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THE LONG-TERM CONTROL OF NITRIC OXIDE ON AORTIC STRUCTURE AND FUNCTION IN UNINEPHRECTOMIZED SPONTANEOUSLY HYPERTENSIVE RATS

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ABSTRACT

Introduction:

Introduction: higher blood pressure and organ damage were always observed in chronic hypertension. Here, we have investigated in female uninephrectomized spontaneously hypertensive rats (UNX-SHRs) the role of NO on changes in blood pressure, renal function, thoracic aortic relaxations and structure.

Materials and Methods: UNX-SHRs have been chronic treated with D-and L-arginine (D-and L-Arg; 1 mg kg-1 day-1 during 6 months in drinking water) alone or in the presence of low doses of Nw-nitro-L-arginine methyl ester (L-NAME; 1 mg kg-1 day-1 the first month and 0.5 mg kg-1 day-1 during the next 5 months in drinking water).

Mean blood pressure (MAP) was measured by the tail-cuff procedure. Plasma and urine levels of creatinine (Jaffé reaction), urinary proteins (Bradford method), as well as, urinary sodium and potassium (flame photometer) were determined for to evaluate renal function.

Nitrite production in plasma was also measured by the Griess reaction. Segments of thoracic aortas were used to analyze vascular reactivity and morphology.

Results: at the end of the study, the MAP was higher in UNX-SHRs treated with L-NAME+D-Arg and L-NAME+L-Arg than D-or L-arginine alone. There were no differences between groups on renal function.

Vascular relaxation induced by acetylcholine (endothelium-depedent relaxation) was higher in animals treated by L-Arg vs. D-Arg, but was similar to L-NAME treated animals. In contract, vascular relaxation induced by sodium nitroprusside (endothelium-independent relaxation) was similar in all treated UNX-SHRs.

Structural changes in thoracic aorta were linked to pressure load, as well as, NO production.

Conclusion: we could suggest in UNX-SHRs treated during 6 months with low doses of NO related drugs that:

a) renal dysfunction may be an independent process from vascular response and vascular reactivity; and
b) functional and structural changes in thoracic aorta seems to be linked to both pressure load, and changes in NO production

INTRODUCTION

Abnormally elevated blood pressure is not the only characteristic of hypertension [1]. Alterations in the function and structure of the cardiovascular organs are also among the hallmarks of this disease. Endothelial dysfunction and hypertrophy of the arterial wall are two well-known, typical features in most types of hypertension [2-4]. The cause-effect relationship between blood pressure and most of these other characteristics is not completely understood. In addition, whereas there is a patent pressure dependent component for most of the structural, and perhaps functional, alterations (or adaptations), there is evidence of a pressure-independent, hormonal component as wall [4]. Accordingly, several studies have reported the appearance of certain cardiovascular alterations already at the pre-hypertensive stage in genetic models of hypertension [5-7].

Spontaneously hypertensive rats (SHRs), a model of genetic hypertension [8, for review see reference 9], has been used in combination with induced renal damage as an animal counterpart to hypertensive patients that, for one cause or another, are subject to nephrectomy (UNX). In this model of glomerular hypertension associated with proteinuria [10], the remnant renal mass undergoes a functional readjustment that allows the maintenance of renal function and control of blood pressure [11]. Perhaps, hormonal changes that appear in the aftermath of this renal adjustment may also reach the periphery and affect the structure and function of, among others, the cardiovascular organs.

Nitric oxide (NO), a heterodiatomic molecule, is produced in vascular endothelium [12, for review see references 13-14] and renal tissue [15, for review see reference 16] by the L-arginine pathway. It is also known that NO participates actively in the functional and the structural homeostasis of the cardiovascular and renal tissue [17-18, for review see references 19-21]. Moreover, several authors have proposed a role of NO in the pathogenesis of hypertension NO [1, 22-23].

Chronic NO synthesis inhibition has been used as a model of experimental hypertension [24, for review see reference 25]. Although, the effect of NO synthesis inhibition has been assessed in UNX-SHRs to study the mechanism leading to hypertension-induced chronic renal failure under NO synthesis inhibition [26-28], there are not studies that correlate both data with aortic vascular relaxations and structure. In some way, it is analyzed genetic hypertension and the importance of NO on adaptive needs of the organism, linking organ damage to blood pressure [29].

Considering that differences in NO production can influence NO actions from a useful cellular signal to a potential toxic oxidant [30], the present study was designed to examine changes in thoracic aortic relaxation and structure (assessed by morphometry) in UNX-SHRs after a 6 months treatment with low doses of L-NAME (Nw-nitro-L-arginine methyl esther, an inhibitor NO) in the presence of D-arginine (D-Arg) and L-arginine (L-Arg; the substrate of NO production) or D-and L-Arg alone. We have used D-Arg-treated UNX-SHRs as control group, because this drug reduced blood pressure, but it is not a substrate for NO formation [31].

MATERIALS AND METHODS

Animals and Surgery

Experimental conditions were published previously [28]. Three month-old female SHR (190-200 g weight at the beginning of the study, Charles-Rives, Barcelona, Spain) were used for this study. Experiments were carried out in female rats, because they have a greater modulation of pressure-induced myogenic tone by NO in large vessels than male SHR [32]. All animals were housed under constant conditions and maintained on a standard rat chow (Panlab A.04) and free access to water. After a laparotomy, the left kidney was removed under ether anesthesia.

After a recovery period of three weeks, the UNX-SHR were randomly allocated into four experimental groups, and were subsequently treated during six months with:

- 1. Group D-Arg (n = 12), which consisted of rats that received D-Arg (1 mg kg-1 day-1) in drinking water.
- 2. Group L-Arg (n = 13), which consisted of rats that received L-Arg (1 mg kg-1 day-1) in drinking water.
- 3. Group L-NAME+D-Arg (n = 14), which consisted of rats that received D-Arg (1mg kg-1 day-1) plus L-NAME (1 mg kg-1 day-1 during the first month and 0.5 mg kg-1 day-1 during the next 5 months) in drinking water.
- 4. Group L-NAME+L-Arg (n = 14), which consisted of rats that received L-Arg (1mg kg-1 day-1) plus L-NAME (1 mg kg-1 day-1 during the first month and 0.5 mg kg-1 day-1 during the next 5 months) in drinking water.

Water intake was continuously monitored to ensure the right dose ingestion.

Blood pressure was measured by the tail-cuff procedure [33]. To analyze renal function, UNX-SHRs were placed into individual metabolic cages 4 days, and afterwards urine free of food and faeces, was collected daily into graduate cylinders under water-equilibrated mineral oil and 0.1% sodium azide during 2 days. A blood sample (150 µl) was taken from the caudal vein of the animals for plasma creatinine measurements. Then, the abdominal aortas were catheterized in UNX-SHRs under ether anaesthesia. Immediately, blood samples (1 ml) were taken into heparinized ice-cold tubes, centrifuged at 4º C (700xg/10 min). Then, plasma samples were stored at -20º C to measure plasma nitrite levels by the Griess reaction [34]. Afterwards, an ice phosphate buffered saline solution was infused into abdominal aortas to wash out the blood, then right kidneys and descending thoracic aortas were removed, cleaned of fat and conective tissues and placed in gassed (95% O2, 5% CO2) Kreb´s solution of the following composition in mM: NaCl 118.0, KCl 4.7, CaCl2 2.5, KH2PO4 1.2, MgSO4 1.2, NaHCO3 25.0 and glucose 11.1, pH of 7.4. Aortas were cut transversally into 6-7 ring segments (3-4 mm in lenght), one of which was assigned to morphologic measurements and the rest to functional studies.

Aortic reactivity

The thoracic aortic rings were mounted between two stainless steel holders and set up in organ baths, filled with Kreb´s solution and continuously gassed (95% O2, 5% CO2) The contractile force was measured with an isometric force-displacement transducer (UF1, Grass Instruments) and registered on a MacLab System (Macintosh Computers). Rings were allowed to equilibrate during 60 min a resting tension of 2 g. Their functional capacity was tested by exposing them to 105 mM KCl. Then, rings were submitted to a repeated washing, to return tension to basal levels, and a new relapse period of 30 min. Cumulative concentration-response curves to 3 x 10-8 - 10-4 M acetylcholine (ACh; endothelium-dependent relaxation) and 10-9 - 3 x 10-6 M sodium nitroprusside (SNP; endothelium-independent relaxation) were obtained, respectively in aorta rings precontracted with a submaximal dose of NA (10-6 M). The thoracic aortic rings were allowed to a 30 min recovery period in resting tension before the use of each relaxing agent. Data are expressed as percentage of dilatation with respect to NA-induced preconstraction.

The IC50 (expressed as negative logarithm of the molar concentration, pD2) of thoracic aortas is the concentrations of the test agents that induce a 50% of relaxation.

Aortic morphology

Segments of thoracic aortas, assigned to morphologic studies, were fixed by immersion in 4% p-formaldehyde and embedded in paraffin. 5 µm thick sections of thoracic aortas were cut with a microtome (E. Leitz Wetzlar, Germany) and placed on slides. The paraffin was removed by heating at 56ºC overnight. The tissues were stained with hematoxylin-eosin. The aortic images were captured with a light Nikon microscopy and photographed using a Nikon FX-35A camera and analyzed with a digitizing board (Kontron model IBAS I). Aortic wall thickness (w, mm) was calculated as the difference between the external aortic radius (Re, mm) and the internal aortic radius (Ri, mm). The media-to-lumen ratio values were calculated as w/Ri. Circumferential wall stress (kPa; 1 mmHg = 133.3 kPa) was calculated as MAP. 2w/Di, where Di (mm) is the internal diameter [35].

Analytic Methods

Plasma and urine levels of creatinine were determined by a colorimetric method based in Jaffé reaction [36]. Urinary proteins were analyzed by the Bradford method [37]. Urinary sodium and potassium were performed using a flame photometer. For to measure nitrite levels by the Griess reaction [34], plasma samples (500 µl) were incubated with Griess reagent (250 µl) during 15 min at room temperature before reading the absorbance. NO2- concentration was determined with reference to a standard curve by using concentrations from 1-75 µM NaNO2. Griess reaction (1879): Nitrite reacts with 1% sulfanilamide in 5% H3PO4/0.1% naphthalene-ethylenediamine dihydrochloride forming a chromophore (pink to dark red color) absorbing at 540 nm.

Statistical analysis

All values are expressed as means ± SEM of the indicated number of individual observations. Comparisons among groups were analyzed by analysis of variance and the Scheffé´s test, for the in vivo study. *P<0.05 was considered statistically significant. For vascular reactivity and structure, comparisons between groups were analyzed by analysis of variance and the Student´s t test. *P<0.05 was considered statistically significant.

Drugs

L-NAME hydrochloride, D-and L-Arg, ACh hydrochloride, NA bitartrate and NSP were all purchased from Sigma-Aldrich (Madrid, Spain). Drug solutions were made in distilled water, except for NA which was prepared in an acid solution (0.01% ascorbic acid w/v). In the case of vascular relaxations, all drug concentrations are expressed as the final molar concentration in the organ chamber for vascular reactivity.

RESULTS

In vivo study

At 6 months of treatment, the survival of the different animal groups was: 100% for D-Arg, 92% for L-Arg, 78% for L-NAME+D-Arg and 64% for L-NAME+L-Arg. There were not significant differences between groups on body weight, water intake, heart rate or organ weights (Table I). Mean blood pressure (MAP) rose significantly in the two groups that received L-NAME when compared with those receiving D-or L-Arg alone (Figure 1a). Plasma nitrite levels were significantly higher in the L-Arg vs. other three groups (Figure 1b).

Table I. Body weights, organ weights and haemodynamics parameters in the four groups of treated uninephrectomized spontanoeusly hypertensive rats (UNX-SHRs).

Treatment	n	BW (g)	WI (ml 24 h -1)	AW/BW (mg g-1)	KW/BW (mg g-1)	HR (bpm)
D-Arg	12	235±4	16.0±1.5	1.73±0.07	5.23±0.20	412±10
L-Arg	13	238±3	16.2±1.1	1.67±0.11	5.31±0.17	409±17
L-NAME+D-Arg	14	238±3	16.4±1.3	1.94±0.11	5.45±0.12	418±7
L-NAME+L-Arg	14	236±4	17.7±1.9	1.98±0.12	5.45±0.22	410±26

D-Arg indicates D-arginine (1 mg kg-1 day-1), L-Arg indicates L-arginine (1 mg kg-1 day-1), L-NAME indicates Nw-nitro-L-arginine methyl ester (1 mg kg-1 min-1 the first month and 0.5 mg kg-1min-1 the next five months), BW indicates body weight, WI indicates water intake; AW indicates aorta weight, KW indicates kidney weight, HR indicates heart rate. Values are means ± SEM *P<0.05 vs. D-Arg; #P<0.05 vs. L-Arg.

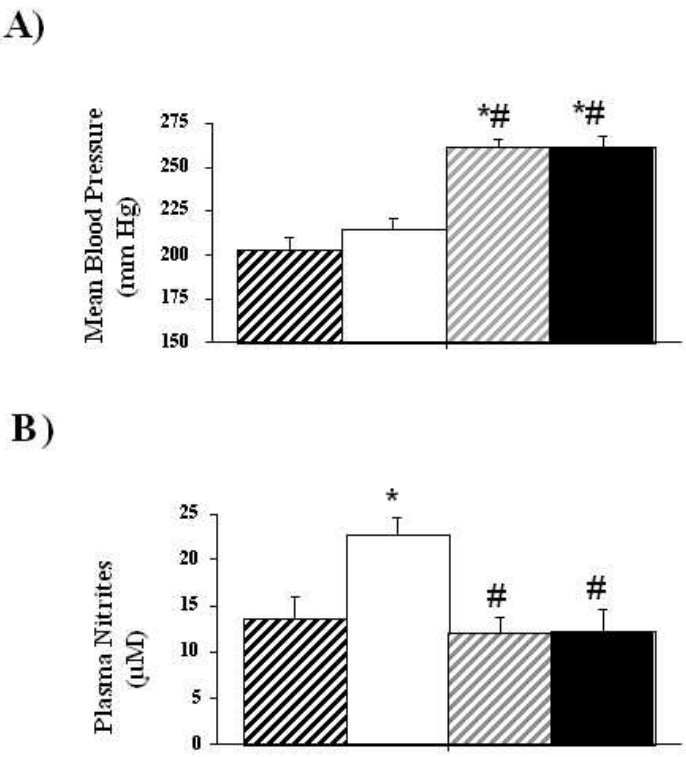

Figure 1. Changes in blood pressure (A) and plasma nitrite levels (B) at the end of the study. UNX-SHRs were treated during six months with: D-Arg (1 mg kg-1 min-1; strong hatch columns), L-Arg (1 mg kg-1 min-1; open columns) alone or in the presence of L-NAME (1 mg kg-1 min-1 the first month and 0.5 mg kg-1 min-1 the next five months; respectively light hatch and close columns). Each symbol represents the mean + SEM of at least 12 animals. *P<0.05 vs. D-Arg, # P<0.05 vs. L-Arg.

Renal parameters were summarized in Table II. The creatinine clearance, urinary flow, proteinuria and sodium excretion were similar in all the treated UNX-SHRs.

Table II. Parameters of renal function in the four groups of treated UNX-SHRs.

		Urinary flow (µl	Ccr (ml min-	PROT (mg 24	UNaV (mEq 24	UKV (mEq 24

Treatment	n	min-1)	1)	h-1)	h-1)	h-1)
D-Arg	12	4.19±0.28	0.827±0.074	3.93±0.60	0.335±0.042	0.256±0.031
L-Arg	13	4.26±0.45	0.963±0.071	4.01±0.59	0.318±0.054	0.253±0.040
L-NAME+D-Arg	14	4.76±0.39	1.009±0.076	5.64±0.86	0.251±0.036	0.240±0.036
L-NAME+L-Arg	14	5.10±0.40	0.917±0.060	6.41±1.14	0.204±0.053	0.167±0.036
Ccr indicates creatinine clearance, PROT indicates proteinuria, UnaV indicates urinary sodium excretion, UKV indicates urinary potassium excretion. Values are means ± SEM.						

Acetylcholine-and nitroprusside-induced vasorelaxations

Addition of NA (10-6 M) to rat thoracic aortic rings induced in different treated UNX-SHRs a contraction of: D-Arg = 2826 + 160 mg (n =18), L-Arg = 2396 + 210 mg (n =15), L-NAME+D-Arg = 2337+149 mg (n =18) and L-NAME+L-Arg = 2435+112 mg (n =18). Data on vascular relaxations were summarized in Figure 2 and and Table III. The response was significantly higher ($p<0.01$) in the D-Arg group in comparison with others. In NA (10-6M)-precontracted rings, the sensitivity and Emax in concentration-responses curves to ACh were significantly reduced in vessels of D-Arg treated-animals vs. other treated-groups. However, in NA (10-6 M)-precontracted rings, although there were differences in the sensitivity between groups in concentration-responses curves to SNP (a nitric oxide-donor) the Emax effects were similar.

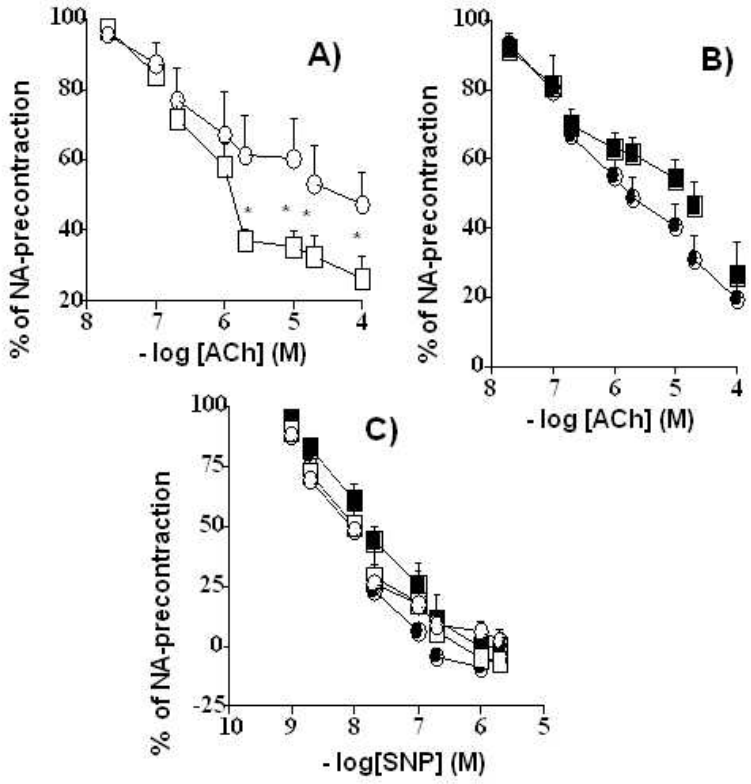

Figure 2. Concentration-response curves for acetylcholine (Ach; A and B) and sodium nitroprusside (SNP; C) in throracic aorta rings of UNX-SHRs. Results are expressed as percentage dilatation after noradrenaline (10-6 M)-induced precontraction. Rats were treated during six months with: D-Arg (1 mg kg-1 min-1), L-Arg (1 mg kg-1 min-1) alone or in the presence of L-NAME (1 mg kg-1 min-1 the first month and 0.5 mg kg-1min-1 the next five months). Each symbol represents the mean + SEM of at least 6 aortic rings. *P<0.05 vs. D-Arg.

Table III. Maximal relaxation, and sensibility of thoracic aortic rings of treated UNX-SHRs.

Treatment	D-Arg		L-Arg		L-NAME+D-Arg		L-NAME+L-Arg	
	pD2	Emax(%)	pD2	Emax(%)	pD2	Emax(%)	pD2	Emax(%)
Relaxation								
ACh	4.22±0.10	52.5±9.0	7.53±0.09	97.4±4.4	5.61±0.20*	73.8±6.5*	7.00±0.05*	106.0±10.6
								†

NPS	5.56±0.05*	80.6±6.7*	7.99±0.02*	104.0±4.0	5.02±0.14*	100.0±5.3	7.68±0.01	100.0±5.3

ACh indicates acetylcholine, NPS indicates sodium nitroprusside. The values are means ± SEM of at least 6 experiments. *P<0.05 vs. D-Arg, #P<0.05 vs. L-Arg and †P<0.05 vs. L-NAME+L-Arg.

Aortic structure

Table IV and Figure 3 show the effects of different treatments in thoracic aortic structure. The mean internal diameter was similar in all treated UNX-SHRs. However, the wall thickness, media-to-lumen ratio and media-cross-sectional area where significantly higher in the L-NAME+D-Arg group in comparison with others. It is interesting to observe some differences between L-NAME+L-Arg treated-animals vs. other groups when MAP related parameters, such as wall thickness/MAP and circumferential wall stress, were analyzed.

Table IV. Morphometric variables measured in thoracic aortas of UNX-SHRs.

Treatment	D-Arg	L-Arg	L-NAME+D-Arg	L-NAME+L-Arg
mean internal diameter (mm)	1.42 + 0.06	1.37 + 0.03	1.44 + 0.03	1.45 + 0.04
wall thickness (mm)	0.129 + 0.001	0.123 + 0.010	0.167 + 0.005*	0.144 + 0.009
media-to-lumen ratio	0.18 + 0.01	0.18 + 0.01	0.23 + 0.01*	0.20 + 0.01
media cross-sectional area (mm2)	0.63 + 0.01	0.56 + 0.06	0.86 + 0.04*	0.72 + 0.05

*Values are means + SEM of at least 4 data. *p<0.05 vs. D-Arg and #p<0.05 vs. L-Arg.*

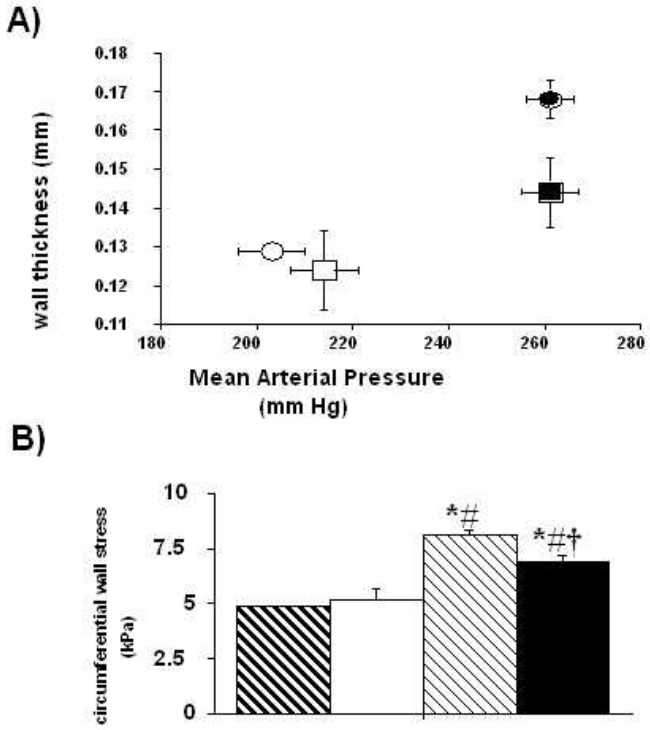

Figure 3. Aortic thickness/mean arterial pressure ratio (A) and circumferential wall stress (B) of UNX-SHRs. Rats were treated during six months with D-Arg (1 mg kg-1 min-1; A: open circles and B: strong hatch columns); L-Arg (1 mg kg-1 min-1; A: open squarts and B: open columns); L-NAME+D-Arg (1 mg kg-1 min-1 the first month and 0.5 mg kg-1 min-1 the next five months plus D-Arg; A: close circles and B: light hatch columns); and L-NAME+L-ARG (1 mg kg-1 min-1 during the first month and 0.5 mg kg-1 min-1 during the next five months plus L-Arg; A: close squarts and B: close columns). Each symbol represents the mean + SEM of at least four data. *P<0.05 vs. D-Arg, #P<0.05 vs. L-Arg and † P<0.05 vs. L-NAME+D-Arg.

DISCUSSION

In view of the kidney and blood pressure are closely related [38] and a renal defect may represent the response of the kidney to the hypertensive progress [39]; we have investigated in this study the long-term role of NO on aortic vessels in a mixed experimental process: a nephron reduction that accelerate renal lesions [40] and SHR, an animal model of essential hypertension [8-9]. According plasma nitrite levels measured at the end of the study, a chronic administration of low doses of L-NAME, were able to blunt NO-production induced by 1 mg kg-1 day-1 of L-Arg. However, L-NAME aggravates blood pressure in this model after 6 months of administration, with or without L-Arg, and indicates that NO is not the main

regulator of blood pressure, after the renal resetting that occurs upon UNX in genetic hypertension. NO synthesis blockade also induced hypertrophy of the aortic wall of UNX-SHRs, but this structural change is more related on blood pressure rise than NO production. This finding is probably one of the most important ones in this work. The other interesting finding of this study is that those UNX-SHRs treated with L-NAME+L-Arg have low survival, perhaps adaptation to maintain high blood pressure makes necessary to preserve the abnormal vascular structure.

Endothelial dysfunction is a typical characteristic of hypertension. It is the result of an imbalance in the normal equilibrium between endothelial contracting and relaxing factors [2, 41-43]. Both pressure-dependent and -independent factors have been implicated in its appearance [2]. Particularly in SHRs, NO production upon stimulation is normal or even elevated, but it is partially neutralized by an abnormally excessive production of oxygen reactive species [44-45]. Here, we show that neither long-term NO synthesis inhibition nor supplementation with L-Arg induces significant changes in the intrinsic relaxation capacity of the aorta (whether endothelium-dependent or -independent). This means that, in our model, the behavior of the aortic wall as regards its capacity of relaxation is not determined by the blood pressure levels and its consequences, but rather by genetic or pre-established determinants. All groups have a similar aortic relaxation profile in spite of being subject to starkly different pressure levels.

When we tested vascular reactivity, we found some differences between groups on NA-induced contraction. If vascular tone and sympathetic response could be dependent on NO release [46], our data on NA-precontracted vessels are clear the consequence of these effects. In the aftermath of the present results on vasorelaxations, we can conclude that this status is not altered by the presence or absence of NO in the aortic wall. The resultant endothelial imbalance seems not to be dependent on further pressure increase or on NO status, since all groups showed a similar response to ACh, except D-Arg. The modification of other endothelium-derived factors, as a consequence of an adaptation pressure load, with a reciprocal regulation of their production with NO would be implicated on this response. In fact, two important local endothelial vasoconstrictors such as arginine II and endothelin-1 would be regulated according NO synthesis [27, 42, 47]. In additional, it is demonstrated that a vasoconstrictor factor derived from cyclooxygenase pathway is up regulated in the vascular wall of UNX-SHRs [48]. Some of these products of arachidonic acid such as 20-hydroxyeicosatetraenoic acid (20-HETE), epoxyeicosatrienoic acids (EETs) or prostaglandin H2 and tromboxane A2 may be also regulated by NO production [49-52]. It is also interesting to observe that the endothelial hyperpolarizing relaxing factor, actually considered as a derivate of EETs, is dependent on cytochrome P450 [53]. If we block NO production with L-NAME that is also able to inhibit EETs formation, because its blocks the reduction of ferric cytochrome C by ferrous ion [54], perhaps we are increasing blood pressure by two different sites.

When morphometric results were analyzed in UNX-SHRs, it is interesting to observe on aortic wall a different degree of MAP with the same internal diameter, as previous reported [55-57], which could be related again to an endothelial dysfunction. Nervertheless, we can not discard a possible role of NO in vascular cell growth [58-59], according data of the L-NAME+D-ARG group on wall thickness and media cross-sectional area; we demonstrate some differences in arterial walls of large arteries as an adaptative process leading to regulate blood flow [35], when data on aortic structural changes were corrected with the MAP. Rat aortic structure has been studied extensively in hypertension in SHRs [60, for review see reference 61] and NO deficiency using high doses of L-NAME [62], but not one has linked essential hypertension with renal mass reduction, chronic NO inhibition and structural changes in large arteries for a long time. Although, our structural changes in aortas of UNX-SHRs were not so large, because doses of different NO drugs were very low; its evidences that NO could modify the adaptative process to hypertension of large arteries by means of a low dose of L-ARG; in fact, L-NAME+L-ARG treated animals had a significant reduction in circumferential wall stress and wall thickness/MAP in comparison with L-NAME + D-ARG group and this could explain the different survival of the animals.

Finally, according our data there are not significant differences in renal function among groups. All groups of UNX-SHRs, regardless of the treatment they have received, reset the remaining kidney to achieve normal renal performance. However, those animals with their NO-pathway blocked by L-NAME administration, needed higher blood pressure to maintain both a normal renal function and a balance in sodium excretion, in agreement with the well-known role of NO on the relationship between blood pressure/intraglomerular pressure and natriuresis/diuresis [63-65]. It is interesting to observe that L-NAME did not inhibit L-Arg effects. Perhaps, L-Arg transport into cells might be altered or reset after UNX, as previous reported in humans with renal damage [66], and the main control of renal function would not depend on NO actions.

In conclusion we could suggest in UNX-SHRs treated during 6 months with low doses of NO related drugs that: a) renal dysfunction may be an independent process from vascular response and vascular reactivity; and b) functional and structural changes in thoracic aorta seems to be linked to both pressure load, and changes in NO production.

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