Differential inhibition by N^G-monomethyl-L-arginine of vasodilator effects of acetylcholine and methacholine in human forearm vasculature

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1 We compared the effects of N^G-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor, on vasodilatation produced by acetylcholine and methacholine in human forearm vasculature.

2 Acetylcholine (83 nmol min⁻¹) infused into the brachial artery of 8 healthy volunteers caused a submaximal increase in forearm blood flow, measured by venous occlusion plethysmography, from 3.3 ± 0.5 (mean ± s.e.mean) to 13.3 ± 1.7 ml min⁻¹ 100 ml⁻¹.

3 Co-infusion of L-NMMA (4 µmol min⁻¹) with acetylcholine (83 nmol min⁻¹) over 6 min resulted in a 58% ± 12% fall in the response to acetylcholine whereas during co-infusion of saline over the same time period in the same subjects (*n* = 8) on a different day the response to acetylcholine fell by only 9% ± 17% (*P* < 0.01).

4 Methacholine (1.5 and 15 nmol min⁻¹) increased forearm blood flow from 2.5 ± 0.4 to 5.9 ± 0.9 and from 3.2 ± 0.4 to 17.0 ± 1.9 ml min⁻¹ 100 ml⁻¹ respectively.

5 Co-infusion of L-NMMA (4 µmol min⁻¹) had no significant effect on the response to methacholine (1.5 or 15 nmol min⁻¹) when compared with saline control (*n* = 8). Co-infusion of a higher dose of L-NMMA (8 µmol min⁻¹) with methacholine (1.5 nmol min⁻¹) did not significantly inhibit the vasodilator response (*n* = 7).

6 These results suggest that, in human forearm vasculature, methacholine acts predominantly through mechanisms other than the L-arginine/nitric oxide pathway.

Keywords: Endothelium; muscarinic receptors; nitric oxide; N^G-monomethyl-L-arginine; acetylcholine; methacholine; human forearm vasculature

Introduction

N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide (NO) synthase, inhibits vasodilator responses to acetylcholine in human forearm resistance vessels (Vallance *et al.*, 1989). This suggests that acetylcholine causes vasodilatation in this vascular bed at least in part through the L-arginine/NO pathway. It has been assumed that other muscarinic agonists such as methacholine also act on this vascular bed through the L-arginine/NO pathway (Creager *et al.*, 1990; 1992; Liao *et al.*, 1991). However, increasing recognition of the heterogeneity of muscarinic receptor subtypes in vasculature (Dauphin & Hamel, 1991) and their actions in producing both endothelium-dependent and endothelium-independent vasodilatation (Brayden & Bevan, 1985; Rubanyi *et al.*, 1986; Nield *et al.*, 1990; Jaiswal *et al.*, 1991) makes this assumption questionable. We demonstrated recently that in patients with hypercholesterolaemia (a condition in which endothelial dysfunction is well documented in animals and in human coronary vessels) forearm resistance vessel responses to acetylcholine are reduced whereas responses to methacholine are not (Chowienzyk *et al.*, 1992). In the present study we therefore compared effects of L-NMMA on vasodilator responses to acetylcholine and methacholine to determine whether or not this inhibitor of NO synthase influences responses to these muscarinic agonists similarly.

Methods

Studies were performed on healthy normotensive, normocholesterolaemic subjects aged 22–47 years, following informed consent and with the approval of the local ethical

committee. Eight subjects were studied on 4 separate occasions in variable order, one week or more apart. They received acetylcholine with saline, acetylcholine with L-NMMA, methacholine with saline and methacholine with L-NMMA. Drugs were obtained from Sigma, UK (methacholine), CooperVision, UK (acetylcholine) and Calbiochem, UK (L-NMMA). L-NMMA was passed through a 0.2 µm filter immediately before use. Experiments were done in a temperature controlled clinical laboratory (20–22°C). Forearm blood flow (ml min⁻¹ per 100 ml forearm volume) was measured in both arms simultaneously by venous occlusion plethysmography (Whitney, 1953) with electronically calibrated temperature compensated mercury in silastic strain gauges (Hokanson *et al.*, 1975). During measurements the hands were excluded from the circulation by inflation of wrist cuffs to 180 mmHg. Upper arm cuffs were inflated intermittently to 40 mmHg. A 27 gauge needle was inserted under sterile conditions into the left brachial artery under local anaesthesia (1% lignocaine) and sterile saline (140 mM sodium chloride), or drugs dissolved in saline, infused at 1 ml min⁻¹. Following insertion of the needle subjects rested for 15 min. Saline was then infused for a further 14 min during which baseline measurements were recorded and a constant rate infusion of acetylcholine (83 nmol min⁻¹) or methacholine (15 nmol min⁻¹) commenced; 4 min after the start of the agonist infusion, either saline or L-NMMA (4 µmol min⁻¹) was co-infused for 6 min. Forearm blood flow was measured during infusion of saline alone, during infusion of agonist alone (from 2 to 4 min) and during co-infusion of agonist with L-NMMA or saline (during the last 2 min of co-infusion). Flows were recorded for 10 s in every 15 s and the mean of 3 to 5 measurements used for analysis. A further group of 8 subjects was studied on two occasions, methacholine (1.5 nmol min⁻¹) with saline being infused on one occasion and methacholine with L-NMMA (4

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$\mu\text{mol min}^{-1}$) on the other. Seven subjects were studied on two further occasions when methacholine ($1.5 \text{ nmol min}^{-1}$) with saline was infused on one occasion and with L-NMMA ($8 \mu\text{mol min}^{-1}$) on the other.

Statistics

Results are presented as means ($\pm$ s.e.mean). Changes in vasodilator response due to L-NMMA or saline (control) were expressed as a percentage of blood flow response (increase above baseline) during agonist administration immediately before co-infusion. Data were analysed by analysis of variance for repeated measures. Differences were considered significant when $P < 0.05$.

Results

Blood flow in the non-infused (control) arm did not change significantly throughout the experiment on any study day, confirming that at the doses used acetylcholine, methacholine and L-NMMA did not cause systemic effects when infused into the brachial artery. Blood flow in the infused arm is shown in Table 1. Acetylcholine (83 nmol min^{-1}) produced approximately a four fold rise in blood flow. Blood flow during methacholine (1.5 and 15 nmol min^{-1}) was approximately 50% lower and 25% higher respectively than during acetylcholine infusion.

During co-infusion of saline there was some tachyphylaxis to both vasodilators ($P < 0.01$) which did not differ significantly for acetylcholine or either dose of methacholine. Percentage change in response to acetylcholine and methacholine during L-NMMA co-infusion relative to saline control is shown in Figure 1. L-NMMA ($4 \mu\text{mol min}^{-1}$) inhibited the response to acetylcholine (83 nmol min^{-1}) by $49\% \pm 15\%$ of saline control ($P < 0.01$). In contrast L-

NMMA ($4 \mu\text{mol min}^{-1}$) had no effect on responses to methacholine (15 nmol min^{-1} , $P = 0.82$ or $1.5 \text{ nmol min}^{-1}$, $P = 0.88$). There was also no significant effect of L-NMMA ($8 \mu\text{mol min}^{-1}$) on the response to methacholine ($1.5 \text{ nmol min}^{-1}$, $P = 0.51$).

Discussion

L-NMMA inhibits vasodilatation caused by brachial artery infusion of acetylcholine (Vallance *et al.*, 1989). These workers infused L-NMMA followed by acetylcholine. A possible confounding factor in this approach is that L-NMMA lowers basal blood flow, and there is a strong positive correlation between basal blood flow and response to acetylcholine in this preparation (Chowienzyk *et al.*, 1993). In the present study we infused vasodilator agonists before starting co-infusion of L-NMMA. The blood flow during L-NMMA infusion was therefore higher than in the study by Vallance *et al.* (1989) reducing the concentration of L-NMMA in the forearm vasculature. Despite this we observed very similar inhibition to that reported by Vallance *et al.* (1989) confirming their conclusion that part, at least, of the vasodilatation caused by acetylcholine in forearm vasculature is mediated by the L-arginine/NO pathway. The order of addition of L-NMMA and acetylcholine markedly influences the development of inhibition of acetylcholine induced relaxation of rings of rabbit aorta (Furchgott *et al.*, 1990). The explanation for this pattern of inhibition is not clear; possible explanations include substrate depletion or agonist dependent access of L-NMMA to its site of action (Furchgott *et al.*, 1990). It is possible that the potency of L-NMMA in the forearm is similarly determined by the order of infusion of acetylcholine and L-NMMA. Alternatively, there might be reduced extraction of L-NMMA in proximal vessels at higher flows. This has been proposed as the explanation of the findings that in general, over a narrow range of flow rates, the response to a drug is more closely related to the dose of drug infused than the calculated concentration (Robinson, 1990).

The principle finding of the present study is that whereas L-NMMA inhibits the vasodilator action of acetylcholine in forearm vasculature substantially it has no effect on responses to methacholine. The doses of acetylcholine and methacholine used cause submaximal vasodilatation, and are similar to those used in previous studies of endothelial function in hypertension and hyperlipidaemia (Linder *et al.*, 1990; Creager *et al.*, 1990). In our first study, blood flow during infusion of methacholine was approximately 25% greater than during acetylcholine infusion, suggesting the possibility that the lack of inhibition of methacholine by L-NMMA was due to greater dilution of the inhibitor. However the second study excluded this possibility by showing that a lower dose of methacholine that caused approximately 50% of the blood

Figure 1 Percentage change in response to acetylcholine (ACh) and methacholine (MCh) relative to saline control after 6 min co-infusion of N^G -monomethyl-L-arginine (L-NMMA) $4 \mu\text{mol min}^{-1}$ (open columns) or $8 \mu\text{mol min}^{-1}$ (hatched column).

Table 1 Effect of N^G -monomethyl-L-arginine (L-NMMA) or saline on vasodilator responses to acetylcholine (ACh) and methacholine (MCh)

Study day†	Forearm bloodflow ($\text{ml min}^{-1} 100 \text{ ml}^{-1}$)				%change with saline/L-NMMA
	Baseline	Agonist	Agonist with saline/L-NMMA		
ACh(83)/saline	3.8 ± 0.5	12.9 ± 1.7	10.7 ± 1.2	-9 ± 17	**
ACh(83)/L-NMMA(4)	3.3 ± 0.5	13.3 ± 1.7	7.3 ± 1.6	-58 ± 12	
MCh(15)/saline	4.0 ± 1.2	14.8 ± 3.0	13.1 ± 2.7	-16 ± 9	NS
MCh(15)/L-NMMA(4)	3.2 ± 0.4	17.0 ± 1.9	15.0 ± 1.7	-13 ± 6	
MCh(1.5)/saline	2.1 ± 0.4	6.4 ± 0.9	5.2 ± 0.7	-25 ± 6	NS
MCh(1.5)/L-NMMA(4)	2.5 ± 0.4	5.9 ± 0.9	5.0 ± 0.8	-23 ± 12	
MCh(1.5)/saline	2.2 ± 0.3	6.4 ± 0.7	5.1 ± 0.7	-20 ± 7	NS
MCh(1.5)/L-NMMA(8)	2.1 ± 0.4	5.5 ± 0.7	4.4 ± 0.6	-29 ± 13	

** $P < 0.01$, NS = not significant.

† Agonist dose in nmol min^{-1} ; L-NMMA dose in $\mu\text{mol min}^{-1}$.

flow response to acetylcholine was also uninhibited by L-NMMA ($4 \mu\text{mol min}^{-1}$). It was possible that a higher dose of L-NMMA would have inhibited the response to methacholine, but in the third series of experiments doubling the dose of L-NMMA did not significantly inhibit the vasodilator response to methacholine ($1.5 \text{ nmol min}^{-1}$).

There are several possible explanations for the differential effect of L-NMMA on acetylcholine and methacholine. Muscarinic receptor subtypes initiating vascular relaxation to acetylcholine *in vitro* are complex (Rubanyi *et al.*, 1986; Nield *et al.*, 1990; Jaiswal *et al.*, 1991), and differential inhibition of different agonists by L-NMMA could result from actions on receptor subtypes linked with more than one effector mechanism, not all of which involve the L-arginine/NO pathway. Endothelium-dependent relaxations resistant to NO synthase inhibitors are well recognised (Nagao *et al.*, 1992; Nagao & Vanhoutte 1992) and are thought to result from endothelium-derived hyperpolarizing factor (Chen *et al.*, 1988; Feletou & Vanhoutte, 1988). Endothelium-independent relaxation may be due to activation of muscarinic receptors on vascular smooth muscle (Brayden & Bevan, 1985; Nield *et al.*, 1990; Jaiswal *et al.*, 1991). Vasoconstriction is also mediated by muscarinic receptors on smooth muscle (Furchgott & Zawadzki, 1980; Jaiswal *et al.*, 1991) and therefore muscarinic receptor subtypes are linked to opposing vasodilator and constrictor pathways. Inhibition of noradrenaline release from nerves and release of prostacyclin from the endothelium have been excluded as important mechanisms of vasodilatation in this preparation for both acetylcholine (Linder *et al.*, 1990) and methacholine (Creager *et al.*, 1990).

Different mechanisms of vasodilatation by acetylcholine

and methacholine may result from the metabolic instability of acetylcholine (Duff *et al.*, 1952) influencing the site of action of the agonist. Destruction of acetylcholine by cholinesterase at the interface between circulating blood and vessel wall or within the vessel wall might favour actions of arterially administered acetylcholine initiated by receptors on the luminal surface of the endothelium and linked with the L-arginine/NO pathway, while preventing access to receptors on the abluminal endothelial cell surface or on nerve endings or vascular smooth muscle. Methacholine is less susceptible to enzymic inactivation than is acetylcholine, so more of its effect might be mediated by actions deep to the luminal surface of the endothelium. There is evidence that sensitivity to inhibitors of the L-arginine/NO pathway varies with size of resistance vessel (Nagao *et al.*, 1992). The instability of acetylcholine in blood might result in a greater fraction of the vasodilator response to acetylcholine being mediated through relatively proximal resistance arteries more sensitive to inhibition by L-NMMA.

Irrespective of the explanation placed on the selective inhibition of acetylcholine by L-NMMA demonstrated in this study, the lack of effect of L-NMMA on responses to methacholine has important implications for the interpretation of studies that have used this agonist as a putative probe of endothelial function in this preparation (Creager *et al.*, 1990; 1992; Liao *et al.*, 1991). The present findings suggest that the vasodilator effect of methacholine in human forearm vasculature is mediated mainly through mechanisms other than the L-arginine/NO pathway.

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