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Polymorphism in *Nos2* Gene Is Absent in Prague Colony of Dahl/Rapp Salt-Sensitive and Salt-Resistant Rats

S. HOJNÁ, M. KADLECOVÁ, J. ZICHA, J. KUNEŠ

Institute of Physiology AS CR and Center for Experimental Research of Cardiovascular Diseases, Prague, Czech Republic

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Summary

We have searched for polymorphism of inducible nitric oxide synthase gene (*Nos2* gene) in the Prague colony of salt-sensitive and salt-resistant Dahl/Rapp rats. Specific primers were used to confirm previously described *Nos2* gene polymorphism because this gene was suggested to be a potential candidate gene for genetic hypertension. Phenotyping (blood pressure, organ weight, plasma lipids) have confirmed the data known from other colonies of Dahl/Rapp rats. However, in our colony we were not able to find any *Nos2* gene polymorphism between salt-sensitive and salt-resistant rats, which was previously described in animals from Harlan colony. Moreover, the genetic homogeneity of our salt-sensitive and salt-resistant rats in terms of *Nos2* gene was the same as in the original Brookhaven colony of Dahl rats. This is surprising because our colony has been established from breeding pairs kindly provided by Prof. J.P. Rapp more than 15 years ago. It seems that the polymorphism found in Harlan colony could be the result of previous contamination or genetic drift during the breeding conditions specific for this colony.

Key words

Salt hypertension • Gene polymorphism • Nitric oxide synthase isoforms • Dahl rats

Introduction

Nitric oxide synthase (NOS) catalyzes the synthesis of nitric oxide (NO) which is a short-lived radical that transmits cellular signals involved in vasorelaxation, neurotransmission and cytotoxicity. There are at least three different isoforms of NOS genes (Deng and Rapp 1997). Two types of NOS, known as *Nos1* (neuronal) and *Nos3* (endothelial), are constitutively expressed and are Ca^{2+}/calmodulin-dependent. The remaining isoform, *Nos2* (inducible), is Ca^{2+}- and calmodulin-independent (Nathan *et al.* 1994). *Nos2* gene is localized on rat chromosome 10 (Deng and Rapp 1995), whereas *Nos1* and *Nos3* genes are on rat chromosome 12 (Deng *et al.* 1995) and chromosome 4 (Hübner *et al.* 1995), respectively. Because NO is involved in blood pressure regulation, NOS genes could be regarded as potential candidate genes for genetic hypertension.

Dahl rats represent a unique model for the study of genetic determinants of salt sensitivity and salt-dependent hypertension. Salt hypertensive Dahl rats have increased sympathetic neural tone (Mark 1991, Kuneš *et al.* 1991) due to enhanced central sympathoexcitation (Huang and Leenen 1994, 1998) and attenuated baroreflex control of sympathetic nerve activity (Gordon

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

et al. 1981, Nedvídek and Zicha 2000). Moreover, this model is characterized by impaired NO bioavailability (Hayakawa *et al.* 1999). We have recently demonstrated altered balance between augmented sympathetic vasoconstriction and insufficiently activated NO-dependent vasodilation in young salt hypertensive Dahl rats (Zicha *et al.* 2001). In the past 30 years, several genetic determinants of high blood pressure (Rapp 2000) and abnormal cardiovascular phenotypes related to hypertension (Moreno *et al.* 2003) were disclosed after the inbreeding of outbred Dahl rats originally selected in Brookhaven (NY). Among them there are the polymorphisms of inducible *Nos2* gene, which were demonstrated in Dahl/Rapp salt-sensitive rats by two different techniques (Deng and Rapp 1995, Chen *et al.* 1998).

Our colony of inbred Dahl/Rapp rats was established from the breeding pairs kindly provided by Prof. J.P. Rapp (Toledo, OH) in 1986. It is evident from the comparison of papers by Deng and Rapp (1995) and Chen *et al.* (1998) that there are some differences between inbred Dahl/Rapp rats from Toledo and Harlan colonies. We have therefore used the same specific primers as the above authors in order to verify the occurrence of earlier reported *Nos2* gene polymorphisms in salt-sensitive animals of our Prague colony.

Methods

Animals

Adult male Dahl/Rapp salt-sensitive and salt-resistant rats (Institute of Physiology AS CR, Prague) were housed under standard laboratory conditions (temperature 23±1 °C, 12-h light-dark cycle), drank tap water *ad libitum* and were fed a low-salt diet containing 0.3 % NaCl. Blood pressure was measured by direct puncture of carotid artery under light ether anesthesia using Statham transducer and Hewlett-Packard recorder. Rats were sacrificed by decapitation, heart and kidney were weighed and the liver was frozen in liquid nitrogen. Plasma lipids were analyzed by commercial kits (Lachema, Brno). All procedures were approved by the Ethical Committee of the Institute of Physiology AS CR and conform to the European Convention on Animal Protection.

Isolation of genomic DNA

Genomic DNA of both genotypes was obtained from liver tissue. Briefly, the samples were incubated at 55 ºC overnight in 700 µl of tail buffer (50 mM Tris/pH 8.0; 25 mM EDTA/pH 8.0; 100 mM NaCl; 1 % SDS) and 35 µl proteinase K (Sigma) was added. The lysates were extracted once with equal volume of phenol, then once with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and subsequently extracted once with chloroform-isoamyl alcohol (24:1). DNA was precipitated in 96 % ethanol, dried and resuspended in 50 µl TE (10 mM Tris/7,4 pH; 1 mM EDTA). DNA was quantitated by UV spectroscopy at A_{260}.

Polymerase chain reaction

Three different sets of primers were used for the detection of *Nos2* gene polymorphism. All these primers were synthetized by Vbc-genomics (Austria).

The first experiment

Two allele-specific forward (5'-CAG CTC TCA CTG ATT GAC TTG G-3') and backward (5'-GTC AGG TTC CCA TGT GCT TA-3') primers (Deng and Rapp 1995) were used to amplify ~269 bp fragment of the *Nos2* gene. The PCR program was run on a Thermocycler (Eppendorf). The PCR conditions for these primers were as follows: 95 °C for 2 min, 30 cycles at 94 °C for 40 s, 55 °C for 30 s, and 72 °C for 1.5 min, with extension for 5 min at 72 °C. *Taq* DNA polymerase (Fermentas) was used in this experiment. PCR products were analyzed by electrophoresis on 2 % agarose gel and 5 % polyacrylamide sequencing gel, visualized using ethidium bromide staining and observed under UV light.

The second experiment

In the second experiment, genotyping of the *Nos2* gene was done in two steps. In the first step PCR products were synthetized and in the second one these products were exposed to restriction (Chen *et al*. 1998). Briefly, forward (5´-AGC AGA ATG TGA CCA TCA TGG AC-3´, corresponding to 1295-1318 nucleotide location) and backward (5´-TTT GAC CCA GTA GCT GCC ACT C-3´, corresponding to 2435-2457 nucleotide location) (GenBank Accession No. NM 012611) primers were used to amplify the expected ~1200 bp fragment of *Nos2* gene. Expand High Fidelity PCR System (Roche), which contains *Taq* and *Tgo* DNA polymerases, was used. This powerful polymerase mixture is able to generate PCR products of high yield, high fidelity and high specificity. PCR was initiated at 94 °C for 2 min., continued 30 cycles at 94 °C for 15 s, 53 °C for 30 s and extension at 72 °C for 7 min. After this amplification,

PCR products were exposed to restriction endonuclease *PleI* (New England Biolabs) overnight at 37 °C. This restriction enzyme should recognized restriction site, which was reported to be different in salt-sensitive and salt-resistant Dahl/Rapp rats. If the amplified product contains this restriction site, two shorter fragments should be produced. Undigested and digested samples were analyzed by electrophoresis on 2 % agarose gel, visualized using ethidium bromide staining and observed under UV light.

The third experiment

The analysis of *Nos2* gene polymorphism was also evaluated by a satellite marker D10Mco40 obtained from the RGD database. Forward (5'-AGC AGA GGC AGA GAT TGA TTG C-3') and backward (5'-GGA CGT TGT TGT GAT TTC CTG G-3') primers amplify 301 bp fragment of the *Nos2* gene. PCR conditions were as followed: 95 °C for 2 min, 45 cycles of 95 °C for 40 s, 61 °C for 30 s and 72 °C for 50 s, with extension for 5 min at 72 °C. PCR products were analyzed by electrophoresis on 2 % agarose gel and visualized using ethidium bromide staining.

Single-strand conformation polymorphism

The technique of single-strand conformational polymorphism (SSCP) was used to confirm the results from normal PCR procedure in Experiment 1. Briefly, 20 µl of PCR products were mixed with 30 µl of SSPC loading buffer (95 % formamide, 10 mM NaOH, 0.05 % bromphenol blue) and denatured for 5 min at 97 °C. After cooling on ice the samples and 10 µl of their non-denatured controls were loaded onto 10 % polyacrylamide gel with 10 % glycerol. Electrophoresis was carried out at 350 V constant power for 2.5 h at room temperature. DNA was detected by silver staining according to the standard procedure.

Statistical analysis

Statistical differences between two genotypes were evaluated by Student t-test. Data were expressed as means ± SEM. $P<0.05$ value was considered as significant.

Table 1. Body weight, organ weight, blood pressure and plasma lipids in Dahl/Rapp salt-resistant and salt-sensitive adult male rats fed a low-salt (0.3 % NaCl) diet

	Salt-resistant	**Salt-sensitive**
Body weight (g)	218±9	271±10*
Heart weight (mg/100 g b.w.)	253±2	259±4
Kidney weight (mg/100 g b.w.)	724±10	639±12*
Systolic blood pressure (mm Hg)	137±3	159±3*
Mean arterial pressure (mm Hg)	113±2	131±3*
Diastolic blood pressure (mm Hg)	91±3	108±3*
Plasma triglycerides (mmol/l)	0.43±0.11	1.20±0.16*
Plasma cholesterol (mmol/l)	1.81±0.18	2.05±0.12

Data are means ± S.E.M., * significantly different ($p<0.05$) from salt-resistant rats

Results

Table 1 summarizes some basic data about salt-sensitive and salt-resistant Dahl/Rapp rats of our colony. Blood pressure of salt-sensitive rats was significantly higher even on low-salt diet when compared to salt-resistant ones. The same was true for plasma triglycerides.

PCR analyses of genomic DNA from Dahl/Rapp salt-sensitive and salt-resistant rats were performed in Experiment 1. PCR amplified 269 bp segment which was the same in both genotypes (Fig. 1). This was also confirmed by SSCP which was not able detected any differences in PCR products of the two genotypes (data not shown).

Using the specific pair of primers, ~1200 bp segment of *Nos2* gene was obtained, which was the same in the two genotypes (Fig. 2 upper panel, Experiment 2). Several other fragments were amplified during this PCR step. However, subsequent restriction of PCR products with *PleI* restriction endonuclease did not display any polymorphism in *Nos2* gene between salt-sensitive and salt-resistant rats (Fig. 2 lower panel).

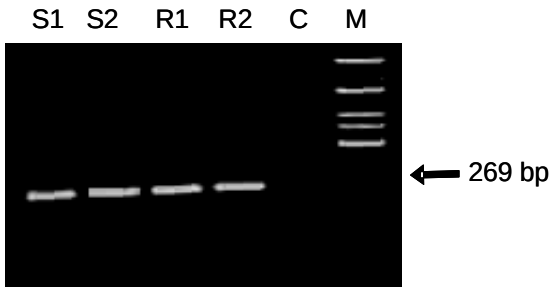

Fig. 1. Representative PCR analysis of genomic DNA by using of specific primers for *Nos2* gene described in Experiment 1. The same 269 bp segments were amplified in Dahl/Rapp salt-sensitive (S1, S2) and salt-resistant (R1, R2) rats by using of these primers. M – marker, C – negative control.

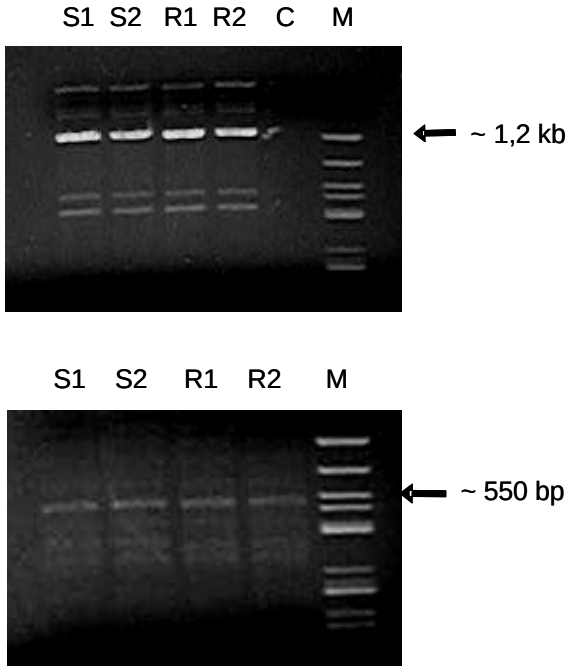

Fig. 2. PCR analysis of genomic DNA by using of combination of specific primers for *Nos2* gene described in Experiment 2. Several fragments were amplified in the first step (2 upper panel), including ~1200 bp segment. Restriction with *PleI* did not display any polymorphism in *Nos2* gene between Dahl/Rapp salt-sensitive (S1, S2) and salt-resistant (R1, R2) rats (2 lower panel).

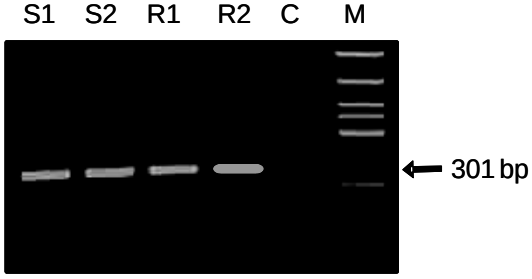

Fig. 3. Representative PCR analysis of genomic DNA by using of specific microsatellite marker D10Mco40, which amplified 301 bp segment of *Nos2* gene in both Dahl/Rapp salt-sensitive (S1, S2) and salt-resistant (R1, R2) rats (Experiment 3).

Finally, we have used microsatellite marker D10Mco40 which amplified 301 bp segment of *Nos2* gene (Fig. 3, Experiment 3), but we also did not detect any polymorphism between the two strains.

Discussion

The Dahl salt-sensitive rats were developed as a model of salt-dependent hypertension (Dahl *et al.* 1963). Since their inbreeding at the beginning of eighties by Rapp and Dene (1985), salt-sensitive and salt-resistant rats represent one of the best characterized and most widely used model of genetic hypertension. However, St. Lezin *et al.* (1994) and Lewis *et al.* (1994) have demonstrated the genetic contamination of salt-sensitive Dahl/Rapp rats from Harlan Sprague Dawley, Inc., one of the biggest commercial supplier of this model. The Prague colony of salt-sensitive and salt-resistant Dahl/Rapp rats was established from breeding pairs kindly provided by John P. Rapp in 1986 and since that time they are bred by a strict brother x sister mating. In the present paper we have searched for a polymorphism in *Nos2* gene, which was demonstrated in salt-sensitive rats from Toledo and Harlan colonies (Deng and Rapp 1995, Chen *et al.* 1998).

Using the same primers and the same techniques of genomic DNA isolation as did the authors mentioned above, no polymorphism in *Nos2* gene was demonstrated between salt-sensitive and salt-resistant Dahl/Rapp rats of Prague colony. Several reasons could explain these discrepant results. As already mentioned, Dahl rats from Harlan colony were genetically contaminated at the beginning of ninetieth (St. Lezin *et al* 1994, Lewis *et al.* 1994). Fortunately, basic stock of foundation colony was not affected (Lewis *et al.* 1994) so that a "new" Harlan Sprague-Dawley Dahl salt-sensitive colony was reestablished from this stock (McGinley 1994). However, these "new" Harlan Dahl rats had several phenotypic characteristics (body weight, mortality, blood pressure response to high salt) different from the original inbred colony of John P. Rapp (Walder *et al.* 1996), suggesting that some new genetic changes were set in a new inbred stock of Harlan Dahl rats. Our present results support this explanation because Prague colony behaves in terms of NOS*2* gene as the original Dahl colony still kept in Brookhaven.

Nevertheless, it should be noted that salt-sensitive Dahl/Rapp rats from Prague colony represent a good model for the study of salt hypertension (Zicha *et al.* 2001, Dobešová *et al.* 2002). The absence of a

polymorphism in NOS*2* gene in our salt-sensitive animals need not be a major problem because later genetic analysis of Dahl/Rapp rats from Toledo colony, which was based upon newly developed congenic strains, localized NOS*2* gene outside of blood pressure QTL(s) on chromosome 10 (Dukhanina *et al.* 1997, Garrett *et al.* 2001). Our failure to disclose any NOS*2* gene polymorphism in our salt-sensitive Dahl/Rapp rats is in a concert with our recent observations that neither acute nor chronic administration of inducible NOS inhibitor, aminoguanidine, induced different blood pressure changes in salt-sensitive than in salt-resistant animals from our colony (Kuneš *et al.* 2004, Zicha *et al.*, unpublished data). This is in contrast with previous reports indicating important pathophysiological role of inducible NOS in the development and/or maintenance of salt hypertension in Dahl rats from Harlan colony (Chen and Sanders 1993, Rudd *et al.* 1999, Tan *et al.* 2000).

In conclusion, the phenotypic characterization of Prague salt-sensitive and salt-resistant Dahl/Rapp rats was not changed from the time of establishing this colony in the Institute of Physiology AS CR in 1986. There is no polymorphism in *Nos2* gene in these rats, suggesting that these animals could be genetically similar to the original stock kept in Brookhaven. Finally, the absence of NOS*2* gene polymorphism in our colony is in agreement with the later exclusion of this gene from the list of candidate genes for salt hypertension.

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References

CHEN PY, SANDERS PW: Role of nitric oxide synthesis in salt-sensitive hypertension in Dahl/Rapp rats. *Hypertension* **22**: 812-818, 1993.

CHEN PY, GLADISH RD, SANDERS PW: Vascular smooth muscle nitric oxide synthase anomalies in Dahl/Rapp salt-sensitive rats. *Hypertension* **31**: 918-924, 1998.

DAHL LK, HEINE M, TASSINARI L: Effects of chronic excess salt ingestion: evidence that genetic factors play an important role in susceptibility to experimental hypertension. *J Exp Med* **115**: 1173-1190, 1963.

DENG AY, RAPP JP: Locus for the inducible, but not a constitutive, nitric oxide synthase cosegregates with blood pressure in Dahl salt-sensitive rat. *J Clin Invest* **95**: 2170-2177, 1995.

DENG AY, RAPP JP: Absence of linkage for „endothelial" nitric oxide synthase locus to blood pressure in Dahl rats. *Hypertension* **29**: 49-52, 1997.

DENG AY, RAPP JP, KATO H, BIHOREAU MT: Linkage mapping of the neuronal nitric oxide synthase gene (*Nos1*) to rat chromosome 12. *Mamm Genome* **6**: 824, 1995.

DOBEŠOVÁ Z, KUNEŠ J, ZICHA J: The altered balance between sympathetic nervous system and nitric oxide in salt hypertensive Dahl rats: ontogenetic and F_2 hybrid studies. *J Hypertens* **20**: 945-955, 2002.

DUKHANINA OI, DENE H, DENG AY, CHOI CR, HOEBEE B, RAPP JP: Linkage map and congenic strains to localize blood pressure QTL on rat chromosome 10. *Mamm Genome* **8**: 229-235, 1997.

GARRETT MR, ZHANG X, DUKHANINA OI, DENG AY, RAPP JP: Two linked blood pressure quantitative trait loci on chromosome 10 defined by Dahl rat congenic strains. *Hypertension* **38**: 779-785, 2001.

GORDON FJ, MATSUGUSHI H, MARK AL: Abnormal baroreflex control of heart rate in prehypertensive and hypertensive Dahl genetically salt-sensitive rats. *Hypertension* **3** (Suppl I): I135-I141, 1981.

HAYAKAWA H, RAIJ L: Relationship between hypercholesterolaemia, endothelial dysfunction and hypertension. *J Hypertens* **17**: 611-619, 1999.

HUANG BS, LEENEN FH: Brain „ouabain" mediates the sympathoexcitatory and hypertensive effects of high sodium intake in Dahl salt-sensitive rats. *Circ Res* **74**: 586-595, 1994.

HUANG BS, LEENEN FH: Both brain angiotensin II and „ouabain" contribute to sympathoexcitation and hypertension in Dahl S rats on high salt intake. *Hypertension* **32**: 1028-1033, 1998.

HÜBNER N, KREUTZ R, RUBATTU S, LEE YA, GANTEN D, ALLEN PD, LINDPAINTNER K: The gene encoding endothelial nitric oxide synthase, *Nos 3*, maps to rat chromosome 4. *Mamm Genome* **6**: 758-759, 1995.

KUNEŠ J, LEONTJEVA GR. BYŠKOVÁ E, POHLOVÁ I, GOVYRIN VA, ZICHA J: Adrenergic innervation of blood vessels in Dahl rats with salt hypertension. *Clin Exp Hypertens A* **13**: 1343-1355, 1991.

KUNEŠ J, HOJNÁ S, KADLECOVÁ M, DOBEŠOVÁ Z, RAUCHOVÁ H, VOKURKOVÁ M, LOUKOTOVÁ J, PECHÁŇOVÁ O, ZICHA J: Altered balance of vasoactive systems in experimental hypertension: the role of relative NO deficiency. *Physiol Res* **53** (Suppl 1): S23-S34, 2004.

LEWIS JL, RUSSELL RJ, WARNOCK DG: Analysis of the genetic contamination of salt-sensitive Dahl/Rapp rats. *Hypertension* **24**: 255-259, 1994.

MARK AL: Sympathetic neural contribution to salt-induced hypertension in Dahl rats. *Hypertension* **17** (Suppl I): I86-I90, 1991.

McGINLEY JR: Response to editorials. *Hypertension* **24**: 254, 1994.

MORENO C, DUMAS P, KALDUNSKI ML, TONELLATO PJ, GREENE AS, ROMAN RJ, CHENG Q, WANG Z, JACOB HJ, COWLEY AW, Jr.: Genomic map of cardiovascular phenotypes of hypertension in female Dahl S rats. *Physiol Genomics* **15**: 243-257, 2003.

NATHAN C, XIE Q: Regulation of biosynthesis of nitric oxide. *J Biol Chem* **269**: 13725-13728, 1994.

NEDVÍDEK J, ZICHA J: Baroreflex control of heart rate in young and adult salt hypertensive inbred Dahl rats. *Physiol Res* **49**: 323-330, 2000.

NUNOKAWA Y, ISHIDA N, TANAKA S: Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem Biophys Res Commun* **191**: 89-94, 1993.

RAPP JP: Genetic analysis of inherited hypertension in the rat. *Physiol Rev* **80**: 135-172, 2000.

RAPP JP, DENE H: Development and characteristics of inbred strains of Dahl salt-sensitive and salt-resistant rats. *Hypertension* **7**: 340-349, 1985.

RUDD MA, TROLLIET M, HOPE S, SCRIBNER AW, DAUMERIE G, TOOLAN G, CLOUTIER T, LOSCALZO J: Salt-induced hypertension in Dahl salt-resistant and salt-sensitive rats with NOS II inhibition. *Am J Physiol* **277**: H732-H739, 1999.

ST. LEZIN EM, PRAVENEC M, WONG A, WANG J-M, MERRIOUNS T, NEWTON S, STEC DE, ROMAN RJ, LAU D, MORRIS RC, KURTZ TW: Genetic contamination of Dahl SS/Jr rats. Impact on studies of salt-sensitive hypertension. *Hypertension* **23**: 786-790, 1994.

TAN DY, MENG S, CASON GW, MANNING RD: Mechanisms of salt-sensitive hypertension: role of inducible nitric oxide synthase. *Am J Physiol* **279**: R2297-R2303, 2000.

WALDER RY, MORGAN DA, HAYNES WG, SIGMUND RD, McCLAIN AM, STOKES JB, MARK AL: Genetic characterization of the "new" Harlan Sprague Dawley Dahl salt-sensitive rats. *Hypertension* **27**: 546-551, 1997.

ZICHA J, DOBEŠOVÁ Z, KUNEŠ J: Relative deficiency of nitric oxide-dependent vasodilation in salt-hypertensive Dahl rats: the possible role of superoxide anions. *J Hypertens* **19**: 247-254, 2001.

Reprint requests

J. Kuneš, Institute of Physiology AS CR, Vídeňská 1083, 142 20 Prague 4, Czech Republic. Fax: +420 24106 2488, e-mail: kunes@biomed.cas.cz