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Hypotrophy of conduit artery walls of the offspring of nitric oxide-defective rats

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Abstract

The objective of the present study was to investigate the structure of the arterial walls of the offspring stemming from nitric oxide (NO)-defective hypertensive parents. The parents were treated with N^G-nitro-L-arginine methyl ester (40 mg kg^{-1} day^{-1}) for 5 weeks. Blood pressure was measured noninvasively in six 30-day-old rats and nine age-matched controls. The cardiovascular system was perfused with glutaraldehyde at 120 mmHg. The thoracic aorta and carotid artery were processed for electron microscopy, and geometry was determined by light microscopy. Endothelial cells, smooth muscle cells (SMC) and extracellular matrix (ECM) were determined by the point counting method in electron micrographs of the carotid artery. The blood pressure of experimental offspring was 150.0 ± 2.3 *vs* 104.6 ± 2.1 mmHg ($P < 0.01$) for the controls and their heart/body weight ratio of 3.9 ± 0.1 *vs* 4.4 ± 0.2 ($P < 0.05$) for the controls indicated cardiac hypotrophy. The wall thickness (tunica intima and media) of the thoracic aorta and carotid artery of experimental offspring was decreased to 78.9% ($P < 0.01$) and 83.8% ($P < 0.01$), respectively, compared to controls, as confirmed by a respective cross-sectional area of 85.3% ($P < 0.01$) and 84.1% ($P < 0.01$). The wall thickness/inner diameter ratio was reduced to 75% ($P < 0.01$) in the thoracic artery and to 81.5% ($P < 0.01$) in the carotid artery. No change in endothelial cell volume density or ECM was observed in the tunica intima of the carotid artery, and SMC volume density was lower in the tunica media (37.6 ± 0.9 *vs* 44.7 ± 1.1% for controls, $P < 0.01$), indicating compromised SMC development. Interference with arginine metabolism, a decrease in NO, and other factors are possible mechanisms underlying the structural alterations of the cardiovascular system of offspring from NO-defective hypertensive rats.

Key words
- Artery
- Nitric oxide
- Offspring
- Hypertension
- Vascular smooth muscle
- Extracellular matrix
- Morphometry

Introduction

Very early after the discovery of the endothelium-derived relaxing factor a series of arginine analogues were found to compete for the key enzyme nitric oxide (NO) synthase and to compromise citrulline and particularly NO production (1). NO synthase competitors elicited blood pressure elevation in acute experiments (1). The simple addition of competitors to drinking water induced a sustained increase in blood pres-

sure (2-4). Adult animals were chosen for this novel experimental model of hypertension and extensive alterations in the structure of the cardiovascular system were found, i.e., cardiac hypertrophy, and increased wall thickness of conduit and resistance arteries (5-8).

In studies started in 1996 and continuing over the subsequent years, we addressed the ontogenetic aspect of the activity of NO synthase and the controlling role of NO in the vascular system (9). In experiments with isolated conduit vessels of canine fetuses and newborns, we detected a distinct response to NO synthase activators (acetylcholine, bradykinin) which was even more marked compared with that of vessels from adult animals (10). Experiments with conduit vessels of canine offspring who received an NO synthase inhibitor for 5 weeks after birth demonstrated only moderate alterations of smooth muscle relaxation, indicating that NO synthase was already fully operative during the early ontogenetic period (9).

We next addressed the characteristics of the cardiovascular system of offspring from parents with sustained NO-defective hypertension (11). The first experiments provided information on high blood pressure in these offspring. Unexpected findings, particularly with respect to high blood pressure, were a low heart weight and heart/body weight ratio. The next natural issue was to determine the structural characteristics of the arterial tree of these newborns, as justified by the fact that no relevant data were available.

Material and Methods

Adult Wistar-Kyoto rats and their male newborns were used for the study. All procedures followed the guidelines of the Guide for the Use of Laboratory Animals issued by the Ethics Committee for Experimental Work, Slovak Academy of Sciences, 1995. The animals (parents) were housed in individual cages on a 12-h dark-light cycle, with constant temperature (22-24ºC) and free access to pellet food and water.

Parents

Experimental group. The parents consisted of 5 females and 5 males aged ten weeks receiving the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) at the dose of 40 mg kg^{-1} day^{-1} in drinking water for a period of 5 weeks. Fertilization occurred during the fifth week of L-NAME administration. In females, L-NAME administration continued during pregnancy and suckling up to the fourth week of offspring age.

Control group. The control group consisted of 3 females and 3 males age matched to the experimental group and housed under the same conditions.

Blood pressure was measured noninvasively in the tail artery weekly in both groups of parents, using the plethysmographic method.

Offspring

Experimental group. This group consisted of 6 newborns from 5 hypertensive NO-deficient parents fed by dams receiving L-NAME continuously.

Control group. The control group consisted of 9 newborns from 3 control parents fed by control mothers.

The blood pressure of the offspring was measured noninvasively in the tail artery twice on the 23rd and 29th day using a miniaturized cuff.

On the 30th day, the newborn pups were sacrificed by an overdose of anesthetic (thiopental, 100 mg/kg body weight, *ip*). The chest was opened and the cardiovascular system was perfused via the left ventricle with 300 mM glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.2-7.4, at a perfusion pressure of 120 mmHg for 10 min. After perfusion, the middle part of the ca-

rotid artery and the middle part of the thoracic aorta were excised, cleaned, divided into segments of about 1 mm in length and fixed in the same fixative overnight at 4º to 8ºC. After fixation, the segments were postfixed with 40 mM OsO_4 in 100 mM sodium phosphate buffer, washed in 100 mM sodium phosphate buffer, and stained *en bloc* with uranyl acetate. The blocks were dehydrated with increasing concentrations of alcohol, washed in propylene oxide and embedded in Durcupan ACM.

Two randomly selected blocks of each artery from each offspring were cut perpendicularly to the longitudinal axis. The inner circumference and arterial wall thickness (tunica intima + tunica media) of individual vessels were measured on semithin sections under light microscopy. Arterial wall thickness was measured at about 45º intervals around the vessel circumference. The cross-sectional area (tunica intima + tunica media) of the arterial wall, the inner diameter, and wall thickness/inner diameter ratio of the vessels were then calculated from these data.

The volume densities of cellular and extracellular components of the tunica media and tunica intima of the carotid artery wall were estimated using the point counting method of Weibel et al. (12). Briefly, the grid was randomly placed on the respective section and 5000 points were counted. Sections from three randomly selected blocks from vessels of control and experimental animals were similarly processed.

Data are reported as means ± SEM. ANOVA and the Bonferroni test for unpaired variables were used to assess statistical significance. Results were considered to be significant when P < 0.05.

Results

The blood pressure of the dams (102.8 ± 1.1 mmHg, N = 5) increased to 129.9 ± 4.3 mmHg (P < 0.001) after the first week of L-NAME administration and remained elevated throughout the pregnancy and suckling period (146.8 ± 7.9 mmHg, P < 0.001 at the end of experiment). The blood pressure of males (107.4 ± 2.8 mmHg, N = 5) increased to 137.0 ± 3.2 mmHg (P < 0.001) after the first week of L-NAME administration and remained at this significantly increased value throughout the first week of pregnancy (154.8 ± 5.2 mmHg, P < 0.001).

All control newborns and 75.5% of the experimental newborns survived until the end of experiment. The blood pressure of offspring measured at the age of 3 and 4 weeks was 140.9 ± 4.6 (P < 0.001) and 150.0 ± 2.3 mmHg, N = 6 (P < 0.001), respectively, significantly higher than the blood pressure of age-matched controls, 94.6 ± 4.5 and 104.6 ± 2.1 mmHg, N = 9.

The body weight of the hypertensive offspring, 68.7 ± 3.2 g, was significantly lower (P < 0.01) than the body weight of control offspring, 83.6 ± 2.7 g. The heart weight of hypertensive offspring, 276.8 ± 15.4 mg, was significantly lower (P < 0.05) than that of control offspring, 367.7 ± 11.4 mg. The heart/body weight ratio (mg/g) of the experimental offspring, 3.9 ± 0.1, was significantly lower (P < 0.05) than that of control offspring (4.4 ± 0.2; Figure 1).

The general parameters of the thoracic aorta and carotid artery are given in Table 1 and Figure 2. As shown in the figure, NO-defective offspring showed a decrease in wall thickness (tunica intima + tunica media) of both thoracic aorta and carotid artery to 78.9 (P < 0.01) and 83.8% (P < 0.01), respectively, compared to controls. This was also confirmed by calculating the cross-sectional area of the arterial wall, which was

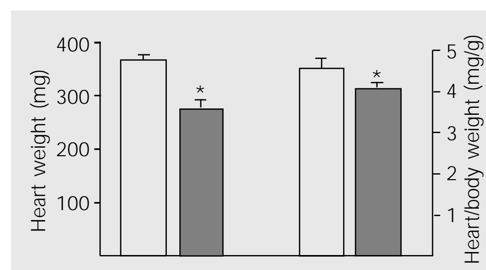

Figure 1. Heart weight and heart/body weight ratio (mg/g) of control (open columns) and NO-defective offspring (closed columns). *P < 0.05 compared to control (ANOVA and Bonferroni test).

Table 1. Geometry of the carotid artery and thoracic aorta of offspring of nitric oxide-defective rats.

	WT (µm)	CSA (µm²) x 10³	ID (µm)	WT/ID x 10⁻²
Carotid artery				
Control	27.37 ± 0.63	46.17 ± 1.45	507 ± 6.85	5.40 ± 0.11
Experimental	22.48 ± 0.66*	38.53 ± 0.99*	525 ± 10.91	4.30 ± 0.20*
Thoracic aorta				
Control	63.53 ± 1.28	203 ± 5.44	954 ± 14.48	6.68 ± 0.16
Experimental	50.18 ± 1.46*	174 ± 4.79*	1052 ± 12.04*	4.78 ± 1.70*

CSA = cross-sectional area (tunica intima plus tunica media); ID = inner diameter; WT = wall thickness (tunica intima plus tunica media).
*P < 0.01 compared to control (ANOVA and Bonferroni test).

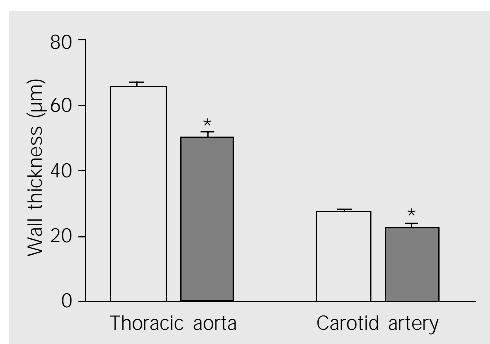

Figure 2. Wall thickness of thoracic aorta and carotid artery (tunica intima and tunica media) of control (open columns) and NO-defective offspring (closed columns). *P < 0.01 compared to control (ANOVA and Bonferroni test).

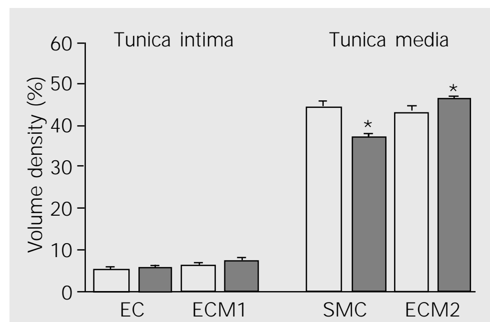

Figure 3. Volume density as percent of individual wall components of the tunica media and tunica intima of the carotid artery. Tunica intima: endothelial cells (EC) and extracellular matrix (ECM1); tunica media: smooth muscle cells (SMC) and extracellular matrix (ECM2). Control offspring (open columns), NO-defective offspring (closed columns). *P < 0.01 compared to control (ANOVA and Bonferroni test).

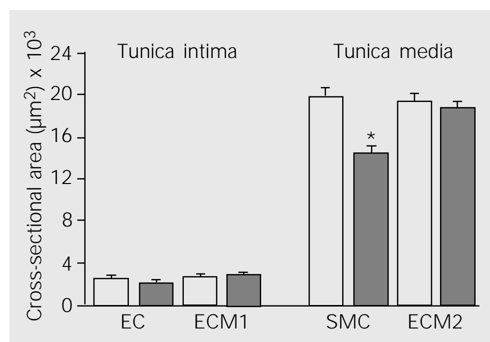

Figure 4. Cross-sectional area (µm²) of individual components of the carotid artery wall. Tunica intima: endothelial cells (EC), extracellular matrix (ECM1); tunica media: smooth muscle cells (SMC) and extracellular matrix (ECM2). Control offspring (open columns), NO-defective offspring (closed columns). *P < 0.01 compared to control (ANOVA and Bonferroni test).

only 85.3% (P < 0.01) of the control thoracic aorta and 84.1% (P < 0.01) of the control carotid artery. The internal diameters were wider in the experimental animals, being 110.2% of control (P < 0.01) in the thoracic aorta and 102.6% (nonsignificant) in the carotid artery. The values of the wall thickness/inner diameter ratio were 75.0% (P < 0.01) in the thoracic aorta and 81.5% (P < 0.01) compared to the respective controls (Table 1).

Abnormalities of individual cellular and non-cellular components of the wall were determined for the carotid artery of NO-defective offspring and of control offspring (Figures 3 and 4). No alterations were found in volume density of the intima components, i.e., endothelial cells and respective extracellular matrix (Figure 3). Remarkable changes were detected in the tunica media, especially a significant decrease in smooth muscle cell volume density. A respective relative increase in extracellular matrix volume density was found. In agreement, the calculated smooth muscle cell cross-sectional areas of the tunica media confirmed the impaired development of the cellular component of the tunica media. No change in extracellular matrix cross-sectional area was detected (Figure 4).

Discussion

The blood pressure of offspring from NO-deficient hypertensive parents at the age of 3-4 weeks was significantly higher than in age-matched offspring from normotensive control rats. In contrast to the high blood pressure, the heart weight and heart-body weight ratio were inappropriately lower in hypertensive newborns than in controls. The main goal of this study was to determine the geometry and structure of the main conduit arteries.

The wall thickness (tunica media and tunica intima) of the aorta and carotid artery was lower in NO-defective offspring com-

pared to controls. Since the cross-sectional area measured and calculated in both vessels was also significantly lower, the low wall thickness value was not due to high intravascular pressure, as one would expect from the increase in the inner diameter, and thus was not a passive consequence of the increase in blood pressure. It seems reasonable to suggest that an inhibition of growth processes in conduit vessel walls was a consequence of compromised arginine metabolism. With respect to the individual layers of the vessel wall, no change was found in volume density of endothelial cells or in the extracellular matrix of the tunica intima of the carotid artery. On the other hand, a significantly lower volume density of smooth muscle cells was found in the tunica media of the carotid artery from NO-defective offspring, supporting the above suggestion. Since the reduced arterial wall thickness was demonstrated to be due to the low volume density of smooth muscle cells, it would be warranted to also consider the role of apoptotic processes in these cells. These considerations are justified by the fact that in adult NO-defective rats apoptotic processes were found even in hypertrophic myocardium and in hypertrophic resistance vessel walls (6,8,13,14). No data on this topic are available for newborn rats from NO-defective hypertensive parents and therefore there is a strong need for experimental proof of the above mechanisms.

However, these findings contradict the respective wall thickness values repeatedly found in the conduit vessels of adult NO-defective hypertensive rats (5,7,15,16). In adult hypertensive NO-defective rats the wall thickness of conduit arteries increased significantly in terms of both extracellular and cellular components. Increased wall thickness in the arteries of adult animals was also repeatedly found in other experimental models of hypertension, i.e., renal or spontaneously hypertensive rats and others (17-20). To understand the unexpected finding of hypotrophy of the wall of conduit arteries in NO-defective hypertensive newborns, and hypotrophy of the heart as well, the following considerations might be of help. Indeed, arginine, which is considered to be a semi-essential amino acid in adults, was demonstrated to be essential in newborns (21). Any interference with the balance of arginine metabolism might have consequences for the structure of the vessel walls and of the heart as well. Furthermore, NO was shown to be essential in cell differentiation (22). Thus, intervention in arginine metabolism by inhibitors of NO synthase causing a lower NO production might be the reason for hypotrophy of the cardiovascular system.

When analyzing the factors involved in the decline of the wall thickness of the aorta and carotid artery it is necessary to consider two further points: since the mothers had long-term sustained hypertension due to NO deficiency, the blood supply for the fetus might be assumed to have been compromised. This would have contributed to low body weight, low heart weight, and low media thickness of the aorta and carotid artery. Nevertheless, the low heart/body weight index indicates a specific inhibition of growth processes of the cardiovascular system.

The second point concerns the level of the NO synthase inhibitor in breast milk. Previous experiments with adult rats demonstrated that high doses of L-NAME (50 mg/kg) increased both blood pressure and aorta wall thickness. However, low doses of L-NAME (20 mg/kg) elicited an increase in blood pressure in adult rats, but did not increase the thickness of the aorta (23). Thus, potential low L-NAME levels in the fetus and low L-NAME concentrations in milk should be considered to explain the difference in the functional response of resistant arteries (implying high blood pressure) and in the structure of conduit arteries. Nevertheless, the paradoxical pattern of decline in wall thickness of the aorta and carotid artery of offspring from NO-defective parents can

hardly be explained by the above analogy.

Four-week-old offspring from L-NAME-treated NO-defective hypertensive parents had high blood pressure and decreased wall thickness of the thoracic aorta and carotid artery, together with low heart weight and heart/body weight ratio. They also had a significantly decreased wall thickness/inner diameter ratio in both conduit arteries studied. Analysis of the rate of involvement of individual components of the vessel wall revealed hypotrophy of vascular smooth muscle in the tunica media with a relative increase in the extracellular matrix in the carotid artery. Several factors, namely the interaction between arginine metabolism and compromised NO levels, suggest that a compromised blood supply to the fetus, as well as other factors might be responsible for the hypotrophy of the cardiovascular system in the offspring studied.

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