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Study of in vivo and in vitro resting vasodilator nitric oxide tone in normotensive and genetically hypertensive rats

José Gil-Longo *, Dolores Fdez-Grandal, Marta Álvarez, Manuela Sieira, Francisco Orallo

Departamento de Farmacoloxía, Facultade de Farmacia, Universidade de Santiago de Compostela, Campus Universitario Sur, E-15706 Santiago de Compostela, Spain

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Abstract

The effects of N^G-nitro-L-arginine (L-NNA) on mean arterial pressure and the effects of both L-NNA and methylene blue on isolated aorta tone, were studied in order to elucidate potential alterations in vasodilator resting nitric oxide (NO) tone in genetic hypertension. L-NNA produced a significantly greater increase of mean arterial pressure in spontaneously hypertensive rats (SHR) than in Wistar Kyoto (WKY) rats; in both cases, L-arginine completely inhibited the L-NNA hypertensive effect. Neither ganglion blockade with hexamethonium nor cyclooxygenase inhibition with indomethacin significantly modified the effect of L-NNA in both rat strains. In intact aorta rings, after submaximally contraction with KCl (25 mM), both L-NNA and methylene blue induced strong dose-dependent contractions. The maximum contractions were, however, significantly greater in WKY rats than in SHR. The mechanical elimination of endothelium markedly inhibited both L-NNA and methylene blue maximum contractions. In intact rings, L-arginine completely inhibited the L-NNA effects in both rat strains; in rubbed rings, the L-arginine inhibitory effects were strong in WKY rats but not important and erratic in SHR. L-Arginine had no effect on the contractions induced only by KCl in any of the preparations. In WKY rat-rubbed rings, sodium nitroprusside was significantly more effective in relaxing the contractions in response to 25 mM KCl than the contractions in response to methylene blue. These results indicate that contractions induced by L-NNA and methylene blue in isolated aorta are principally due to the inhibition of an important endothelial resting vasodilator NO tone. They also show that hypertension reduces the resting vasodilator NO tone in isolated rat aorta, in spite of enhancing the total vasodilator NO tone in anaesthetized rat.

Keywords: N^G-Nitro-L-arginine; Methylene blue; Endothelium; Nitric oxide (NO); Hypertension; Aorta, rat; Arterial pressure

1. Introduction

It is now recognised that the vascular endothelium importantly determines vascular tone, through the release of different relaxing and constricting factors (Rees et al., 1990; Vane et al., 1990; Lüscher, 1993). One of these factors is nitric oxide (NO), or a molecule containing NO (for review, see Moncada et al., 1991). NO can also be generated by other cell types including smooth muscle cells, circulating blood cells and neurones, within the central nervous system (also Moncada et al., 1991). NO is formed from the terminal guanidino nitrogen atom(s) of the non-essential amino acid L-arginine by the action of at least three different isoforms of NO synthase (Förstermann et al., 1994). The endothelial cell isoform and the neuronal isoform are constitutive enzymes which require calcium and calmodulin. The third type is a inducible enzyme, which is Ca^{2+}-independent and can be found in a multitude of different cells after activation by endotoxin or cytokines. Both constitutive and inducible enzymes can be inhibited by certain closely related derivatives of L-arginine, such as N^G-nitro-L-arginine (L-NNA; Ishii et al., 1990; Moncada et al., 1991; Mayer et al., 1993b), and thus the role of NO in the regulation of vascular tone can be investigated (Santiago et al., 1994). The administration of L-NNA and other NO synthase inhibitors to rats (Lacolley et al., 1991a; Kobayashi et al., 1991; Fozard and Part, 1991; Schleiffer et al., 1991) and other animals results in a substantial increase in blood pressure. Moreover, the NO synthase inhibitors induce constrictor effects in different vascular beds (Fozard and Part, 1991; Moncada et al., 1991) and the contraction of different isolated vascular ring arteries (see Moncada et al., 1991), suggesting that the increase in blood pressure is due largely, if not entirely, to

* Corresponding author. Tel.: 34 81 59 46 30; fax: 34 81 59 45 95.

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an increase in peripheral resistance. These results also demonstrate that the vasculature is in a constant state of vasodilatation due to the continuous basal release of NO from the endothelium (Lüscher, 1993). NO specifically stimulates soluble guanylate cyclase and thus increases intracellular cGMP production; in the vasculature this provokes, after a decrease in intracellular Ca^{2+}, vasodilatation (Moncada et al., 1991). Methylene blue, an inhibitor of soluble guanylate cyclase activation, prevents the rise in cGMP levels and blocks vasorelaxant responses to NO (Martin et al., 1985). Therefore, methylene blue can be used as a pharmacological probe to specify the role of NO in mediating vascular responses (Hofman et al., 1992).

Evidence, albeit indirect, suggests that the evoked NO-dependent dilator system may be impaired in the established hypertension (Moncada et al., 1991; Lüscher, 1993; Panza et al., 1993). However, contradictory results were obtained when studying to see if a major factor contributing to the elevated blood pressure was a reduced basal release of NO in hypertension. Thus, with similar in vivo studies, some authors have found that the total vasodilator resting NO tone is reduced in SHR (Schleiffer et al., 1991), whereas others have found no alterations (Fozard and Part, 1991) or an enhanced release (see for example, Lacolley et al. (1991a). There are also conflicting reports with in vitro studies, Dohi et al. (1990) and Pourageaud and Freslon (1992) noted that the release of NO on resistance arteries is impaired in hypertension while Randall et al. (1991) and Adeagbo et al. (1994) did not.

The aim of this work was to examine potential alterations in the resting vasodilator NO tone in SHR as compared to WKY rats and to try to discover the origin of any possible alteration. To do this, we have defined the resting NO tone in SHR and WKY rats employing L-NNA and methylene blue as probes in both in vivo and in vitro conditions.

2. Materials and methods

Experiments were conducted on male Wistar Kyoto (WKY) rats weighing 300–350 g and age-matched spontaneously hypertensive rats (SHR) weighing 250–300 g, purchased from Iffa-Credo (Barcelona, Spain). The systolic blood pressure (tail-cuff method; conscious rats) was tested in all the rats. If a WKY rat pressure exceeded 110 mm Hg or if a supposed SHR developed no significant hypertension before experimental procedure (systolic blood pressure > 170 mm Hg) it was rejected.

2.1. Studies in anaesthetized rats

WKY rats and SHR were anaesthetized with urethane (1.26 g kg^{-1}, i.p.) and the rectal temperature was kept at 36.5–37.5°C with an overhead lamp. A tracheotomy was made to facilitate spontaneous respiration. Polyethylene cannulae, containing heparinised saline solution (120 iu ml^{-1}), were surgically inserted in the left carotid artery (for arterial pressure and heart rate measurement) and in the left femoral vein (for i.v. administration of drugs). Systolic and diastolic arterial pressures were monitored by means of a TRA 021 Letica Unigraph 1000-506 device. Heart rate was obtained from the arterial pulse wave on a digital counter (Panlab 0602) connected to the polygraph output. The preparation was allowed to equilibrate, generally 30 min, after surgical operations. All experiments were performed on both WKY rats and SHR.

In the first group of experiments, saline solution (1 ml kg^{-1}, for control group) or N^G-nitro-L-arginine (L-NNA; 5 mg kg^{-1}, for treated group) were injected intravenously in order to observe the effects on the blood pressure. When the response was stable, L-arginine (100 mg kg^{-1}, i.v.) was injected to the treated group.

In another group of experiments, the animals were first treated with an injection of hexamethonium (5 mg kg^{-1}, i.v.) or indomethacin (5 mg kg^{-1}, i.v.), depending on the experiments, and 20 min later L-NNA was added to the same dose as the first group, in order to study if the effect in mean arterial pressure was modified by the above-mentioned drugs.

2.2. Contraction studies in rat isolated thoracic aorta rings

2.2.1. General procedure

Both WKY rats and SHR were killed by stunning and exanguination. The thoracic aorta was rapidly removed and placed in a Petri dish with Krebs bicarbonate solution (composition mM = NaCl, 119; $CaCl_2 \cdot 2H_2O$, 1.5; $NaHCO_3$, 25; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; EDTA, 0.029; ascorbic acid, 0.56; glucose, 11; pH 7.4; room temperature), oxygenated with carbogen (95% O_2 + 5% CO_2), cleaned of adherent connective tissue, stripped (if necessary) of endothelium by gentle rubbing of the intimal surface with a wide cotton thread and cut into 5 mm long cylindrical rings. Then, aorta rings were immediately transferred to an organ bath containing 20 ml of the above-mentioned solution thermoregulated at 37°C and bubbled with carbogen. Two stainless steel pins were inserted through the lumen of each arterial segment: one pin was fixed to the organ bath and the other was connected to a CPOL force-displacement transducer to record the isometric tension, using a computerized Celaster IOS 1 system. Before initiating specific experimental protocols, aortae were equilibrated at a resting tension of 2 g for at least 1 h, during which the physiological solution was replaced every 10 min. The endothelial integrity was determined by the relaxation in response to acetylcholine (1 μM) after the contraction by phenylephrine (1 μM). Thereafter, the preparations were equilibrated again following the above-mentioned protocol. All the studies were

performed in both intact and rubbed rings from WKY rats and SHR, unless otherwise specified.

2.2.2. Phenylephrine-induced contractions

In some endothelium intact rings, once endothelial integrity was verified by the relaxation in response to acetylcholine and after the corresponding washout and equilibration period, the same procedure was performed again, however rings were preincubated with L-NNA (0.1 mM) or methylene blue (10 μM) for at least 1 h to see if the acetylcholine relaxation had been modified by the drugs. In another group of experiments, and after two reproducible phenylephrine control contractions were obtained, the tissues were then incubated with the protein-synthesis inhibitors cycloheximide (10 μM) or puromycin (100 μM) during 20 min and a third phenylephrine contraction was obtained.

2.2.3. L-NNA and methylene blue concentration-response curves

After an incubation period of 45 min in KCl (12, 25 or 33 mM, depending on the studies), L-NNA (0.1 μM to 0.1 mM) or methylene blue (10 nM to 10 μM) were added to the tissue bath and the isometric tension was monitored until a plateau response was obtained. In some experiments, once the contractile response to L-NNA was obtained, the effect of subsequent addition of D-arginine (1 μM to 1 mM) or L-arginine (1 μM to 1 mM) was recorded. In rubbed rings of WKY rats, after the contractile response to methylene blue was stabilized, the effect of a subsequent addition of sodium nitroprusside was also recorded. Furthermore, L-arginine and sodium nitroprusside effects were studied in rings only contracted with KCl (25 mM). In another group of experiments with intact rings of WKY rats, the L-NNA and methylene blue concentration curves were also obtained in the presence of indomethacin (5 μM), which was added to the tissue bath saline solution. In addition, in rubbed rings of WKY rats, L-NNA and methylene blue concentration-response curves were obtained after incubation for 20 min in the presence of cycloheximide and puromycin.

2.3. Drugs

The following drugs were used: acetylcholine chloride (acetylcholine), D- and L-arginine, cycloheximide, heparin, hexamethonium bromide, indomethacin, methylene blue, N^G-nitro-L-arginine (L-NNA), L-phenylephrine hydrochloride, puromycin dihydrochloride, sodium nitroprusside and urethane, which were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Acetylcholine, D- and L-arginine, cycloheximide, hexamethonium bromide, methylene blue, L-NNA, L-phenylephrine hydrochloride, potassium chloride, puromycin dihydrochloride and sodium nitroprusside were prepared daily in de-ionized water (drugs used on isolated aorta) or saline solution (drugs used on anaesthetized rats) from stock solutions kept at −20°C. Indomethacin, which was freshly prepared for each experiment, was dissolved over a 3 min period in 0.05 M Na_2CO_3 heated to 40°C. Heparin (12 000 iu 100 ml^{-1}) and urethane (25 g 100 ml^{-1}) were dissolved in a saline solution and kept at 4°C. All reagents used in the preparation of physiological solutions were of analytical grade.

2.4. Statistical analysis and data presentation

Results shown in the text and figures are expressed as means ± S.E.M.; n indicates the number of observations, one in each animal. Student's two tailed t-tests for unpaired data were used to compare the difference between two groups. An analysis of variance was used for comparisons among three or more experimental data groups; if significant differences were found the Newman-Keuls test was used to make specific comparisons. Results were considered to be significant at $P < 0.05$.

The blood pressure and heart rate values are expressed in mm Hg and beats min^{-1}, respectively. Mean arterial pressure was calculated according to the formula: (2 diastolic pressure + systolic pressure)/3. Increases or decreases of mean blood pressure and heart rate values are expressed in mm Hg or as a percentage with respect to resting values of each animal.

The response is expressed in mg for agents that elicit constriction of aorta rings. The response is expressed as the percentage reduction of tension in the preconstricted state for agents that elicit relaxation of isolated aorta rings preconstricted by a drug. In several cases, the 50% effective concentration (EC_{50}) was also calculated from the cumulative dose response curves.

3. Results

3.1. Arterial blood pressure studies in anaesthetized rat

Resting values for mean arterial pressure and heart rate were 87 ± 3.0 mm Hg ($n = 20$) and 357 ± 10.8 beats min^{-1} in WKY rats, and 94 ± 3.8 mm Hg ($n = 20$) and 317 ± 20.1 beats min^{-1} in SHR. The differences observed between both urethane-anaesthetized SHR and WKY rats were not found to be significant. The values of mean arterial pressure and heart rate were stable in the absence of treatment. Moreover, the control vehicle injections produced no changes in the resting values.

The administration of N^G-nitro-L-arginine (L-NNA; 5 mg kg^{-1}; i.v.) produced a gradual sustained increase in mean arterial pressure. The maximum increase was achieved about 20 min after the administration of L-NNA, and was significantly greater ($P < 0.01$) in SHR than in WKY rats (Fig. 1). The pulse pressure did not change in

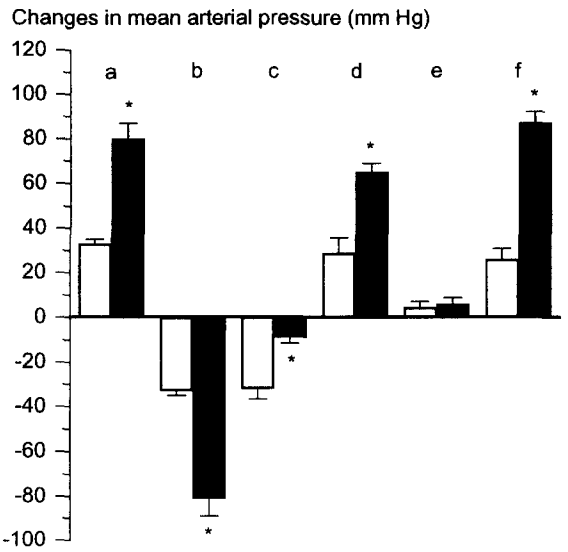

Fig. 1. Changes in mean arterial pressure induced by (a) N^G-nitro-L-arginine (L-NNA; 5 mg kg^{-1}, i.v.), (b) L-arginine (100 mg kg^{-1}, i.v.), after the hypertensive effect of L-NNA was stabilized, (c) hexamethonium (5 mg kg^{-1}, i.v.), (d) L-NNA, after the hypotensive effect of hexamethonium, (e) indomethacin (5 mg kg^{-1}, i.v.) and (f) L-NNA, after indomethacin. The effects of these drugs were studied in normotensive Wistar-Kyoto rats (open columns) and spontaneously hypertensive rats (closed columns). Each column is the mean of 5 experiments with S.E.M. shown by vertical bars. * $P < 0.05$ versus WKY rats with the same treatment.

either of the strains. Expressed as a percentage, the increases observed were $36 \pm 2.2\%$ ($n = 5$) in WKY rats and $87 \pm 12.7\%$ ($n = 5$) in SHR. The increases in mean arterial pressure were accompanied by slight but non-significant increases in heart rate in both strains. After the hypertensive effect of L-NNA was stabilized, the administration of L-arginine (100 mg kg^{-1}; i.v.) produced a slow and sustained fall in mean arterial pressure until the resting values in both strains (Fig. 1).

Hexamethonium (5 mg kg^{-1}, i.v.) caused a decrease in the resting mean arterial pressure, significantly lower in SHR than in WKY rats ($P < 0.01$). This decrease was maintained for the remainder of the experiment. The previous administration of hexamethonium did not modify the hypertensive effects of L-NNA (5 mg kg^{-1}, i.v.) in either of the strains ($P > 0.05$; Fig. 1). After hexamethonium, the L-NNA-induced increase in mean arterial pressure was $45 \pm 5.6\%$ ($n = 5$) in WKY rats and $85 \pm 12.7\%$ ($n = 5$) in SHR.

The injection of indomethacin (5 mg kg^{-1}, i.v.), did not significantly modify the mean arterial pressure in both WKY rats and SHR. In addition, the cyclooxygenase inhibitor did not significantly alter ($P > 0.05$) the increase of mean arterial pressure induced by L-NNA (5 mg kg^{-1}, i.v.), which was $40 \pm 7.9\%$ ($n = 5$) in WKY rats and $83 \pm 4.3\%$ ($n = 5$) in SHR (Fig. 1).

3.2. Contraction studies in rat isolated thoracic aorta rings

In aortic rings with intact endothelium of WKY rats, the accumulative administration of L-NNA (0.1 μM to 0.1 mM) produced a very small contraction, of which the maximum tension (E_{max}) was 52 ± 8 mg ($n = 5$). In the same rings, the KCl (25 mM) caused a contractile response in WKY rats (1177 ± 261 mg, $n = 13$) and in SHR (585 ± 130 mg, $n = 12$). After the 25 mM KCl precontraction, the administration of L-NNA (0.1 μM to 0.1 mM) induced a strong slow-onset dose-dependent contraction in both strains (Fig. 2). The E_{max} was significantly greater in normotensive (327%) than in hypertensive rats ($P < 0.01$; Fig. 3). Expressed as percentage of the reference constrictor response to KCl (25 mM) obtained in each ring, the E_{max} evoked by L-NNA was also greater ($P < 0.05$) in WKY rats ($285 \pm 36\%$, $n = 7$) than in SHR ($175 \pm 25\%$, $n = 6$). In intact rings of WKY rats, 12 mM KCl induced a contractile response ($E_{max} = 543 \pm 81$ mg, $n = 12$) similar to that induced by 25 mM KCl in intact rings of SHR;

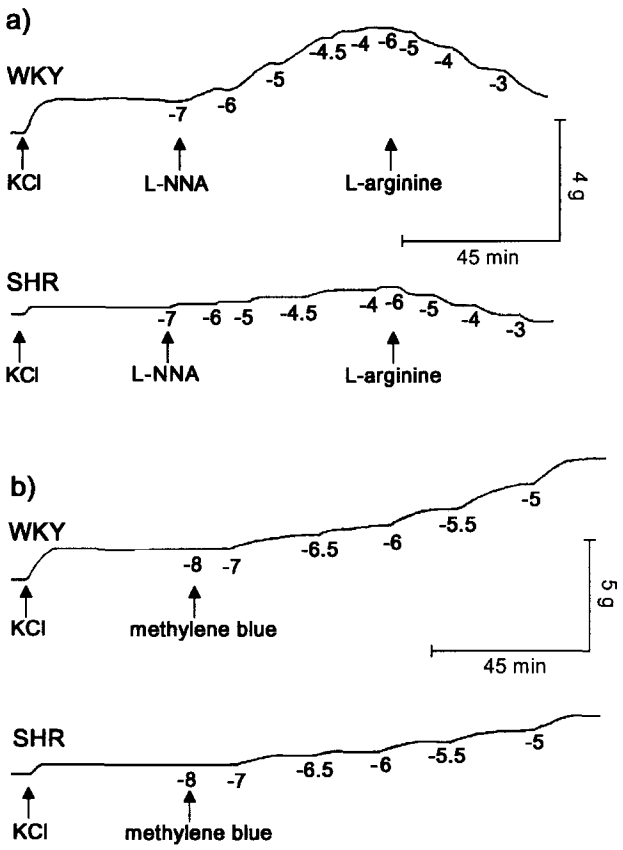

Fig. 2. Rat aorta rings with intact endothelium taken from normotensive Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR): typical effects of (a) N^G-nitro-L-arginine (L-NNA) and (b) methylene blue on submaximally KCL (25 mM) precontracted rings. After constrictor responses to L-NNA had stabilized, aortic rings were exposed to L-arginine. The different contractions are expresed as logarithms to base 10 of final bath concentration.

after 12 mM KCl precontraction, L-NNA-induced dose-dependent contractions (E_{max} = 3250 ± 316 mg, n = 6; EC_{50} = 7.0 ± 0.84 μM) reached a greater E_{max} ($P < 0.01$) than in 25 mM KCl precontrated intact rings of SHR. On the other hand, in intact rings of SHR, 33 mM KCl induced a contractile response (1230 ± 245 mg, n = 12) similar to that induced by 25 mM KCl in intact rings of WKY rats; after this 33 mM KCl precontraction, L-NNA-induced dose-dependent contractions (E_{max} = 1132 ± 187 mg, n = 6; EC_{50} = 7.7 ± 0.73 μM) reached a smaller E_{max} ($P < 0.01$) than in 25 mM KCl-precontracted intact rings of WKY rats. In presence of 25 mM KCl precontraction, and after obtaining a stable E_{max} with L-NNA, the accumulative administration of L-arginine (1 μM to 1 mM), but not D-arginine, completely inhibited the L-NNA effects in endothelium-containing aortic rings of normotensive and hypertensive rats. The EC_{50} were 28 ± 2.1 μM (n = 4) in WKY rats and 77 ± 12.3 μM (n = 4) in SHR. L-Arginine had no effect on the preparations treated only with KCl (25 mM).

The exposure to increasing concentrations of methylene blue (10 nM to 10 μM) caused a supplementary strong dose-dependent contraction in both rat strains in similar 25 mM KCl-pretreated intact rings (Fig. 2). The E_{max} was significantly greater in WKY rats (252%) than in SHR (Fig. 3). In terms of percentage of the reference contrictor response to KCl (25 mM) obtained in each ring, the E_{max} evoked by methylene blue was also greater ($P < 0.05$) in WKY rats (352 ± 30%, n = 6) than in SHR (255 ± 22%, n = 6). In intact rings of WKY rats, after 12 mM KCl precontraction, methylene blue-induced dose-dependent contractions (E_{max} = 3580 ± 410 mg, n = 6; EC_{50} = 1.8 ± 0.22 μM) reached a greater E_{max} ($P < 0.01$) than in 25 mM KCl-precontrated intact rings of SHR. Conversely, in intact rings of SHR, after 33 mM KCl precontraction, methylene blue-induced dose-dependent contractions (E_{max} = 1461 ± 194 mg, n = 6; EC_{50} = 1.9 ± 0.15 μM) reached a smaller E_{max} ($P < 0.01$) than in 25 mM KCl-precontracted intact rings of WKY rats.

In the presence of indomethacin (5 μM) and after preconstriction with KCl (25 mM), the maximum L-NNA-induced contraction in intact rings of WKY rats was smaller than in the absence of cyclooxygenase inhibitor, 46 ± 6.1% (n = 5; $P < 0.05$). In the same preparations and conditions, methylene blue-induced contractions were also inhibited by the cyclooxygenase inhibitor, 44 ± 7.2% (n = 5; $P < 0.05$): the inhibition was not significantly different ($P > 0.05$) to that obtained with L-NNA.

Phenylephrine (1 μM) elicited contractions in endothelium intact aorta rings in both WKY rats (E_{max} = 2305 ± 151.3 mg, n = 27) and SHR (E_{max} = 1327 ± 206.4 mg, n = 12). Once E_{max} had been reached, the addition of acetylcholine (1 μM) produced a rapid and pronounced decrease in tension (73 ± 2.1%, n = 27, in WKY rats and 64 ± 4.8%, n = 6, in SHR rats; $P < 0.05$). However, if endothelium intact aorta rings were preincubated with L-NNA (0.1 mM) or methylene blue (10 μM), the endothelium-dependent relaxation was eliminated.

In rubbed rings, the KCl (25 mM) evoked a contractile response in both WKY rats (2663 ± 363 mg, n = 12) and SHR (1663 ± 278 mg, n = 11). After KCl precontraction, the addition of L-NNA (0.1 μM to 0.1 mM) evoked

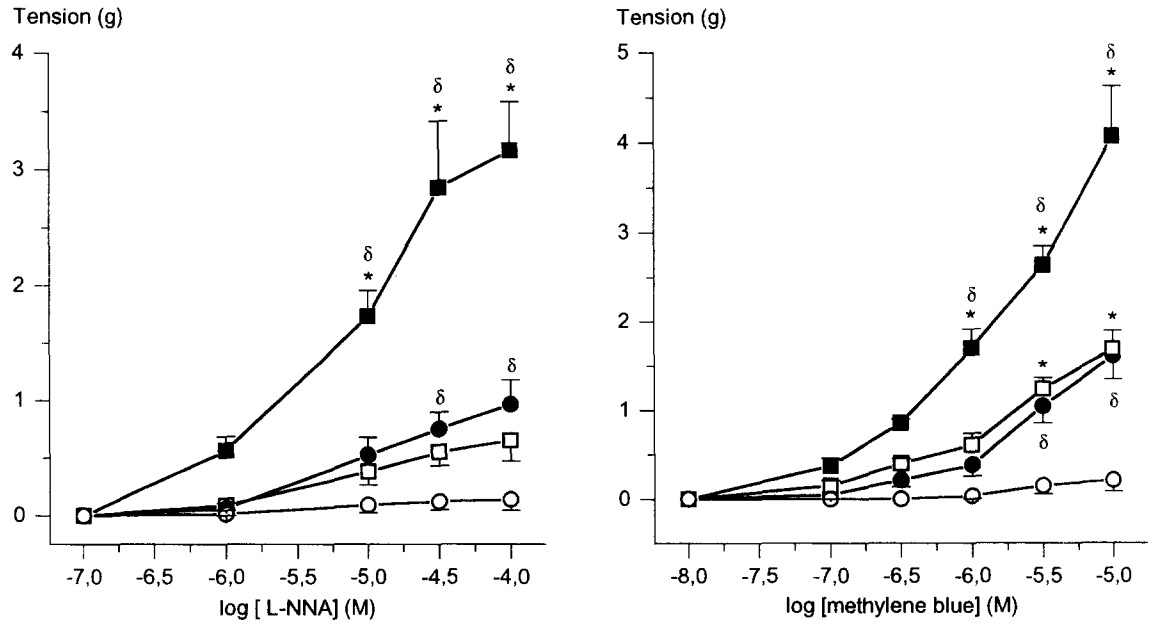

Fig. 3. Concentration-response curves of N^G-nitro-L-arginine (L-NNA) and methylene blue in intact (closed symbols) and rubbed (open symbols) rings of thoracic aorta taken from normotensive Wistar-Kyoto (WKY) rats (squares) and from spontaneously hypertensive rats (SHR; circles). Each point represents the mean response of at least 5 aorta rings; S.E.M. is represented by vertical lines. * $P < 0.05$ for WKY rats versus corresponding SHR rings; $^{\delta}$ $P < 0.05$ for intact versus corresponding rubbed rings.

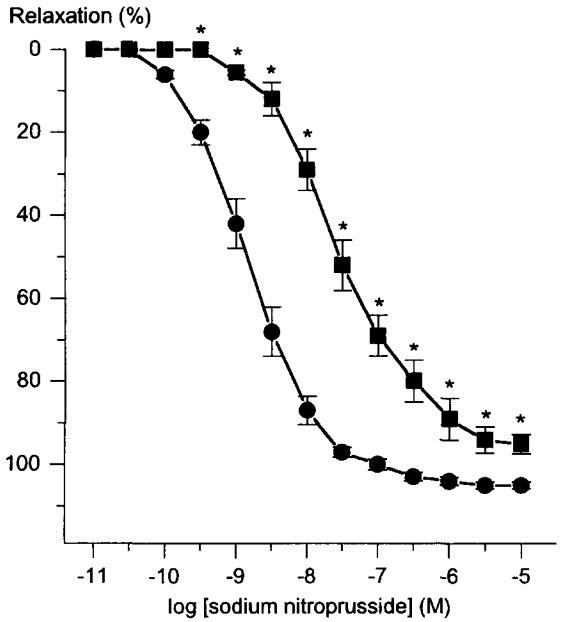

Fig. 4. Concentration-response curves showing the relaxation induced by sodium nitroprusside in rubbed rings taken from normotensive Wistar-Kyoto rats expressed as the percentage reduction of tension in the preconstricted state. Circles, relaxation control response to sodium nitroprusside when it was added to rings that had only been contracted with 25 mM KCl. Squares, relaxation response to sodium nitroprusside after submaximal tone induced with 25 mM KCl and methylene blue (10 nM to 10 μM) concentration-response curves were stabilized. Each point represents the mean response of 5 aorta rings; S.E.M. is represented by vertical lines. * $P < 0.05$ compared with control.

dose-dependent contractions in both strains, although the effect in SHR was of little importance (Fig. 3) and several preparations had no response. The E_{max} evoked by L-NNA was significantly weaker than in intact rings, 79% in normotensive rats and 85% in SHR. In these rubbed rings, L-arginine (1 μM to 1 mM) produced a relaxing effect in WKY rats which averaged 62 ± 16.9% ($n = 6$) at 1 mM although two rings had no relaxing response; in SHR, the relaxing effect of L-arginine was not important and erratic. L-Arginine had no effect in the preparations treated only with KCl (25 mM). The administration of methylene blue (10 nM to 10 μM) to KCl-precontracted rubbed rings also caused a contraction, which was small and erratic in hypertensive rats but important in WKY rats (Fig. 3). In both strains the E_{max} was smaller than in intact rings, 58% in normotensive rats and 87% in SHR. After the contractile response of methylene blue was stabilized in rubbed rings of WKY rats, the administration of sodium nitroprusside (10 pM to 1 μM) induced a completely dose-dependent relaxation ($EC_{50} = 31.5 \pm 3.81$ nM, $n = 5$; Fig. 4). However, the sodium nitroprusside-induced relaxation was significantly more potent ($P < 0.01$) when it was added to rings that had only been contracted with KCl (25 mM; $EC_{50} = 1.01 \pm 0.15$ nM, $n = 5$; Fig. 4).

In endothelium-denuded rings of WKY rats, incubation with the protein-synthesis inhibitors cycloheximide (10 μM) and puromycin (100 μM), resulted in a complete inhibition of contraction to L-NNA and methylene blue after preconstriction with KCl (25 mM). However, the contraction induced by phenylephrine (1 μM) in resting rings was only inhibited 13 ± 2.1% ($n = 5$) and 8 ± 2.3% ($n = 4$) by pretreatment with cycloheximide and puromycin, respectively.

4. Discussion

In our study, the systemic administration of N^G-nitro-L-arginine (L-NNA), a NO synthase inhibitor, in accordance with Lacolley et al. (1991a), produced a markedly exaggerated hypertensive response in urethane-anaesthetised SHR compared to age-matched WKY rats. The response of L-NNA can be specifically attributed to the inhibition of NO because L-arginine completely restored the observed hypertensive effect in both strains. If it is borne in mind that the resting values of mean arterial pressure in WKY rats and SHR are quite similar when they are specifically anaesthetized with urethane (see also other authors, for example, Lacolley et al., 1991a), a different vascular tone in both strains is unlikely to be the responsible for the differences as was suggested by Vargas et al. (1990). In our experiments, L-NNA did not significantly change the heart activity and therefore, the total vasodilator resting NO tone may be particularly enhanced in the SHR. This conclusion is in agreement with similar results obtained by Lacolley et al. (1991a), although it disagrees with the results of Schleiffer et al. (1991) and Fozard and Part (1991). Our results, moreover, are in accordance with the fact that the cGMP pathway seems to be activated more in SHR than in WKY rats (Mourlon-Le Grand et al., 1993).

The origin of the overproduction of NO in SHR is unknown. It has been described that the sympathetic nervous system plays an important role in modulating the synthesis or release of vascular NO (Lacolley et al., 1991b). Nevertheless, a major role for the autonomic nervous system can be ruled out since the haemodynamic changes induced by L-NNA in the anaesthetised animals were broadly similar to those seen in anaesthetized hexamethonium ganglion-blocked animals. In the presence of an increased release of cyclooxygenase-dependent contracting factors, as occurs in hypertension (Lüscher, 1993), NO synthase inhibitors could produce an exaggerated hypertensive effect with a normal release of NO. However, in our experiments, the L-NNA hypertensive effect was not inhibited by indomethacin in WKY rats or SHR, suggesting that cyclooxygenase-constricting products are not responsible for the exaggerated effect of L-NNA in SHR. In accordance with our results, Ruiz et al. (1994) found that indomethacin does not modify the pressor response to N^G-nitro-L-arginine methyl ester in awake SHR.

Another possible explanation for the exaggerate vasodilator NO tone in SHR is an intrinsic, independent of in vivo stimulus, overproduction of NO by vascular cells. In our in vitro study, L-NNA produced only a very weak contraction in the quiescent aortic rings of WKY rats. These data suggest that NO may not be importantly spontaneously released under the in vitro basal conditions. Nevertheless, another interpretation is that in vitro quiescent conditions vasodilatation is favoured and that the basal release of NO is not necessary to achieve a complete relaxation. In fact, in this study, quiescent rubbed rings were very weakly relaxed with sodium nitroprusside (data not shown). Therefore, to observe a possible resting vasodilator NO tone in aortic rings, it would be necessary to prestimulate to counteract the intrinsic vasodilatation; thus, basal NO could have already participated in the vasodilator final tone. To prestimulate aorta rings we precontracted with KCl 25 mM. It is unlikely that KCl has a direct effect on the NO release, as proved by Collins et al. (1988), who showed that 120 mM potassium did not elevate the cGMP in rat aorta. Moreover, increased potassium concentration did not elevate intracellular calcium ion concentration in endothelial cells (Colden-Stanfield et al., 1987) which is considered to be a prerequisite for the release of NO from vascular tissue. KCl (25 mM) also inactivates endothelial-derived hyperpolarizing factor (EDHF)-related mechanisms (Adeagbo and Triggle, 1993). Therefore, we were able to work without the complications arising from the EDHF interference. Following the preconstriction of intact aortic rings with KCl (25 mM) L-NNA caused marked vasoconstriction in both aortic rings of SHR and WKY rats, indicating that the continuous basal release of NO represents, also in in vitro conditions, an important factor regulating the vascular tone. Although it cannot be assumed that the vasoconstrictor effects of the L-arginine analogues are exclusively due to the inhibition of NO synthesis (Peterson et al., 1992; Wang and Pang, 1994), the vasoconstriction does not seem to be a non-specific effect of the L-NNA because L-arginine, but not D-arginine, restored the observed vasoconstriction.

Methylene blue is thought to prevent the activation of soluble guanylate cyclase (Martin et al., 1985). It has been employed widely in studies using isolated arteries to specify the role of NO in mediating vascular responses (see for example Martin et al., 1985 and Hofman et al., 1992). Probably only NO stimulates soluble guanylate cyclase in our in vitro experimental conditions and therefore, the magnitude of this basal release can be determined indirectly by measuring the degree of contraction induced by methylene blue. Unfortunately, the effects of methylene blue are not only specific to guanylate cyclase inhibition but include the inhibition of NO synthesis (Mayer et al., 1993a), the generation of superoxide anion (Marczin et al., 1992; Mayer et al., 1993a) or a possible prostacyclin inhibition (Martin et al., 1989). In our study in intact rings of WKY rats, indomethacin inhibited the L-NNA- and methylene blue-induced contraction to a similar extent, suggesting that methylene blue effects are not due specifically to a prostacyclin inhibition and other proposed effects, all of them due to the inhibition of the L-arginine-NO-cGMP pathway, could be responsible. Moreover, methylene blue probably was specific in the L-arginine-NO-cGMP pathway because it abolished the relaxation induced by acetylcholine and had an inhibitory effect on the relaxation induced by sodium nitroprusside in our experiments but had no effect on those induced by isoproterenol and atrial natriuretic factor (Clozel, 1991). Our data with methylene blue in intact aortic rings precontracted with KCl 25 mM, showing a marked vasoconstriction in both aortic rings of SHR and WKY rats, also indicates, in agreement with the L-NNA data, that the continuous basal release of NO is an important factor regulating the vascular tone in isolated aortic rings.

An important finding in our work was the progressive rise in tension evoked by L-NNA and methylene blue being more important in the WKY rat aorta than in SHR. The impaired contractile response to L-NNA and methylene blue in aortic rings from SHR does not seem to be a reflection of a decreased smooth muscle contractile function, because in the standardized responses (expressed as a percentage of the reference constrictor response to 25 mM KCl obtained in each ring), the effects of L-NNA and methylene blue are also greater in WKY rats. Moreover, the decrease in smooth muscle contraction to L-NNA and methylene blue in SHR does not seem to be caused by previous differences in the smooth muscle tone, because the effects of L-NNA and methylene blue in intact rings of WKY rats preconstricted with KCl (12 mM; in order to achieve the same relatively lower level of precontraction as in intact rings of SHR), are greater than in SHR. Conversely the effects of L-NNA and methylene blue in intact rings of SHR preconstricted with a high concentraction of KCl (33 mM; in order to achieve the same relatively higher precontraction as in intact rings of WKY rats), are also weaker than in WKY rats. Particularly, the methylene blue results ruled out that the L-NNA effects were caused by a hypothetical different sensitivity to L-NNA or by a different availability of L-arginine between the two strains. Together these results suggest that the release of basal NO in rat aorta is less important in arteries in SHR compared to WKY rats, and may discard an overproduction of NO in SHR vascular cells as responsible for the in vivo exaggerated effect of L-NNA in SHR. This happens in spite the fact that arterial beds of hypertensive animals have an increased wall-to-lumen ratio (Folkow, 1990) and an enhanced release of endothelium-derived contracting factors (Lüscher, 1993) in aortic rings of SHR. Our conclusion agrees with other results on resistance and coronary arteries (Dohi et al., 1990; Pourageaud and Freslon, 1992) but not with the results of Adeagbo et al. (1994) and Randall et al. (1991), who noted, working with mesenteric vascular beds, that the release of NO is not impaired

in hypertension. Furthermore, other in vivo studies (Li and Joshua, 1993), also using a NO synthase inhibitor as a probe for vascular basal NO production, indicate that an overproduction of NO by the vascular cells is not the cause of the enhanced total basal release of NO in SHR.

In in vitro conditions, the origin of NO in rat aorta of both strains is probably endothelial to a great extent because the mechanical removal of endothelium strongly inhibited the L-NNA and methylene blue concentration-dependent contractions. Nevertheless, our results also show that in the denuded rings of WKY rats, L-NNA and methylene blue induced a significant contraction over the same concentration range as employed in the intact rings (10 nM to 10 μM), suggesting a similar action mechanism of the drugs in both preparations. The L-NNA contraction was importantly reversed by L-arginine in WKY rats, indicating that L-NNA contractions are principally due to the inhibition of NO synthesis. In any case, because the reversion was not complete in WKY rats or not important and erratic in SHR, some small contractile effects of L-NNA may not be due to the inhibition of the continuous release of NO (Wang and Pang, 1994). The most likely cells in the endothelium-denuded arterial rings which may be responsible for the generation of NO in WKY rats are the vascular smooth muscle cells. Wood et al. (1990) and Charpie and Webb (1993) support this argument by showing that NO is released by smooth muscle cells. L-NNA- and methylene blue-induced contractions in rubbed rings were abolished by cycloheximide and puromycin, suggesting that an inducible smooth muscle NO synthase may be responsible for the basal NO production in smooth muscle cells in in vitro conditions. The cycloheximide and puromycin effects seem to be specific because these drugs only weakly inhibit contractions induced by phenylephrine. The experiments with denuded rings also show that the magnitude of contraction induced by L-NNA and methylene blue tended to be important in denuded rings of WKY rats but very small in SHR, suggesting that the production of NO by the smooth muscle cells is not responsible for the in vivo exaggerated effect of L-NNA in SHR, and disagrees with the idea of a general activation of the NO synthesis system in SHR (Xiao and Pang, 1994).

Taken together our in vivo and in vitro results, it seems that hypertension enhances the total resting vascular NO tone in anaesthetized rat, although inhibits it in isolated rat aorta. In agreement with these aorta data, the basal release of NO also seems to be less important in the resistance and coronary arteries of SHR when compared to WKY rats (Pourageaud and Freslon, 1992), indicating a general arterial phenomenon. In this context, fluid shear stress could be considered as responsible for the differences between in vitro and in vivo studies. However, the NO flow-dependent dilation of arterioles of SHR is also significantly reduced compared with that of the normotensive arterioles (Koller and Huang, 1994). Since NO is potentially derived from multiple non-vascular sources, the exaggerated response to L-NNA in anaesthetised rats may be due to an overproduction of NO on these pools of cells. For example, an overproduction of NO in SHR macrophages has been reported by Xiao and Pang (1994).

To summarize, the present studies indicate that there is an important endothelial resting vasodilator NO tone in isolated rat aorta. Moreover, our results show that genetic hypertension reduces the resting vasodilator NO tone in rat aorta, although it enhances the total vasodilator NO tone in anaesthetized rat.

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References

Adeagbo, A.S.O. and C.R. Triggle, 1993, Varying extracellular [K^+]: a functional approach to separating EDHF-related and EDNO-related mechanisms in perfused rat mesenteric arterial bed, J. Cardiovasc. Pharmacol. 21, 423.

Adeagbo, A.S.O., R. Tabrizchi and C.R. Triggle, 1994, The effects of perfusion rate and N^G-nitro-L-arginine methyl ester on cirazoline- and KCl-induced responses in the perfused mesenteric arterial bed of rats, Br. J. Pharmacol. 111, 13.

Charpie, J.R. and R.C. Webb, 1993, Vascular myocyte-derived nitric oxide is an autocrine that limits vasoconstriction, Biochem. Biophys. Res. Commun. 194, 763.

Clozel, M., 1991, Mechanism of action of angiotensin converting enzyme inhibitors on endothelial function in hypertension, Hypertension 18, II-37.

Colden-Stanfield, M., W.P. Schilling, A.K. Ritchie, S.G. Eskin, L.T. Navarro and D.L. Kunze, 1987, Bradykinin-induced increases in cytosolic calcium and ionic currents in cultures bovine aortic endothelial cells, Circ. Res. 61, 632.

Collins, P., A.H. Henderson, D. Lang and M.J. Lewis, 1988, Endothelium-derived relaxing factor and nitroprusside compared in noradrenaline- and K^+-contracted rabbit and rat aortae, J. Physiol. (London) 400, 395.

Dohi, Y., M.A. Thiel, F.R. Buhler and T.F. Lüscher, 1990, Activation of endothelial L-arginine pathway in resistance arteries. Effect of age and hypertension, Hypertension 15, 170.

Folkow, B., 1990, 'Structural factor' in primary and secondary hypertension, Hypertension 16, 89.

Förstermann, U., E.I. Closs, J.S. Pollock, M. Nakane, P. Schwarz, I. Gath and H. Kleinert, 1994, Nitric oxide synthase isozymes – characterization, purification, molecular cloning, and functions, Hypertension 23, 1121.

Fozard, J.R. and M. Part, 1991, Haemodynamic responses to N^G-monomethyl-L-arginine in spontaneously hypertensive and normotensive Wistar-Kyoto rats, Br. J. Pharmacol. 102, 823.

Hofman, W.F., H.A. El-Kashef, J. Endrédi and I.C. Ehrhart, 1992, Effect of methylene blue on vasoreactivity in dog lung, Am. J. Physiol. 263, H587.

Ishii, K., B. Chang, J.F. Kerwin, Z.J. Huang and F. Murad, 1990, N^ω-Nitro-L-arginine: a potent inhibitor of endothelium-drived relaxing factor formation, Eur. J. Pharmacol. 176, 216.

Kobayashi, Y., K. Ikeda, E. Shinozuka, Y. Nara, Y. Yamori and K. Hattori, 1991, Comparison of vasopressor effects of nitro arginine in stroke-prone spontaneously hypertensive rats and Wistar-Kyoto rats, Clin. Exp. Pharmacol. Physiol. 18, 599.

Koller, A. and A. Huang, 1994, Impaired nitric oxide-mediated flow-induced dilation in arterioles of spontaneously hypertensive rats, Circ. Res. 74, 416.

Lacolley, P.J., S.J. Lewis and M.J. Brody, 1991a, L-N^G-Nitro arginine produces an exaggerated hypertension in anesthetized SHR, Eur. J. Pharmacol. 197, 239.

Lacolley, P.J., S.J. Lewis and M.J. Brody, 1991b, Role of sympathetic nerve activity in the generation of vascular nitric oxide in urethane-anesthetized rats, Hypertension 17, 881.

Li, F. and I.G. Joshua, 1993, Decreased arteriolar endothelium-derived relaxing factor production during the development of genetic hypertension, Clin. Exp. Hyperten. 15, 511.

Lüscher, T.F., 1993, The endothelium as a target and mediator of cardiovascular disease, Eur. J. Clin. Invest. 23, 670.

Marczin, N., U.S. Ryan and J.D. Catravas, 1992, Methylene blue inhibits nitrovasodilator- and endothelium-derived relaxing factor-induced cyclic GMP accumulation in cultured pulmonary arterial smooth muscle cells via generation of superoxide anion, J. Pharmacol. Exp. Ther. 263, 170.

Martin, W., K. Drazan and A.C. Newby, 1989, Methylene blue but not changes in cyclic GMP inhibits resting and bradykinin-stimulated production of prostacyclin by pig aortic endothelial cells, Br. J. Pharmacol. 97, 51.

Martin, W., G.M. Villani, D. Jothianandan and R.F. Furchgott, 1985, Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta, J. Pharmacol. Exp. Ther. 232, 708.

Mayer, B., F. Brunner and K. Schmidt, 1993a, Inhibition of nitric oxide synthesis by methylene blue, Biochem. Pharmacol. 45, 367.

Mayer, B., M. Schmid, P. Klatt and K. Schmidt, 1993b, Reversible inactivation of endothelial nitric oxide synthase by N(G)-nitro-L-arginine, FEBS Lett. 333, 203.

Moncada, S., R.M.J. Palmer and E.A. Higgs, 1991, Nitric oxide: physiology, pathophysiology, and pharmacology, Pharmacol. Rev. 43, 109.

Mourlon-Le Grand, M.C., J. Benessiano and B.I. Levy, 1993, Endothelium, mechanical compliance, and cGMP content in the carotid artery from spontaneously hypertensive rats, J. Cardiovasc. Pharmacol. 21 (Suppl. 1), S26.

Panza, J.A., P.R. Casino, C.M. Kilcoyne and A.A. Quyyumi, 1993, Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension, Circulation 87, 1468.

Peterson, D.A., D.C. Peterson, S. Archer and E.K. Weir, 1992, The non specificity of specific nitric oxide synthase inhibitors, Biochem. Biophys. Res. Commun. 187, 797.

Pourageaud, F. and J.L. Freslon, 1992, Endothelium function in resistance and coronary arteries of spontaneously hypertensive compared to WKY rats: effects of nitro-L-arginine, Gen. Hypertens. 218, 39.

Randall, M.D., G.R. Thomas and C.R. Hiley, 1991, Effect of destruction of the vascular endothelium upon pressure/flow relations and endothelium-dependent vasodilatation in resistance beds of spontaneously hypertensive rats, Clin. Sci. 80, 463.

Rees, D.D., R.M. Palmer, R. Schulz, H.F. Hodson and S. Moncada, 1990, Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo, Br. J. Pharmacol. 101, 746.

Ruiz, F.J., A.C. Ingles, T. Quesada, M.G. Salom and L.F. Carbonell, 1994, Indomethacin does not modify the role of nitric oxide on blood pressure regulation of SHR, Gen. Pharmacol. 25, 103.

Santiago, J.A., E.A. Garrison and P.J. Kadowitz, 1994, Comparative effects of N^{ω}-nitro-L-arginine and N^{ω}-nitro-L-arginine methyl ester on vasodilator responses to acetylcholine, bradykinin, and substance P, Eur. J. Pharmacol. 254, 207.

Schleiffer, R., F. Pernot, B. Vanoverloop and A. Gairard, 1991, In vivo involvement of endothelium-derived nitric oxide in spontaneously hypertensive rats – effects of N^G-nitro-L-arginine methyl ester, Hypertension 9, S192.

Vane, J.R., E.E. Anggard and R.M. Botting, 1990, Regulatory functions of the vascular endothelium, New Engl. J. Med. 323, 27.

Vargas, H.M., L.I. Ignarro, G. Chaudhuri and G. Chaudhuri, 1990, Physiological release of nitric oxide is dependent on the level of vascular tone, Eur. J. Pharmacol. 190, 393.

Wang, Y.X. and C.C.Y. Pang, 1994, N^G-nitro-L-arginine contracts vascular smooth muscle by an endothelium-independent mechanism, J. Cardiovasc. Pharmacol. 24, 59.

Wood, K.S., G.M. Buga, R.E. Byrns and L.J. Ignarro, 1990, Vascular smooth muscle-derived relaxing factor (MDRF) and its close similarity to nitric oxide, Biochem. Biophys. Res. Commun. 170, 80.

Xiao, J. and P.K.T. Pang, 1994, Does a general alteration in nitric oxide synthesis system occur in spontaneously hypertensive rats?, Am. J. Physiol. 266, H272.