

Changes of inducible nitric oxide synthase in aortic cells during the development of hypertension: Effect of angiotensin II

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Key words: hypertension, inducible nitric oxide synthase, angiotensin II, spontaneously hypertensive rats, vascular smooth muscle cells.

ABSTRACT: Nitric oxide (NO) generation by inducible nitric oxide synthase (iNOS) in the vascular smooth muscle cells (VSMC), may play a role in blood vessel tone regulation. Lipopolysaccharide (LPS) induced iNOS activity and subsequent nitrite production by cultured aortic VSMC, from SHR with an established chronic blood pressure elevation (adult SHR) or during the period preceding the development of hypertension (young SHR) and from age-matched normotensive Wistar (W) rats were compared. Angiotensin II (Ang II) effect was also evaluated. Both basal LPS-induced iNOS activity and nitrite accumulation were significantly lower in young SHR VSMC compared to young W rat cells. In contrast, adult hypertensive and normotensive rat cells did not differ in NO generation. Besides, young SHR cells exhibited a significant smaller iNOS activity and nitrites than adult SHR cells. After 24h-incubation with Ang II, both variables were markedly reduced in all groups. The proportional reduction of iNOS activity and nitrites by Ang II was not different between hypertensive and normotensive rat cells, at any age. However, this Ang II inhibitory effect was greater in both adult SHR and W cells than in VSMC from young rats. In conclusion, a reduced LPS-induced iNOS activity and NO generation was observed in VSMC from spontaneously hypertensive rats before the raise of blood pressure, but not in adult hypertensive rat cells. Additionally, an inhibitory effect of angiotensin II on these variables is described. We can speculate that the impairment in vascular smooth muscle NO production precedes the development of hypertension in SHR and may play a pathophysiologic role in the early blood pressure elevation in genetically hypertensive rats.

Introduction

Nitric oxide (NO) generated from L-arginine by nitric oxide synthases (NOS) is an important signal and effector molecule in the regulation of various cell functions (Moncada *et al.*, 1991), including inhibition of vascular smooth muscle cells (VSMC) proliferation (Ignarro *et al.*, 2001).

In the vascular wall, endothelial cells express a constitutive calcium/calmodulin-dependent NOS (eNOS) (Lamas *et al.*, 1992). Another calcium/calmodulin-independent NOS (iNOS) is present in VSMC and its expression can be induced by cytokines such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF α) or bacterial lipopolysaccharides (LPS) (Busse and Mulsch, 1990; Dinerman *et al.*, 1993). Expression of iNOS in adventitial tissue of the vascular wall has been described (De Meyer *et al.*, 2000a). The expression of a neuronal NOS (nNOS) has been also demonstrated in rat VSMC from carotid artery (Boulanger *et al.*, 1998).

The role of NO generated by iNOS in the control of blood pressure is not well established. NO causes

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vascular smooth muscle relaxation through activation of soluble guanylate cyclase and subsequent increasing cyclic guanosine 3', 5'-monophosphate (cGMP) levels, causing a decrease in intracellular Ca^{2+} (Förstermann *et al.*, 1986). Therefore, alterations in NO synthesis by iNOS may be an important factor in the pathogenesis of hypertension.

Several attempts have been made seeking to see differences in the vascular NO-iNOS system between hypertensive and normotensive rats, but the results are contradictory, with reports showing an increase (Chou *et al.*, 1998; Briones *et al.*, 2000), a decrease (Dubois, 1996) or no change (Singh *et al.*, 1996) in NO generated by iNOS or in the enzyme expression.

Angiotensin II (Ang II) is a regulator of vascular tone as well as a promotor of vascular cell growth. There is a whole body of evidence regarding the countervailing influences between NO and Ang II in the cardiovascular system. It has been suggested that Ang II and NO could be integrated in a homeostatic mechanism aimed at regulating vascular structure and function (Fernández-Alfonso and González, 1999). Conflicting reports also exist on Ang II effects on NO-NOS systems. An inhibitory effect of Ang II on IL-1 β stimulated iNOS expression in VSMC from normal rats has been described (Nakayama *et al.*, 1994). Besides, evidences exist supporting the notion that Ang II may directly be associated with cardiovascular alterations in hypertension, independently of its blood pressure-elevating effects. Therefore we found of interest to examine Ang II effect on NO generation in VSMC during the development of hypertension.

The current study was designed to investigate the possibility of changes in iNOS activity induced by LPS in non-stimulated and Ang II-stimulated cultured VSMC obtained from spontaneously hypertensive rats (SHR) with an established chronic blood pressure elevation or during the period preceding the development of hypertension, comparing with age-matched normotensive animals.

Material and Methods

Male SHR of 3- to 4- weeks- old (young SHR) and 12- weeks- old (adult SHR) and their age-matched Wistar normotensive control rats (young W and adult W) (n=8 in each group) were used for this study. SHR were acquired from the Veterinary School, University of La Plata, Argentina. They were housed under standardized conditions of controlled temperature (20°C)

and humidity (60%) and a 12-hour light/dark cycle. Animals were fed regular commercial pelleted rat chow and given tap water *ad libitum*. All procedures were performed in accordance with institutional guidelines for animal experimentation (Animal Experimentation Committee, School of Medicine, Universidad Nacional de Cuyo). Systolic blood pressure was monitored indirectly in conscious prewarmed slightly restrained rats by the tail-cuff method and recorded on a Grass model 7 polygraph (Grass Medical Instruments). The average of three pressure readings was recorded and systolic pressure was measured four times in each rat. There was not statistic difference between prehypertensive young SHR systolic pressure (116 ± 4 mmHg) and normotensive young W (104 ± 4 mmHg). Old SHR systolic pressure (183 ± 3 mmHg) was significantly greater than that of old W (114 ± 3 mmHg) ($P < 0.001$).

Cell Culture

Unless otherwise noted, all reagents were obtained from Sigma Chemical Co. The animals were killed by decapitation under ether anesthesia and arterial vessels were aseptically excised and placed in chilled Hank's Buffered Saline Solution (HBSS) and antibiotic mixture for further dissection. Aortic cells were isolated according to a technique previously described (Cruzado *et al.*, 1998).

Aortic SMC were obtained from two to three thoracic aortas by enzyme dispersion with 1.5 mg/mL collagenase (Class II Worthington Biochemical Corp, NJ, USA) in F-12 modified Eagle's medium (MEM) with 10% fetal calf serum (FCS) (Gen S.A., Buenos Aires, Argentina). After a 2 to 3 h period in an oscillating water bath at 37°C, isolated cells were grown in 10% FCS/F-12 MEM, incubated at 37°C under humid 5% CO_2 -air conditions, and passaged every 5 to 7 days. Aortic SMC between the fourth and sixth passages were used for the experiments.

Characterization of Cultured SMC

Although cultured aortic VSMC exhibited the characteristic hill-and-valley growth pattern on reaching confluence (Chamley-Campbell JH *et al.*, 1981), cultures were identified by the presence of positive staining with anti-smooth muscle α -actin (Pang, 1989). A negative staining with anti-factor VIII antibodies (Boehringer Mannheim-Biochem) assessed a complete removal of endothelial cells from aortic arteries.

Experimental Protocol

Aortic SMC (4×10^4 cells/well) were plated onto 12-well tissue culture dishes and grown in 10% FCS/F-12 MEM. When the cells reached confluence, they were washed with 0.1% FCS/F-12 MEM during 48 h and then the cells were incubated for 24 h in fresh 0.1% FCS/F-12 MEM with LPS (10 $\mu\text{g}/\text{mL}$) divided in two groups, according to the addition or not of 100 nmol/L Ang II ($n=6$ per group).

Nitrite Measurement

Nitrites, stable oxidation products of NO, were determined in medium removed from cultured VSMC. Aliquots of cell supernatants were mixed with an equal volume of Griess reagent (prepared by adding 1 part 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride to 1 part 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was measured and nitrite concentration determined using sodium nitrite as a standard. Culture medium was used as diluent and as blank to avoid medium phenol red interference. Values were corrected to the amount of protein, measured by Bradford method, (Bradford, 1976), present in the respective cell homogenate ($\mu\text{mol}/\text{mg}$ protein).

Determination of iNOS Activity

iNOS activity was measured in the cell lysate, by the conversion of ^3H -L-arginine in ^3H -citrulline according to the Bredt method (Bredt and Snyder, 1990). The cells were removed with a plastic scraper and then sonicated in a buffer (pH 7.40, 37°C) containing 50 mmol/L Tris, 20 mmol/L HEPES, 250 mmol/L sucrose, 1 mmol/L dithiothreitol, 10 $\mu\text{g}/\text{mL}$ leupeptin, 10 $\mu\text{g}/\text{mL}$ soybean trypsin inhibitor, 5 $\mu\text{g}/\text{mL}$ aprotinin and 0.1 mmol/L phenyl methyl sulphonyl fluoride. After centrifugation of the homogenates (1,000 \times g, 5 min, 4°C), aliquots of 50 μL were added to 100 μL of a cocktail reaction buffer containing 50 mmol/L Tris, 20 mmol HEPES, 1 mmol/L dithiothreitol, 1 mmol/L NADPH, 0.1 mmol/L tetrahydrobiopterine, 50 $\mu\text{g}/\text{mL}$ FAD, 50 $\mu\text{g}/\text{mL}$ FMN and 10 $\mu\text{Ci}/\text{mL}$ L-[2,3- ^3H]-arginine (New England Nuclear, Boston MA, USA) and incubated for 30 min at 37°C in a shaking bath in the presence of 3 mmol/L EGTA and absence of Ca^{2+} /calmodulin. The reaction was stopped by adding 1 mL cold distilled water and the mixture applied to a anion-exchange chromatography column containing Dowex AG 50W-X8 (200/400

Mesh) resin previously saturated with 50 μL of 100 mmol/L L-citrulline and 2 mL 50 mmol/L Tris, 20 mmol/ HEPES buffer (pH 7.40) and eluted with 2 mL of distilled water. Concentration of the specifically eluted ^3H -citrulline was determined by liquid scintillation counting. Values were corrected to the amount of protein (Bradford method) present in the homogenates and the incubation time (dpm/mg protein/min).

Statistical and data analysis

Data are presented as mean \pm SEM. The statistical significance was assessed with unpaired student's t test for comparison of 2 samples values or one way ANOVA and a Bonferroni post-test. A P value <0.05 was considered statistically significant.

Results

LPS- induced iNOS activity and nitrites production by cultured aortic VSMC, obtained from SHR in a period previous to the blood pressure elevation (3-4 week old) and from older hypertensive SHR (12-week old), and age-matched normotensive Wistar rats, with or without the addition of Ang II, were examined.

Figure 1 shows the results when iNOS activity was measured in non stimulated basal conditions, incubating with 0.1% FCS. VSMC from young SHR displayed a significant ($P<0.001$) lesser basal iNOS activity that

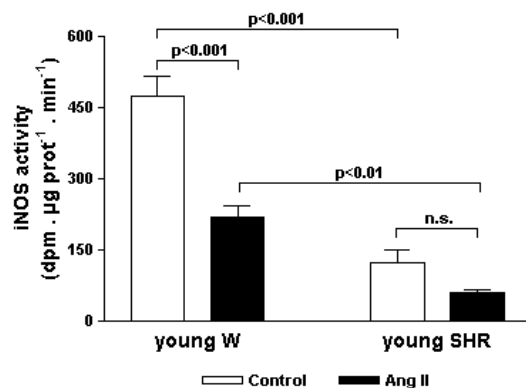


FIGURE 1. Effect of angiotensin II (Ang II) on LPS-induced iNOS activity in cultured vascular smooth muscle cells (VSMC) from young SHR and young W rats. Aortic VSMC were incubated with 0.1% FCS/F-12 MEM plus 100 nmol/L Ang II or not (control) for 24 h. Data are mean \pm SEM ($n=6$ in all groups).

those of control rats of the same age. The incubation with 100 nmol/L Ang II produced an important and significant reduction of iNOS activity in VSMC from normotensive control rats ($P < 0.001$). In cells from young SHR Ang II reduced the enzyme activity but this difference was not significant. There was a significant smaller iNOS activity in Ang II incubated young SHR cells compared with young W cells ($P < 0.01$).

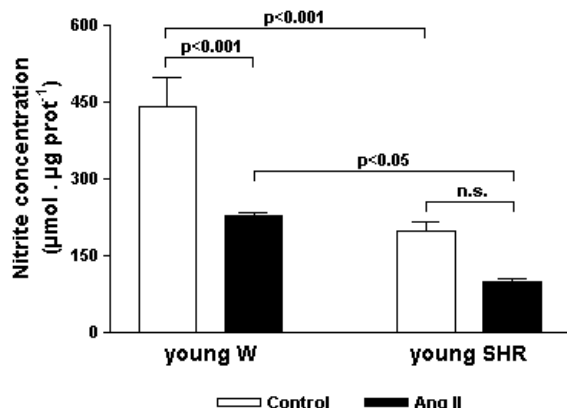


FIGURE 2. Effect of angiotensin II (Ang II) on LPS-induced nitrite production in cultured vascular smooth muscle cells (VSMC) from young SHR and young W rats. Aortic VSMC were incubated with 0.1% FCS /F-12 MEM plus 100 nmol/L Ang II or not (control) for 24 h. Data are mean \pm SEM ($n=6$ in all groups).

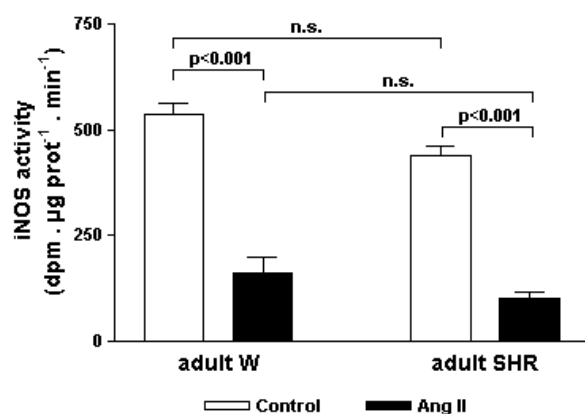


FIGURE 3. Effect of angiotensin II (Ang II) on LPS-induced iNOS activity in cultured vascular smooth muscle cells (VSMC) from adult SHR and adult W rats. Aortic VSMC were incubated with 0.1% FCS /F-12 MEM plus 100 nmol/L Ang II or not (control) for 24 h. Data are mean \pm SEM ($n=6$ in all groups).

A similar pattern of changes was observed when nitrite concentration was measured in the medium (Fig. 2). In basal conditions, there was also a smaller nitrite production in young SHR cells than in young W cells ($P < 0.001$). When Ang II effect was examined, nitrite concentration was significantly decreased in control VSMC ($P < 0.001$). In VSMC from young SHR Ang II produced a non significant reduction compared with its own non stimulated control, being Ang II effect smaller than in young W cells ($P < 0.05$).

Figure 3 shows the results when LPS-induced iNOS activity was measured in VSMC from adult hypertensive rats and their normotensive age-matched controls. No significant difference in the enzyme activity was observed between SHR and W cells in both basal or stimulated with Ang II groups. However Ang II produced a significant decrease of iNOS activity in VSMC from both control ($P < 0.001$) and hypertensive ($P < 0.001$) rats.

When levels of nitrites in the medium of cultured VSMC from adult SHR or W rats were evaluated (Fig. 4) the results followed the same pattern than iNOS activity. There was no significant difference between nitrite released from SHR and W cells in basal or Ang II stimulated conditions. Ang II treatment produced a significant reduction of nitrite production in SHR ($P < 0.001$) and W cells ($P < 0.001$).

When data from unstimulated young and adult rat cells were compared, there was no difference of iNOS activity and nitrites between VSMC obtained from young and adult W rats. However, cultured cells from young SHR showed a significant smaller iNOS activity ($P < 0.001$) and nitrite production ($P < 0.001$) than old SHR cells.

In order to compare proportional Ang II effects on young and adult rat cells, results with Ang II treatment were calculated as percent of the respective 0.1% FCS control incubation and data expressed as the remained value of iNOS activity and nitrite concentration. Proportional reduction of both variables, induced by Ang II, did not differ between young SHR and young W cells or between adult SHR and adult W rat cells. When iNOS activity data from young and adult rats were compared, the downregulation by Ang II was significantly greater in adult SHR ($23.0 \pm 3.1\%$) versus young SHR cells (49.0 ± 6.2) ($P < 0.001$) and it was also greater in VSMC from adult W ($28.3 \pm 2.6\%$) than that from young W rats ($43.0 \pm 4.5\%$) ($P < 0.05$). The reduction of nitrite production by Ang II was also greater in VSMC from adult SHR ($34.4 \pm 4.2\%$) than in those from young SRH ($49.6 \pm 3.1\%$) ($P < 0.05$). There was also a greater re-

duction in nitrite accumulation in adult W ($44.3 \pm 5.2\%$) versus young W cells ($52.0 \pm 1.1\%$), but in this case the difference did not reach statistical significance.

Discussion

One important physiological role of the arginine-NO pathway is to protect cardiovascular system against pathophysiological insults that can lead to chronic diseases such as hypertension, stroke injury and atherosclerosis (De Meyer and Herman, 2000b).

Vascular NO production by NOS activity has emerged as an important factor in the blood vessel tone regulation in physiological and pathological conditions. A number of disorders are associated with reduced synthesis and /or increased degradation of vascular NO, including hypercholesterolemia, diabetes mellitus and hypertension (Li and Förstermann, 2000).

In hypertension, the NO-iNOS system may be abnormal, contributing to differences in NO generation or action in the vascular wall and hence to changes in blood vessel tone and blood pressure elevation.

The present study was designed to examine the NO-iNOS system of aortic cultured VSMC from genetically normotensive and hypertensive rat strains during the period preceding the elevation of blood pressure and the period of well established hypertension. Since it is known that angiotensin II, the principal mediator of the physiological functions of the renin-angiotensin system, through its autocrine/paracrine properties, influences the

vascular wall by modulating cell growth and contributing to biologic responses and an integrated mechanism between Ang II and NO in the regulation of vascular structure and function has been suggested (Fernández-Alfonso and González, 1999), the effect of Ang II on the NO-iNOS system in cells obtained from young and adult hypertensive rats was also studied.

Bacterial LPS is one of the different stimuli that leads to induction of iNOS. LPS-induced iNOS activity in the cell layer and NO production, indicated by nitrite accumulation in the medium, were measured. The present results indicate that unstimulated VSMC obtained from young SHR, before the development of hypertension, exhibited a significant smaller LPS-induced iNOS activity and nitrite production than young normotensive control rat cells. On the other hand, there were no differences in these variables between hypertensive adult SHR and age-matched normotensive rat aortic cells. Besides, levels of LPS-induced iNOS activity and nitrite accumulation were markedly lower in young prehypertensive SHR than in those from mature SHR cells, not being observed this difference between young and adult normotensive rat cells. These results suggest that the weaker induction of iNOS activity by LPS and the subsequent reduced NO production, evaluated by nitrites, appear as an early defect during the development of hypertension. The lower iNOS activity in cells obtained from SHR in a period previous to the blood pressure elevation is in accordance with other reports. Aortic VSMC from prehypertensive SHR produced less NO than age-matched rat cells, after a long-term incubation with IL-1 β , without difference in iNOS expression (Singh *et al.*, 1996). Similar results were obtained in cultured mesenteric VSMC from prehypertensive stroke-prone SHR (Malinski *et al.*, 1993) or in aortic VSMC stimulated for proliferation with 5% FCS (Dubois, 1996). However, there are reports with opposite results, as the one of Vaziri *et al.* that described an upregulation of renal and vascular iNOS activity and proteins in tissue homogenates from young SHR (Vaziri *et al.*, 1998). Probably these contradictory results could be explained by the use of different methodology including rat's age, the use of cultured VSMC or vessel homogenates, the incubation time with the inductor, and different inductors, among others. We used cultured arterial smooth muscle cells, which allows to study variables in the tunica media, excluding other vascular components.

In contrast with young SHR, when VSMC from 12-week-old SHR, with a well established hypertension, were used, LPS-induced iNOS activity and nitrite production were almost the same found in cells derived from

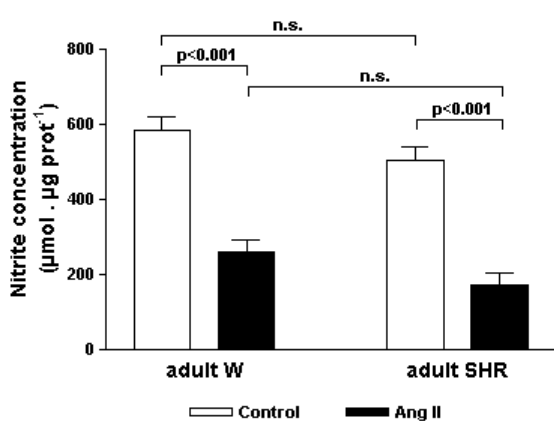


FIGURE 4. Effect of angiotensin II (Ang II) on LPS-induced nitrite production in cultured vascular smooth muscle cells (VSMC) from adult SHR and adult W rats. Aortic VSMC were incubated with 0.1% FCS /F-12 MEM plus 100 nmol/L Ang II or not (control) for 24 h. Data are mean \pm SEM ($n=6$ in all groups).

age-matched normotensive rats, and the enzyme activity and nitrite levels were significantly greater than in cells from prehypertensive SHR. We can postulate that in adult SHR the iNOS activity and hence the NO production augments to normotensive rats levels, as an adaptative response to increased blood pressure. Several studies have found evidence for increased NO production in mature SHR. Junquero *et al.* demonstrated that cultured aortic VSMC from 20-week-old SHR respond to IL-1 β with more production of NO than did those from WKY rats of the same age (Junquero *et al.*, 1992), and that IL-1 β elicited a greater production of nitrites in aortic rings from SHR, comparing with those of control rats (Junquero *et al.*, 1993). Wu *et al.* have detected a slightly higher plasma nitrite concentration and a greater iNOS expression in aortic homogenates in 16-week-old SHR than in control rats at baseline and after a 3 hours LPS stimulation (Wu *et al.*, 1996; Wu and Yen, 1999). In homogenized mesenteric arteries from much older SHR (6-month-old), Briones *et al.* found a greater induction of iNOS protein expression and activity by LPS than in age-matched WKY arteries (Briones *et al.*, 2000). Direct comparison of these studies with ours cannot be made since different methodologies and/or older rats were used, and some of the changes they found may be secondary to a chronic and sustained elevated blood pressure.

In order to examine whether angiotensin II could modulate NO production in VSMC, Ang II effect on LPS-induced iNOS activity and subsequent nitrite accumulation was evaluated. Basal levels of both variables were markedly reduced in cells from all groups by treatment with Ang II. When data were calculated as percent of its own non stimulated control, the proportional reduction of iNOS activity and nitrites in the presence of Ang II was not different between hypertensive and normotensive rat cells, at any age. However, the comparison between young and adults rat cells showed that this inhibitory effect of Ang II was greater in both adult SHR and W cells than in VSMC from young rats. This inhibitory effect of Ang II on the NO-NOS system is in accordance with some reports. Nakayama *et al.* have

described a dose-dependently inhibition by Ang II of IL-1 β - induced nitrite production, by blocking iNOS expression, in cultured aortic smooth muscle cells from normal rats, possible through angiotensin AT₁ receptors (Nakayama *et al.*, 1994). Similar results have been obtained with aortic VSMC from adult Sprague-Dawley rats, showing an inhibitory effect of Ang II on IL-1 β -induced nitrite production. In contrast, Ang II augmented NO synthesis in IL-1 β stimulated cardiac myocytes (Ikeda *et al.*, 1995). As far as we know, the present results show for the first time this inhibitory effect of angiotensin II on VSMC from either prehypertensive or hypertensive rats, being this effect the same as in cells from normotensive rats of the same age. A possible explanation of this Ang II effect could be the fact that NO generated by LPS treatment significantly decreases Ang II binding to cultured VSMC, suggesting that NO regulates Ang II receptors *in vitro* (Cahill *et al.*, 1995). In rat cardiac fibroblasts a downregulation of the expression of Ang II AT₂ receptors by LPS- or cytokine- dependent production of NO has been also described (Tamura *et al.*, 1999).

In summary, our results show that vascular smooth muscle cells from spontaneously hypertensive rats previous to the development of hypertension, before the raise of blood pressure, exhibit a reduced LPS-induced iNOS activity and NO generation, and that this characteristic disappears in cells from adult hypertensive rats. Additionally, an inhibitory effect of angiotensin II on these variables is described, which was of the same degree for hypertensive or normal rat cells.

We can speculate that the impairment in vascular smooth muscle NO production precedes the development of hypertension in SHR and may play a pathophysiological role in the early blood pressure elevation in genetically hypertensive rats.

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