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Vascular acetylcholine response during chronic NO synthase inhibition: in vivo versus in vitro

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Abstract

Objective: The aim of this study was to compare the response to NO-mediated vasodilators in vivo and in vitro during chronic NO synthase inhibition. **Methods:** N^G-Nitro-L-arginine-methyl ester (L-NAME, 0.4 g/l) or vehicle was administered in the drinking water for 6 weeks to male Wistar rats weighing 220–240 g. The effect of acetylcholine and sodium nitroprusside was examined in vivo, on systemic blood pressure and heart rate and in vitro, on the precontracted isolated mesenteric artery. The in vivo response to both vasodilators was examined in awake rats monitored by an indwelling catheter in the femoral artery. Isolated segments of the third-generation mesenteric artery were examined in vitro with a Mulvany dual myograph after precontraction with noradrenaline. **Results:** In isolated mesenteric arteries obtained from rats chronically treated with L-NAME, the initial relaxant response to acetylcholine was significantly decreased whereas that to sodium nitroprusside was enhanced. A late acetylcholine-induced contractile response was present and abolished by indomethacin. In vivo, the hypotensive action of sodium nitroprusside was also enhanced in the L-NAME-treated rats. Acetylcholine reduced blood pressure in the L-NAME-treated hypertensive animals more than in normotensive controls, but less than in control rats infused intravenously with noradrenaline at a dose increasing their blood pressure to hypertensive levels. **Conclusions:** The NO-mediated vasodilation induced by acetylcholine is attenuated during chronic NO synthase inhibition, both in vivo and in vitro. The blunted hypotensive response to acetylcholine can be demonstrated only if blood pressure of control rats is acutely increased to hypertensive levels.

Keywords: Nitric oxide; N^G-Nitro-L-arginine-methyl ester (L-NAME); Acetylcholine; Nitrates; Rat, arteries

1. Introduction

The production of nitric oxide (NO) by the vascular endothelium is known to play an important role in regulating the tone of arterial resistance vessels [1–10]. Through the activation of NO synthase, acetylcholine enhances the release of NO from the endothelium and induces a relaxation of the vascular smooth muscle cell [1,8,9]. In vitro, this endothelium-dependent response has been shown to be inhibited in a dose-dependent manner by NO synthase inhibitors and abolished after mechanical removal of the endothelium [1,11]. However, in vivo, NO synthase inhibition does not always decrease the hypotensive response to acetylcholine [11–14]. Thus, there seems to be a discrepancy between the in vitro and in vivo responses to acetylcholine.

The present study was designed to investigate further this apparent discrepancy by comparing the in vivo and in vitro effects of aceylcholine in rats chronically treated with the NO synthase inhibitor, N^G-nitro-L-arginine-methyl ester (L-NAME). In a first set of experiments, we assessed in normotensive Wistar rats whether chronic treatment with L-NAME alters the responsiveness of their isolated mesenteric arteries to acetylcholine, sodium nitroprusside and noradrenaline. Additional in vitro experiments were performed to explore the contribution of prostaglandins to the arterial responsiveness during NO synthase inhibition. In complementary in vivo experiments, the response to the same hypotensive agents (e.g., acetylcholine and sodium nitroprusside) was tested in intact rats also rendered hypertensive by chronic NO synthase inhibition. These results were compared with those obtained in normotensive Wistar rats and in Wistar rats exhibiting an increase in blood pressure after the acute infusion of noradrenaline.

2. Methods

2.1. In vitro studies

Normotensive male Wistar rats weighing 220–240 g (Iffa Credo, Lyon, France) had free access for 6 weeks to

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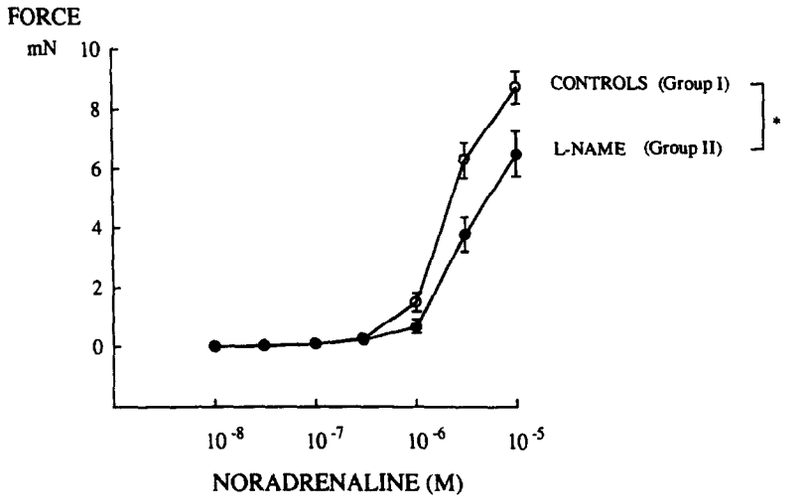

Fig. 1. Dose–response curve to noradrenaline in isolated mesenteric arteries obtained from L-NAME-treated rats and their controls. * $P < 0.05$, L-NAME vs. controls (mean ± s.e.m.).

drinking fluid containing either L-NAME (0.4 g/l of tap water, Group I, $n = 8$) or tap water (Group II, $n = 7$). L-NAME was obtained from Sigma Chemie (Buchs, Switzerland). The animals were housed in a conditioned environment, with constant temperature and humidity and regular light/dark cycles, and they were fed a regular sodium diet (38 mg/day NaCl). At the end of the treatment period, all animals had their right femoral artery

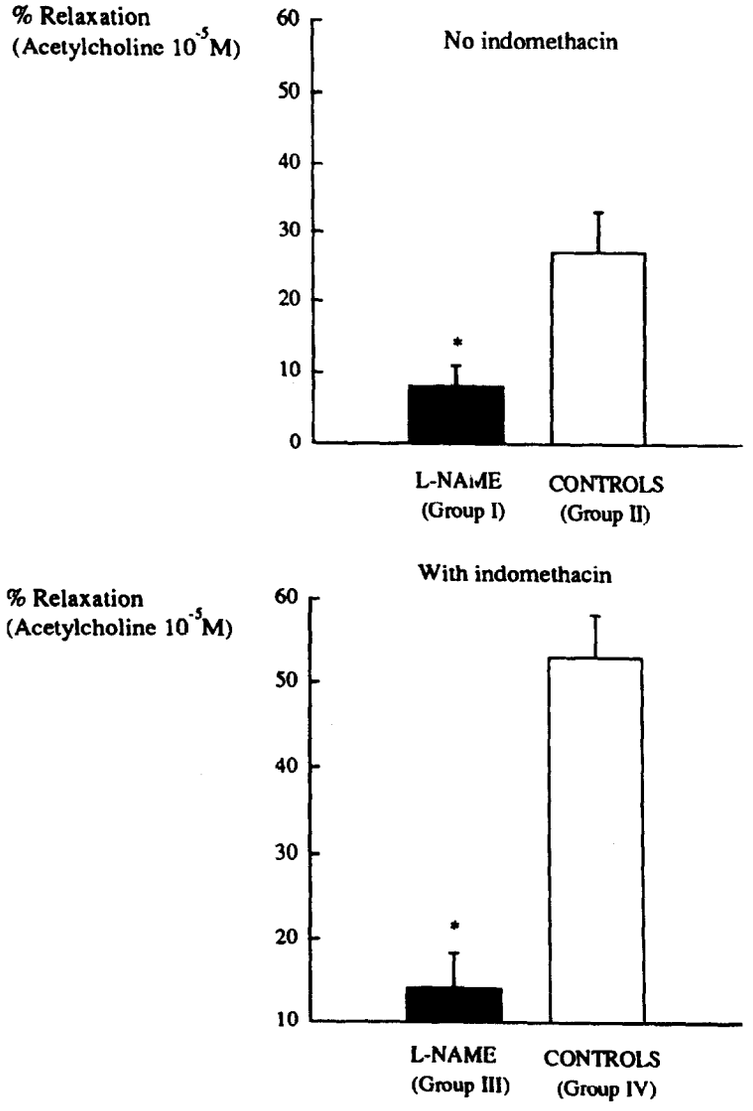

Fig. 2. Percent relaxation induced by acetylcholine (10^{-5} M) in isolated mesenteric arteries precontracted with 10^{-5} M noradrenaline, obtained from L-NAME-treated rats and their controls and pretreated or not with indomethacin. * $P < 0.05$, L-NAME vs. controls (mean ± s.e.m.).

Table 1
Characteristics of the study groups examined in vitro (mean ± s.e.m.)

	n	BW (mg)	MAP (mmHg)	HR (bpm)	L-NAME (in vivo)	Indomethacin (in vitro)
I	8	378 ± 13.9 *	192 ± 3.2 *	462 ± 19	+	−
II	7	414 ± 6.5	126 ± 3.5	444 ± 7	−	−
III	7	344 ± 9.5 *	193 ± 7.0 *	498 ± 15 *	+	+
IV	8	405 ± 13.6	130 ± 14	391 ± 14	−	+

BW = body weight; MAP = mean intra-arterial pressure; HR = heart rate; + = procedure done; − = procedure not done. * $P < 0.05$ versus corresponding controls.

Table 2
Characteristics of the study groups examined in vivo (mean ± s.e.m.)

	n	BW (mg)	MAP (mmHg)	HR (bpm)
Control	9	403 ± 6	134 ± 2	369 ± 7
L-NAME	7	395 ± 9	181 ± 5 *†	406 ± 18 †
Noradrenaline	7	407 ± 3	160 ± 3 *	329 ± 16

BW = body weight; MAP = mean intra-arterial pressure; HR = heart rate. * $P < 0.05$ versus controls. † $P < 0.05$ versus noradrenaline.

instrumented with a catheter (PE50) containing a heparinized 0.9% NaCl solution. This was done under halothane anesthesia the day before hemodynamic measurements were performed. The animals were then returned to their cage.

On the study day, they were placed in a plastic tube for partial restriction of their movements. Intra-arterial pressure and heart rate were monitored after 1 h rest using a computerized data acquisition system [15]. The animals

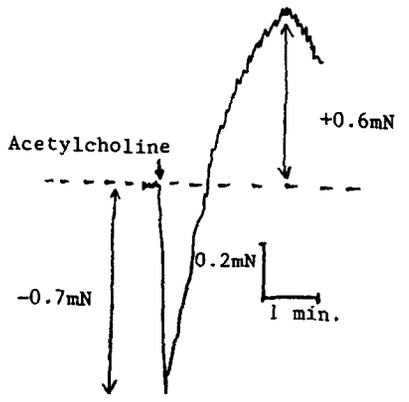

Fig. 4. Biphasic response induced by acetylcholine (10^{-5} M) in an isolated mesenteric artery precontracted with 10^{-5} M noradrenaline. This artery was obtained from a L-NAME-treated rat. In this example, baseline was at 6.8 mN. Acetylcholine induced a relaxation (−0.7 mN from baseline) followed by a contraction (+0.6 mN from baseline).

were then anesthetized with halothane and a 2 mm length segment of the third-generation mesenteric artery (100–150 μm diameter) was dissected and suspended on two tungsten wires (25 μm diameter) and mounted on stainless steel gains in a Mulvany dual myograph [16]. The bath medium surrounding the arteries consisted of an oxygenated, warmed (37°C) physiological salt solution of the following composition (mM): NaCl 119, KCL 4.7, KH_2PO_4 1.17, $NaHCO_3$ 25, $MgSO_4$ 1.17, $CaCl_2$ 2.5, glucose 5.5. After having been stretched in steps under micrometer control, each artery was set at an internal circumference of $0.9 \times L_{100}$ where L_{100} is the internal

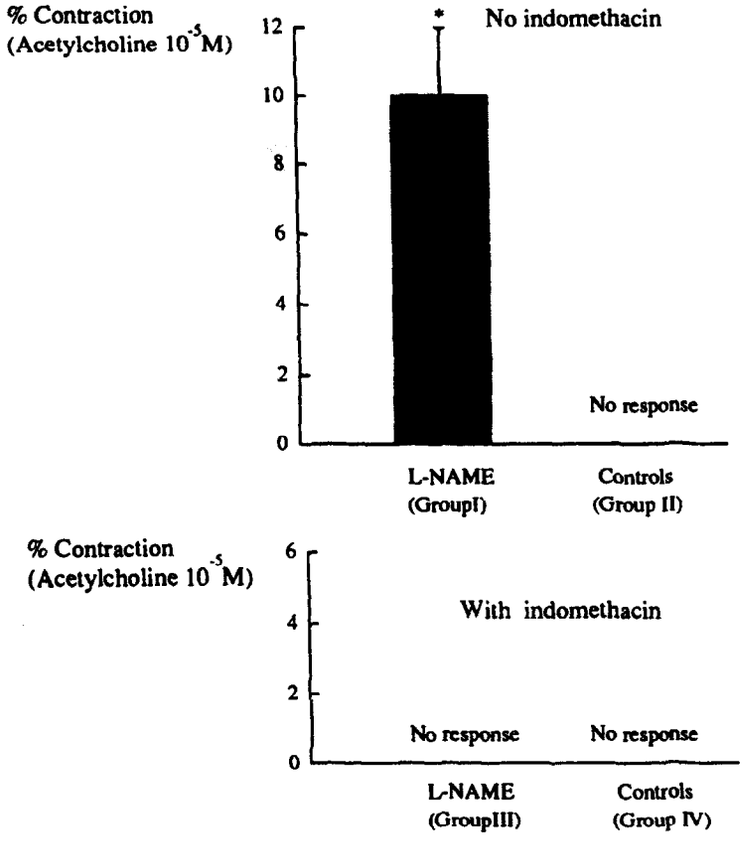

Fig. 3. Percent contraction induced by acetylcholine (10^{-5} M) in isolated mesenteric arteries precontracted with 10^{-5} M noradrenaline, obtained from L-NAME-treated rats and their controls and pretreated or not with indomethacin. * $P < 0.05$, L-NAME vs. controls (mean ± s.e.m.).

circumference the artery would have had in vivo when relaxed and under a transmural pressure of 100 mmHg [16]. The starting tension of each artery was the same and the active tension induced by noradrenaline was expressed as a function of the starting tension. After 1 h rest in the drug-free physiological salt solution, the arteries were challenged with increasing doses of noradrenaline (10^{-8}–10^{-5} M, Sigma). Once the contraction with noradrenaline 10^{-5} M was achieved, acetylcholine 10^{-5} M (Sigma) was introduced into the bath. The peak relaxation and/or contraction was calculated according to the maximal contraction with noradrenaline. The same experiment was performed with sodium nitroprusside 10^{-5} M (Hofmann-La Roche, Basel, Switzerland) instead of acetylcholine after a wash-out period of 1 h.

Identical segments of the mesenteric arteries were examined from other rats treated either with L-NAME (Group III, $n = 7$) or tap water (Group IV, $n = 8$). In order to test the influence of prostaglandins on the response to the vasoactive agents, the same experiments were performed after introduction of the cyclo-oxygenase inhibitor indomethacin at 10^{-5} M (Merck Sharp, Dohme-Chibret, Glattbrugg, Switzerland) into the physiological salt solution.

A separate experiment was performed in order to study the ratio *% acetylcholine-induced relaxation / % sodium nitroprusside-induced relaxation* as a marker of NO synthase inhibition. As acute NO synthase inhibition decreases acetylcholine-induced relaxation and does not modify or increases sodium-nitropruside-induced relaxation, it can be assumed that this ratio decreases during NO synthase inhibition. Four segments of the third-generation mesenteric artery obtained from control rats were mounted in the Mulvany myograph as described above. The relaxant response to acetylcholine 10^{-5} M and to sodium nitroprusside 10^{-5} M was tested on the arteries pretreated with indomethacin 10^{-5} M and precontracted with noradrenaline 10^{-5} M. After a wash-out period of 1 h, the same experiments were repeated with L-NAME 10^{-6} M in the bath medium. Indomethacin and L-NAME were introduced into the bath medium 5 min before noradrenaline. The relaxation induced by acetylcholine and sodium nitroprusside was expressed as the percent decrease from the contraction induced by noradrenaline 10^{-5} M. The ratio described above was then calculated twice for each artery: with and without L-NAME in the bath medium.

2.2. *In vivo studies*

Normotensive male Wistar rats weighing 220–240 g were treated for 6 weeks with L-NAME (0.4 g/l of drinking water, $n = 7$) or tap water ($n = 16$). At the end of

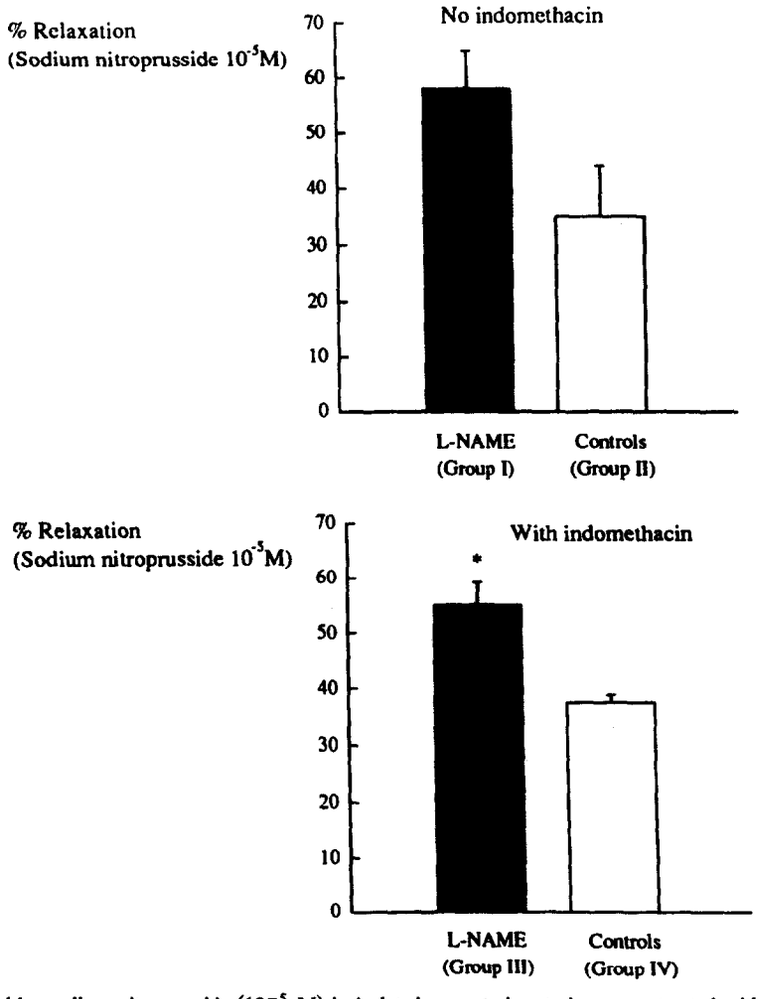

Fig. 5. Percent relaxation induced by sodium nitroprusside (10^{-5} M) in isolated mesenteric arteries precontracted with 10^{-5} M noradrenaline, obtained from L-NAME-treated rats and their controls and pretreated or not with indomethacin. * $P < 0.05$, L-NAME vs. controls (mean ± s.e.m.).

the treatment period, all animals were instrumented with a catheter (PE50) into the right femoral artery and vein, respectively. This was done under halothane anesthesia the day before the hemodynamic measurements were performed. On the study day, the animals were placed in a plastic tube for partial restriction of their movements. Intra-arterial pressure and heart rate were monitored after a 1 h rest. In 7 of the rats receiving tap water, noradrenaline was infused intravenously for 15 min at a rate of 500 ng/min (5 μl/min) in order to increase the baseline blood pressure by approximately 30 mmHg. It was felt unethical to increase acutely the blood pressure in these conscious rats to the levels similar to those found after chronic NO synthase inhibition.

In all rats, sodium nitroprusside was then infused via the intravenous catheter at increasing doses (1.25, 2.5, 5 μg/min). Each dose was given during a period of 5 min. The same procedure was done after 1 h rest with increasing doses of acetylcholine (3.75, 7.5, 15 μg/min). Both test agents were diluted in 0.9% NaCl (sodium nitroprusside, 1.25 μg/10 μl; acetylcholine, 3.75 μg/10 μl).

2.3. Statistics

The dose–response curves to norepinephrine in vitro, acetylcholine and sodium nitroprusside in vivo were analysed by an analysis of variance for repeated measures. When two sets of values were compared as with the values of blood pressure, body weight, heart rate and relaxation to acetylcholine or sodium nitroprusside in vitro, Student's unpaired t-test was used A value of $P < 0.05$ was considered significant. Data are reported as mean ± s.e.m.

3. Results

3.1. In vitro studies

Body weight (BW), mean arterial blood pressure (MAP) and heart rate (HR) of treatment Groups I to IV are shown in Table 1. L-NAME-treated rats gained less weight than the controls. L-NAME induced a marked increase in MAP and a slight rise in heart rate when the groups with similar treatments in vivo were grouped together (I + III 481 ± 13 bpm, $n = 15$ vs. II + IV 412 ± 12 bpm, $n = 15$; $P < 0.001$).

In vitro, mesenteric arteries obtained from the L-NAME-treated rats contracted less in response to noradrenaline than those obtained from controls (Fig. 1). The acetylcholine-induced relaxation was significantly attenuated in the precontracted arteries obtained from the L-NAME-treated rats (Fig. 2). With indomethacin, the relaxation tended to be enhanced in both groups.

A contractile response to acetylcholine was only observed in the arteries obtained from the L-NAME-treated rats (Fig. 3). This was preceded by a small vasodilatory

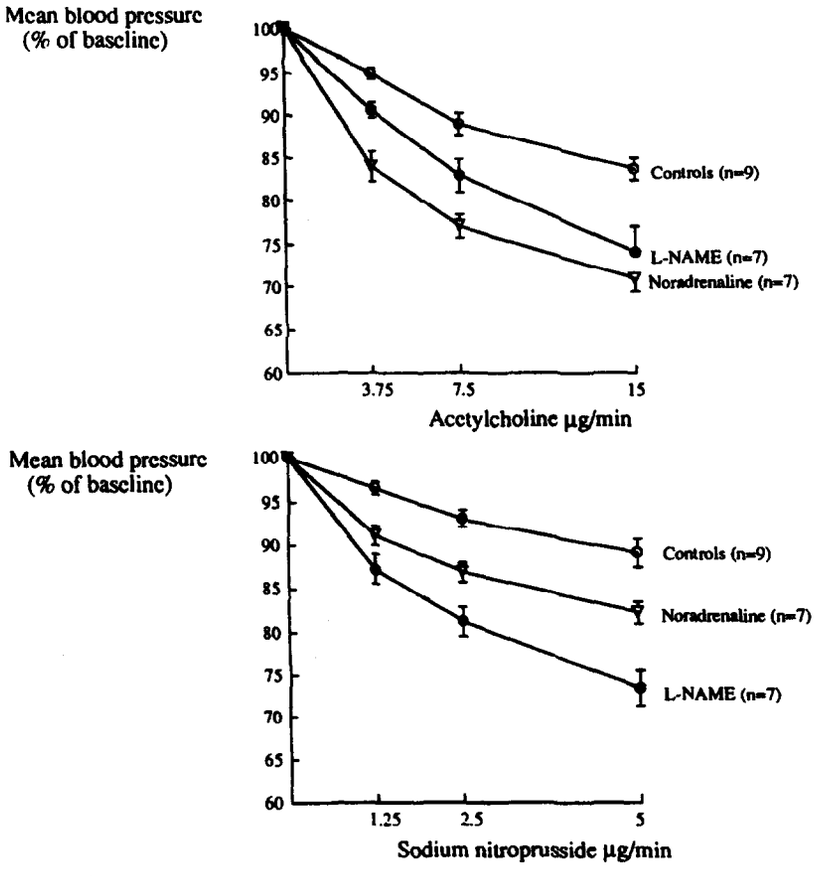

Fig. 6. Changes in mean blood pressures, expressed in % of baseline, induced in concious L-NAME, control and noradrenaline-infused rats by increasing doses of acetylcholine and sodium nitroprusside (mean ± s.e.m.).

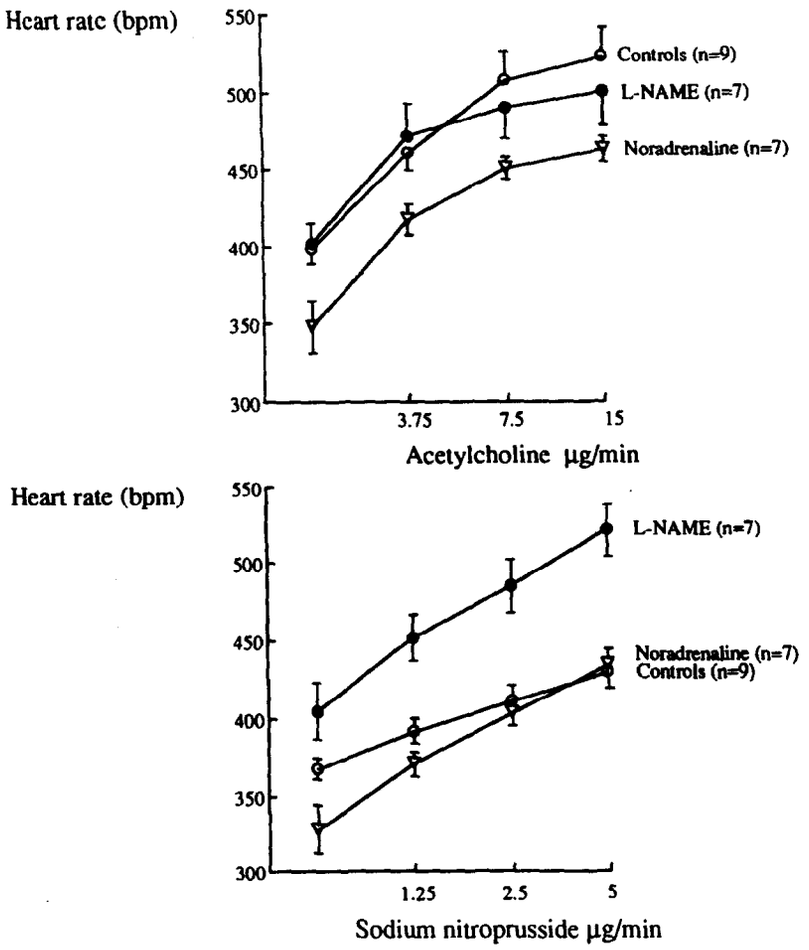

Fig. 7. Effect of increasing doses of acetylcholine (upper panel) and sodium nitroprusside (lower panel) on heart rate of L-NAME, control and noradrenaline-infused rats (mean ± s.e.m.).

response (Fig. 4). The contractile response was no longer present with indomethacin, suggesting a prostaglandin-mediated response (Fig. 3).

The relaxant response to sodium nitroprusside was enhanced in the arteries obtained from the L-NAME-treated rats and indomethacin did not change the response (Fig. 5).

When mesenteric arteries obtained from control rats are exposed to L-NAME 10^{-6} M in vitro, the *% acetylcholine-induced relaxation / % sodium nitroprusside-induced relaxation* ratio was significantly decreased compared to the values obtained with the same arteries held in the bath without L-NAME (L-NAME vs. controls, 0.49 ± 0.07 vs. 0.96 ± 0.14, $P < 0.05$). When the ratio was compared between the arteries obtained from the L-NAME-treated rats (L-NAME only given in vivo) and control rats, the difference was highly significant (L-NAME vs. controls, without indomethacin (Groups I and II): 0.14 ± 0.05 vs. 0.88 ± 0.18; L-NAME vs. controls, with indomethacin (Groups III and IV): 0.25 ± 0.08 vs. 1.42 ± 0.12, $P < 0.001$). The ratio was decreased to a larger extent in the arteries obtained from the rats treated with L-NAME in vivo than in those treated with L-NAME only in vitro.

3.2. *In vivo studies*

The characteristics of the study groups are presented in Table 2. Sodium nitroprusside (5 μg/min) reduced blood pressure in the L-NAME-treated rats by 26.5% as compared to the 10.8% reduction observed in the controls and the 17.8% reduction in the noradrenaline-infused rats $P < 0.001$, L-NAME vs. controls and L-NAME vs. noradrenaline) (Fig. 6). Surprisingly, acetylcholine (15 μg/min) also reduced blood pressure markedly more in L-NAME-treated animals (26%) than in the controls (16.3%). However, the noradrenaline-infused rats exhibited an even blood pressure fall of 29% (Fig. 6). The dose–response curves with acetylcholine were all significantly different from each other when analysed by repeated measures analysis of variance ($P < 0.05$). This was also the case for the dose–response curves established with sodium nitroprusside. The decrease in blood pressure was associated with a comparable increase in heart rate in the three groups (Fig. 7).

4. Discussion

Chronic administration of L-NAME in drinking water (0.4 g/l) during 6 weeks decreases NO synthase activity in the rat brain to less than 16% [17]. It has however not yet been demonstrated whether NO synthase remains inhibited in vitro when the arteries are obtained from rats chronically treated with an NO synthase inhibitor. In vascular preparations, the acetylcholine-induced relaxation disap-

pears completely after endothelial removal [1]. With an intact endothelium, it is inhibited in a concentration-dependent manner by adding NO synthase inhibitors in vitro [11]. The response to acetylcholine is often used as an indicator of NO synthase activity, but acetylcholine may act on the endothelium by ways which are not NO-synthase-dependent, such as by releasing the endothelial-derived contracting factor (EDCF) or the endothelial-derived hyperpolarizing factor (EDHF). This is why we also examined the response to sodium nitroprusside which is known to be enhanced during NO synthase inhibition [18,19]. Our data show that the in vitro response to one dose of acetylcholine is blunted and that to one dose of sodium nitroprusside is enhanced in the mesentric arteries obtained from rats chronically treated with L-NAME. These data should be interpreted with caution since full dose–response curves were not available and the degree of contraction during exposure to noradrenaline 10^{-5} M was lower in L-NAME-treated than in control rats. However, considering these limitations, these results suggest that some NO synthase inhibition still persists in vitro. This is further supported by the observation that the ratio *% acetylcholine-induced relaxation / % sodium nitroprusside-induced relaxation* used as an indicator of NO synthase inhibition accentuated the differences between the L-NAME-treated group and the control group. The ratio was lower when L-NAME was administered chronically to the rat than when L-NAME was only administered in vitro. This discrepancy could be related to a difference in L-NAME concentrations at the level of the artery in vivo and in vitro or to possible endothelial damage occurring as a consequence of chronic hypertension in the L-NAME-treated rats.

Incubation with the cyclo-oxygenase inhibitor indomethacin has been reported to alter the relaxation response to acetylcholine and to reduce the inhibitory effects of NO synthase inhibition [20,21]. In our experiment, indomethacin enhanced the relaxant response to acetylcholine in arteries obtained from the L-NAME-treated rats and from controls, but did not change the response to sodium nitroprusside in both groups. In the arteries obtained from the control rats and exposed to indomethacin, the relaxant response was higher with acetylcholine than with sodium nitroprusside. This difference may be related to another relaxant factor than NO released by acetylcholine, such as the hyperpolarizing factor (EDHF). This hypothesis was not tested in our study.

Interestingly, only the arteries obtained from the L-NAME-treated rats exhibited a contractile response to acetylcholine. Under normal circumstances, acetylcholine may release sufficiently high quantities of NO to mask the effect of EDCF. During NO synthase inhibition, acetylcholine-induced contraction is observed as a late response, presumably reflecting the inability of NO synthase to release enough NO. The fact that this contractile response is inhibited with the cyclo-oxygenase inhibitor, indomethacin, strongly suggests that the action of EDCF is prostaglandin-mediated. Recent studies [21,22] using specific prostaglandin inhibitors demonstrated that prostaglandin H_2 (PGH_2) is a strong candidate for the vasoconstriction caused by acetylcholine. In these experiments performed on the rat aorta, indomethacin presumably inhibited the production of PGH_2 and thereby prevented the contractile response induced by high concentrations of acetylcholine ($> 10^{-7}$ M). Thus, the inhibition of EDCF by indomethacin may result in an increased relaxant response to acetylcholine, as observed in our study.

In vitro, both removal of the endothelium and acute NO synthase inhibition increases the catecholamine-induced vasoconstriction [18,20]. Such an effect on noradrenaline responses was not found in our arteries obtained from chronically L-NAME-treated rats. Quite to the contrary, the arterial responsiveness to α-adrenoreceptor stimulation was blunted by in vivo NO synthase inhibition. In the rats chronically treated with a NO synthase inhibitor, there might be a counter-regulatory phenomenon decreasing the responsiveness to catecholamines. In our L-NAME-treated rats, a 3-fold increase in plasma noradrenaline levels was observed [17]. Persistent high levels of catecholamines can lead to a down-regulation of α-adrenoreceptors [23] and could explain the decreased contractile response to noradrenaline in this model.

The hypotensive response to sodium nitroprusside was significantly enhanced in the L-NAME-treated rats. These results are similar to those reported previously [18,24,25] and to the response found in vitro in this study. The attenuated response to acetylcholine could only be demonstrated when comparing the L-NAME-treated rats with the noradrenaline-infused rats. Indeed, with the infusion of noradrenaline, the baseline levels of blood pressure are more comparable to those of the L-NAME-treated rats. Since the baseline blood pressure was still significantly lower in the noradrenaline-infused group than in the L-NAME-treated group, it can be speculated that the hypotensive response to acetylcholine might have been even greater with a dose of noradrenaline inducing the same increase in blood pressure as L-NAME. Furthermore, as the baseline tension was the same in all arteries in vitro, it seems particularly logical to compare the results obtained in vitro with those obtained in the groups having the closest baseline blood pressure levels in vivo. Indeed, the response to acetylcholine in vivo involves the whole cardiovascular system with its counter-regulatory mechanisms and in many ways can differ from the local action of acetylcholine on vascular tone. Nevertheless, these results demonstrate that the response to acetylcholine in vivo parallels that found in vitro when the blood pressure of the control animals is somewhat comparable to that of the L-NAME-treated rats.

In conclusion, chronic administration of L-NAME for 6 weeks induced severe hypertension. NO synthase inhibition persisted in isolated mesenteric arteries obtained from rats chronically treated with the NO synthase inhibitor. This was indicated by the fact that the acetylcholine-induced relaxation remained reduced, the sodium-nitroprusside-induced relaxation was enhanced and the late acetylcholine-induced contractile response occurred only in the L-NAME-treated rats. This latter effect was prevented when the isolated arteries were pretreated with indomethacin, suggesting a prostaglandin-mediated mechanism. In vivo, the attenuated response to acetylcholine could be demonstrated in the control hypertensive rats

infused with noradrenaline but not in the normotensive control rats. These results demonstrate that there is no discrepancy between the response to acetylcholine in vivo and in vitro after NO synthase inhibition if the intra-arterial blood pressure of the control animals is increased to levels close to those of L-NAME-treated animals.

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