Physiol. Res. 48: 259-265, 1999

Influence of Active Immunization against Angiotensin AT_1 or AT_2 Receptor on Hypertension Development in Young and Adult SHR

B. ŽELEZNÁ, L. VESELSKÝ, J. VELEK[1], Z. DOBEŠOVÁ[2], J. ZICHA[2], J. KUNEŠ[2]

Institute of Molecular Genetics, [1]Institute of Organic Chemistry and Biochemistry and [2]Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Received January 25, 1999
Accepted June 22, 1999

Summary

The influence of long-lasting blockade of angiotensin AT_1 or AT_2 receptors by antibody against the particular receptor peptides on blood pressure and relative heart and kidney weight was studied in spontaneously hypertensive rats (SHR). Young and adult SHR were repeatedly immunized against the sequence 14-23 of angiotensin AT_1 receptor from the age of either 1 or 3 months. Other groups of young and adult SHR were immunized against the sequences 37-43 and 106-116 of angiotensin AT_2 receptor. Synthetic peptides conjugated to bovine gamma globulin were used as antigens. After 5 months of immunization, blood pressure was measured by the direct method. All immunized animals produced antibodies against the particular peptides. At the end of immunization, the blood pressure was significantly decreased in SHR immunized in youth against angiotensin AT_1 receptor peptide, although no difference in heart and kidney hypertrophy was observed compared to sham-immunized SHR. The immunization against angiotensin AT_1 receptor peptide in adulthood as well as the immunization against angiotensin AT_2 receptor peptides in youth or in adulthood affected neither blood pressure nor heart and kidney weight. No influence of immunization on the studied parameters was observed in normotensive WKY rats. Angiotensin AT_1 receptors play a more important role in the pathogenesis of spontaneous hypertension than angiotensin AT_2 receptors. The blockade of angiotensin AT_1 receptors by active immunization against the receptor peptide attenuated hypertension development in young SHR but did not modify the already established hypertension in adult SHR.

Key words

Spontaneously hypertensive rats • Blood pressure • Active immunization • Angiotensin AT_1 receptor • Angiotensin AT_2 receptor • Heart hypertrophy • Kidney weight

Introduction

Angiotensin II, which participates in the pathogenesis of renovascular as well as genetic hypertension, has two main angiotensin receptor types, AT_1 and AT_2 (Chiu *et al.* 1989, Timmermans *et al.* 1993).

The angiotensin AT_1 receptor is abundantly expressed in organs and tissues connected with blood pressure regulation and control of the fluid-electrolyte balance such as vascular smooth muscle, kidney, heart and adrenals. It is also found in specific brain areas involved in the neurogenic control of blood pressure

Fax +420 2 293 894
E-mail physres@biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

(De Gasparo *et al.* 1995). Through various G-proteins, angiotensin AT_1 receptor activates phospholipase C, inhibits adenylate cyclase and opens G-protein-coupled calcium channels (Ohnishi *et al.* 1992). Losartan and other nonpeptide angiotensin AT_1 receptor antagonists act as targeted antihypertensive drugs in various forms of experimental hypertension including spontaneous hypertension of the rat (Bunkenburg *et al.* 1991, Wong *et al.* 1991, Morton *et al.* 1992).

Angiotensin AT_2 receptor takes part in the activation of protein tyrosine phosphatase (Bottari *et al.* 1992) and is coupled with G_i protein (Hayashida *et al.* 1996). It is expressed abundantly in fetal tissues, but only at low levels in adulthood. The physiological role of the angiotensin AT_2 receptor seems to be rather complicated. Recently, the opposite growth modulating actions of angiotensin AT_1 and AT_2 receptors were reported in coronary endothelial cells, where both receptor types are present. Angiotensin AT_1 receptor stimulation caused cell proliferation, whereas the activation of angiotensin AT_2 receptor had an antiproliferative effect (Stoll *et al.* 1995). If angiotensin AT_2 receptors had been transfected to smooth muscle cells, which normally do not express these receptors, they antagonized the growth effect of angiotensin II mediated by angiotensin AT_1 receptors (Nakajima *et al.* 1995).

Both angiotensin AT_1 and AT_2 receptors belong to the family of seven transmembrane receptors. For angiotensin II binding as well as for binding of antibody that would recognize the receptor on the cell surface, only the N-terminal part and three extracellular loops of the receptor are apparently involved. A peptide corresponding to an extracellular sequence of the receptor could serve as an antigen for the production of an antibody aimed to a particular extracellular part of the receptor. Antibodies against the N-terminal sequence 14-23 (Železná *et al.* 1992) and 15-24 (Ishida *et al.* 1995) of angiotensin AT_1 receptor recognized the receptor *in vitro* in membrane lysates of receptor producing tissues and cultivated cells. When administered intracerebroventricularly, the antibody against the sequence 14-23 of angiotensin AT_1 receptor was proven to antagonize angiotensin II-induced increase of blood pressure and water drinking (Richards *et al.* 1993). Moreover, we have demonstrated that the repeated immunization against the sequence 14-23 prevented the development of two-kidney, one-clip hypertension in Wistar rats (Železná *et al.* 1994).

The aim of the present study was to evaluate the influence of repeated immunization against angiotensin AT_1 or AT_2 receptors on blood pressure and relative organ weights in young and adult spontaneously hypertensive rats. The peptide corresponding to the sequence 14-23 of angiotensin AT_1 receptor, or two peptides identical with the sequence 37-43 from the N-terminal part and sequence 106-116 from the first extracellular loop of angiotensin AT_2 receptor, served as antigens. The influence of immunization against particular receptors on hypertension development in immature SHR, or on the maintenance of established hypertension in adult SHR, was followed in this study.

Methods

Experimental groups

Spontaneously hypertensive (SHR) and normotensive Wistar-Kyoto (WKY) rats were bred in the Institute of Physiology AS CR, Prague. To study the effects of active immunization against angiotensin AT_1 or AT_2 receptors, four groups of SHR were established for each type of angiotensin receptor. The first group was immunized from the age of 1 month (young), the second group from the age of 3 months (adult), the third group was injected with adjuvans only (sham-immunized) and the fourth group did not undergo any treatment (non-immunized). Due to the absence of significant differences between non-immunized and sham-immunized SHR (data not shown), these two experimental groups were considered as a single control SHR group. The young (one-month-old) WKY were treated in the same manner.

Preparation of peptides

Peptides corresponding to the sequence 14-23 of angiotensin AT_1 receptor and 37-43 and 106-116 of angiotensin AT_2 receptor were prepared by the solid phase method according to Merrifield (1963). The 4-methylbenzhydrylamine resin (substitution 0.81 mmol/g) was used for synthesis. The N-amino terminal and side chain groups of amino acids were provided with protecting groups that had been compatible by the Boc/Bzl protection strategy. Synthesis was performed in the 0.3 mmol schedule. The peptides were cleaved from the resin and the protecting groups were split off with a mixture of liquid hydrogen fluoride:anisol (9:1). The peptides were purified by preparative reverse phase HPLC using 250x16 mm column of Sepharon SG X C18 (10 μm). The H_2O-methanol gradient was 1 %/min, at flow rate 8.5 ml/min. The purity of all peptides prepared was higher than 95 %. The amino acid analysis and FAB

mass spectrometry of the particular peptides confirmed the expected peptide composition.

Immunization of animals

The peptide corresponding to the sequence 14-23 of angiotensin AT_1 receptor was conjugated to bovine gamma globulin (BGG). Briefly, 3 mg of the peptide in 0.6 ml phosphate buffered saline (PBS) was mixed with 6 mg BGG in 0.9 ml PBS. Dropwise, 25 μl of 8 % glutaraldehyde was added and the mixture was kept for 2 h at room temperature and then dialyzed against PBS at 4 °C overnight. One hundred microliters of the conjugate containing 0.1 mg of peptide were used as a dose for the immunization of each animal.

An identical procedure was used for the preparation of the conjugate of angiotensin AT_2 receptor peptides 37-43 and 106-116 with bovine gamma globulin. In this case, 3 mg of each peptide in 0.6 ml PBS were reacted with 6 mg BGG in 0.9 ml PBS. As a dose for immunization, 100 μl of the conjugate containing 0.1 mg of each peptide were applied.

Before immunization, the conjugate was suspended in an equal volume of adjuvans and the suspension was used for subcutaneous immunization. The animals were immunized five times at one-month intervals. For the first immunization, the complete Freud's adjuvans was used, for the second and third ones, the incomplete Freud's adjuvans was applied, the fourth and fifth antigen administrations were not accompanied by the adjuvans.

ELISA

The antibody titer was checked by ELISA method in the serum obtained 7-10 days after each immunization. Briefly, 1 μg of the particular peptide was conjugated to 1 mg of bovine serum albumin *via* 1 % glutaraldehyde in each well. Blocking was performed with 1 % bovine serum in PBS, then serially diluted rat sera were applied for one hour. The second antibody, goat antirat IgG conjugated with horseradish peroxidase (Sigma) diluted 1:5000, was added for 30 min. Bound peroxidase activity was detected using *o*-phenylene-diamine and H_2O_2 as substrate. The absorbance was determined at 492 nm.

Blood pressure and organ weight

At the end of the experiment, blood pressure was measured by direct puncture of the carotid artery under light ether anesthesia (Železná *et al.* 1994). After killing the animals by decapitation, the heart and kidney weight were determined.

Statistics

The data are expressed as means ± S.E.M. Statistical differences were evaluated by means of one-way ANOVA with the subsequent least significant difference test (Snedecor and Cochran 1968).

Results

All SHR and WKY animals immunized against the peptide 14-23 from angiotensin AT_1 receptor in youth (from the age of 1 month) developed 1:1600 antibody titer after the third immunization. The antibody titer was kept at this level after two further antigen injections. Similar antibody formation had been found in the animals immunized against peptides 37-43 and 106-116 from angiotensin AT_2 receptor. All SHR animals immunized in adulthood (from the age of 3 months) developed antibodies after the second immunization, with a titer 1:800 to 1:1600 for both peptides from the angiotensin AT_2 receptor and 1:1600 for the angiotensin AT_1 receptor peptide. The animals maintained this antibody titer after three further antigen boosts.

Table 1. Influence of immunization against angiotensin AT_1 or AT_2 receptor on relative heart and kidney weight in SHR subjected to this treatment in youth or in adulthood.

Groups	Controls	anti AT_1	anti AT_2
Immunization from			
	Heart weight (mg/100 g b.w.)		
Youth	342±5 (16)	338±4 (10)	338±5 (8)
Adulthood	353±6 (8)	340±5 (8)	358±4 (6)
	Kidney weight (mg/100 g b.w.)		
Youth	554±10 (16)	545±7 (10)	553±10 (8)
Adulthood	570±8 (8)	564±7 (8)	558±13 (6)

Data are means ± S.E.M. The number of animals is given in parentheses. Controls represent non-immunized and sham-immunized animals taken together.

At the end of the experiment, SHR immunized against angiotensin AT_1 receptor peptide in youth had lower blood pressure than SHR controls (Fig. 1). However, no changes in heart or kidney weight were found (Table 1). On the contrary, the immunization against angiotensin AT_1 receptor peptide in adulthood did not affect the blood pressure levels in SHR (Fig. 1). The immunization against two angiotensin AT_2 receptor peptides did not influence either the blood pressure or relative heart and kidney weight in SHR, irrespective of whether they were immunized from youth or in adulthood only (Fig. 1, Table 1).

The normotensive WKY immunized in youth also did not show any change in blood pressure in comparison with non-immunized WKY controls (Fig. 1).

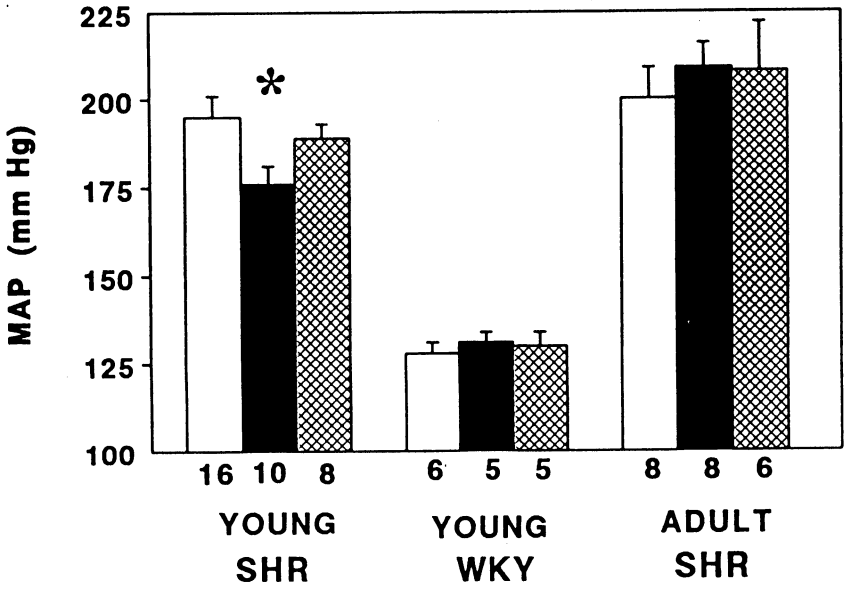

Fig. 1. *Influence of repeated immunization of young SHR and WKY or adult SHR against angiotensin AT_1 (full columns) or AT_2 receptor (cross-hatched columns) on mean arterial pressure at the end of the experiment, i.e. after 5 months of immunization. Controls (open columns) represent non-immunized and sham-immunized animals taken together. Data are means ± S.E.M. Numbers at the bottom of respective columns indicate the number of animals. Asterisk indicates a significant difference ($p<0.05$) from SHR controls.*

Discussion

Our study demonstrated that repeated immunization of young SHR against angiotensin AT_1 receptor attenuated the development of genetic hypertension, whereas immunization of adult animals with established hypertension had no significant effect on blood pressure. On the other hand, the immunization against angiotensin AT_2 receptor did not influence the development or maintenance of spontaneous hypertension. A greater effect of immunization against angiotensin AT_1 receptor in young than in adult SHR is compatible with similar age-dependent blood pressure effects of chronic treatment of SHR with either angiotensin-converting enzyme inhibitors (Harrap *et al.* 1990, Adams *et al.* 1990, Gohlke *et al.* 1992, Kost *et al.* 1995) or AT_1 receptor antagonists (Oddie *et al.* 1992, 1993, Gillies and Lee 1996, Gillies *et al.* 1997).

The antibody against the peptide 14-23 from angiotensin AT_1 receptor (with an antagonistic action to peripheral receptors only) was able to prevent completely the blood pressure increase in Wistar rats subjected to two-kidney, one-clip procedure (Železná *et al.* 1994). Since circulating angiotensin II levels are significantly increased in the early phase of two-kidney, one-clip hypertension, it seems that the antibody competed with circulating angiotensin II for angiotensin AT_1 binding sites.

The antibody against the peptide 14-23 from angiotensin AT_1 receptor only partly reduced hypertension development in SHR and did not affect heart and kidney hypertrophy. Under physiological conditions,

this antibody does not cross the blood-brain barrier. Therefore, the antibody does not compete with angiotensin II formed in the brain and also probably not with angiotensin II locally produced in cardiovascular tissues, which might play a more important role in the pathogenesis of spontaneous hypertension than circulating angiotensin II.

Recently, the long-term prevention of genetic hypertension development was achieved in SHR by the delivery of angiotensin AT_1 receptor antisense to 5-day-old animals (Iyer *et al.* 1996, Lu *et al.* 1997, Martens *et al.* 1998). This procedure is thus more effective in blood pressure control than the immunization against angiotensin AT_1 receptor because of two reasons. Firstly, the above antisense, but not our antibody, acts on brain angiotensin AT_1 receptors, the density of which is higher in SHR than in WKY (Raizada *et al.* 1993). Secondly, the antisense acts from the earliest postnatal age, whereas the production of antibody does not occur in our animals prior to the age of one month.

The blockade of angiotensin AT_2 receptors with the corresponding antibody did not induce any significant changes in blood pressure and organ weight of SHR. This is not surprising because there is still no positive evidence on either AT_2 receptor participation in acute blood pressure control of SHR (Toney and Porter 1993a,b) or on their involvement in the pathogenesis of genetic hypertension (Makino *et al.* 1997). Furthermore, contradictory blood pressure changes were reported in mice subjected to targeted angiotensin AT_2 receptor disruption (Hein *et al.* 1995, Ichiki *et al.* 1995).

In conclusion, repeated immunization against angiotensin AT_1 receptor lowers the blood pressure of SHR during the development of hypertension but not in the established phase of hypertension. It is evident that angiotensin AT_1 receptors play a more important role in the pathogenesis of spontaneous hypertension than angiotensin AT_2 receptors.

Acknowledgements

Supported by the grant of Ministry of Education of the Czech Republic OK 170 (1997), CIPA CT 94-0239 (EC Program COPERNICUS) and A7011711 (Grant Agency of the Academy of Sciences CR).

References

ADAMS MA, BOBIK A, KORNER PI: Enalapril can prevent vascular amplifier development in spontaneously hypertensive rats. *Hypertension* **16:** 252-260, 1990.

BOTTARI SP, KING IN, REICHLIN S, DAHLSTROEM I, LYDON N, DE GASPARO M: The angiotensin AT_2 receptor stimulates protein tyrosine phosphatase activity and mediates inhibition of particulate guanylate cyclase. *Biochem Biophys Res Commun* **183:** 206-211, 1992.

BUNKENBURG B, SCHNELL C, BAUM HP, CUMIN F, WOOD JM: Prolonged angiotensin II antagonism in spontaneously hypertensive rats. Hemodynamic and biochemical consequences. *Hypertension* **18:** 278-288, 1991.

CHIU AT, HERBLIN WF, MCCALL DE, ARDECKY RJ, CARINI DJ, DUNCIA JV, PEASE LJ, WONG PC, WEXLER RR, JOHNSON AL, TIMMERMANS PB: Identification of angiotensin II receptor subtypes. *Biochem Biophys Res Commun* **165:** 196-203, 1989.

DE GASPARO M, BOTTARI S, LEVENS NR: Characteristics of angiotensin II receptors and their role in cell and organ physiology. In: *Hypertension: Pathophysiology, Diagnosis and Treatment.* JH LARAGH, BM BRENNER (eds). Raven Press, New York, 1995, pp 1695-1720.

GILLIES LK, LEE RM: Effects of chronic blockade of angiotensin II receptor on the maintenance of hypertension and vascular changes in spontaneously hypertensive rats. *Can J Physiol Pharmacol* **74:** 1061-1069, 1996.

GILLIES LK, LU M, WANG H, LEE RM: AT_1 receptor antagonist treatment caused persistent arterial functional changes in young spontaneously hypertensive rats. *Hypertension* **30:** 1471-1478, 1997.

GOHLKE P, STOLL M, LAMBERTY V, MATTFELD T, MALL G, VAN EVEN P, MARTORANA P, UNGER T: Cardiac and vascular effects of chronic angiotensin converting enzyme inhibition at subantihypertensive doses. *J Hypertens* **10** (Suppl 6): S141-S144, 1992.

HARRAP SB, VAN DER MERWE WM, GRIFFIN SA, MACPHERSON F, LEVER AF: Brief angiotensin converting enzyme inhibitor treatment in young spontaneously hypertensive rats reduces blood pressure long-term. *Hypertension* **16:** 603-614, 1990.

HAYASHIDA W, HORIUCHI M, DZAU VJ: Intracellular third loop domain of angiotensin II type-2 receptor. Role in mediating signal transduction and cellular function. *J Biol Chem* **271:** 21985-21992, 1996.

HEIN L, BARSH GS, PRATT RE, DZAU VJ, KOBILKA BK: Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature* **377:** 744-747, 1995.

ICHIKI T, LABOSKY PA, SHIOTA C, OKUYAMA S, IMAGAWA Y, FOGO A, NIIMURA F, ICHIKAWA I, HOGAN BL, INAGAMI T: Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature* **377:** 748-750, 1995.

ISHIDA M, MARRERO MB, SCHIEFFER B, ISHIDA T, BERNSTEIN KE, BERK BC: Angiotensin II activates $pp^{60c\text{-}src}$ in vascular smooth muscle cells. *Circ Res* **77:** 1053-1059, 1995.

IYER SN, LU D, KATOVICH MJ, RAIZADA MK: Chronic control of high blood pressure in the spontaneously hypertensive rat by delivery of angiotensin type 1 receptor antisense. *Proc Natl Acad Sci USA* **93:** 9960-9965, 1996.

KOST CK, LI P, JACKSON EK: Blood pressure after captopril withdrawal from spontaneously hypertensive rats. *Hypertension* **25:** 82-87, 1995.

LU D, RAIZADA MK, IYER S, REAVES P, YANG H, KATOVICH MJ: Losartan versus gene therapy: chronic control of high blood pressure in spontaneously hypertensive rats. *Hypertension* **30:** 363-370, 1997.

MAKINO N, SUGANO M, OTSUKA S, HATA T: Molecular mechanism of angiotensin II type I and type II receptors in cardiac hypertrophy of spontaneously hypertensive rats. *Hypertension* **30:** 796-802, 1997.

MARTENS JR, REAVES PY, LU D, KATOVICH MJ, BERECEK KH, BISHOP SP, RAIZADA MK, GELBAND CH: Prevention of renovascular and cardiac pathophysiological changes in hypertension by angiotensin II type 1 receptor antisense gene therapy. *Proc Natl Acad Sci USA* **95:** 2664-2669, 1998.

MERRIFIELD RB: Solid phase peptide synthesis. I. The synthesis of tetrapeptide. *J Am Chem Soc* **85:** 2149-2154, 1963.

MORTON JJ, BEATTIE EC, MACPHERSON F: Angiotensin II receptor antagonist losartan has persistent effects on blood pressure in the young spontaneously hypertensive rat: lack of relation to vascular structure. *J Vasc Res* **29:** 264-269, 1992.

NAKAJIMA M, HUTCHINSON HG, FUJINAGA M, HAYASHIDA W, MORISHITA R, ZHANG L, HORIUCHI M, PRATT RE, DZAU VJ: The angiotensin II type 2 (AT_2) receptor antagonizes the growth effects of the AT_1 receptor: gain-of-function study using gene transfer. *Proc Natl Acad Sci U S A* **92:** 10663-10667, 1995.

ODDIE CJ, DILLEY RJ, BOBIK A: Long-term angiotensin II antagonism in spontaneously hypertensive rats: effects on blood pressure and cardiovascular amplifiers. *Clin Exp Pharmacol Physiol* **19:** 392-395, 1992.

ODDIE CJ, DILLEY RJ, KANELLAKIS P, BOBIK A: Chronic angiotensin II type 1 receptor antagonism in genetic hypertension: effects on vascular structure and reactivity. *J Hypertens* **11:** 717-724, 1993.

OHNISHI J, ISHIDO M, SHIBATA T, INAGAMI T, MURAKAMI K, MIYAZAKI H: The rat angiotensin II AT_{1A} receptor couples with three different signal transduction pathways. *Biochem Biophys Res Commun* **186:** 1094-1101, 1992.

RAIZADA MK, SUMNERS C, LU D: Angiotensin II type 1 receptor mRNA levels in the brains of normotensive and spontaneously hypertensive rats. *J Neurochem* **60:** 1949-1952, 1993.

RICHARDS EM, LU D, ŽELEZNÁ B, PHILLIPS MI, TROLLIET M, SUMNERS C, RAIZADA MK: Inhibition of central angiotensin responses by angiotensin type-1 receptor antibody. *Hypertension* **21:** 1062-1065, 1993.

SNEDECOR GW, COCHRAN WG: *Statistical Methods.* Iowa State University Press, Ames, IA, 1968, pp 258-298.

STOLL M, STECKELINGS UM, PAUL M, BOTTARI SP, METZGER R, UNGER T: The angiotensin AT_2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J Clin Invest* **95:** 651-657, 1995.

TIMMERMANS PB, WONG PC, CHIU AT, HERBLIN WF, BENFIELD P, CARINI DJ, LEE RJ, WEXLER RR, SAYE JAM, SMITH RD: Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev* **45:** 205-251, 1993.

TONEY GM, PORTER JP: Effects of blockade of AT_1 and AT_2 receptors in brain on the central angiotensin II pressor response in conscious spontaneously hypertensive rats. *Neuropharmacology* **32:** 581-589, 1993a.

TONEY GM, PORTER JP: Functional roles of brain AT_1 and AT_2 receptors in the central angiotensin II pressor response in conscious young spontaneously hypertensive rats. *Dev Brain Res* **71:** 193-199, 1993b.

WONG PC, PRICE WA, JR., CHIU AT, DUNCIA JV, CARINI DJ, WEXLER RR, JOHNSON AL, TIMMERMANS PB: In vivo pharmacology of DuP 753. *Am J Hypertens* **4:** 288S-298S, 1991.

ŽELEZNÁ B, RICHARDS EM, TANG W, LU D, SUMNERS C, RAIZADA MK: Characterization of a polyclonal anti-peptide antibody to the angiotensin II type-1 (AT_1) receptor. *Biochem Biophys Res Commun* **183:** 781-788, 1992.

ŽELEZNÁ B, VESELSKÝ L, VELEK J, ZICHA J, KUNEŠ J: Angiotensin AT_1 receptor blockade by specific antibody prevented two-kidney, one-clip renal hypertension in the rat. *Eur J Pharmacol* **260:** 95-98, 1994.

Reprint requests

Dr. J. Kuneš, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20 Prague 4, Czech Republic. e-mail: kunes@biomed.cas.cz