increased Ca^{2+} sensitization in hypertensive animals [55]. At that time Michal Behuliak developed a new approach to evaluate the contribution of Ca^{2+} sensitization and Ca^{2+} influx to BP control in conscious rats. In rats, which were pretreated with captopril and pentolinium to abolish endogenous angiotensin-dependent and sympathetic vasoconstriction, he administered the increasing doses of BAY K8644 (L-VDCC opener) and he monitored BP increase both in the absence or presence of fasudil, i.e. under the conditions of preserved or reduced Ca^{2+} sensitization (Fig. 6). His results clearly demonstrated a surprising attenuation of BP component dependent on Ca^{2+} sensitization in SHR compared to normotensive WKY rats. This was a great surprise facing the previous finding of the enhanced BP reduction elicited by dose-dependent fasudil administration in intact SHR [55].To convince us (and the reviewers) that his findings were correct, he used the rats subjected to acute RAS and SNS blockade (by captopril and pentolinium) in which he measured BP response to norepinephrine (NE).

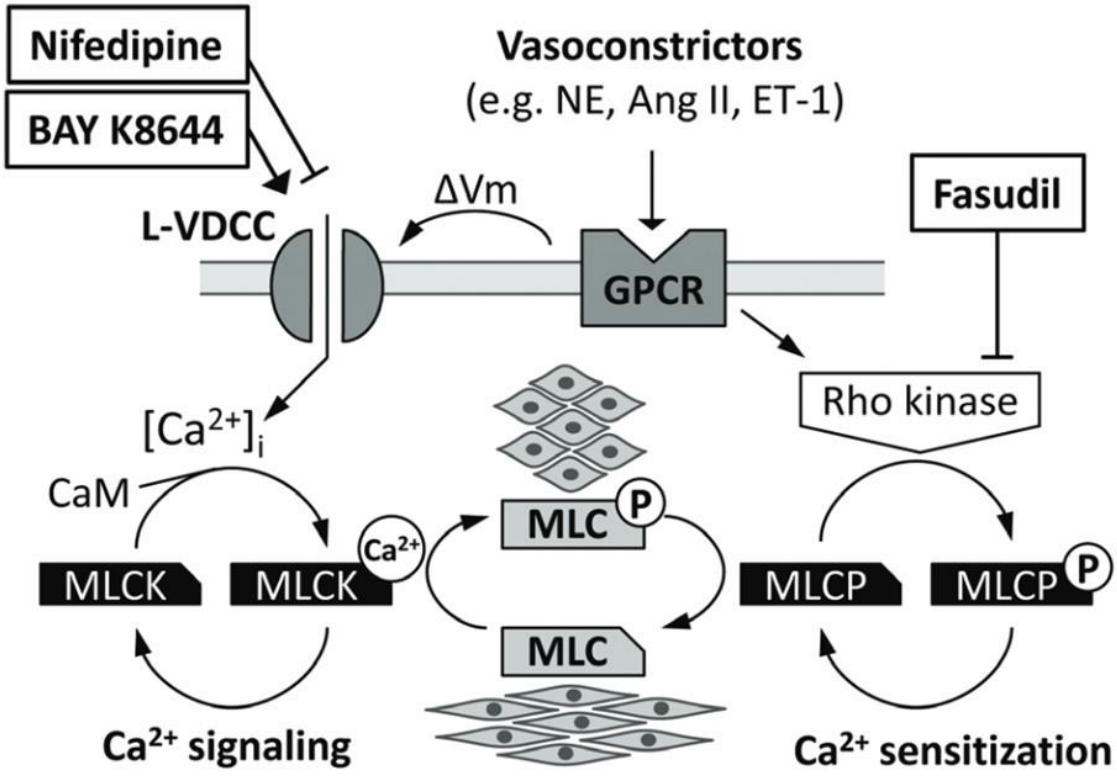

Fig. 5. The schematic representation of the principal pathways (Ca^{2+} signaling and Ca^{2+} sensitization) involved in the control of vascular smooth muscle tone. GPCR – G protein-coupled receptors, ΔVm – membrane potential change, L-VDCC – L type voltage-dependent calcium channels, MLC – myosin light chain, CaM – calmodulin, MLCK – myosin light chain kinase, MLCP – myosin light chain phosphatase, NE – norepinephrine, Ang II – angiotensin II, ET-1 – endothelin-1 [56].

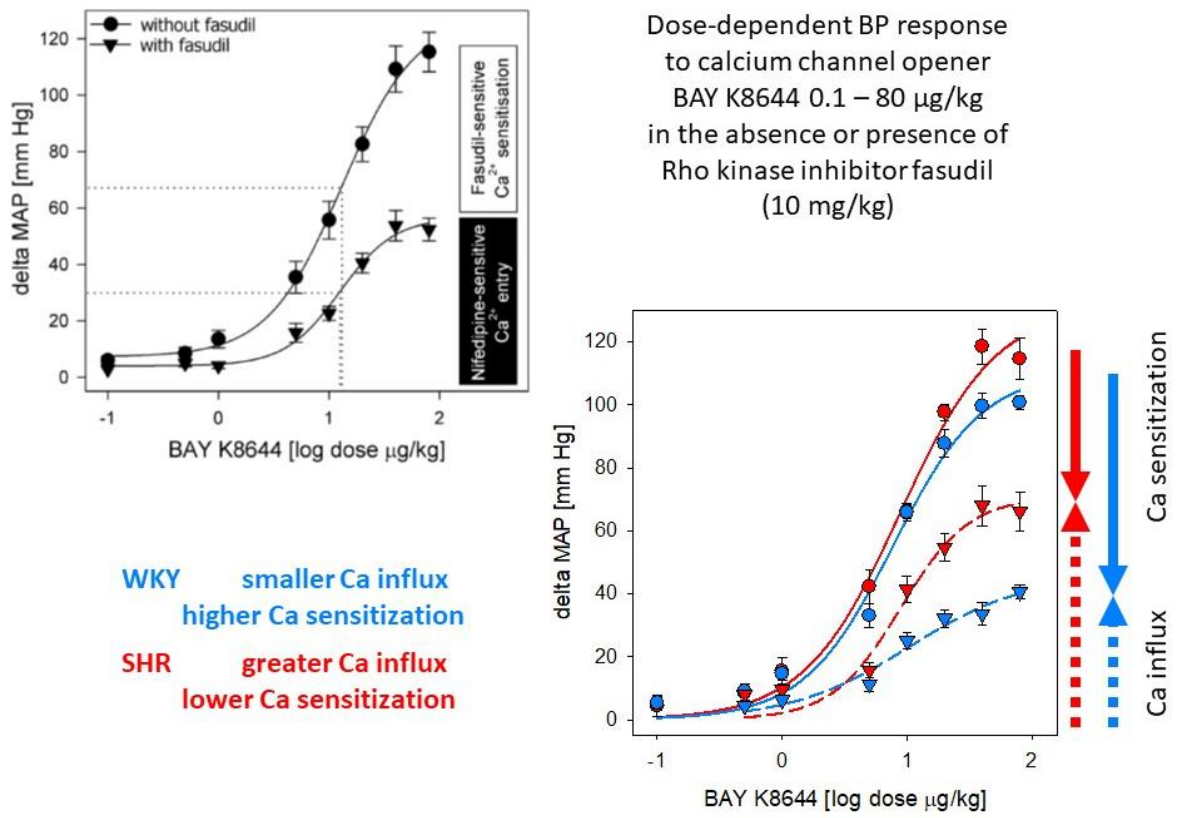

Fig. 6. The method developed by Michal Behuliak for the estimation of the complementary role of Ca^{2+} sensitization and Ca^{2+} influx in conscious rats (left panel) and their contribution to blood pressure response to L-VDCC opener BAY K 8644 (right panel) in SHR and WKY rats (modified from [55]).

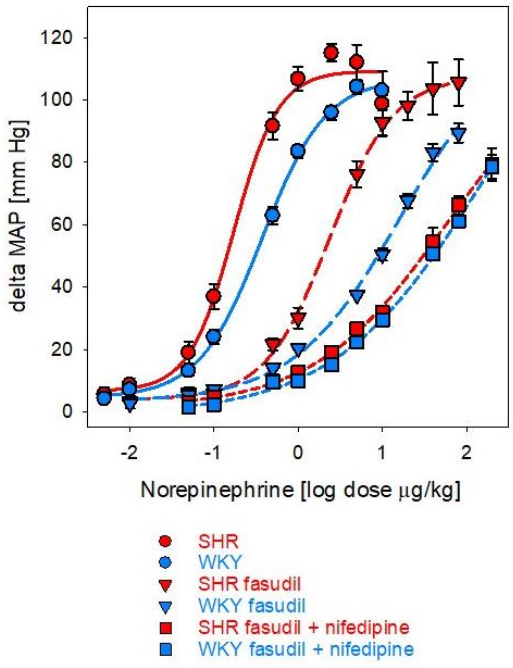

Fig. 7. Fasudil- and nifedipine-induced shifts of norepinephrine dose-response curves in conscious SHR and WKY rats (modified from [55]).

This response was slightly greater in SHR than in WKY rats prior to fasudil administration. A more pronounced attenuation (greater rightward shift) of BP response to NE was observed in WKY rats than in SHR after fasudil administration. The subsequent addition of nifedipine caused a further attenuation of BP response, which was greater in SHR than in WKY rats (Fig. 7). These results helped us to suppose that in genetic hypertension the Ca^{2+} sensitization is less important than Ca^{2+} influx through L-VDCC. Moreover, the relative fasudil-induced BP reduction (expressed in percentage of initial BP) was similar in SHR and WKY rats, whereas the relative BP response to nifedipine was greatly enhanced in SHR [55]. These findings were then tested by Michal Bencze, who joined us in 2010, using wire myography in small femoral arteries and in mesenteric resistance arteries. He demonstrated that i) fasudil pretreatment attenuated norepinephrine-induced contraction of arteries with intact endothelium in both strains, and ii) fasudil dose-dependently attenuated the wall tension of phenylephrine-precontracted arteries of both types. However,

fasudil-induced relaxation was greater in SHR mesenteric arteries but smaller in SHR femoral arteries. The findings in mesenteric arteries were similar to BP changes elicited by acute fasudil administration in conscious rats. Furthermore, Michal Bencze observed that Rho kinase inhibitor Y-27632 attenuated phenylephrine-induced contraction more in endothelium-denuded arteries from WKY rats than in those from SHR animals [55,56].

Further study by Behuliak *et al.* [57] was focused on the ontogenic changes in the role of Ca^{2+} sensitization and Ca^{2+} influx in BP maintenance of the developing SHR and WKY rats (Fig. 8). BP response to fasudil (contribution of Ca^{2+} sensitization) was smaller in 3-week-old prehypertensive SHR than in age-matched WKY rats, but this response was enhanced in SHR aged 3 months or older. On the other hand, BP response to nifedipine (contribution of Ca^{2+} influx) was significantly greater in SHR than in WKY rats already from the age of 5 weeks. Figure 8 also shows that BP responses to fasudil or nifedipine did not change with age in WKY rats, whereas it was increasing in the developing SHR in parallel with their BP increase. The data obtained in the developing SHR suggest a more important role of Ca^{2+} influx than Ca^{2+} sensitization in the pathogenesis of genetic hypertension [57]. Myographic study of vascular response to Rho kinase inhibition or L-VDCC blockade confirmed a smaller fasudil-induced attenuation of wall tension in phenylephrine-precontracted femoral arteries of 3-week-old SHR as compared to age-matched WKY rats. This was absent in the arteries of adult SHR aged 26 weeks. In contrast, vascular relaxation elicited by nifedipine was enhanced in arteries isolated from both age groups of SHR [57].

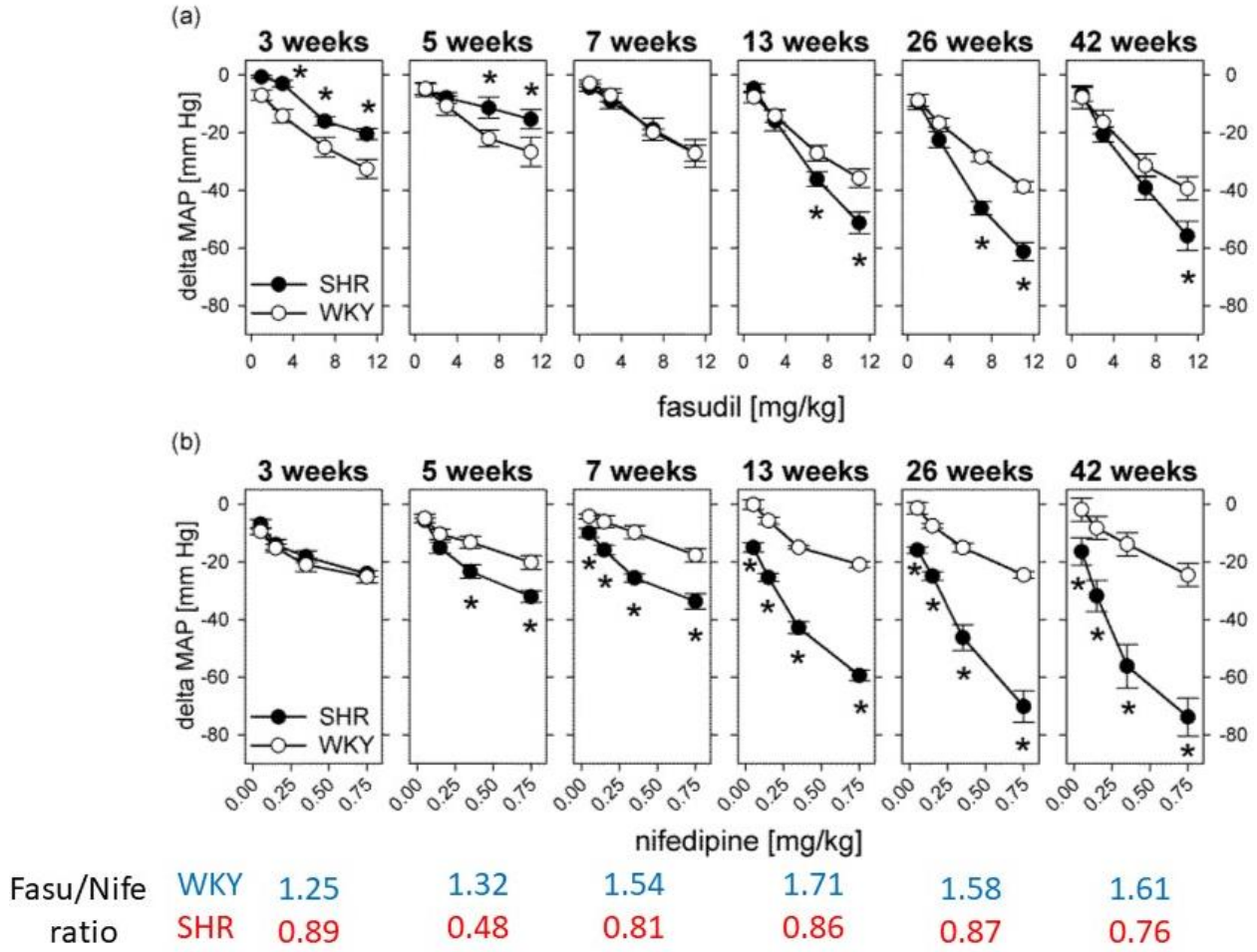

Fig. 8. The development of BP response to acute administration of fasudil (Fasu, upper panel) or nifedipine (Nife, lower panel) in intact SHR and WKY rats. Fasu/Nife represents the ratio of the magnitude of BP responses to the highest cumulative doses of fasudil and nifedipine (modified from [57]).

Another part of this developmental study was devoted to the molecular biology of RhoA/Rho kinase pathway in young prehypertensive SHR and adult SHR with established hypertension, i.e. rats aged 3 or 24 weeks. The mRNA expression of selected Rho-GEFs (Arhgef1, Arhgef11 and Arhgef12) was decreased only in adult SHR, whereas p63RhoGEF and CPI-17 expressions were reduced in both age groups of SHR. Active RhoA and phosphorylated CPI-17 were increased in adult but not in young SHR. In conclusion, the importance of RhoA/Rho-kinase pathway for the control of vascular tone and BP is attenuated in SHR from prehypertensive stages. The enhanced RhoA activation and/or CPI-17 phosphorylation might be counteracted by the reduced expression of upstream activators of Rho-kinase (Rho-GEFs) together with lower expression of CPI-17 (in downstream cascade of Rho-kinase) [57].

Our further effort in the research of the complementary action of Ca^{2+} sensitization and Ca^{2+} influx in vascular tone control continued by the evaluation of BP effects elicited by acute blockade of particular system under the conditions of the attenuation of the other system. Logically, L-VDCC blockade by nifedipine induces a smaller BP reduction under the conditions when Ca^{2+} sensitization is attenuated by pretreatment with Rho kinase inhibitor fasudil. Similarly, the inhibition of Rho kinase by fasudil has smaller BP-lowering effects if Ca^{2+} influx is reduced by previous nifedipine-induced L-VDCC blockade. Indeed, we demonstrated that if nifedipine or fasudil were given as the first drug, they lowered BP more than in the case when they were administered as the second drug. These observations were made not only in SHR but also in WKY rats [56]. At that time Brunová *et al.* [58] reported that sympathetic nervous system plays a decisive role in the magnitude of fasudil-induced BP reduction because SNS activation during hypotension elicited by acute fasudil administration leads to a compensatory increase of heart rate and/or sympathetic tone. In this study carried out in normotensive animals we have seen for the first time that a combined RAS, SNS and NO synthase blockade augmented BP reduction elicited by fasudil more than two times compared to animals with intact vasoactive systems [58].

Even if we studied Ca^{2+} sensitization and Ca^{2+} influx mostly in SHR, we also paid some attention to other hypertensive models such as heterozygous Ren-2 transgenic rats and salt hypertensive Dahl rats. Behuliak *et al.* [59] reported enhanced fasudil-induced as well as nifedipine-induced BP reductions in both forms of experimental hypertension studied under the conditions of intact RAS and SNS. Nevertheless, they also evaluated Ca^{2+} sensitization and Ca^{2+} influx in these hypertensive models using his original BAY K8644 method in animals subjected to previous RAS and SNS blockade. He demonstrated the attenuation of Ca^{2+} sensitization in heterozygous Ren-2 transgenic rats, but in Dahl rats he described a significant Ca^{2+} sensitization only in salt-sensitive SS/Jr animals, whereas it was almost absent in salt-resistant SR/Jr animals (Fig. 9). The most surprising finding was the absence of any significant effect of high salt intake on either Ca^{2+} sensitization or Ca^{2+} influx in SS/Jr rats, although the salt-sensitive rats fed a high-salt diet developed a severe salt hypertension [59]. To explain the discrepancy of results obtained after fasudil administration in rats with intact or inhibited endogenous RAS and SNS, we proposed a hypothesis on so-called "basal" and "activated" Ca^{2+} sensitization, referring to the data obtained by BAY K8644 method or by dose-dependent fasudil administration [59].

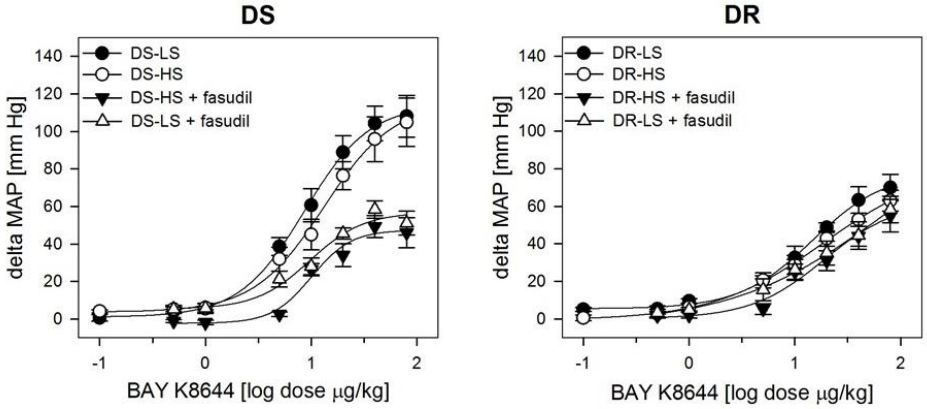

Fig. 9. The estimation of Ca^{2+} sensitization and Ca^{2+} influx in salt-sensitive (DS) and salt-resistant (DR) Dahl rats fed either a low-salt (LS) or high-salt (HS) diet which were subjected to the blockade of renin-angiotensin and sympathetic nervous system (modified from [59]).

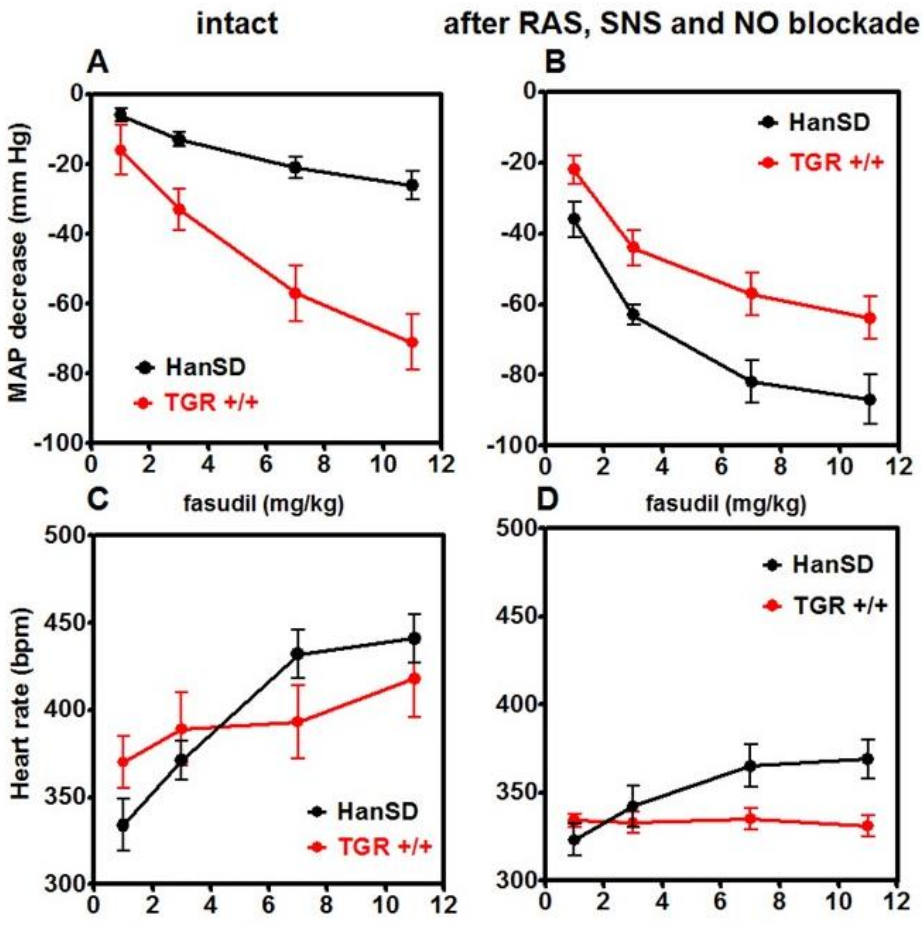

Fig. 10. Blood pressure and heart rate responses to increasing doses of Rho kinase inhibitor fasudil in TGR and HanSD rats – a comparison of intact rats and animals subjected to a combined blockade of renin-angiotensin system, sympathetic nervous system and NO synthase (modified from [60])

The paper by Vaněčková *et al.* [60] in which we studied Ca^{2+} sensitization and Ca^{2+} influx of homozygous Ren-2 transgenic rats, was crucial for the solution of this problem. The study of developing Ren-2 transgenic rats revealed important ontogenetic changes in both Ca^{2+} sensitization and Ca^{2+} influx, which were similar to those reported in SHR by Behuliak *et al.* [57]. Using adult Ren-2 transgenic rats we determined BP lowering effects of fasudil and nifedipine in intact rats as well as in rats subjected to the combined RAS, SNS and NO synthase blockade. The main finding was that both Ca^{2+} sensitization and Ca^{2+} influx seemed to be substantially greater (at least four times) in intact Ren-2 transgenic rats than in normotensive Han-SD rats. However, the reverse was true for fasudil-induced or nifedipine-induced BP reduction in rats subjected to the combined blockade, which augmented BP responses only in normotensive but not in hypertensive rats. Concomitant changes in heart rate clearly indicated that combined RAS, SNS and NO synthase blockade attenuated the efficiency of arterial baroreflex BP control in Han-SD rats (Fig. 10). Thus, the sympathetic activation occurring in intact Han-SD rats during fasudil- or nifedipine-induced hypotension might efficiently counteract this BP reduction. This was not the case of hypertensive Ren-2 transgenic rats, which are characterized by considerably decreased baroreflex efficiency compared to normotensive Han-SD controls [60].

Reduced baroreflex efficiency is a characteristic feature in most hypertensive models. This is also true for salt hypertensive Dahl rats [11]. Based on our experiments in Ren-2 transgenic rats we tried to evaluate the potential role of arterial baroreflex for the magnitude of BP response to fasudil or nifedipine in salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) Dahl rats fed either a low-salt (LS) or a high-salt (HS) diets [61]. BP-lowering responses to both fasudil or nifedipine were greater in intact SS/Jr than in SR/Jr rats. Moreover, salt hypertensive SS/Jr fed HS diet had more pronounced BP responses than SS/Jr fed LS diet with lower BP (Fig. 11). If the same experiments were

done in Dahl rats pretreated with NO synthase inhibitor L-NAME, BP responses were augmented in all experimental group but substantially more in SS/Jr than in SR/Jr rats. However, the difference between SS/Jr rats fed HS or LS diet persisted. Nevertheless, a combined blockade of RAS, SNS and NO synthase, which prevented baroreflex-mediated sympathetic hyperactivity, abolished the difference in BP responses to fasudil between salt hypertensive SS/Jr rats and their SS/Jr controls fed LS diet, while the strain difference between SS/Jr and SR/Jr rats persisted even after ganglionic blockade (Fig. 11). Thus, the increased Ca^{2+} sensitization seems to be a hallmark of salt sensitivity but not of salt hypertension in Dahl rats. Our further experiments indicated that increased Ca^{2+} influx potentiated by sympathoexcitation is the abnormality essential for salt hypertension in this model [61].

Figure 12 depicts the relationship between basal BP in Dahl rats and absolute or relative BP changes induced by the acute administration of either nifedipine or fasudil. It is clear that the acute blockade of Ca^{2+} influx or Ca^{2+} sensitization lowered BP proportionally to basal BP. However, the contribution of these two mechanisms to BP maintenance was considerably enhanced in hypertensive animals so that even the relative BP changes elicited by nifedipine or fasudil were increased proportionally to basal BP (Fig. 12).

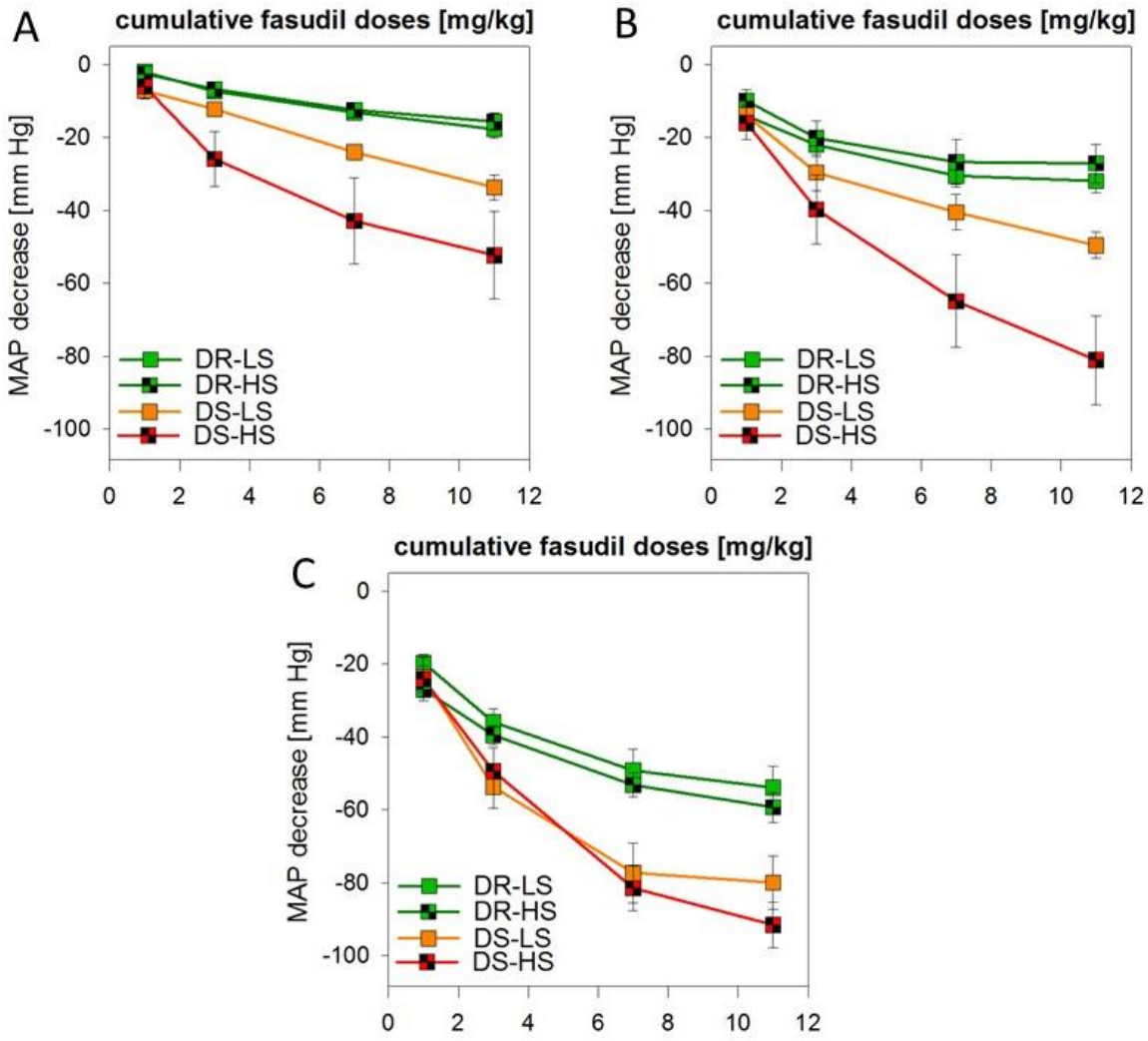

Fig. 11. Dose-dependent BP responses to cumulative doses of fasudil in salt-sensitive (DS) and salt-resistant (DR) Dahl rats fed either a low-salt (LS) or high-salt (HS) diet which were studied either intact (A), after acute NO synthase inhibition (B) or after a combined blockade of renin-angiotensin system, sympathetic nervous system and NO synthase (modified from [61]).

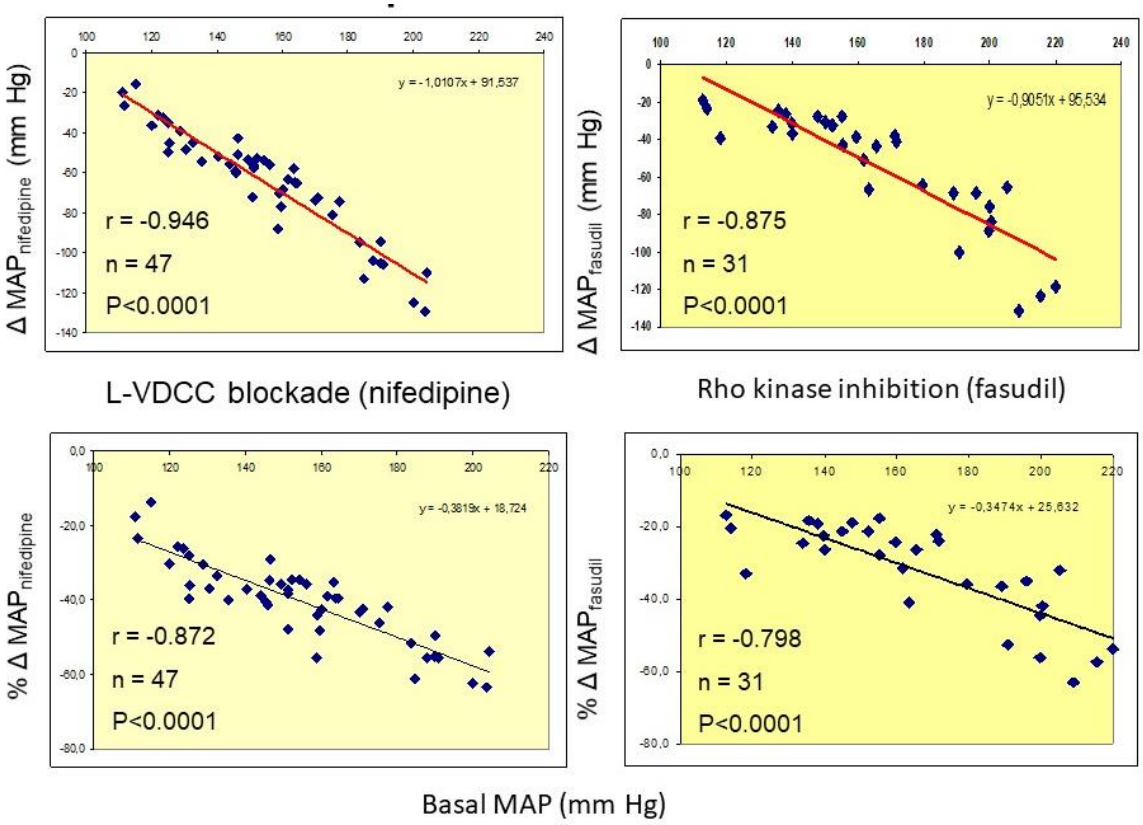

Fig. 12. The relationships of basal BP and BP responses to acute nifedipine or fasudil administration in conscious Dahl rats – absolute BP changes (upper panels) and relative BP changes (lower panels) (Zicha *et al.*, unpublished data).

Ca^{2+} sensitization mediated by RhoA/Rho kinase pathway is often considered as a Ca^{2+}-independent mechanism of vasoconstriction. Our experiments indicated its different role in genetic or salt hypertension. The attenuated Ca^{2+} sensitization in SHR or Ren-2 transgenic rats seems to compensate (at least partially) for the enhanced Ca^{2+} influx. The abnormal contribution of Ca^{2+} sensitization to BP maintenance appears in rats with genetic hypertension already in the prehypertensive stage. On the other hand, the enhanced Ca^{2+} sensitization in Dahl rats is a marker of salt sensitivity, whereas salt hypertension is based upon the additive BP effects of enhanced Ca^{2+} influx.

Myographic studies of arterial vasoconstriction and vasodilation in hypertension

Although we focused most of our attention to the control of vascular tone in resistance arteries of conscious rats, we cannot forget the effort of Ľudovít Paulis, Silvia Líšková, Michal Bencze and Pavol Valovič, who performed numerous myographic studies in femoral and mesenteric arteries isolated from rats with L-NAME hypertension [62-64], hereditary hypertriglyceridemic rats [65] or SHR [65-69].

Paulis *et al.* [62] studied a very interesting topic, i.e. vascular changes occurring during the regression of L-NAME-induced hypertension with a special respect to the role of nitric oxide (NO) and endothelium-derived constricting factor (EDCF). They reported a rather fast improvement of NO-dependent vasorelaxation after the cessation of L-NAME treatment but the enhanced formation of EDCF (cyclooxygenase product) persisted in femoral arteries for much longer time. During the spontaneous recovery from L-NAME hypertension a functional restoration of NO signaling took place in all parts of the vascular tree. However, the increases in systolic blood pressure, EDCF formation, and cyclooxygenase expression as well as the arterial structural alterations (reduction in femoral artery diameter) were not completely restored [62]. A further study [63] concerned the effects of chronic melatonin administration on the development of L-NAME hypertension, the magnitude of oxidative load and the changes in femoral and mesenteric arteries. Although melatonin abolished the increase of

oxidative load in L-NAME hypertensive rats, melatonin-induced BP reduction was modest and there were minimal effects of melatonin on the decreased NO synthase activity, augmented EDCF-mediated vasoconstriction, increased cyclooxygenase-2 expression and a reduced inner diameter which are characteristic alterations in L-NAME hypertensive rats [63]. The third study [64] examined the effects of melatonin administration during the regression of L-NAME hypertension. While the NO-signaling was restored spontaneously within three weeks after L-NAME cessation, the EDCF-signaling, oxidative load and arterial remodeling were completely restored only when melatonin treatment was administered during the recovery period. Although melatonin did not accelerate blood pressure reduction, it attenuated EDCF-contractions and oxidative load and enlarged arterial diameter.

Ľudovít Paulis and Silvia Líšková also studied the abnormalities of norepinephrine-induced contraction of isolated femoral arteries of young SHR and WKY rats. They compared blood vessels of untreated rats with those of animals subjected to captopril treatment from the 4th to the 10th week of age [35]. Both phasic (dependent on internal calcium stores) and tonic (dependent on calcium influx) contractions to NE were augmented in SHR femoral arteries *in vitro*. Nifedipine attenuated only the tonic contractions and this attenuation was larger in SHR than in WKY arteries. Nifedipine effect was greater after endothelium removal, which augmented the tonic but not the phasic contractions after NE. Chronic captopril treatment of SHR prevented hypertension development by suppression of their sympathetic vasoconstriction including its nifedipine-sensitive component, but failed to influence enhanced NE-induced arterial contractions or increased relaxation to nifedipine *in vitro*. The contribution of nifedipine-sensitive component to noradrenergic vasoconstriction was enhanced during excessive NE stimulation (increased sympathetic tone of SHR *in vivo* or supramaximal NE stimulation *in vitro*) [35]. Further experiments performed by Silvia Líšková demonstrated that intact endothelium attenuated more the tonic than the phasic NE-induced arterial contraction and this augmented contraction of de-endothelized femoral arteries was almost completely blocked by nifedipine [36]. Moreover, she demonstrated that pertussis toxin (PTX) pretreatment attenuated more α_2-adrenergic than α_1-adrenergic contraction of vascular smooth muscle of femoral arteries. It seems that α_2-adrenergic vasoconstriction is entirely based upon Ca^{2+} influx through L-VDCC blocked by nifedipine [36].

Michal Behuliak studied a similar problem in conscious rats using prazosine and yohimbine for the blockade of α_1-adrenergic and α_2-adrenergic vasoconstriction. He demonstrated that 1) α_1-adrenergic vasoconstriction was more important than α_2-adrenergic vasoconstriction in both SHR and WKY rats, 2) α_2-adrenergic vasoconstriction contributed to BP maintenance more in SHR than in WKY rats, and 3) nifedipine-induced blockade of L-VDCC lowered the sensitivity of BP response to NE by about 10 times and this effect was mediated by the changes of α_1-adrenergic vasoconstriction. On the other hand, nifedipine lowered not only the sensitivity but also the maximal BP response to NE if he studied α_2-adrenergic vasoconstriction in the presence of α_1-adrenoceptor blocker prazosine [36].

Líšková *et al.* [65] examined the influence of the blockade of either NO synthase and/or large conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}) on NE-induced contraction of isolated femoral artery in normotensive Wistar rats, moderately hypertensive hereditary hypertriglyceridemic (hHTG) rats and severely hypertensive SHR. NE-induced contractions of femoral arteries were augmented in both hypertensive strains compared with Wistar rats, but acetylcholine-induced relaxation was impaired in hHTG rats only. The increase of basal vascular tone of isolated arteries after BK_{Ca} channel blockade was similar in all rat strains, but a subsequent NO synthase inhibition increased basal vascular tone more in vessels from both hypertensive rat strains. NO synthase inhibition increased the sensitivity to NE in all strains, but BK_{Ca} channel blockade only in SHR. Neither treatment enhanced the maximal NE-induced contraction. NO-dependent attenuation of NE-induced contractions was greater in SHR arteries than in hHTG or Wistar vessels, whereas BK_{Ca} channels may play a greater role in modulating vascular contraction in the severe form of hypertension in SHR [65].

The activation of Ca^{2+}-dependent Cl^- channels during norepinephrine-induced contraction of vascular smooth muscle was suggested to depolarize cell membrane and to increase Ca^{2+} entry. Líšková *et al.* [67] studied the effects of ageing, hypertension and NO synthase inhibition on the contribution of these channels to NE-induced contraction of femoral artery. They found that Ca^{2+}-dependent Cl^- channels are important for the maintenance of normal vascular tone, while their inactivation/closing might be a pathological mechanism. In contrast, the contribution of Ca^{2+}-dependent Cl^- channels to NE-induced contraction diminished with age, hypertension

development, and/or NO synthase inhibition. The effect of hypertension and ageing on the participation of EDCF in the NE-induced contraction of femoral artery was also investigated in another paper by Líšková *et al.* [66]. They demonstrated that EDCF participation in vascular contraction is similarly enhanced in conduit arteries of adult SHR and aged normotensive WKY rats.

Michal Bencze started his myographic experiments within the frame of the studies on Ca^{2+} sensitization [55,57] and gabapentin [70]. In parallel, he examined the effects of broad-range transient receptor potential (TRP) channel inhibitors on the contraction of resistance and conduit arteries of the rat [71]. TRP channels were proposed to contribute to membrane depolarization and Ca^{2+} influx into vascular smooth muscle cells. TRP channel inhibitors – 2-aminoethoxy-diphenyl borate (2-APB), flufenamic acid (FFA) and SKF-96365 – attenuated K^+-induced contraction less than nifedipine. In contrast, phenylephrine-induced contraction was more influenced by 2-APB in resistance arteries, while FFA completely prevented U-46619-induced contraction in both sizes of arteries. The absence of extracellular Na^+ prevented the inhibitory effects of 2-APB, but not those of FFA. The observed effects of broad-range TRP channel inhibitors, which were dependent on the size of the artery, confirmed the involvement of TRP channels in agonist-induced vascular contractions [71].

He also studied the mechanisms responsible for the enhanced contractility of isolated arteries from SHR and WKY rats [68]. He compared the contractions of endothelium-denuded femoral arteries elicited by i) exogenous NE, ii) NE released from neural varicosities by tyramine or iii) by membrane depolarization induced by high K^+ concentration. There was no strain difference in the contraction elicited by exogenous NE, but SHR arteries responded more to tyramine-induced endogenous NE release. K^+-induced contraction was enhanced in SHR arteries with no involvement of endogenous catecholamines. The α_1-adrenoceptor blockade lowered tyramine-induced contraction more in SHR arteries. Partial depolarization of WKY arteries by 20 mM K^+ enhanced their contraction to SHR level. The blockade of β-adrenoceptors by propranolol or selective β_2-antagonist ICI-118,551 induced the contraction of SHR endothelium-denuded arteries but was without significant effects on WKY arteries unless they were stimulated with K^+. Both tyramine-induced and propranolol-induced contractions were attenuated by K^+ channel opener flupirtine and abolished by nifedipine.

There were plans for a promising study on excitation-contraction coupling and excitation-transcription coupling in blood vessels [72], which remained unfinished, although this topic is highly interesting. We speculated that abnormal vascular smooth muscle cell phenotype reported in rats with genetic hypertension (such as SHR) might be partially caused by a shift from contractile to proliferative phenotype of vascular smooth muscle cells.

The last study of the myographic series concerned the β-adrenergic relaxation of conduit and resistance arteries from normotensive WKY rats or SHR [69]. We confirmed the impaired β-adrenergic relaxation of SHR femoral arteries due to the absence of its endothelium-independent component, whereas the endothelium-dependent component of β-adrenergic smooth muscle relaxation was similar in both strains. Conversely, the isoprenaline-induced relaxation of resistance mesenteric arteries was similar in both strains and this was true for endothelium-dependent and endothelium-independent components. Further experiments in conscious rats indicated that the increasing isoprenaline doses elicited a similar BP decrease in both rat strains, although BP sensitivity to isoprenaline was slightly decreased in SHR. The blockade of cyclooxygenase (indomethacin) and NO synthase (L-NAME) further reduced BP sensitivity to isoprenaline in SHR. We concluded that the attenuated β-adrenergic vasodilatation of conduit arteries of SHR but similar β-adrenergic relaxation of resistance mesenteric arteries from WKY and SHR, together with the similar BP response to β-adrenergic agonists in conscious rats of both strains, do not support a major role of altered β-adrenergic vasodilatation in the maintenance of high BP in genetic hypertension [69].

Numerous abnormalities of vascular contraction and relaxation were disclosed in the arteries of different diameter isolated from hypertensive animals. Nevertheless, it is well known that the contraction or relaxation of conduit arteries is important for the maintenance of their diameter but not for the control of vascular resistance. Thus, neither conduit nor mesenteric resistance arteries represent appropriately the vascular beds decisive for the increased peripheral resistance and high blood pressure such as musculo-cutaneous vascular bed of the hindlimb. Therefore, the results obtained in isolated blood vessels should always be verified by *in vivo* experiments in conscious rats.

Autonomic nervous system in genetic hypertension

As mentioned above, enhanced sympathetic vasoconstriction is a common feature in most forms of experimental hypertension. This is true for salt-dependent [6,12,23], NO-deficient [19,23] and spontaneous forms of genetic hypertension [13,16,23]. Michal Behuliak devoted most of his research activity to this vasoactive system, which he studied especially in SHR. He started with the evaluation of the contribution of α_1-adrenergic and α_2-adrenergic vasoconstriction to BP maintenance in SHR and WKY rats [36]. This was followed by a comparison of three different vasodilating systems (NO, prostacyclin PGI_2 and BK_{Ca} channels) which oppose sympathetic vasoconstriction in SHR, Dahl salt hypertensive rats, and L-NAME-treated hypertensive rats [23]. Furthermore, Michal Bencze and Michal Behuliak designed a study on the effects of particular classes of anesthetics on the mechanisms regulating BP with special respect to the sympathetic tone [16]. This study revealed a major BP reduction elicited by isoflurane, ketamine and chloralose-urethane, which was mediated by the attenuation of sympathetic BP component. The interference of pentobarbital anesthesia with cardiovascular parameters was smaller as compared to other anesthetics.

After the study of the interaction of Ca^{2+} sensitization and Ca^{2+} influx in BP control [55,57,59], Michal Behuliak focused his attention on the problem of participation of Ca^{2+} influx in the regulation of the sympathetic nervous system in genetic hypertension [70]. The initial stimulus were two papers by Bannister *et al.* [73,74] that the ligands of auxiliary α_2,δ subunit of voltage-dependent Ca^{2+} channels (VDCC) such as gabapentin might decrease the surface expression of L-type VDCCs in arterial myocytes and thus reduce arterial contraction in SHR. Initially, he studied the influence of chronic gabapentin exposure on the contraction of endothelium-denuded femoral arteries, but the research was soon focused on the effects of acute gabapentin administration on BP control in conscious SHR with neurogenic hypertension [70]. He demonstrated that the acute gabapentin administration lowered BP and HR more in conscious SHR than in WKY rats. The ability of gabapentin to abolish the nitroprusside-induced reflex tachycardia within the frame of baroreceptor-HR control is similar to the effects of N-type VDCCs blocker ω-conotoxin but not to the effects of L-type VDCCs blocker nifedipine. Hypotensive effects of gabapentin were accompanied by the reduction of 1) plasma NE level, 2) depressor response to ganglionic blocker pentolinium, 3) power of low frequency component of systolic BP variability (LF-SBPV), and 4) pressor response of mesenteric vascular bed to periarterial nerve stimulation, suggesting a decrease of peripheral sympathetic nerve transmission. Besides the known L-type VDCCs involvement in the vascular effect of gabapentin, our data revealed the important role of N-type VDCCs in the acute gabapentin effect on sympathetic control of BP. Gabapentin-induced changes in sympathetic tone represent the major hemodynamic mechanism of the acute cardiovascular response to this drug [70].

In 2014 Anna Vavřínová joined our group and her research interest was focused on the changes of sympathoadrenal and sympathoneural systems in the developing SHR. Her cooperation with Michal Behuliak yielded numerous interesting papers on the abnormalities of the sympathetic nervous system in genetic hypertension. It was demonstrated [75] that mRNA and protein expression of catecholamine-synthesizing enzymes was decreased in adrenals of prehypertensive 4-week-old SHR. This was surprisingly accompanied by higher dopamine, noradrenaline and adrenaline content in adrenals of young SHR. These early changes in catecholamine synthesis were absent in adrenals of adult SHR in which the expression of catecholamine synthesizing enzymes was even more reduced and this was accompanied by decreased adrenal dopamine and noradrenaline contents. It was concluded that this downregulation might be a compensatory mechanism that counteracts the vascular sympathetic hyperinnervation seen in SHR of both ages [75].

Further study by Vavřínová *et al.* [76] examined the effects of guanethidine-induced sympathectomy carried out in adult SHR. Radiotelemetric measurements revealed only a transient BP reduction in sympathectomized SHR, which was in contrast to the important role of the sympathetic nervous system in BP maintenance. However, sympathectomy permanently lowered HR, improved baroreflex sensitivity and decreased LF-SBPV (a marker of vascular sympathetic activity) in sympathectomized SHR. It also attenuated cardiovascular response to acute restraint stress. The effect of sympathectomy on BP was counteracted by the increased vascular sensitivity to catecholamines in WKY rats and SHRs and/or by the enhanced secretion of adrenaline, which was more pronounced in WKY rats [76]. However, Michal Behuliak (unpublished data)

demonstrated later that BP maintenance in sympathectomized SHR was dependent on the enhanced renin-angiotensin system as indicated by the profound BP reduction induced by chronic captopril administration in these animals (Fig. 13).

Recently, Vavřínová *et al.* [77] summarized the current knowledge on the abnormalities of the adrenomedullary hormonal system in genetic hypertension and their contribution to altered regulation of blood pressure. The participation of the sympathoadrenal system in the pathogenesis of genetic hypertension and BP maintenance in SHR is still discussed. However, the persistence of moderately elevated blood pressure in SHR subjected to neonatal sympathectomy as well as the resistance of adult SHR to the treatment with sympatholytic drugs suggests that other factors (including enhanced activity of the adrenomedullary hormonal system) are involved in the pathogenesis of hypertension of SHR. The abnormalities in the adrenomedullary hormonal system of SHR range from the hyperactivity of brain centers regulating sympathetic outflow, through the exaggerated activation of sympathoadrenal preganglionic neurons, up to the local changes in chromaffin cells of the adrenal medulla. All the above alterations might contribute to the enhanced release of epinephrine and/or norepinephrine from adrenal medulla. It should be mentioned that SHR are hyper-responsive to various stressful stimuli and hence the repeated excessive activation of the adrenomedullary system and the enhanced catecholamine release might promote other pathological changes observed in this rat strain [77].

The last years of the work of Michal Behuliak and his coworkers were devoted to the study of the complex control of the autonomic nervous system, the involvement of arterial baroreflex changes and ganglionic transmission under basal and stress conditions. The results of this work were published in his last three papers [78-80]

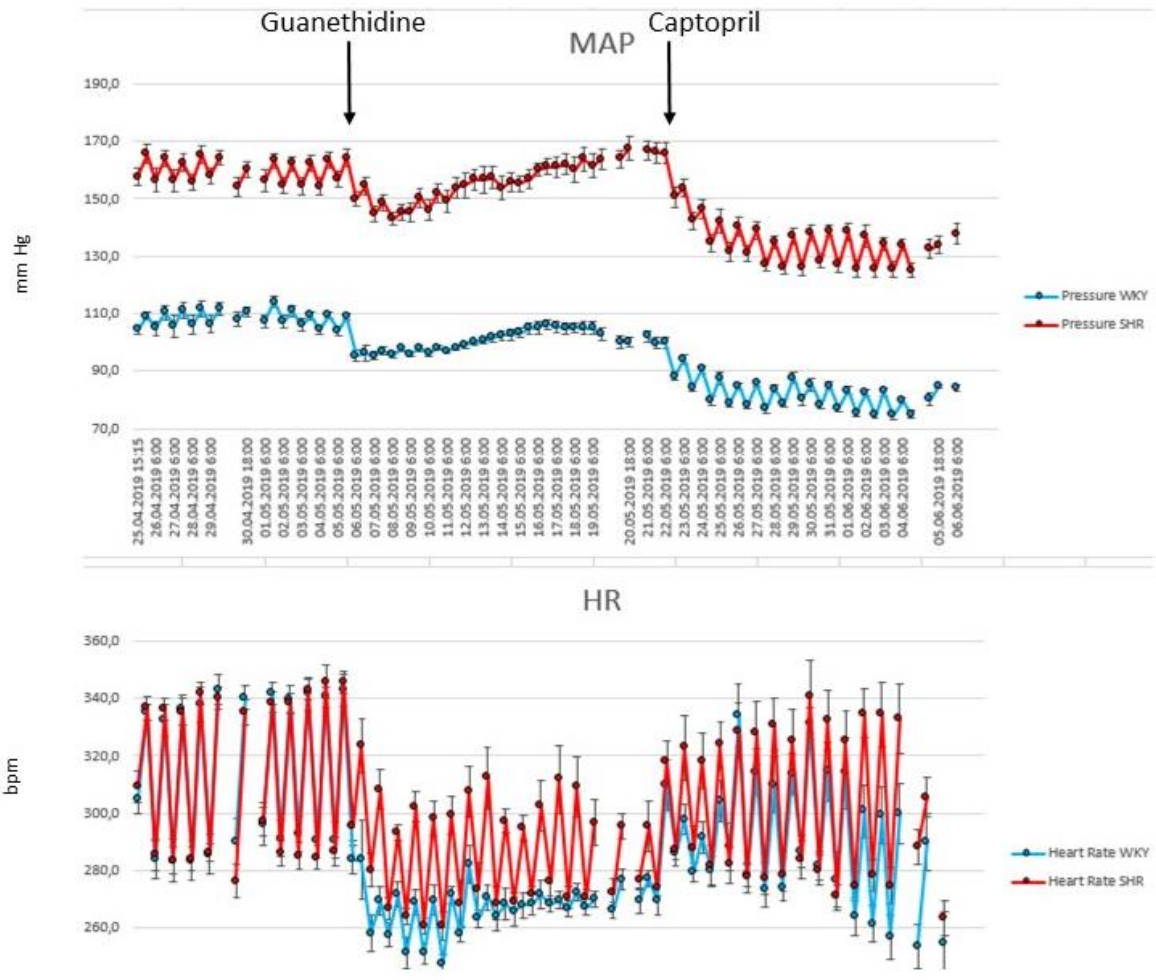

Fig. 13. Mean arterial pressure (MAP) and heart rate (HR) changes in SHR and WKY rats subjected to sympathectomy by guanethidine treatment and subsequent RAS inhibition by captopril (Behuliak *et al.*, unpublished data).

Radiotelemetric study of BP and HR in adult SHR and WKY rats subjected to chronic treatment with ACE inhibitor captopril revealed a parallel decrease of BP and LF-SBPV, which was more pronounced in SHR than in WKY rats [78]. This was true not only under basal conditions but also during acute restraint stress. HR variability (HRV) was greater in WKY rats than in SHR, indicating an increased parasympathetic tone in normotensive rats. Surprisingly. chronic captopril administration decreased HRV but only in WKY rats. Chronic ACE blockade improved the impaired baroreflex-HR control in SHR by increasing the sensitivity but not the capacity of the vagal arm of the arterial baroreflex. Finally, Michal Behuliak demonstrated that chronic captopril treatment attenuated BP changes elicited by dimethylphenylpiperazinium (DMPP, agonist of nicotinic acetylcholine receptors), especially in SHR, indicating that sympathetic nerve transmission was facilitated by angiotensin II more in hypertensive than in normotensive animals. Thus, chronic ACE inhibition lowered BP through both central and peripheral attenuation of sympathetic tone [78].

The simultaneous study [79] was aimed at the changes in cardiovascular autonomic control induced by chronic inhibition of acetylcholinesterase (AChE) using two different inhibitors – pyridostigmine (peripheral AChE inhibitor) or donepezil (peripheral and central AChE inhibitor). Chronic treatment with either acetylcholinesterase inhibitor decreased HR and increased HRV in both SHR and WKY rats, but neither drug significantly altered BP under basal or stress conditions. Regarding sympathovagal balance, the acute blockade of acetylcholine muscarinic receptors by acute methylatropine administration caused a greater increase of HR in WKY than in SHR. The chronic AChE inhibition by pyridostigmine or donepezil enhanced HRV and HR response to methylatropine (vagal tone) in WKY rats, whereas similar changes were elicited only by pyridostigmine but not by donepezil treatment in SHR. In conclusion, the vagal tone was lower in SHR compared with WKY, but it was enhanced by chronic pyridostigmine treatment in both strains. Thus, chronic peripheral, but not central, acetylcholinesterase inhibition has major effects on HR and its variability in both normotensive and hypertensive rats [79].

The greatest effort on this topic was given in the study of the adaptation of SHR and WKY rats to the repeated restraint, which was applied once daily for seven consecutive days [80]. It should be noted that adult SHR show several symptoms of chronic stress such as adrenal hypertrophy, hyperthermia and increased plasma corticosterone levels which correspond to their higher reactivity to a novel stressor. Stress-induced pressor response and vascular sympathetic activity (LF-SBPV) were enhanced in SHR subjected to a single restraint compared to WKY rats, whereas stress-induced tachycardia was similar in both strains. SHR exhibited attenuated cardiac parasympathetic activity (decreased HRV) and blunted baroreflex sensitivity compared to WKY rats. Repeated restraint did not affect the stress-induced BP increase. However, cardiovascular response during the post-stress recovery period on the last day of repeated restraint was reduced in both strains. The repeatedly restrained SHR showed lower HR compared to stress-naive SHR. SHR subjected to repeated restraint also exhibited attenuated stress-induced tachycardia, augmented cardiac parasympathetic activity, attenuated vascular sympathetic activity, and improved baroreflex sensitivity during the last 7th restraint compared to single-stressed SHR. Thus, SHR exhibited enhanced cardiovascular and sympathetic responsiveness to novel stressor exposure (single restraint) compared to WKY. Unexpectedly, the adaptation of cardiovascular and autonomic responses to repeated restraint was more effective in SHR than in WKY rats [80]. These autonomic nervous and cardiovascular differences in the adaptation to chronic homotypic stress between SHR and WKY rats should be kept in mind when interpreting the results of cardiovascular experiments comprising repeated measurements of blood pressure or other stressful manipulations with animals.

Special attention was also paid to strain differences in the adaptation to repeated restraint stress which was studied in Fischer 344 and Lewis rats, i.e. rats with either hyper-reactivity or hypo-reactivity of hypothalamic-pituitary-adrenal axis. The study by Vodička *et al.* [81] reported that F344 rats exhibited higher BP response during restraint than Lewis rats. Moreover, repeatedly restrained F344 rats showed elevated heart rate and impaired baroreflex sensitivity. It can be concluded that poor adaptation to repeated stress in F344 rats is not only limited to the neuroendocrine response but also has important cardiovascular consequences.

The alterations of central and peripheral mechanisms regulating the autonomic nervous system play an important role in the pathogenesis of various forms of experimental hypertension. They include the abnormal maturation of sympathoneural and sympathoadrenal

systems during the development of spontaneous hypertension in SHR. The available data suggest a complex interaction of RAS and SNS at both central and peripheral levels. The analysis of variability in systolic blood pressure and heart rate enabled us to observe the changes in the autonomic nervous system (sympathetic and parasympathetic) in both normotensive and hypertensive rats under various conditions such as sympathectomy, stress or chronic treatment with AChE or ACE inhibitors. Our results suggest that sympathetic and parasympathetic tone are rather independently regulated as indicated by the influence of chronic captopril treatment on blood pressure and sympathetic tone, and by the effects of chronic acetylcholine esterase inhibition by pyridostigmine on parasympathetic tone and heart rate.

Fig. 14. Dr. Michal Behuliak is receiving the Award of Otto Wichterle for young scientists from Professor Jiří Drahoš, the President of Czech Academy of Sciences, in 2016.

Michal Behuliak (Figs 1 and 13) participated in the work of our research group for 15 years. It was a very productive time but he could have done much more work if his life had been longer. We shall remember him for his scientific and human qualities for a long time.

Conflict of Interest

There is no conflict of interest.

Acknowledgement

Valuable comments of our former coworkers (Anna Vavřínová, Michal Bencze, Silvia Líšková and Mária Pintérová) were highly appreciated. This study was supported by grants from the Czech Science Foundation (GACR 21-03810S), institutional support RVO:67985823, and by the project National Institute for Research of Metabolic and Cardiovascular Diseases (Programme EXCELES, ID Project No. LX22NPO5104) – Funded by the European Union – Next Generation EU.

Abbreviations

ACE, Angiotensin converting enzyme; ACF, Aorto-caval fistula; AChE, Acetylcholine esterase; BK_{Ca}, Large-conductance Ca^{2+}-activated potassium channel; BN.lx, Brown Norway rats with luxation; BP, Blood pressure; cAMP, Cyclic adenosine monophosphate; cGMP, Cyclic guanosine monophosphate; CPI-17, Myosin phosphatase inhibitor protein; DBH, *Dbh*, Dopamine β-hydroxylase; DDC, DOPA decarboxylase; DOCA, Deoxycorticosterone acetate; EDCF, Endothelium-derived constricting factor; ET_A, Endothelin receptor type A; HanSD, Hannover Sprague-Dawley rats; HS, High-salt diet; hHTG, Hereditary hypertriglyceridemic rats; HR, Heart rate; HRV, Heart rate variability; K_V, Voltage-gated potassium channel; L-NAME, N-Nitro-L-arginine methylester; L-VDCC; L type voltage-dependent calcium channel; LF-SBPV, low-frequency systolic blood pressure variability; LS, Low-salt diet; NE, Norepinephrine; NO, Nitric oxide; PGI_2, Prostaglandin I_2 (prostacyclin); *Pnmt*, Phenylethanolamine N-methyltransferase; PTX, Pertussis toxin; RAS, Renin-angiotensin system; Ren-2, Murine renin gene; sEH, Soluble epoxide hydrolase; SHR, Spontaneously hypertensive rats; SNS, Sympathetic nervous system; SR/Jr, Inbred salt-resistant Dahl rats; SS/Jr, Inbred salt-sensitive Dahl rats; TGR, Transgenic rats; TRP, Transient receptor potential channel; WKY, Wistar-Kyoto rats

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