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Altered Balance of Vasoactive Systems in Experimental Hypertension: The Role of Relative NO Deficiency

J. KUNEŠ, S. HOJNÁ, M. KADLECOVÁ, Z. DOBEŠOVÁ, H. RAUCHOVÁ, M. VOKURKOVÁ, J. LOUKOTOVÁ, O. PECHÁŇOVÁ[1], J. ZICHA

CECR and Department of Experimental Hypertension, Institute of Physiology AS CR, Prague, Czech Republic, and [1]Institute of Normal and Pathological Physiology SAS, Bratislava, Slovak Republic

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Summary

This review summarizes our findings concerning the altered balance of vasoactive systems (namely sympathetic nervous system and nitric oxide) in various forms of experimental hypertension – genetic hypertension (SHR, HTG rats), salt hypertension (Dahl rats) and NO-deficient hypertension (L-NAME-treated rats). An attempt is made to define relative NO deficiency (compared to the existing level of sympathetic vasoconstriction), to describe its possible causes and to evaluate particular indicators of its extent. A special attention is paid to reactive oxygen species, their interaction with NO metabolism, cell Ca^{2+} handling and blood pressure regulation. Our current effort is focused on the investigation of abnormal regulation of cytosolic Ca^{2+} levels in smooth muscle and endothelium of hypertensive animals. Such a research should clarify the mechanisms by which genetic and/or environmental factors could chronically modify blood pressure level.

Key words

Nitric oxide • Sympathetic nervous system • Reactive oxygen species • Vasoactive system balance • Nifedipine-sensitive BP component • Calcium influx

Introduction

Both structural and functional factors are responsible for the elevation of peripheral resistance which is characteristic for all known forms of experimental or human hypertension. It is well documented that wall hypertrophy and/or remodeling of resistance arterioles together with altered sensitivity and/or reactivity to enhanced activity of vasoconstrictor systems or to attenuated efficiency of vasodilator systems belong to principal factors augmenting systemic resistance in hypertensive subjects. Despite a considerable effort to detect a wide range of alterations of arterial structure and function in hypertensive animals, less attention was paid to the relative contribution of these abnormalities to the development and/or maintenance of hypertension. The missing information can be obtained by a combination of chronic interventions modifying the effects of principal vasoactive systems in hypertensive animals with the determination of participation of given vasoactive systems in actual BP maintenance. The quantification of the impact exerted by particular vasoactive systems in various forms of experimental hypertension might direct

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

our future effort towards the analysis of the most important abnormalities. Since vascular effects of both vasoconstrictor and vasodilator systems are mediated by the changes of intracellular calcium concentration, the attention should also be paid to abnormal cell calcium handling in vascular smooth muscles (VSMC) derived from hypertensive rats as well as to BP changes induced by systemic blockade of Ca^{2+} channels in hypertensive and normotensive animals.

Age factor in salt-dependent hypertension: the role of pressor systems

Increased susceptibility of immature organism to excess salt intake is a traditional research topic in the Laboratory of Experimental Hypertension in our institute (for review see Jelínek *et al*. 1986, Zicha *et al*. 1986, Zicha and Kuneš 1999a). In the past our attention was focused to age-dependent differences in the participation of various pressor systems in the development and/or maintenance of high blood pressure in animals subjected to chronic salt loading before sexual maturation or only in adulthood. It was demonstrated that sympathetic nervous system and digoxin-like factor play a more important role in immature rats (Kuneš *et al*. 1985, Zicha *et al*. 1985, 1987), whereas reserve pressor systems such as vasopressin (Zicha *et al*. 1989), angiotensin II (Zicha *et al*. 1987) or endothelin (Dobešová *et al*. 2003) are highly significant for salt-dependent hypertension elicited in adult animals. It should be noted that antidiuretic effect of vasopressin is essential for the occurrence of DOCA-salt induced BP elevation in adult but not young rats, although the severity of this form of hypertension is dependent on antidiuretic vasopressin effects in both age groups. In addition, pressor effects of vasopressin contribute to the maintenance of high BP only in adult DOCA-salt treated rats (Zicha *et al*. 1989).

On the contrary, the study of the role of vasodilating systems in experimental hypertension was rather neglected because of the lack of appropriate pharmacological tools in the eighties.

Dysbalance of vasoconstrictor and vasodilator systems in experimental hypertension

In the last five years we have focused our attention to the alterations in the balance of two major vasoactive systems – sympathetic nervous system (SNS) and nitric oxide (NO) which seem to be well-balanced counterparts of BP regulation in normotensive animals. Using a sequential blockade of renin-angiotensin system (RAS), SNS and NO synthase (NOS) originally described by Minami *et al*. (1995) we revealed a characteristic dysbalance between sympathetic hyperactivity and relative NO deficiency in most of the examined forms of experimental hypertension, i.e. in animals with spontaneous (hereditary hypertriglyceridemic rats – HTG), salt-induced (Dahl salt-sensitive rats) and NO-deficient hypertension (L-NAME-treated rats).

The initial analysis was performed in young salt-sensitive Dahl rats in which elevated residual BP (measured after the blockade of both RAS and SNS) and the predominance of sympathetic vasoconstriction over NO-dependent vasodilation was demonstrated (Zicha *et al*. 2001). Greater BP reduction after acute tempol administration indicated increased importance of enhanced superoxide production for BP maintenance in young salt hypertensive Dahl rats. Our data revealed that the impairment of NO-dependent vasodilation in young salt hypertensive rats was due to enhanced NO inactivation by augmented superoxide formation rather than due to a defective NO synthesis (Zicha *et al*. 2001). Further study (Dobešová *et al*. 2002) disclosed that the elevation of residual BP, enhanced superoxide production and relative NO deficiency are absent in adult Dahl rats with a less pronounced form of salt hypertension. Subsequent genetic analysis (Dobešová *et al*. 2002), which was carried out in young salt-loaded F_2 hybrids derived from salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) Dahl rats, revealed several important associations between basal BP and its particular components disclosed by a consecutive blockade of RAS, SNS and NOS. It is evident from the evaluation of the slopes of highly significant relationships between basal BP values and BP response to the blockade of particular vasoactive systems that the role of angiotensin II in BP maintenance is almost negligible (less than 10 % of BP elevation), whereas the impact of residual BP (probably reflecting structural changes in resistance vessels) was somewhat greater (almost 20 % of BP elevation seen in salt hypertensive animals). There is no doubt that sympathetic nervous system exerts the most important contribution in salt hypertension maintenance (more than 70 % of BP elevation). This is evidenced not only by a rather high correlation coefficient but also by a very steep slope of the relationship between basal BP and pentolinium-induced BP changes compared to the relationship

between basal BP and L-NAME-induced BP changes. It is clear that NO-dependent vasodilation is unable to adequately compensate for the sympathetic hyperactivity, especially in animals with elevated BP values (Dobešová *et al*. 2002).

Similar findings were made in Prague hereditary hypertriglyceridemic (HTG) rats and their HTG x LEW F_2 hybrids (Kuneš *et al*. 2002) in which enhanced SNS-dependent vasoconstriction and unchanged NO-dependent vasodilation (relative NO deficiency in rats with high BP) were also revealed.

Recent study of Pecháňová *et al*. (2004) was focused on the balance of vasoactive systems in NO-deficient hypertension elicited by chronic L-NAME treatment. Surprisingly, SNS had again a major role in BP maintenance, although it is well known that this form of experimental hypertension can be substantially ameliorated by chronic RAS blockade (Arnal *et al*. 1993, Kalliovalkama *et al*. 1999, Jover *et al*. 2001). The most plausible explanation for this apparent contradiction is that the altered CNS interaction between NO and angiotensin II in L-NAME hypertension results in the elevated sympathetic tone which might be "normalized" when not only central NOS but also central RAS is inhibited (Tsuchihashi *et al*. 2000, Bergamaschi *et al*. 2002). The same study also revealed that NO deficiency in this form of experimental hypertension need not be so profound as it was originally supposed. It was repeatedly observed that compared to normotensive intact controls the catalytic NOS activity in L-NAME hypertensive rats was suppressed by 30-60 % only (Pecháňová *et al*. 1997, Bernátová *et al*. 1999). Such a reduction of NOS activity probably does not prevent the involvement of this vasoactive system in BP regulation because we have demonstrated a highly significant inverse correlation between NOS activity and basal BP level just within a group of L-NAME hypertensive rats (Zicha *et al*. 2003). Moreover, in L-NAME hypertensive animals there should be an up-regulation of reserve vasodilator mechanisms such as iNOS (Luvara *et al*. 1998, Pecháňová *et al*. 2004) and/or endothelium-derived hyperpolarizing factor (Vargas *et al*. 1996, Ruiz-Marcos *et al*. 2001).

Nitric oxide formation in experimental hypertension

Within the frame of the search for reasons why NO system of hypertensive rats is not capable to compensate fully the sympathetic overactivity, we have tried to look for abnormalities of particular NOS isoforms ranging from potential gene polymorphisms up to differences in BP response to specific inhibitors of given NOS isoforms. Three distinct nitric oxide synthase isoforms exist in mammalian cells: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). We have investigated possible polymorphisms for these NOSs between HTG and control rats (Lewis), but no mutation for any NOS isoform was detected (Kadlecová *et al*. 2003). Currently, we are studying protein expression (Western blot) of particular NOS isoforms in left ventricle and aorta of both strains.

In salt-sensitive Dahl rats various polymorphisms of iNOS gene (*Nos2*) were described (Deng and Rapp 1995, Chen *et al*. 1998), whereas no polymorphisms were detected for *Nos1* and *Nos3* genes coding nNOS and eNOS, respectively (Deng and Rapp 1995, 1997). Our effort was therefore concentrated on *Nos2* gene using Dahl rats of our colony established from the initial breeding pairs provided by Prof. J.P. Rapp in 1986. No polymorphism between SS/Jr and SR/Jr Dahl rats was found in *Nos2* gene fragment defined by gene-specific primers described by Deng and Rapp (1995). Moreover, two-step PCR procedure according to Chen *et al*. (1998) did not also disclose any difference between SS/Jr and SR/Jr rats in restriction site for *PleI* restriction endonuclease (Hojná *et al*. 2003). Our failure to find the above described *Nos2* polymorphisms might be based upon the differences between our colony and those in Toledo (OH) or Harlan. Nevertheless, two facts should be mentioned. First, Rapp´s group constructed a congenic strain by replacing a small chromosome region containing *Nos2* of salt-sensitive Dahl rat with the homologous region of the contrasting normotensive strain. It should be noted that blood pressures in this congenic strain and SS/Jr rats were not significantly different (Dukhanina *et al*. 1997). Second, Chen *et al*. (1998) were not able to find their *Nos2* polymorphism in salt-sensitive rats of the original Dahl-Brookhaven strain from which Dahl-Rapp (SS/Jr) rats were inbred.

The expression and activity of particular NOS isoforms in Dahl rats were studied by numerous investigators. There is a remarkable agreement concerning neuronal NOS the activity of which is decreased in the kidney of salt hypertensive Dahl rats (Ikeda *et al*. 1995). Its mRNA expression is reduced in both brain and kidney of salt-sensitive Dahl rats (Castrop and Kurtz 2001). Even under the conditions of low salt intake nNOS mRNA expression is decreased in the outer

medulla of SS/Jr rats (Yuan and Cowley 2001). It is therefore not surprising that chronic nNOS inhibition by 7-nitroindazole elevated BP in salt-loaded SR/Jr but not SS/Jr rats (Tan *et al*. 1999). Less consistent are the data on endothelial NOS. Its activity in the kidney of salt hypertensive Dahl rats seems to be unaltered (Ikeda *et al*. 1995), its protein expression is unchanged in the kidney but reduced in the aorta of these animals (Ni *et al*. 1999) and its mRNA expression in most organs is similar to that of SR/Jr rats (Castrop and Kurtz 2001) except of a reduction in outer medulla of SS/Jr rats (Yuan and Cowley 2001, Castrop and Kurtz 2001). Our measurements of total NOS activity in the kidney and left ventricle of Dahl rats revealed its reduction not only in salt hypertensive SS/Jr rats but also in salt-loaded SR/Jr rats which remained normotensive (Pecháňová *et al*., unpublished data).

The most discrepant are the findings concerning inducible NOS in Dahl rats. Its activity in the kidney is negligible in both genotypes (Ikeda *et al*. 1995) and its protein expression in the heart, aorta and kidney of salt hypertensive rats yielded very low values compared to those obtained in salt-resistant animals (Ni *et al*. 1999). Nevertheless, Rudd *et al*. (1999) and Tan *et al*. (2000) reported that under the conditions of high salt intake a systemic blockade of iNOS by AMT (2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine) or aminoguanidine elevated BP not only in SS/Jr but also in SR/Jr rats. Recently, Tian *et al*. (2003) described that BP of salt-loaded SS/Jr but not SR/Jr rats can be raised by intramedullary iNOS blockade by aminoguanidine infusion. On the other hand, our experiments in young salt-loaded Dahl rats drinking aminoguanidine solution (1 g/l) for one month did not disclose any BP change in either rat strain (Zicha *et al.*, unpublished data).

In order to reveal the role of NO produced by the individual NOS isoforms in blood pressure maintenance of HTG rats we have used three different NOS inhibitors in conscious chronically cannulated rats in which pressor systems (RAS and SNS) were acutely blocked by losartan and pentolinium, respectively. There was no strain difference in BP response to L-NAME (30 mg/kg i.v.) which inhibits almost completely eNOS. On the contrary, we have observed enhanced BP response to dimethylguanidine (combined eNOS and iNOS inhibitor, 50 mg/kg i.v.) in HTG than in Lewis rats (+116±6 vs. +81±4 mmHg) (Kadlecová *et al*. 2003). Further experiments suggested an up-regulation of iNOS in HTG because BP response to a more specific iNOS inhibitor – aminoguanidine (50 mg/kg i.v.) was increased in HTG rats (+68±4 vs. +41±3 mm Hg). Finally, we have found the augmented contribution of nNOS to BP maintenance in HTG rats in which enhanced BP response to S-methyl-L-thiocitrulline (1 mg/kg i.v.) was observed (Kadlecová *et al*., data to be published). Similar analysis on the involvement of NOS isoforms is currently being performed in Dahl rats in which we have recently demonstrated enhanced BP response of SS/Jr rats to acute aminoguanidine. This response was, however, not augmented in salt-loaded animals (Zicha *et al*., data to be published).

Reactive oxygen species and relative NO deficiency in experimental hypertension

Enhanced production of reactive oxygen species (ROS) might be one of the causes for decreased NO bioavailability observed in various forms of experimental hypertension. It is well known that superoxide reacts with NO to form toxic peroxynitrite, but it can also influence many cell constituents. The main source of ROS in hypertension seems to be membrane-bound NAD(P)H oxidase, but there are also other important sources of oxygen free radicals such as xanthine oxidase, cyclooxygenase, lipoxygenase, mitochondrial respiration and NO synthase which produces superoxides when deprived of adequate supply of its substrate, L-arginine, and/or cofactor, tetrahydrobiopterin (Schnackenberg 2002, Maxwell 2002).

Our experiments with acute administration of tempol (membrane-permeable superoxide dismutase mimetic) revealed a considerable BP fall in salt hypertensive Dahl rats (Zicha *et al*. 2001) which was more pronounced in young than in adult rats (Dobešová *et al*. 2002). In young but not in adult Dahl rats the tempol pretreatment also augmented BP response to subsequent NOS inhibition, indicating increased NO bioavailability after lowering superfluous superoxide levels (Zicha *et al*. 2001). Moderate but significant BP reduction after acute tempol administration were also observed in HTG rats (Kuneš *et al*. 2002) and L-NAME hypertensive rats (Rauchová *et al*., submitted).

Our subsequent studies brought further evidence for increased ROS production in rats with spontaneous, salt or L-NAME-induced hypertension. Superoxide formation (measured by lucigenin chemiluminescence) was found to be enhanced in the aorta of hypertensive animals in which elevated levels of conjugated dienes

(reflecting long-term increase in ROS production) were detected in the heart and kidney. It should be noted that in both Dahl and L-NAME hypertensive rats there was a remarkable positive association between values of lucigenin chemiluminescence and conjugated diene levels (Rauchová *et al*. 2003). These findings are in good agreement with other reports on enhanced ROS production in salt hypertension of Dahl rats (Swei *et al*. 1997, Trolliet *et al*. 2001, Meng *et al*. 2002) or in L-NAME hypertension (Bauersachs *et al*. 1998, Kitamoto *et al*. 2000, Attia *et al*. 2001).

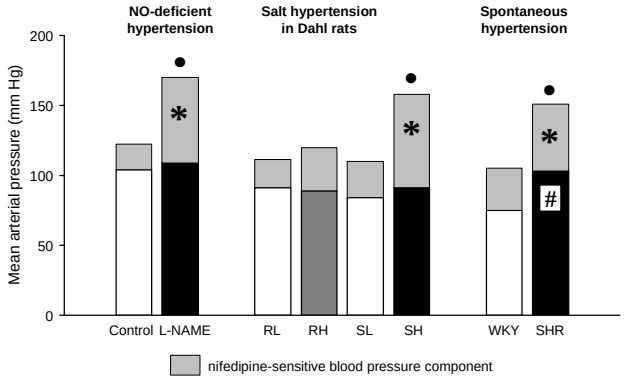

Fig. 1. Basal blood pressure and its nifedipine-sensitive component (hatched bars) in three different forms of experimental hypertension – Wistar rats with NO-deficient hypertension (L-NAME), salt-sensitive Dahl rats with salt hypertension (SH) and spontaneously hypertensive rats (SHR). Data are mean values of about 8 animals per group. Significant differences ($P<0.05$) from corresponding normotensive controls are indicated by full dots (for MAP values), asterisks (for nifedipine-sensitive MAP component) and double cross (for nifedipine-insensitive MAP component).

In the following experiments we have tried to lower ROS production by chronic administration of antioxidants and/or radical scavengers such as tempol or N-acetylcysteine (NAC). It is important to point out that chronic tempol treatment was substantially less efficient compared to NAC administration. In fact, drinking of 2 mmol/l tempol solution for 4 weeks did not modify BP of young salt hypertensive Dahl rats (Dobešová *et al*., unpublished data) and had only a borderline effect on BP of L-NAME-treated rats, although it lowered their ROS production and increased NOS activity (Rauchová *et al*., submitted). On the contrary, chronic NAC treatment completely prevented hypertension development in both young salt-loaded Dahl rats (Kuneš *et al*., data to be published) and adult L-NAME-treated rats (Rauchová *et al*. 2003). This treatment also attenuated the development of spontaneous hypertension if the drinking of NAC solution (20 g/l) started at weaning. In contrast, such a treatment had only a minimal effect on high BP of adult SHR with established hypertension (Pecháňová *et al*. 2003). The antihypertensive effects of NAC treatment are usually ascribed to the reduction of ROS production, the augmentation of antioxidant defense and a concomitant increase of NO bioavailability (Cabassi *et al*. 2001, Girouard *et al*. 2003). Our observations in SHR and L-NAME hypertensive rats are compatible with this mechanism of NAC action, but it should be pointed out that NOS activity was considerably augmented in our animals subjected to chronic NAC treatment. It seems that chronic NAC administration is a useful tool for attenuation or prevention of developing hypertension, but further experiments are necessary to test whether this treatment can substantially influence blood pressure in rats with established salt or L-NAME hypertension.

Voltage-dependent Ca^{2+} channels and experimental hypertension

The Laboratory of Experimental Hypertension has a long tradition in the research of ion transport abnormalities in hypertension. In the past we have used rat erythrocytes for the study of Na^+ and K^+ transport (for review see Zicha *et al.* 1991, Zicha 1993) and rat platelets for investigation of structural and functional membrane abnormalities (for review see Zicha *et al.* 1999). In recent years our research interest extended to cell Ca^{2+} handling in vascular smooth muscle cells (VSMC) cultured in vitro (Loukotová *et al.* 2002a) because numerous alterations of intracellular Ca^{2+} metabolism were reported in rats with genetic hypertension.

We have focused our attention on voltage-dependent Ca^{2+} channels of L type (VDCC) because there is some evidence that enhanced Ca^{2+} influx might play an important role in the pathogenesis of hypertension (for review see Zicha and Kuneš 1999b). Using a systemic administration of Ca^{2+} channel antagonists (nifedipine or verapamil) we have examined BP response to the acute blockade of these Ca^{2+} channels in rats with various forms of experimental hypertension (Kuneš *et al.* 2003). The acute i.v. injection of nifedipine (0.4 mg/kg) to conscious rats lowers blood pressure more in hypertensive than in normotensive rats, the effect being proportional to initial BP level.

Figure 1 shows that nifedipine completely abolished BP difference between L-NAME hypertensive rats and control Wistar rats as well as between salt

hypertensive Dahl rats and their normotensive control groups. In SHR nifedipine removed only about 40 % of the existing BP difference compared to WKY rats, indicating the presence of other hypertensive mechanisms. Figure 2 depicts three highly significant inverse correlations between initial BP level and nifedipine-induced BP reduction obtained in particular forms of experimental hypertension – NO-deficient hypertension induced in Wistar rats by chronic L-NAME treatment, salt hypertension in Dahl rats and genetic hypertension in SHR. In all three hypertensive models such significant correlations can also be found if hypertensive animals are considered separately from normotensive ones (L-NAME rats: r = –0.776, n=18, p<0.001; Dahl salt-sensitive rats: r = –0.907, n=10, p<0.001; SHR: r = –0.504, n=16, p<0.05).

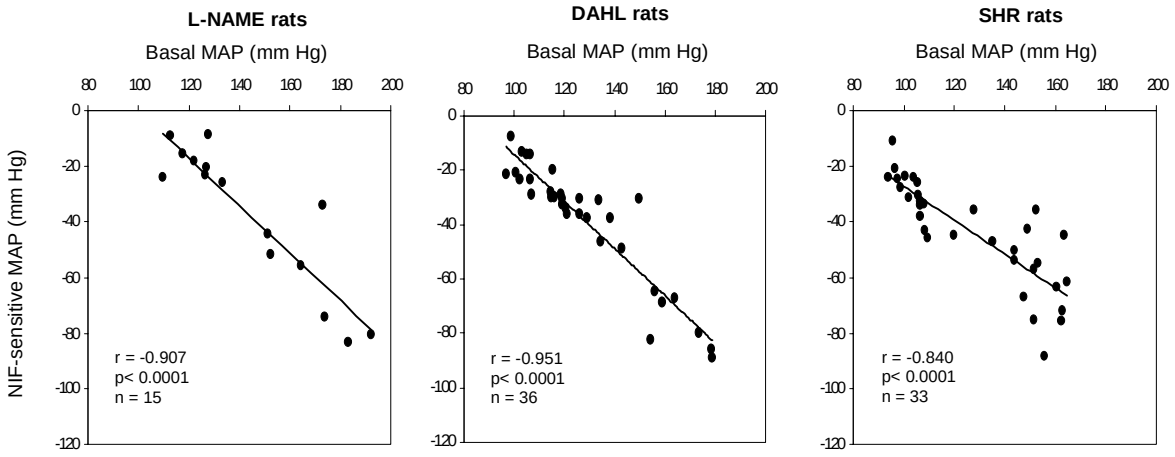

Fig. 2. The relationship of nifedipine-sensitive BP component to basal blood pressure in Wistar rats with NO-deficient hypertension, Dahl rats with salt hypertension and spontaneously hypertensive rats.

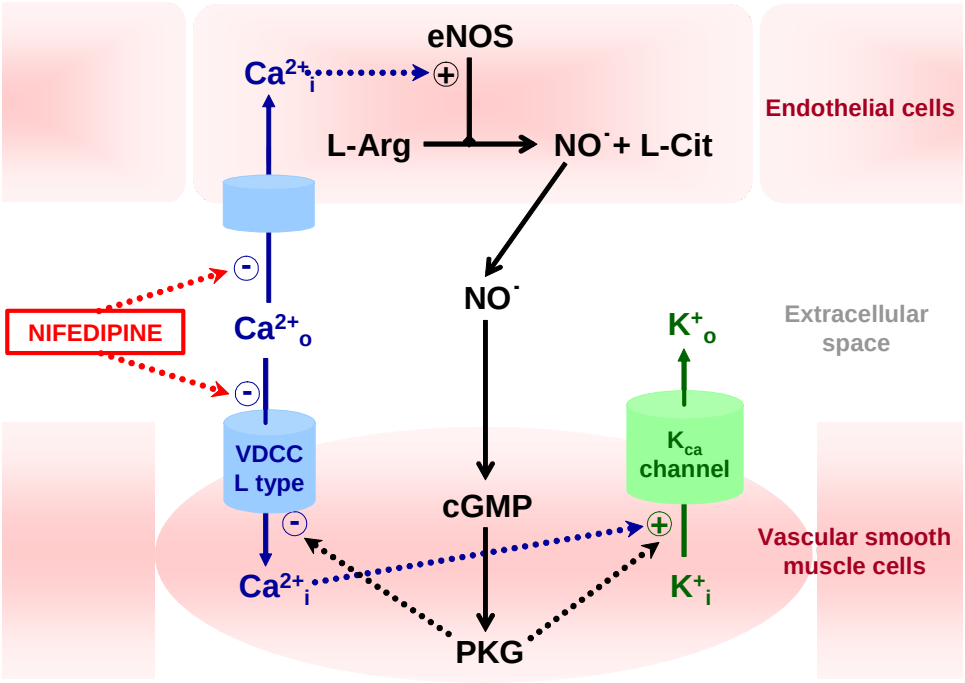

Fig. 3. Scheme of the proposed mechanisms by which nifedipine modifies blood pressure – blockade of NO-dependent vasodilation at the endothelial level and blockade of Ca^{2+}_i-dependent vasoconstriction at the level of vascular smooth muscle.

Although we have originally interpreted our results solely as the consequence of the blockade of Ca^{2+} influx into smooth muscle of resistance arterioles (Kuneš *et al.* 2003), it is evident that BP decrease observed after systemic nifedipine administration is more complex. It seems that Ca^{2+} channel antagonists also block Ca^{2+} entry into endothelial cells (Iouzalen *et al.* 1995), thus removing an essential stimulus for NO formation by the constitutive NO synthase (Lantoine *et al.* 1998). Consequently, NO release decreases and VSMC begin to be deprived of its relaxing effects. Thus the observed BP decrease after nifedipine injection is a sum of two contrasting processes: i) vasodilation occurring after the blockade of Ca^{2+} entry into VSMC through VDCC, and ii) vasoconstriction resulting from the absence of NO and its lowering $[Ca^{2+}]_i$ action in VSMC through its influence on VDCC and Ca^{2+}-dependent K^+ channels (Fig. 3). Consequently, the magnitude of BP response to nifedipine depends on the ratio between constrictor and relaxation mechanisms governing the tone of resistance vessels prior to the injection of Ca^{2+} channel antagonists. Nifedipine therefore causes only minimal BP changes in normotensive animals in which the balance between vasoconstrictor and vasodilator systems is not altered. In contrast, enhanced vasoconstriction and/or attenuated vasodilation in hypertensive animals explains profound BP reduction occurring after acute nifedipine administration (Fig. 2). Figure 4 shows that nifedipine-induced BP reduction might be proportional to the degree of relative NO deficiency which is augmented progressively with increasing basal blood pressure.

To our knowledge, nifedipine-induced BP response seems to be the best "indicator" of the dysbalance between vasoconstrictor and vasodilator mechanisms because its magnitude is directly related to the existing vasoconstriction and inversely related to the actual vasodilation. Moreover, it reflects the changes of intracellular Ca^{2+} levels in both tissues directly involved in the control of vascular tone – endothelium and smooth muscle. It is important to note that the same phenomenon was observed in a wide range of genetic and induced forms of experimental hypertension. Moreover, our recent findings suggest that this "indicator" also reflects BP changes induced in genetically hypertensive rats by environmental factors such as dietary NaCl intake or antihypertensive therapy. On the other hand, nifedipine-induced BP change is not a simple clear-cut marker for the degree of Ca^{2+} influx into VSMC, although it should be pointed out that this parameter reflects the abnormalities of VSMC Ca^{2+} handling better in hypertensive than in normotensive animals.

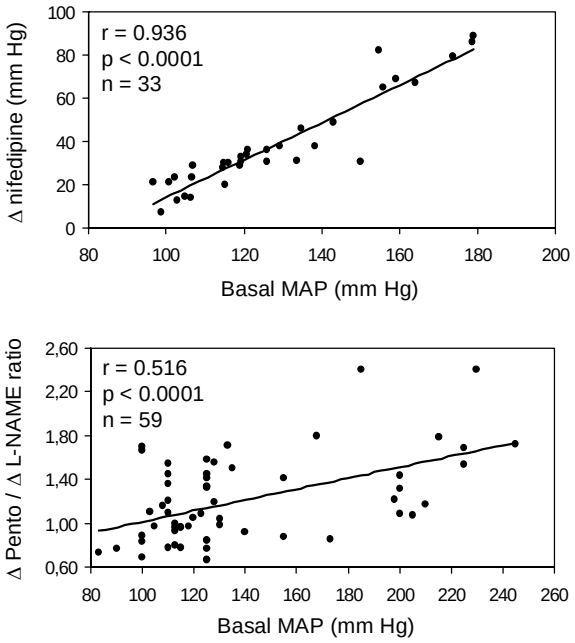

Fig. 4. The relationship of basal blood pressure in Dahl rats to nifedipine-sensitive BP component (upper panel) and to vasoactive dysbalance (lower panel) expressed as the ratio of BP responses to pentolinium and L-NAME.

Calcium influx and gender difference in genetic hypertension

In order to search for the mechanisms responsible for sexual dimorphism in blood pressure, we have studied growth and cell calcium handling in vascular smooth muscle cells (VSMC) isolated from SHR. The experimental data obtained in SHR (Loukotová *et al.* 1998) have indicated that VSMC isolated from males proliferate more rapidly than those isolated from females, both under basal conditions and after stimulation with angiotensin II (Ang II). Later we have demonstrated substantially greater response of free cytosolic calcium ($[Ca^{2+}]_i$) to Ang II in aortic VSMC isolated from male SHR compared to those isolated from females. This gender-dependent difference has been further evaluated by studying the role of thapsigargine-sensitive intracellular calcium stores and calcium influx in VSMC isolated from male and female SHR.

We have shown that thapsigargin-sensitive calcium stores are higher in aortic VSMC isolated from female SHR as compared to male SHR. Since the

difference in thapsigargin-sensitive calcium stores in VSMC cannot explain the previously described gender-dependent difference in $[Ca^{2+}]_i$ response to Ang II, the role of calcium influx has been evaluated. This gender difference in $[Ca^{2+}]$i response to Ang II seen in the presence of extracellular calcium was abolished in the absence of extracellular calcium, indicating the important role of Ca^{2+} influx in this gender difference (Loukotová *et al.* 2002a). This finding was further evaluated by using calcium channel blocker nifedipine. Nifedipine caused a decrease in resting $[Ca^{2+}]_i$, which was significantly greater in VSMC isolated from male SHR compared with VSMC isolated from females. Furthermore, nifedipine attenuated Ang II-stimulated $[Ca^{2+}]_i$ response more in VSMC isolated from male SHR than from female SHR (Loukotová *et al.* 2002b,c). These results suggest that gender-dependent difference in $[Ca^{2+}]_i$ response to Ang II is dependent on Ca^{2+} influx, which seems to be greater in male SHR. Our findings also suggest that Ca^{2+} influx via L-type voltage-dependent calcium channels is increased in VSMC isolated from male SHR compared with those isolated from female animals.

Cytosolic calcium, NO and ROS in cultured VSMC of hypertensive rats

Free cytosolic calcium as a primary determinant of contractile tone has been shown to be increased in SHR (Bendhack *et al.* 1992). Oxygen free radicals are found to disrupt the cellular calcium homeostasis leading to the elevation of free cytosolic calcium (Bielefeldt *et al.* 1997). Sarcoplasmic reticulum Ca^{2+} pump (SERCA) in membranes isolated from arterial smooth muscle can be inactivated by reactive oxygen species (ROS) (Grover and Samson 1988). Moreover, superoxide anions stimulate IP_3-induced Ca^{2+} release from vascular smooth muscle reticulum (Suzuki and Ford 1992). On the other hand, a variety of mechanisms have been suggested by which NO participates in the regulation of smooth muscle tone, through the modulation of $[Ca^{2+}]_i$ (Karaki *et al.* 1997). NO-induced inhibition of Ca^{2+}-influx through L-type Ca^{2+} channels has been proposed, including their inhibition by membrane hyperpolarization due to cGMP-dependent activation of Ca^{2+}-dependent K^+ channels. Nitric oxide is known to accelerate the sequestration of $[Ca^{2+}]_i$ via SERCA (Cohen *et al.* 1999). NO has also been proposed to reduce $[Ca^{2+}]_i$ by inhibiting the agonist-induced release of Ca^{2+} from intracellular stores (Hirata *et al.* 1990).

Since aortic VSMC isolated from SHR are characterized both by greater $[Ca^{2+}]_i$ and by enhanced superoxide formation, we have investigated the influence of oxidative stress reduction by means of tempol (SOD mimetic) on basal $[Ca^{2+}]_i$. In the VSMC isolated from SHR we have observed that tempol reduced $[Ca^{2+}]_i$ to the levels of their normotensive controls WKY which were not significantly altered by tempol (Loukotová *et al.* 2003a,b). Furthermore, we have investigated the influence of tempol on $[Ca^{2+}]_i$ response of VSMC to Ang II. The effects of this potent vasoconstrictor are known to be mediated in part through ROS, since Ang II stimulates the production of superoxide and H_2O_2 *via* NADH/NADPH oxidase-sensitive pathways. Recently we have shown that tempol attenuated angiotensin II-stimulated $[Ca^{2+}]_i$ response more in VSMC isolated from SHR than from their normotensive controls (Loukotová *et al.* 2003a,b).

Production of NO and superoxide radicals is gender-dependent. It is commonly accepted that estrogens maintain NO synthesis in arterioles of female spontaneously hypertensive rats (Huang *et al.* 1997). In addition to the enhanced release of NO from arterioles of female SHR, it has been shown that the aorta from male rats produced significantly more superoxide radicals (by about 34 %) than the aorta from female animals (Brandes and Mugge 1997).

Our previous studies indicated that aortic VSMC isolated from male SHR are characterized by augmentation of $[Ca^{2+}]_i$ response to Ang II administration as compared to VSMC isolated from female SHR (Loukotová *et al.* 2002a). This gender-dependent difference was abolished by tempol (Loukotová *et al.*, data to be published). Altogether these results suggest that increased $[Ca^{2+}]_i$ level in VSMC isolated from SHR is dependent on the increased amount of superoxide anion in SHR.

Perspectives of further research

An attempt was made to define relative NO deficiency as the inability to compensate adequately for the existing sympathetic vasoconstriction, to describe its possible causes, and to evaluate its extent in hypertensive animals using particular indicators such as nifedipine-sensitive BP response or the ratio of BP responses elicited by pentolinium and L-NAME (ΔSNS/ΔNO ratio). It is evident that relative NO deficiency is a common denominator of many forms of experimental

hypertension, ranging from genetic to induced ones. One of the reasons for the failure of NO-dependent vasodilation to keep pace with enhanced sympathetic vasoconstriction might be the absence of central regulatory control elements which would augment NO production under the conditions of high BP. This is further aggravated by decreased NO bioavailability due to superfluous ROS production. In later stages of hypertension development the vicious circle of endothelial dysfunction is completed by endothelial damage resulting from deleterious impact of high BP on vascular wall. Both enhanced vasoconstriction and attenuated vasodilation are coupled with augmented levels of cytosolic $[Ca^{2+}]_i$ in vascular smooth muscle of resistance vessels. Using the acute administration of antagonists of L-type channels we have revealed a promising tool for evaluation of the extent of existing dysbalance of vasoactive systems. Nifedipine-sensitive BP component, which is directly proportional to the current vasoconstriction and inversely proportional to the actual vasodilation, represent that part of blood pressure which is modified by environmental factors or antihypertensive therapy, whereas nifedipine-insensitive BP component is far less susceptible to interventions modulating BP level. Future effort should be aimed to careful investigation of genetic and environmental factors chronically modulating cell Ca^{2+} handling at the level of vascular smooth muscle (responsible for vasoconstriction) and endothelium (responsible for vasodilation).

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Reprint requests

Dr. J. Zicha, Institute of Physiology AS CR, Videňská 1083, CZ-142 20 Prague 4, Czech Republic. E-mail: zicha@biomed.cas.cz