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Short communication

Effect of nitric oxide synthase inhibition on the acetylcholine response in the perfused hind limb of rats

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Abstract

The effect of nitric oxide (NO) synthase inhibition on acetylcholine-induced vasodilation in the perfused rat hind limb was investigated to find the contribution of NO to such relaxation. Although NO synthase inhibition with 150 μg L-nitro-arginine increased vascular resistance considerably, from 7.28 ± 0.29 to 10.83 ± 0.44 mm Hg · min/ml ($n = 7$), the acetylcholine responses were not attenuated. Acetylcholine (10 μg) induced a peak relaxation of 66 ± 4% before, and 63 ± 7% after L-nitro-arginine. The duration of the peak and total responses, examined in separate sets of animals ($n = 12$), was similar in both circumstances (61 ± 4 s before vs. 53 ± 5 s after, and 7.45 ± 0.61 min before vs. 7.48 ± 0.64 min after respectively). These results suggest that a non-NO factor is responsible for acetylcholine-stimulated relaxation in the rat hind limb vascular bed.

Keywords: Endothelium; Acetylcholine; Nitric oxide (NO); Hind limb, rat; EDHF (endothelium-derived hyperpolarizing factor)

1. Introduction

Acetylcholine is routinely chosen for the assessment of endothelial function. Although acetylcholine responses are often equated with stimulated nitric oxide production, various vasoactive factors, including prostaglandins and an unidentified endothelium-derived hyperpolarizing factor (EDHF) are released concomitantly upon acetylcholine stimulation. The extent to which nitric oxide (NO) actually contributes to this response in various vessels and vascular beds is not clear. Recent studies have shown the role of NO to vary greatly, depending on agonist, vascular bed, and vessel size (Hwa et al., 1994; Vargas et al., 1994; Zygmunt et al., 1994; Garland et al., 1995).

The present study was undertaken to investigate the contribution of NO to acetylcholine-stimulated relaxation in the buffer-perfused hind limb vascular bed of the rat at physiological flows by examining the effect of NO synthase inhibition on acetylcholine vasodilator responses.

2. Materials and methods

2.1. Hind limb perfusion

Male Wistar rats (Iffa Credo, Netherlands), 305–320 g, were anaesthetized (sodium pentobarbital; 60 mg/kg i.p.) and subjected to hind limb perfusion as described previously (Nelissen-Vrancken et al., 1992). Briefly, after cannulation of the abdominal aorta and vena cava, perfusion with an isotonic, oxygenated Krebs-Henseleit buffer containing 10 μM indomethacin was initiated. After the animal was killed (intracardiac injection of KCl), the flow was adjusted to 7.5 ml/min (electromagnetic probe, Skalar, Delft, Netherlands). An on-line monitoring program (Hemodynamic Data Acquisition Systems, Instrumental Services, University of Limburg) was utilized to monitor flow (F = ml/min) and pressure (P = mm Hg) and subsequently calculate resistance ($R = P/F$). The values were sampled at a frequency of 10 ms intervals and averaged over 1 s. Experiments were conducted at 37°C. Mediators were added into the perfusion circuit proximal to a stirring chamber.

2.2. Protocol

The hind limb was precontracted (20 mm Hg) by phenylephrine infusion (7.5 μM). After a non-cumulative

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acetylcholine dose-response curve (0.10 ng–10 μg) had been made, NO synthase was inhibited with 150 μg L-nitro-arginine, a dose higher than the ED_{50} (see Section 3). When this reaction plateaued, a submaximal dose of acetylcholine (10 μg) was again administered.

A separate set of experiments ($n = 12$) served to test the effect of L-nitro-arginine on the time course of the acetylcholine response. To this end, the peak percentage relaxation (the maximum relative change in resistance), the duration of the peak relaxation, and the duration of the total response were analyzed. The progression of the response was plotted by averaging the percent relaxation for the first 10 s of every minute and subsequently averaging these values for the various experiments.

2.3. Materials

The Krebs-Henseleit buffer was of the following composition (mM): 111 NaCl, 5 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.25 $CaCl_2$, 11.1 glucose, and 40 g/l dextran-70, pH 7.4. All salts were purchased from Merck (Amsterdam, Netherlands); dextran-70, indomethacin and phenylephrine from Sigma (St. Louis, MO, USA); L-nitro-arginine from Research Biomedicals International (Natick, MA, USA); acetylcholine from Ciba Vision Ophta (Breda, Netherlands).

2.4. Data analysis

The data are expressed as means ± S.E.M. Half-maximal effective dose (ED_{50}) and maximal response values were obtained from non-linear regression of individual dose-response curves. The values for all parameters defining the response induced by 10 μg acetylcholine before and during NO synthase inhibition were compared by paired Student's t-test, and considered to be statistically different at a value of $P < 0.05$.

3. Results

Perfusion of the rat hind limb produced a low vascular tone (3.53 ± 0.15 mm Hg · min/ml) which was increased to 6.23 ± 0.21 mm Hg · min/ml by infusion of 7.5 μM phenylephrine. Acetylcholine (0.10 ng–10 μg) relaxed the phenylephrine-precontracted hind limb in a dose-dependent manner with an ED_{50} of 8.2 ± 2.2 ng and a maximal relaxation of 71 ± 2% ($n = 7$).

Pilot experiments ($n = 3$) in which cumulative dose-response curves for L-nitro-arginine were made, yielded a maximal increase in resistance of 9.98 ± 1.31 mm Hg · min/ml and an ED_{50} of 62 ± 32 μg. The maximal contraction could be substantially reversed (85 ± 10%) with an infusion of excess of L-arginine (10 μM).

Injection of 150 μg L-nitro-arginine after completion of the acetylcholine curve induced a contraction which developed slowly over approximately 15 min and remained stable for the remainder of the experiment. Resistance increased by 3.67 ± 0.39 mm Hg · min/ml (Fig. 1).

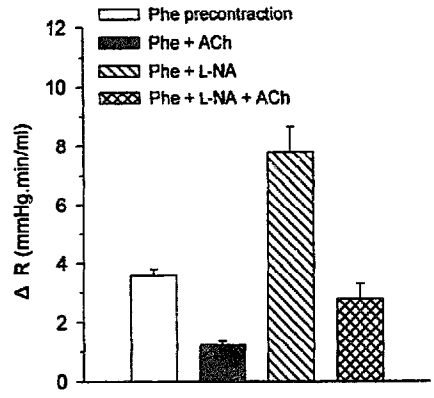

Fig. 1. Vasodilatory effect of 10 μg acetylcholine (ACh) in the phenylephrine (Phe)-precontracted perfused rat hind limb before and after NO synthase inhibition with 150 μg L-nitro-arginine (L-NA). ΔR represents absolute changes in basal vascular resistance. Data are presented as means ± S.E.M.

As shown in Fig. 1, acetylcholine (10 μg) reduced the precontracted tone 66 ± 4% before NO synthase inhibition. Treatment with L-nitro-arginine had no effect on the peak response, as subsequent injection of 10 μg acetylcholine produced an unattenuated relaxation of 63 ± 7%.

In rats in which the time course of the acetylcholine relaxation was studied, the peak response was significantly increased after L-nitro-arginine (77 ± 3% vs. 84 ± 3% respectively) (Fig. 2 insert). The relative relaxation as a function of time was not significantly altered by NO synthase inhibition (Fig. 2). The duration of the peak response and that of the total relaxation were similar irrespective of NO synthase inhibition (61 ± 4 s before vs. 53 ± 5 s after, and 7.45 ± 0.61 min before vs. 7.48 ± 0.64 min after, respectively).

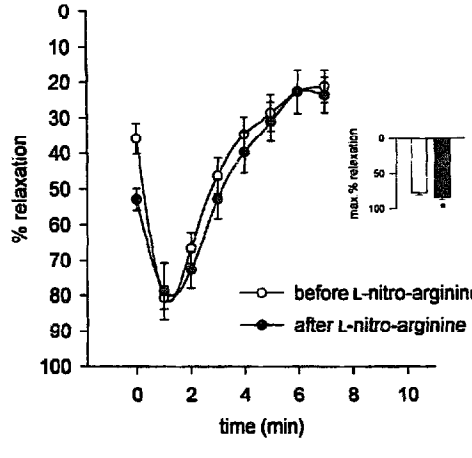

Fig. 2. Effect of NO synthase inhibition with 150 μg L-nitro-arginine on the time course of the acetylcholine (10 μg) response in the phenylephrine-precontracted perfused rat hind limb. % relaxation is the relative decrease in precontracted tone. Insert shows maximum percent relaxation. Data are presented as means ± S.E.M.

4. Discussion

In this study, the effect of NO synthase inhibition on vasodilator responses to acetylcholine in the perfused rat hind limb was examined in order to find the contribution of NO to acetylcholine-stimulated relaxation in this vascular bed. Although NO synthase inhibition with L-nitro-arginine generated a considerable increase in vascular resistance, it had no effect on acetylcholine-induced responses.

The unaffected acetylcholine vasodilatory capability during NO synthase inhibition that was now demonstrated is in agreement with several in vivo studies (Zambetis et al., 1991; Hu et al., 1994) and with the results of Müggе et al. (1991) for the blood-perfused hind limb of the rabbit. In our preparation, however, possible interference from blood-borne substances or central effects could be avoided. Furthermore, our study supplements recent results in isolated vessels, which show a non-NO component of acetylcholine vasodilation (Hwa et al., 1994; Zygmunt et al., 1994; Garland et al., 1995), by further demonstrating that NO plays only a minor role in these endothelium-dependent responses in an entire vascular bed at physiological flows.

Acetylcholine stimulates the release of several vasoactive agents including NO, EDHF and prostaglandins. Although several studies have shown that prostacyclin contributes insignificantly to acetylcholine vasodilation (Müggе et al., 1991; Vargas et al., 1994), indomethacin was included in these experiments to avoid any interference from prostaglandins.

The production of NO has been shown to be specifically inhibited by L-arginine analogues, with L-nitro-arginine being one of the most potent (Vargas et al., 1991). As L-arginine was able to largely reverse the actions of L-nitro-arginine in our pilot experiments, it is most likely that specific inhibition of NO synthase occurred in this preparation. The incomplete irreversibility of the L-nitro-arginine-induced contraction and the large excesses of L-arginine required to attain it are probably due to the high affinity of L-nitro-arginine for NO synthase. These pilot experiments likewise demonstrated that the dose of L-nitro-arginine chosen was sufficient to ensure a considerable inhibition of NO synthase.

Nonetheless, the effects of NO synthase inhibition on the acetylcholine response might be tempered if an increased sensitivity to stimulated NO develops upon withdrawal of constitutively released NO. Although this has been noted by several authors (Müggе et al., 1991; Vargas et al., 1994), an inverse relationship between vasoconstrictor-induced tone and acetylcholine responsiveness has also been demonstrated (Dainty et al., 1990). In our preparation, although we have observed a positive correlation between vascular tone and absolute changes in resistance, percentage changes remain unaffected. We can not exclude the possibility that supersensitivity to NO contributed to the present findings, but the fact that L-nitro- arginine induced a considerable increase in resistance indicates that supersensitivity was not sufficient to outweigh the effects of inhibition.

It has been suggested that EDHF contributes to a transient component and NO to a longer-lasting component of the acetylcholine response (Hayashi et al., 1994; Vicaut et al., 1994). As such, NO synthase inhibition may only affect the duration and magnitude of the total response and have little effect on peak responses. In the present study, however, we found that NO synthase inhibition had no effect on the duration of either the total response or the peak response. Furthermore, the magnitude of the relative relaxation as a function of time remained unchanged. This suggests that NO plays a minor role in acetylcholine-stimulated relaxations in this preparation.

By default, our results suggest the importance of a non-NO, non-prostanoid component of endothelium-dependent relaxation in the rat hind limb vasculature. Recent studies have suggested that EDHF, which is probably a cytochrome P450-derived arachidonic acid metabolite (Bauersachs et al., 1994; Hecker et al., 1994), plays a role in acetylcholine-induced vasodilation in isolated vessels and the perfused kidney (Hwa et al., 1994; Vargas et al., 1994; Zygmunt et al., 1994). Although NO appears to be the primary mediator of agonist-induced endothelium-dependent dilation in the aorta, it is becoming more apparent that its role is lessened as the periphery is approached (Zygmunt et al., 1994; Garland et al., 1995). Indeed, in isolated vessels of the rat mesentery, Hwa et al. (1994) have shown that, whereas acetylcholine-stimulated relaxation in the superior mesenteric arteries is largely due to NO, EDHF becomes the primary endothelium-dependent dilator in resistance vessels. As the total response of a vascular bed is primarily determined by resistance vessels, it seems likely that EDHF also participates in acetylcholine-induced vasodilations of the perfused hind limb.

In conclusion, the results of this study suggests that a non-NO, non-prostacyclin factor(s), such as EDHF, plays a major functional role in the acetylcholine vasodilator response of the non-blood-perfused rat hind limb at physiological flows.

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