Chronic inhibition of NO synthesis *per se* promotes structural intimal remodeling of the rat aorta

Marcos A. Rossi and Massimo Colombini-Netto

Objective **We characterized, using histomorphometry and transmission and scanning electron microscopy, the intimal remodeling of the thoracic aorta of normocholesterolemic young rats chronically-treated with L-NAME and examined the question whether these changes were caused by the lack of NO *per se* or by the hypertension which L-NAME administration induces.**

Methods **Male Wistar rats were divided randomly into three sets: control group, standard diet/L-NAME-treated group, and standard diet/L-NAME + captopril-treated group.**

Results **The treatment of rats with L-NAME for 4 weeks resulted in increased blood pressure (by 32% at the end of the treatment) as compared with the control value and intimal remodeling comprising a continuous layer of enlarged endothelial cells with irregular nuclear and cytoplasmic contours, lying over a thickened layer of fibrocollagenous support tissue focally expanded with lymphomononuclear cells and mainly diffuse foci of smooth muscle cells. In addition, the NO synthase inhibition caused a marked thickened tunica intima (150% thicker than the control value) and a significantly augmented intima:media ratio (126% higher than the control value). On the other hand, captopril prevented hypertension in rats simultaneously treated with L-NAME as compared with controls, and induced intimal remodeling comprising the same qualitative changes as those observed in L-NAME-treated rats. The tunica intima of L-NAME + captopril-treated rats was moderately thickened (60% increase in comparison with that of controls and 65% thinner as compared with L-NAME-treated rats). In the same way, the mean intima:media ratio of rats concomitantly treated with L-NAME and captopril was moderately increased (45% more) as compared with controls and significantly lower in comparison with rats administered L-NAME alone (36% less).**

Conclusions **Chronic inhibition of NO synthesis *per se* promotes structural intimal remodeling of the rat aorta, which is potentiated by L-NAME-induced hypertension. Most important, the present findings favor the idea that blockade of NO synthesis by causing intimal remodeling might be a primary cause, as individual biologic phenomenon, in the development of an atherosclerotic plaque. *J Hypertens* 19:1–13 © 2001 Lippincott Williams & Wilkins.**

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Introduction

Atherosclerosis is now believed to be an inflammatory process driven by inflammatory cells, in particular macrophages, in association with subendothelial (intimal) deposition of lipids in the artery wall [1]. The characteristic lesion of atherosclerosis represents the result of cycles of accumulation of monocyte-derived macrophages and migration and proliferation of smooth muscle cells that occur principally in the tunica intima of large and medium-sized elastic and muscular arteries. Endothelial dysfunction is an early feature in atherosclerosis that manifests as impaired local nitric oxide (NO) production and increased expression of adhesion molecules [2,3]. It has been clear that risk factors for atherosclerosis, such as hypercholesterolemia and hypertension, profoundly affects endothelial function that manifests as diminished release of nitric oxide into the arterial wall either because impaired local production or excessive oxidative degradation [4–6]. Importantly, however, it remains unclear whether this endothelial dysfunction manifestation is a cause or a consequence of lipid accumulation since fatty streaks are also present from a young age.

Recent studies have shown that chronic administration of L-arginine analogues such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) to rats induces a dose dependent systemic arterial hypertension, decreased intracellular cGMP levels (the second messenger of NO) in vascular smooth muscle, and inflammatory phenotypic

changes in the coronary vascular wall [7–14]. Since NO-donor compounds have been shown to inhibit vascular smooth muscle cell proliferation, protein synthesis, and production of extracellular matrix in culture [15,16] and to restrict proliferation and mitogenesis of cultured endothelial cells [17], the present study was designed to determine the effect of prolonged NO synthase inhibition with L-NAME on the intima of the aorta in rats. We characterized, using histomorphometry and transmission and scanning electron microscopy, the intimal remodeling of the thoracic aorta of normocholesterolemic young rats and examined the question whether these changes were caused by the lack of NO *per se* or by the hypertension which L-NAME administration induces. This way, the main objective was to detail the reduction of NO availability, as individual biological phenomenon, on early aortic intimal remodeling.

Methods

Male Wistar albino rats, weighing an average of 87 g, were obtained from the breeding colony at the Faculty of Medicine of Ribeirão Preto. The animals were housed individually in cages constructed of polypropylene with stainless steel mesh tops washed twice weekly. The rats were fed solid laboratory rat food in stainless steel feeding dishes and liquid in Richter graduated drinking tubes. Their liquid intake was recorded thrice weekly and their solid food consumption was recorded twice weekly. The approximate composition of the commercial standard diet (g/100 g) was: protein 21.0, fat 3.5, carbohydrate 60.0, salts 5.3, vitamin mixture 2.0, and remainder ash (the data were provided by the supplier). This diet provided approximately 3.5 kcal/g. The animals were divided randomly into three sets: (1) a control group, drinking untreated water and receiving a dose of placebo (1 ml of sterile saline solution administered intra-peritoneally); (2) a standard diet/L-NAME-treated group, drinking water containing 45 mg/100 ml of N^{ω}-nitro-L-arginine methyl ester, L-NAME and a daily dose of placebo (1 ml of sterile saline solution administered intra-peritoneally); (3) and a standard diet/L-NAME + captopril-treated group, drinking water containing 45 mg/100 ml of L-NAME and administered intra-peritoneally a daily dose of captopril, an angiotensin converting enzyme inhibitor, at 40 mg/kg of body weight. The animals were weighed twice weekly. Captopril was freshly prepared by dissolving the drug powder in 1 ml of sterile saline solution and administered daily via intra-peritoneal injection. The administration of captopril began 3 days before L-NAME administration in the drinking water. The dosage used was based on pilot experiments and was found to be effective for blood pressure control, as previously reported [18–20]

The serum of rats from control, L-NAME-treated, and L-NAME + captopril groups at the beginning (considered as a unified 0 week time point, $n = 35$) and at the end of the experiment (4 week time points relatives to control, $n = 7$, L-NAME-treated, $n = 11$, and L-NAME + captopril-treated, $n = 9$, groups) were used to measure the concentration of total and HDL cholesterol by a spectrophotometric method using a commercially available kit (Cobas Mira, Roche). Serum proteins and albumin levels were estimated at the end of the experimental period by means of the biuret reaction.

Mean arterial blood pressures were measured once a week by a tail cuff method without anesthesia. The mean of three measurements was considered to be the mean for the week. A tail cuff was placed over the tail with the systolic and diastolic blood pressure being measured on a NARCO physiograph (E & M Instruments Co., Inc, Houston, Texas, USA). Measurements were made after the rat had been resting for a few minutes in a hot box.

After 4 weeks, the rats were killed in light ether anesthesia by exsanguination from the abdominal aorta, between 0900 and 1100 h. The thoracic cavity was opened exposing the still beating heart. The hearts were rapidly removed, rinsed in ice-cold 0.9% NaCl solution (saline), blotted and weighed. The heart weight index was calculated by dividing the heart weight to body weight (heart ratio). The hearts were fixed as a whole in neutral 10% formalin for histological study. Both ventricles of each heart were isolated and cut into three fragments by two coronal sections at equal intervals. Each block was serially cut at 6 μm in the same direction, and sections were stained with hematoxylin and eosin and picrosirius red technique. The quantitative examination of the left myocardium was carried out on medium power light-microscopic field (× 320): 100-indexed square graticle (Carl Zeiss, Jena, Germany) was used to estimate the volume fraction (% of fibrosis in picrosirius red-stained sections. Fields ($n = 48$) of subepicardial and midmyocardial zones were analyzed for each heart (seven hearts from control group, 11 hearts from L-NAME group and six hearts from L-NAME + captopril group).

The aortas ($n = 6$) were rinsed at a mild pressure-perfusion of 100 mmHg with phosphate buffered saline (PBS) through the ascending aorta followed by perfusion fixation with 2.5% glutaraldehyde in cacodylate buffer (pH 7.3) for 3 min. The thoracic aortas were then immediately harvested ($n = 6$ in each group) and the adventitial tissue was removed. The aortic tubes were transversally cut into 5–6, 2 mm-long fragments (sampling from the upper to the lower part of the thoracic aorta) and fixed in the same fixative for 2 h. The samples were rinsed in cacodylate buffer, postfixed in 1% osmium tetroxide buffered in cacodylate for 2 h,

dehydrated in ascending concentrations of acetone, and subsequently embedded in Araldite®. Morphometric analysis was performed using videomicroscopy with the Kontron Image System KS-100 software (Kontron Elektronik GmbH, Germany) in conjunction with a Zeiss microscopy, video camera, and an on-line computer. Cross sections ($n = 5–6$) of good technical quality, exactly perpendicular to the long axis of the aorta from each vascular segment, were chosen for morphometric evaluation. The absolute thickness of the intima, as defined as the endothelium and the subendothelial layer to the inner edge of the internal elastic lamina (IEL), the absolute thickness of the media, as defined by the tissue between the IEL and the external elastic lamina (EEL), and the cross sectional area of the aorta lumen were measured. The number of measurements to estimate the intima and media thickness was 100 around the vessel circumference for each aorta from each rat. This number was chosen arbitrarily. The use of thin plastic sections stained with toluidine blue allowed adequate resolution of structural details. Measurements were made by a skilled observer blinded to the treatment groups. The intima:media ratio was calculated by dividing the intima thickness to media thickness and multiplying per 100. Intima and media thickness was determined with the use of an overall microscope magnification of $\times 400$ whereas the cross-sectional area was determined at $\times 100$. Findings were averaged for each group. Ultrathin sections were obtained from selected areas with a diamond knife in a Sorvall MT-5000 ultramicrotome (Du Pont Co., Wilmington, Delaware, USA) stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 109 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) at 80 kV.

The thoracic aortas of four animals from each group were immediately harvested and cut into anterior and posterior halves. Fragments 5–6 mm long were fixed by immersion in phosphate buffer 2.5% glutaraldehyde (pH 7.3) for 2 h, postfixed in osmium tetroxide in phosphate buffer for 2 h, dehydrated in ascending concentrations of ethanol, and dried in liquid carbon dioxide by the critical point method. To improve our visualization of the endothelial cells, the specimens already fixed were put into 1% HCl for 30 s with gentle shaking. The dried specimens were then glued on aluminum stubs with silver paste, sputter-coated with gold, and examined in a Zeiss 940A scanning electron microscope at 15 kV. The segments were fixed under atmospheric pressure rather than physiological pressure to prevent eventual artifacts due to overstretching. It is felt that qualitative assessment of this data is justified, as all segments were prepared under similar conditions.

Data were analyzed using a GraphPad Prism statistic program (GraphPad Software Inc., San Diego, California, USA) for an IBM PC computer. For analysis, one-way analysis of variance (ANOVA) and the Bonferroni test (to correct for multiple comparisons) were used. One value for each case entered into the analysis. A level of significance of 5% was chosen to denote the difference between group means. Unless specified, data are presented as mean ± standard error.

Results

Rats treated with L-NAME or L-NAME + captopril remained in good health, with no signs of nutritional deficiencies. However, the L-NAME- and L-NAME + captopril-treated rats gained less weight (6.65 and 6.27 g/day per rat, respectively) as compared with controls (8.07 g/day per rat) over the 4-week period. The mean final body weight of control animals was 314.10 g, respectively 13% and 16.5% higher than the final body weight of L-NAME- (273.40 g) and L-NAME + captopril-treated rats (261.16 g).

The animals in the present study received L-NAME dissolved in the drinking water, 45 mg/100 ml. Mean water intake in the control, L-NAME- and L-NAME - + captopril groups decreased from 19.27, 19.96, and 21.77 ml/day per 100 g of body weight at the beginning of the experiment to 11.63, 15.02, and 13.51 ml/day per 100 g at the end, respectively. The average intake of drinking water did not differ significantly in both L-NAME- (18.16 ml/day per 100 g) and L-NAME + captopril-treated animals (19.04 ml/day per 100 g while the average intake of drinking water in controls (15.98 ml/day per 100 g) was slightly smaller. The mean intake of L-NAME during the experimental period decreased from 8.98 and 9.80 mg/day per 100 g of body weight at the beginning of the experiment to 6.75 and 6.07 mg/day per 100 g at the end in L-NAME- and L-NAME - + captopril-treated rats, respectively. The average intakes of L-NAME in both L-NAME-tested rats were similar (8.17 ml/day per 100 g in L-NAME-treated and 8.57 ml/day per 100 g in L-NAME + captopril-treated rats). In contrast, average consumption of solid food in control rats (19.54 g/day per rat) was higher than the average consumption in L-NAME- (17.37 g/day per rat) and in L-NAME + captopril-treated rats (16.32 g/day per rat).

At the first day preceding the beginning of intraperitoneal injections of captopril and placebo, the plasma concentration of total and HDL cholesterol (0 week time point) was 59.80 ± 4.54 mg/100 ml, and 39.27 ± 2.34 mg/100 mg, respectively. These values were closely similar to those found at the 4 week time point of control (60.33 ± 5.32 mg/100 ml of cholesterol and 43.63 ± 2.60 mg/100 ml of HDL cholesterol), of L-NAME-treated rats (58.14 ± 3.72 mg/100 ml of cholesterol and 43.51 ± 1.87 mg/100 ml of HDL cholesterol),

and L-NAME + captopril-treated rats (62.63 ± 4.73 mg/100 ml of cholesterol and 41.05 ± 3.11 mg/100 ml of HDL cholesterol. The total plasma proteins and albumin levels of L-NAME-treated rats (5.76 ± 0.41 g/100 ml and 3.42 ± 0.13 g/100 ml, respectively) and L-NAME + captopril-treated rats (6.33 ± 0.42 g/100 ml and 3.57 ± 0.12 g/100 ml, respectively) were closely similar to those of controls (5.55 ± 0.54 g/100 ml of total plasma proteins and 3.53 ± 0.18 g/100 ml of albumin).

Treatment with L-NAME only, increased moderately the mean arterial blood pressure, by approximately 32% in comparison with that of untreated controls.s and 3.53 ± 0.18 g/100 ml of albumin).

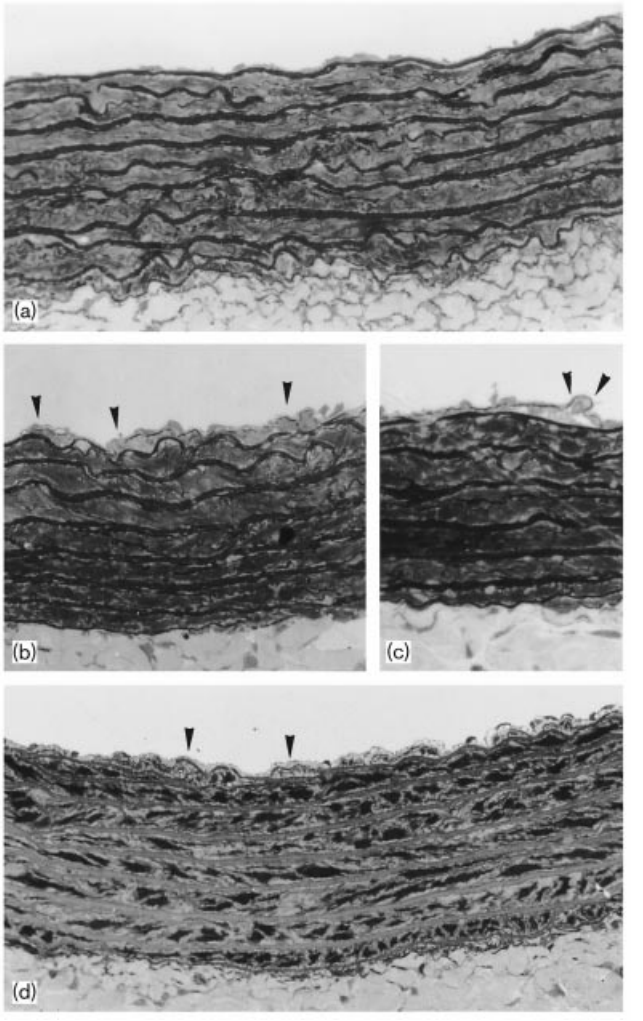
Treatment with L-NAME only, increased moderately the mean arterial blood pressure, by approximately 32% in comparison with that of untreated controls. The concomitant treatment with L-NAME and captopril decreased the mean arterial blood pressure by approximately 4% versus control animals. The hypertensive response was time-dependent reaching a maximum at the week 4. Captopril was able to inhibit the development of high blood pressure since the first week of treatment with L-NAME.

The hearts from rats of groups control, L-NAME, and L-NAME + captopril were similar on gross examination, and no differences were found in their weights expressed in g/100 g body weight: 3.35 ± 0.10 and 3.41 ± 0.11 and 3.42 ± 0.07, respectively. Since the body weight of animals is an important source of variability of organ weight, it seems obvious that the organ weight should be corrected for differences in body weights. Body and heart weight data on a large group of normal male rats in the body weight range of 50 g and 450 g were collected, and expressed as a weight curve of heart relative to body weight [21]. By using this method, it was possible to compare the wet heart weight of rats from both groups to wet heart weight of equal body weight predicted controls. The wet heart weight of L-NAME-treated (924.82 ± 15.12 mg), L-NAME + captopril-treated (897.40 ± 36.13 mg), and control (1050.08 ± 29.63 mg) were not different from the predicted wet heart weight of equal body weights controls, 920, 882, and 1005 mg, respectively. The analysis of picrosirius red-stained sections revealed mild myocardial fibrosis manifested by discrete increase in amount of pericellular collagen (endomysial matrix) and mild perivascular fibrosis. The volume fraction of fibrosis of the controls was 2.56 ± 0.30% whereas the volume fraction of L-NAME and L-NAME + captopril hearts increased to 4.07 ± 0.18% and 4.75 ± 0.28%

Grossly, the thoracic aortas from both experimental groups appeared similar to those of controls. The mean cross-sectional luminal area of aortas from L-NAME- and L-NAME + captopril-treated rats was 1.34 ± 0.04 mm^2 and 1.30 ± 0.03 mm^2, respectively, quite similar to the mean cross-sectional area of control aortas, 1.27 ± 0.04 mm^2.

The light microscopic study of plastic-embedded aortas could demonstrate striking intimal changes in L-NAME-treated and L-NAME + captopril-treated groups as compared with control group (Fig. 1). The changes in the L-NAME-treated groups were characterized by enlarged endothelial cells lying over a thick extracellular toluidine blue stained support tissue and

Fig. 1



Light microscopy. Representative views of aorta walls from (a) control, (b,c) L-NAME-and (d) L-NAME + captopril-treated rats. Glutaraldehyde-fixed, plastic-embedded tissue section stained with toluidine blue. (a) illustrates the delicate intima of control rats. A clearly expanded intima can be seen in b, c and d. Clusters of smooth muscle cells (b and d arrowheads) and mononuclear cells (c arrowheads) within the intima can be seen. (Mag. × 410).

foci of intimal thickening. This was composed of mononuclear cells or multilayered cushions of smooth muscle cells in contrast to the delicate structure of the intima in the control group. Simultaneous treatment of the rats with L-NAME and captopril resulted in clearly attenuated qualitatively similar intimal changes. This could be clearly demonstrated by evaluating the tunica intima and tunica media thickness and the intima: media ratio by means of light microscopy morphometry. Light microscopy sampling in morphometry was preferred because large test areas could be analyzed. The use of thin plastic allowed adequate resolution of structural details. The histomorphometric analysis showed that the mean intima thickness of the aorta from rats receiving L-NAME was 4.32 ± 0.33 μm, 150% higher than the value 1.73 ± 0.11 μm determined for the controls, and 54% higher than the value 2.81 ± 0.17 μm determined for the rats concomitantly given L-NAME and captopril. In the same way, the intima:media ratio in L-NAME-treated rats (4.22 ± 0.38) was 126% increased as compared with control rats (1.87 ± 0.14) or 56% increased as compared with the mean ratio for rats administered L-NAME and captopril (2.71 ± 0.19). On the other hand, the mean media thickness of the thoracic aorta from L-NAME-treated (103.00 ± 3.99 μm), L-NAME + captopril-treated (104.3 ± 4.92 μm), and control rats ($92{,}80 \pm 1.27$ μm) were not statistically different.

The body weight (initial and final), growth rate, heart weight, heart ratio, and mean arterial blood pressure (initial and final) from the three groups are tabulated in Table 1. The average daily intake of water and L-NAME, solid food consumption, and the mean plasma concentration of total and HDL cholesterol, and the total plasma proteins and albumin levels for all three groups are given in Table 2. Table 3 shows the values of tunica intima and tunica media thickness, intima:media ratio and cross sectional luminal area of the aorta from control, L-NAME-treated, and L-NAME + captopril-treated groups. This can be clearly seen when the frequency distribution of intima and media thickness and intima: media ratios in each group were plotted (Fig. 2).

The transmission electron microscopic appearance of the intima of control aortas did not differ from that reported in the literature. The intima was composed of a continuous layer of endothelial cells sitting on a basal lamina and a very thin layer of fibrocollagenous support tissue. This is contiguous with the media, composed of smooth muscle cells reinforced by organized layers of elastic fibers forming elastic laminas and collagen fibers. Just below the intima there is a broad continuous layer of elastic tissue interrupted by fenestras, the internal elastic lamina (Fig. 3). In the L-NAME-treated rats the intima was diffusely expanded. The endothelial cells appeared enlarged with irregular nuclear and cytoplasmic contours resting on a basement membrane-like material. Beneath this there was a thicker layer of fibrocollagenous support tissue. Clusters of smooth muscle cells appeared within the intima, randomly arranged, and surrounded by basement membrane-like material, collagen and young elastic fibers. Migration of smooth muscle cells from the media into the intima through the fenestras of the internal elastic lamina could be also seen. Diffuse focal accumulation of mononuclear cells was detected in the expanded intimal layer. The media remained intact and the medial smooth muscle cells appeared unaltered and retained their original orientation to the vessel (Fig. 4).

In the L-NAME + captopril-treated rats the intima was also thickened as compared with controls. Ultrastructural changes were qualitatively the same as those found in the L-NAME-treated animals, although less pronounced as clearly suggested by the histomorphometric study (Fig. 5).

The scanning electron microscopic evaluation revealed that the endothelial surface was lightly wavy. The endothelial cells throughout the aorta of control animals were elongated in shape with a major axis parallel to the direction of the blood flow. Marginal folds marked the margins. The luminal surface had microvilli spread out, mainly clustered over the nuclear hillock. The endothelial surface of the aorta of L-NAME-treated rats was highly irregular with a striking nodular pattern with deep grooves between the nodules while the endothelial surface of the aorta of rats concomitantly treated with L-NAME and captopril was moderately irregular with discrete nodular pattern. In both groups, the endothelial cells were ill-defined, with less evident margins due to the paucity of marginal folds (Fig. 6).

Discussion

In the present study, we show that inhibition of NO synthesis by chronic administration of L-NAME into rats induces hypertension and intimal remodeling in the absence of elevated plasma cholesterol levels. In addition, and importantly, we found that animals made normotensive by simultaneous L-NAME and captopril treatment had reduced, although qualitatively unchanged, intimal remodeling.

In our studies, N^{ω}-nitro-L-arginine methyl ester (L-NAME) was given in the drinking water containing 45 mg/100 ml, resulting in a mean daily intake of approximately 8 and 8.5 mg/100 g of body weight for L-NAME- and L-NAME + captopril-treated rats, respectively. However, intake of L-NAME decreased from the beginning of the treatment period to the end, as previously reported [22]. It has been demonstrated that endothelial NO synthesis is inhibited by L-NAME

Table 1 Body weights (initial and final), growth rates, heart weights, heart ratios, and mean arterial blood pressure (initial and final) from control, L-NAME-treated, and L-NAME + captopril-treated rats

	Body weight (g)		Growth rate	Heart weight (mg)			Heart ratio	Mean blood pressure**	
Group	Initial	Final	g/day/rat	Observed	Predicted	P*	(g/100 g)	Initial	Final
Control ($n = 7$)	88.14 ± 1.30	314.10 ± 8.48	8.07	1050.08 ± 29.63	1005	NS	3.35 ± 0.10	77.82 ± 7.48	107.5 ± 7.46
L-NAME-treated ($n = 11$)	87.14 ± 1.02	273.40 ± 8.25	6.65	924.82 ± 15.12	920	NS	3.41 ± 0.11	79.69 ± 8.46	141.7 ± 7.46
L-NAME + captopril-treated ($n = 9$)	86.05 ± 1.22	261.16 ± 6.11	6.27	897.40 ± 36.13	882	NS	3.42 ± 0.07	80.20 ± 9.21	101.4 ± 6.43
Control × L-NAME	NS	< 0.01		< 0.01			NS	NS	< 0.001
P Control × L-NAME + captopril	NS	< 0.01		< 0.01			NS	NS	NS
L-NAME × L-NAME + captopril	NS	NS		NS			NS	NS	< 0.001

Values are mean ± standard error. *Measured versus predicted. **Mean ± standard deviation. NS, not significant.

Table 2 Average daily intake of water and L-NAME, solid food consumption, and mean plasma concentration of total and HDL cholesterol, and total plasma proteins and albumin levels from control, L-NAME-treated, and L-NAME + captopril-treated rats.

Group	Water intake (ml/day per 100 g)	L-NAME intake (mg/day per100 g)	Solid food consumption (g/day per rat)	*Total cholesterol (mg/100 ml)	*HDL cholesterol (mg/100 ml)	*Total plasma proteins (g/100 ml)	*Albumin (g/100 ml)
Control ($n = 7$)	15.98	–	19.54	60.33 ± 5.32	43.63 ± 2.60	5.76 ± 0.41	3.42 ± 0.13
L-NAME-treated ($n = 11$)	18.16	8.17	17.37	58.14 ± 3.72	43.51 ± 1.87	6.33 ± 0.42	3.57 ± 0.12
L-NAME + captopril-treated ($n = 9$)	19.04	8.57	16.32	62.63 ± 4.73	41.05 ± 3.11	5.55 ± 0.54	3.53 ± 0.18

*Values are mean ± standard error.

Table 3 Tunica intima and media thickness, intima:media ratio, and cross sectional area of the aorta from control, L-NAME-treated, and L-NAME + captopril-treated rats

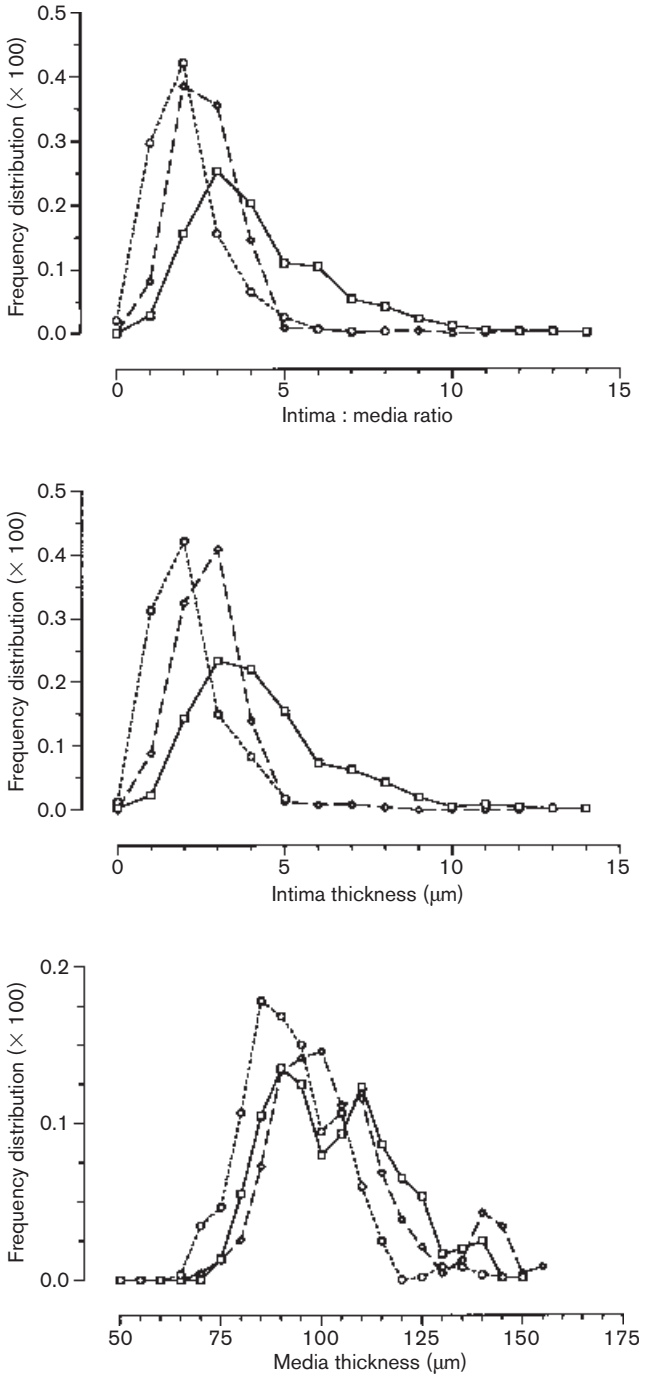
Group	Intima thickness (m)	Media thickness (m)	Intima:media ratio	Aorta lumen area (mm^2)
Control ($n = 6$)	1.73 ± 0.11	92.80 ± 1.27	1.87 ± 0.14	1.27 ± 0.04
L-NAME ($n = 6$)	4.32 ± 0.33	103.00 ± 3.99	4.22 ± 0.38	1.34 ± 0.04
L-NAME + captopril ($n = 6$)	2.81 ± 0.17	104.3 ± 4.92	2.71 ± 0.19	1.30 ± 0.03
Control × L-NAME	< 0.001	NS	< 0.001	NS
P Control × L-NAME + captopril	< 0.05	NS	NS	NS
L-NAME × L-NAME + captopril	< 0.001	NS	< 0.01	NS

Values are mean ± standard error of the mean. NS, not significant

administration. Treatment of adult rats with a daily dose of 4 mg/day per 100 g of body weight for 4 weeks, alone or with captopril [23,24], has been shown to result in a significant attenuation of NO synthase activity in the heart (69% less), aorta (26% less), brain (73% less), and kidney (30% less). L-NAME administered in the drinking water (50 mg/100 ml) to rats markedly reduced the levels of NO metabolites in serum and urine while increasing blood pressure. The simultaneous administration of an angiotensin converting enzyme inhibitor completely normalized the blood pressure without affecting the decreased NO production [25]. Furthermore, the NO production from the aortas of rats treated with L-NAME (10 mg/day per 100 g of body weight) was markedly less than in control group aortas, whereas L-arginine normalized the L-NAME-induced decrease in NO production [13].

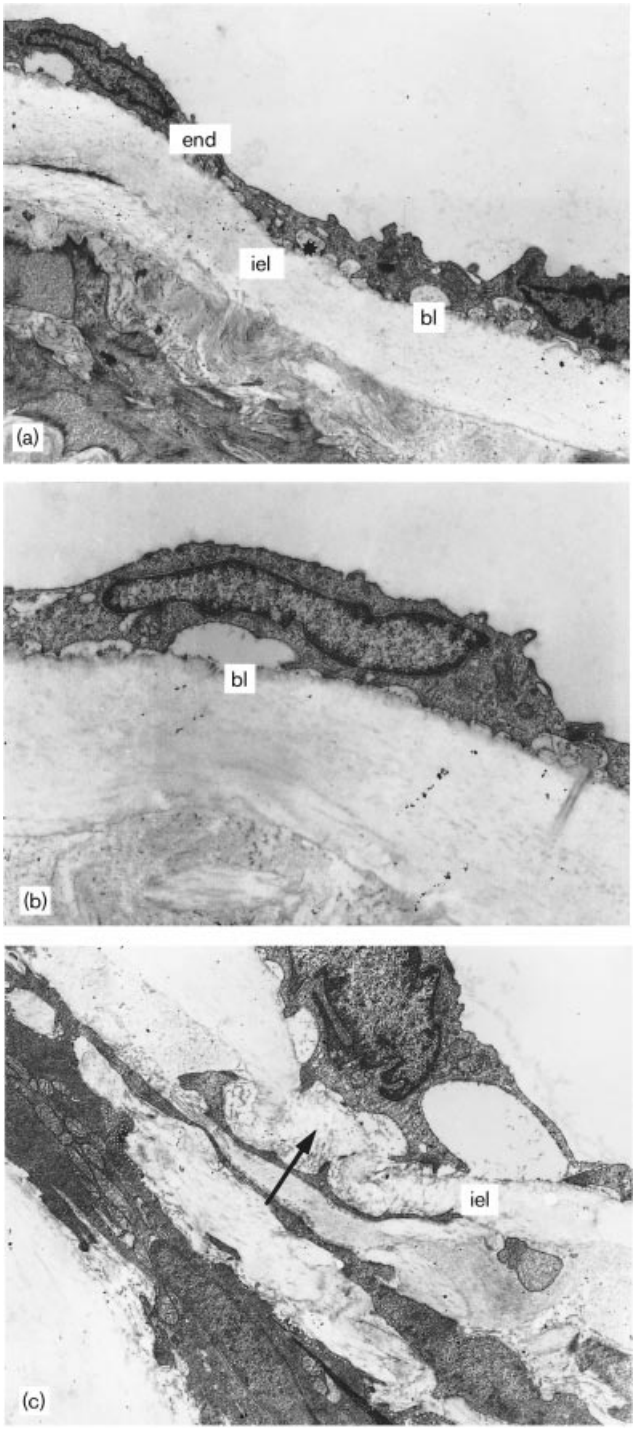
L-NAME-treated rats as compared with controls experienced mild retardation of body weight gain. It could be argued that the effect of NO synthase inhibition could be related to insufficient nutrition. This is very unlikely since the serum albumin levels of L-NAME-treated rats were closely similar to those of control rats and hypoalbuminemia is an invariable finding in developed protein-calorie under nutrition [26]. Similarly, rats chronically treated with 20–80 mg/100 ml in the drinking water of L-NAME or rabbits chronically treated with 80 mg/100 ml of L-NAME in the drinking water, did not shown alterations of serum levels of albumin

Fig. 2



Frequency distribution of intima:media ratio and tunica intima and media thickness in L-NAME-treated (————), L-NAME + captopril-treated (– – –), and control (·····) groups.

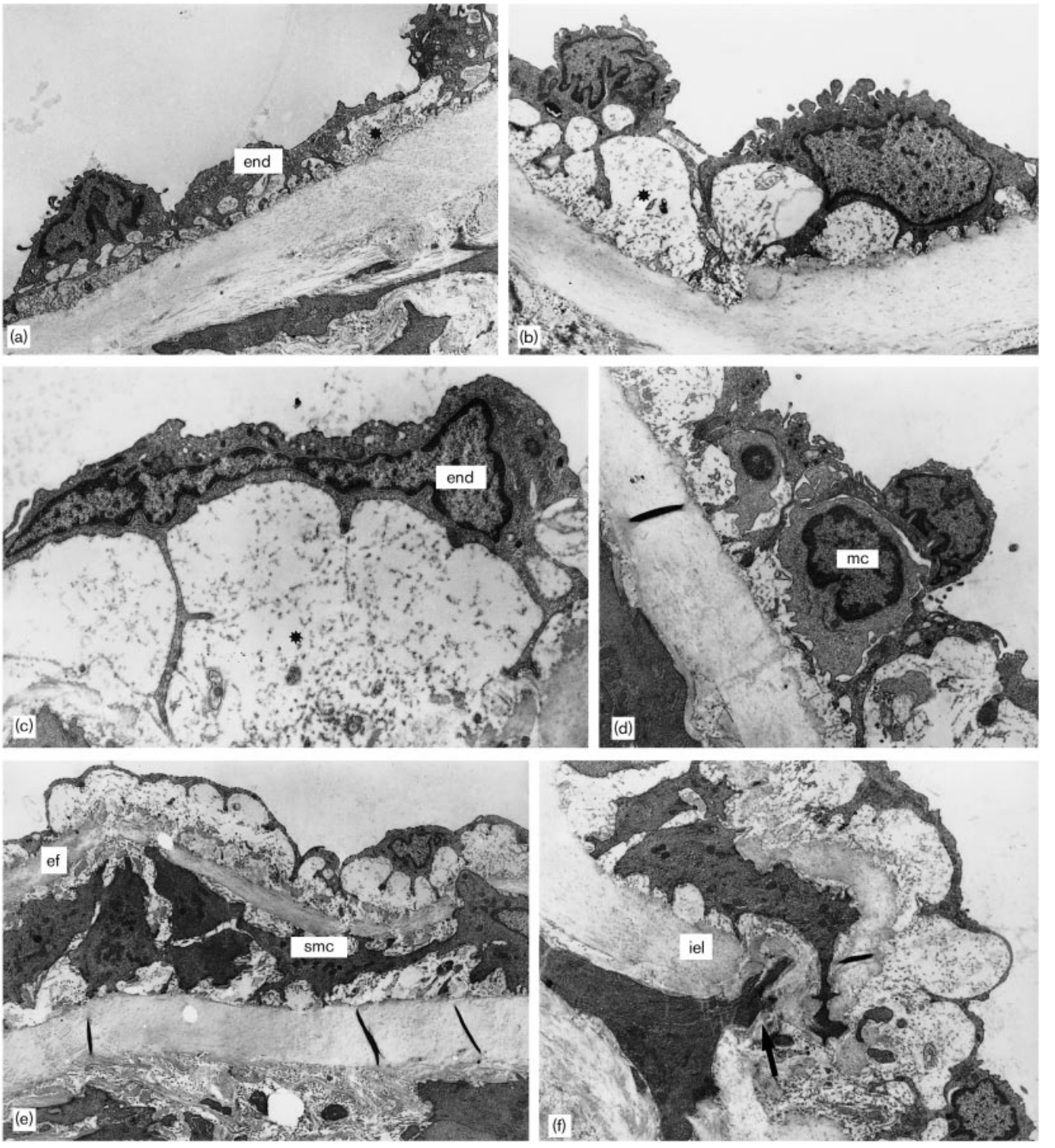
Fig. 3



Transmission electron microscopy. Control aorta. (a) The delicate structure of the intima contains flattened endothelial cells (end) sitting on a basal lamina (bl) and a very thin layer of support tissue (*); (iel), internal elastic lamina. (b) a higher magnification of one of the endothelial cells seen in (a). (c) The intima is delimitated by the internal elastic lamina (iel) interrupted by fenestras (arrow). (Mags: a, × 4350; b, × 10450; c, × 5800.)

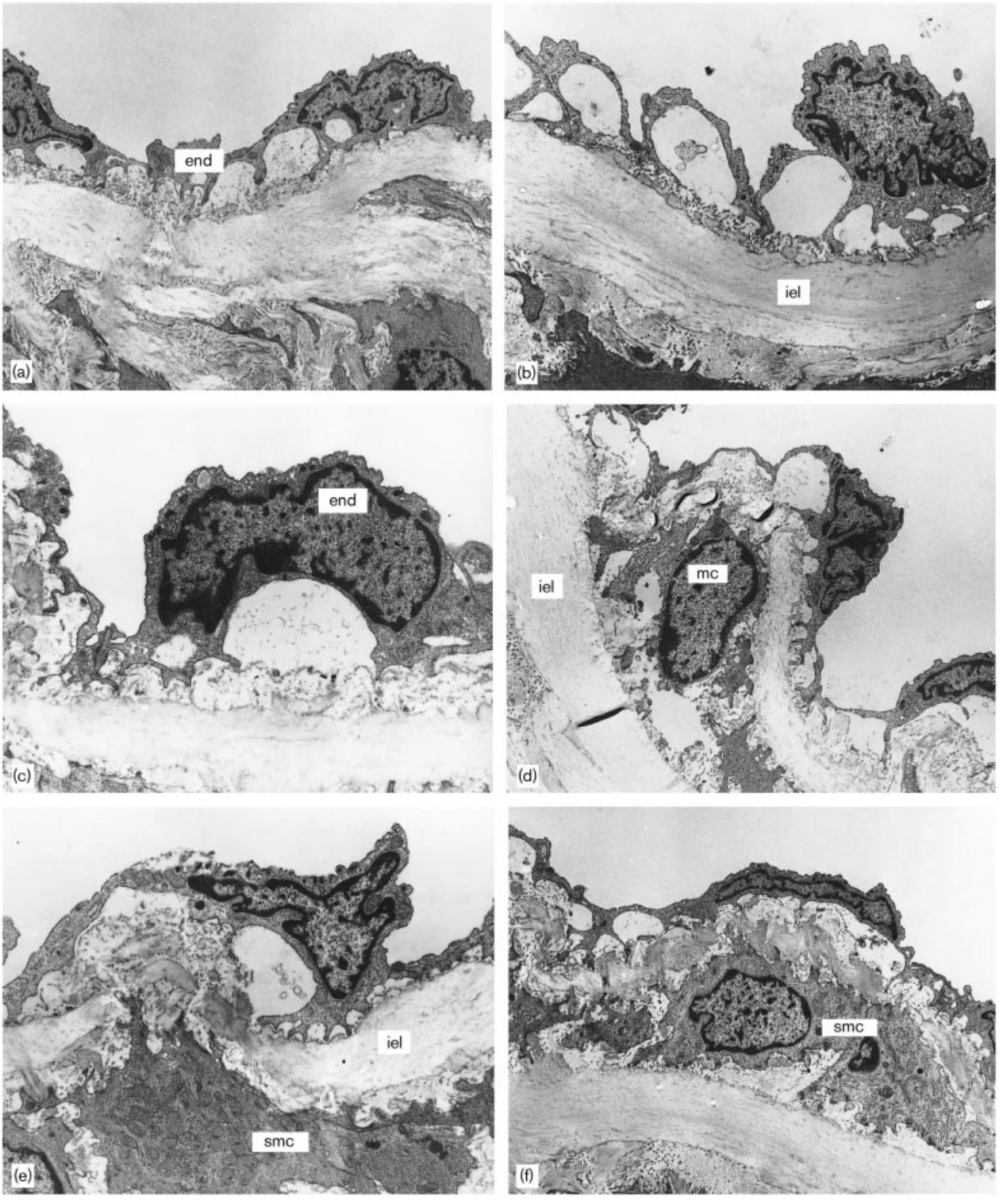
[27,28]. In addition, the observed daily food consumption was in accord with the assumed adequate dietary intake of 15 g/day per rat for growing rats or adult rats at maintenance. [29]. The slightly higher average consumption of solid food in control rats, in comparison

Fig. 4



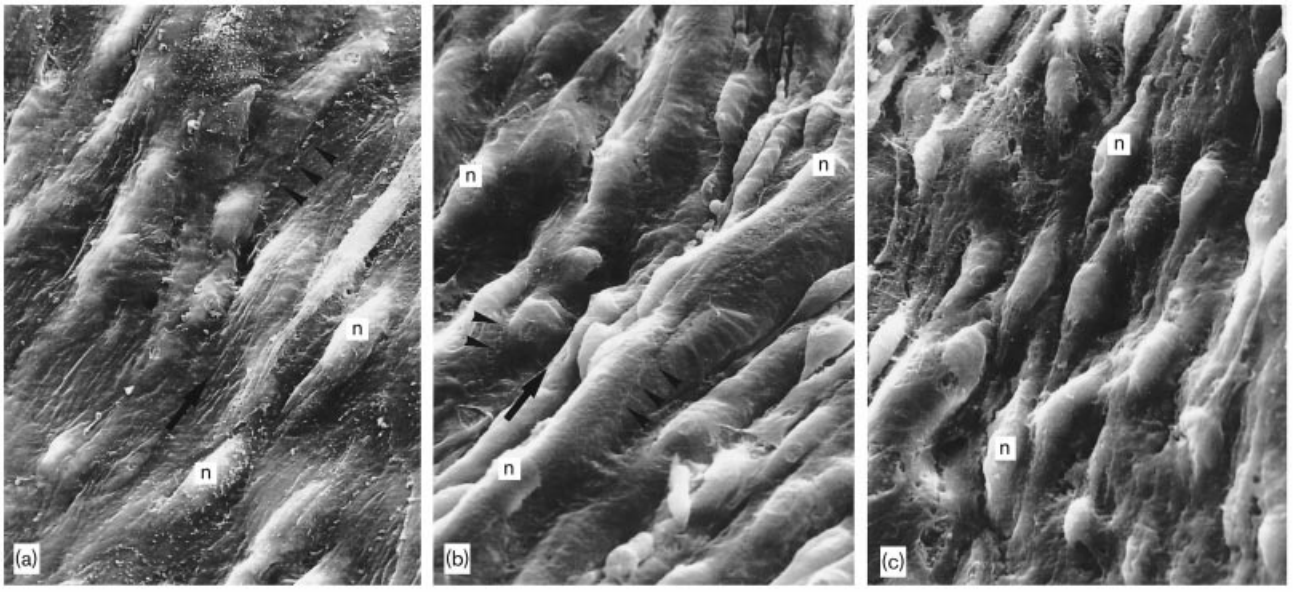
Transmission electron microscopy. L-NAME-treated aorta. (a,b) The intima is thickened. The endothelial cells (end) are heterogeneous, most of them enlarged with convoluted nuclear and cytoplasmic contours. The endothelial cells are sitting over a thicker layer of support tissue (*). (c) a higher magnification of an enlarged endothelial cell clearly showing tortuous nuclear and cytoplasmic contours. Focal accumulation of mononuclear cells (mc) could be detected in the expanded intimal layer, as seen in (d). (e) Clusters of smooth muscle cells (smc) appear within the intima, randomly arranged, and surrounded by basement membrane-like material, collagen and young elastic fibers (ef). Migration of smooth muscle cells from the media into the intima (arrow) through the fenestras of the internal elastic lamina (iel) can be also seen (panel F). (Mags. a, × 4350; b, × 6900; c, × 10450; d, × 5750; e, × 3500; f, × 5750.)

Fig. 5



Transmission electron microscopy. L-NAME + captopril-treated aorta. The ultrastructural changes are qualitatively similar to those found in the L-NAME-treated animals, although less pronounced. (a,b,c) The tunica intima layer is expanded. The endothelial cells (end) are heterogeneous, most of them enlarged with tortuous nuclear and cytoplasmic contours. A mononuclear cell (mc) is seen in the thickened intimal layer in (d). A smooth muscle cell migrating through the fenestra of the internal elastic lamina is illustrated in panel e and clusters of smooth muscle cells (smc) surrounded by collagen and elastic fibers can be seen in (f). iel, internal elastic lamina. (Mags. a, × 5300; b, × 6500; c, × 9600; d, × 5800; e, × 4850; f, × 8550.)

Fig. 6



Scanning electron microscopy. (a) Control aorta. The endothelial cells are elongated in shape with major axis parallel to the direction of the blood flow (thick arrow). Marginal folds (arrowheads) distinctly mark the margins. The nuclear hillocks can be clearly seen (n). (b) L-NAME-treated aorta. The endothelial surface is highly irregular with striking nodular pattern with deep grooves between the nodules. The endothelial cells are ill- defined with less evident margins due to paucity of marginal folds (arrowheads). The nuclear hillocks are more pronounced in comparison to controls (n). (c) L-NAME + captopril-treated aorta. The endothelial surface is moderately irregular with discrete nodular pattern, although qualitatively similar to the appearance shown in panel (b). (Mag. × 2050.

with the average consumption in L-NAME-tested rats, may be related to the ability of NO regulating appetite [30]. The mean wet heart weights of rats given either L-NAME or L-NAME and captopril were significantly decreased in comparison with those of controls while the heart ratios of all three groups were not different. However, the mean wet heart weights of both L-NAME-tested rats and of control animals were not different from the predicted heart weight of equal body weight controls. Similarly, several authors did not find any hypertrophy of the whole heart [10,31] or the left ventricle [32,33] after oral L-NAME treatment for 4–8 weeks. Negative metabolic effects of L-NAME on protein synthesis in spite of the induced sustained pressure overload [33] would mediate this blunted hypertrophic response. This same mechanism could also contribute to the weight gain retardation of animals from both L-NAME-tested groups. In contrast, treatment with L-NAME for 6 and 8 weeks has been shown to result in significant increase in heart ratio [9,10,34]. In addition, diffuse myocardial fibrosis was noted in all animals chronically given L-NAME. The patterns of fibrosis in rats from both L-NAME- and L-NAME - + captopril-treated groups were similar and manifested by discrete increase in amount of pericellular collagen weave fibers (endomysial matrix) and mild perivascular fibrosis. Many authors reported that chronic inhibition of NO synthesis resulted in significant fibrosis characterized by areas of dense and replacement fibrosis consistent with organized myocytolytic necrosis and striking perivascular fibrosis [8–11,25]. The interstitial myocardial fibrosis would be related to L-NAME-induced vasoconstriction with consequent myocardial ischemia [8–10]. Previous studies addressing the question whether the myocardial fibrosis during NO synthase inhibition was caused by the consequent hypertension or the lack of NO *per se* reported conflicting results. Concomitant administration of hydralazine and L-NAME prevented arterial hypertension and attenuated myocardial fibrosis without affecting cardiac hypertrophy and microvascular remodeling induced by chronic inhibition of NO synthesis [9]. Co-treatment with enalapril (angiotensin converting enzyme inhibitor) and L-NAME prevented arterial hypertension and left ventricular hypertrophy, but could not prevent myocardial fibrosis [8]. Administration of another angiotensin converting enzyme inhibitor, ramipril, simultaneously with L-NAME prevented the development of both blood pressure increase and structural fibrotic alterations of the ventricle [35,36]. Contrasting, recent study showed that captopril (100 mg/day per kg body weight) simultaneous with L-NAME (4 mg/day per 100 g of body weight) caused regression of hypertension and left ventricular hypertrophy while the myocardium concentration of collagenous fractions remained increased [37]. Our results give support to the

idea that the fibrotic changes in the myocardium of rats chronically-treated with NO synthase inhibitor depend on the process associated with NO insufficiency *per se*.

An important question is concerned with the effect of blood pressure elevation on aorta remodeling. In the present investigation chronic inhibition of NO synthesis induced a time-dependent hypertension that reached a maximum at the fourth week. Blood pressure increase in animals chronically treated with L-NAME has been described in numerous studies and serves as a reliable indicator of NO synthesis inhibition [7,38]. Decreased NO-synthase activity [23] with consequent reduced NO production [13,25] associated with increased renin-angiotensin system activation [39,40] seem to be the mechanisms involved in this type of hypertension. Captopril prevented hypertension in rats simultaneously treated with L-NAME since the first week of treatment. Captopril is an angiotensin converting enzyme inhibitor that lowers blood pressure and improves endothelial function. There are two possible mechanisms by which angiotensin converting enzyme inhibitors exert their effect: (1) decreased local production of angiotensin II and (2) decreased bradykinin breakdown, release of NO, and increased prostacyclin levels [41–43]. Experimental studies have been demonstrating that angiotensin converting enzyme inhibitors improve endothelium function by increasing NO activity [44–46]. Actually, a solid basis underlies the thesis that NO production plays a key role in determining endothelial cell function; and defective endothelial NO synthase activity is a crucial parameter characterizing endothelial cell dysfunction [47]. The initial remodeling of the thoracic aorta from both hypertensive L-NAME-treated and normotensive L-NAME + captopril-treated rats were qualitatively indistinguishable and characterized by endothelial cell changes, intimal accumulation of mononuclear cells, and focal accumulation and migration of medial smooth muscle cells into the tunica intima. However, we could clearly demonstrate that the thoracic aorta from hypertensive L-NAME-treated rats developed significantly worse intimal changes in comparison with animals made normotensive by concomitant L-NAME and captopril administration. It could be argued that captopril could have had this same beneficial effect on intimal remodeling without lowering blood pressure. In fact, long-term angiotensin converting enzyme inhibitors administration has been shown to reduce the extent of minimal intimal lesions in the abdominal aorta of normal rats, demonstrating a regulation of the rat aortic intima by the renin–angiotensin system [48]. However, in the present study this mechanism is very unlikely because L-NAME-induced inhibition of NO synthase activity can not be restored by angiotensin converting enzyme inhibition in relatively short period experiments. Endothelial dysfunction of a conduit artery in hypertension can only be reversed after active long-term therapy with angiotensin converting enzyme inhibitors [49]. Treatment of rats with L-NAME and captopril for 4 weeks resulted in the same level of decrease of NO synthase activity and persistent low levels of cGMP within the aortic walls of rats treated with L-NAME only [20]. Furthermore, L-N^G monomethyl arginine (L-NMMA), a specific NO synthase inhibitor, abolished the potentiation of endothelial function in the femoral circulation with enaprilat in patients with atherosclerosis of the coronary circulation [50]. Accordingly, our findings clearly indicate that hypertension alone was not the causative factor of the intimal remodeling, but acted as a worsening factor. This harmonizes with the previous study, demonstrating that the inflammatory phenotype observed in the aortic intima from L-NAME hypertensive rats (4 mg/day per 100 g body weight) for 18 days could be reversed by L-arginine administration for the latter 10 days of the experiment, although the hypertensive response was not reduced [12].

Endothelial cell changes, intimal focal accumulation of mononuclear cells, and focal accumulation and migration of medial smooth muscle cells into the intima of the thoracic aorta were a striking response to L-NAME treatment, alone or associated with captopril. In our study, the endothelial cells were heterogeneous, most of them enlarged, with convoluted nuclear and cytoplasmic contours. Considering that there is a close correlation between endothelial structure and function [51], this damage may manifest itself as endothelial dysfunction. Previous study could demonstrate that the endothelial cells associated with elevated lesions of the aorta of rabbits fed a high-cholesterol diet were chaotically-oriented and their sizes were markedly increased with the concurrent appearance of multinucleated giant cells, while their shapes were altered [52]. Regarding the focal intimal accumulation of mononuclear cells, the inhibition of NO synthesis has been shown to cause leukocyte recruitment in the pulmonary [53] and coronary circulation [54] and increase leukocyte adhesion to postcapillary endothelium [55]. Decreased basal NO release by hypercholesterolemic rabbit coronary endothelium is associated with a three-fold increase in leukocyte adherence to endothelial cells [56]. In this same study, pharmacological reversal of the hypercholesterolemia increased basal NO production and decreased leukocyte adhesiveness to coronary endothelium. Enhanced leukocyte adhesion to endothelium during infusion of NO synthase inhibitors could be reversed by L-arginine or by an antibody to leukocyte adhesion molecule CD11b/CD18 [57]. A few studies have dealt with the structural changes of conduit arteries after NO synthase activity chronic blockade. Using morphological criteria to measure cellular changes, NO inhibition in adult rats given L-NAME for 18 days has been shown to specifically and markedly

promote increased adhesion of monocytes/macrophages to the vascular endothelium and increased medial thickness [12]. In a recent study [13], chronic blockade of NO synthesis activity with L-NAME (10 mg/day per 100 g body weight) in adult rats resulted in early (3 days after L-NAME administration) inflammatory changes in large coronary arteries characterized by infiltration of monocytes (60% , myofibroblasts (10% , and a few T cells. The thoracic aorta was infiltrated with monocytes. In addition, the coronary inflammatory changes were associated with the expression of monocyte chemoattractant protein-1 (MCP-1). The inflammatory changes and the MCP-1 expression declined after 4 weeks treatment with L-NAME replaced by vascular (fibrosis and medial thickening) and myocardial (fibrosis) remodeling. These late structural changes in rat hearts were ascribed to the early inflammatory changes induced by NO synthase blockade. In another recent study [14], aortas from adult rats were examined after 8 weeks of L-NAME treatment (5 mg/100 g body weight per day). This treatment was able to induce the accumulation of inflammatory cells, mainly macrophages, in the arterial wall. In addition, the infiltration by inflammatory cells was associated with the expression of the proinflammatory protein inducible nitric oxide synthase (iNOS) localized in smooth muscle cells and overexpression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in the endothelium. Moreover, the concomitant administration of L-NAME and ibersartan, an angiotensin II antagonist drug, was able to prevent the development of the vascular inflammatory process. With respect to the role of NO as an endogenous inhibitor of smooth muscle cells, although not well-defined, there is a growing body of experimental and clinical evidence pointing out that NO inhibits smooth muscle cell proliferation and migration and does so via a variety of intracellular mechanisms [58–60].

In conclusion, chronic inhibition of NO synthesis with L-NAME administration for 4 weeks promotes striking intimal remodeling of the thoracic aorta of normocholesterolemic rats. This remodeling was caused by the prolonged blockade of NO synthase activity *per se* and potentiated by L-NAME-induced hypertension. Most important, the present findings favor the idea that blockade of NO synthase activity by causing intimal remodeling might be a primary cause, as individual biologic phenomenon, in the development of an atherosclerotic plaque.

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