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Quantitative aspects of the inhibition by N^G-monomethyl-L-arginine of responses to endothelium-dependent vasodilators in human forearm vasculature

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1 N^G-monomethyl-L-arginine (L-NMMA) constricts human forearm resistance vasculature and selectively attenuates vasodilator responses to endothelium-dependent vasodilators. Incomplete inhibition of such responses could be due to an inadequate dose of L-NMMA or to NO-independent vasodilator mechanisms.

2 This study sought to determine doses of L-NMMA that are maximally effective in reducing basal and stimulated forearm blood flow. Drugs were infused *via* the brachial artery in 32 healthy men. Acetylcholine (11–330 nmol min^{-1}) was compared with albuterol (0.33–10 nmol min^{-1}), and nitroprusside (1.7–20 nmol min^{-1}).

3 The effect of L-NMMA on basal flow approached maximum ($53 \pm 2\%$ reduction) at a dose of 16 μmol min^{-1}. L-NMMA (16 μmol min^{-1}) did not significantly influence responses to nitroprusside, but antagonized acetylcholine and albuterol (each $P < 0.001$, by repeated measures analysis of variance).

4 Inhibition of acetylcholine by L-NMMA (16 μmol min^{-1}) was strongly influenced by acetylcholine dose ($73 \pm 7\%$ inhibition at 11 nmol min^{-1}, $P < 0.01$; $4 \pm 11\%$ inhibition at 330 nmol min^{-1}, $P =$ NS, Student's paired *t*-test). Significant inhibition of albuterol was observed at all doses.

5 A higher dose of L-NMMA (64 μmol min^{-1}) did not significantly inhibit the response to acetylcholine (330 nmol min^{-1}). Responses to this dose of acetylcholine were unaffected by a cyclo-oxygenase (COX) inhibitor (indometacin) alone but combined COX and NO inhibition attenuated acetylcholine responses by $42 \pm 19\%$, implying that there is a compensatory increase in the contribution of prostaglandins or NO to acetylcholine-induced dilatation when one or other pathway is inhibited.

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Abbreviations: ANOVA, repeated measures analysis of variance; COX, cyclo-oxygenase; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; L-NMMA, N^G-monomethyl-L-arginine; NE, norepinephrine; NO, nitric oxide

Introduction

Following the original account of the effect of N^G-monomethyl-L-arginine (L-NMMA) on human forearm vasculature (Vallance *et al*., 1989), this drug has been used extensively to investigate the L-arginine/nitric oxide (NO) pathway in this vascular bed. Inhibition by L-NMMA of vasodilator responses is consistent with a mechanism mediated by endothelium-derived NO. Vasodilatation to acetylcholine is attenuated but not abolished by brachial artery administration of L-NMMA (4 μmol min^{-1}) in forearm vasculature (Vallance *et al*., 1989). L-NMMA resistant vasodilatation could result from use of a dose of L-NMMA that only partly inhibits endothelial NO synthase (eNOS), or from NO-independent vasodilator mechanisms. Such mechanisms include release of vasodilator prostaglandins (Beetens *et al*., 1983; Forstermann *et al*., 1986); endothelium-derived hyperpolarizing factor (EDHF) (Bolton *et al*., 1984; Edwards & Weston, 1998; Mombouli & Vanhoutte, 1997); or activation of muscarinic receptors on vascular smooth muscle (Brayden & Bevan, 1985; Jaiswal *et al*., 1991; Neild *et al*., 1990).

It has been stated that a dose of L-NMMA of 4 μmol min^{-1} causes near-maximal inhibition of acetylcholine in the forearm (Calver *et al*., 1992), but full dose response data have not been reported. In view of the importance of this readily accessible vascular bed for investigations of endothelial function in humans (Benjamin *et al*., 1995), the present study sought to investigate further L-NMMA resistant vasodilatation to acetylcholine. Effects of L-NMMA and of a cyclo-oxygenase inhibitor, indometacin (Wilson & Kapoor, 1993) on basal and agonist-stimulated forearm blood flow were measured. A dose response relation between L-NMMA and basal forearm blood flow was first established. A maximally effective dose of L-NMMA was then co-infused with rising doses of acetylcholine and compared to other endothelium-dependent (albuterol) and endothelium-independent (nitroprusside) agonists. Vasodilator-induced increases in forearm blood flow could dilute coinfused L-NMMA. A supra-maximal dose of L-NMMA was therefore used to

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establish whether incomplete inhibition of acetylcholine was due to an inadequate dose of L-NMMA.

Methods

The St Thomas' Hospital Research Ethics Committee approved the protocols. Thirty-two healthy, normotensive (blood pressure by sphygmomanometry $123 \pm 3/79 \pm 3$ mmHg, mean $\pm$ s.e.mean), normocholesterolaemic (fasting serum cholesterol 4.5 ± 0.3 mmol l^{-1}) men (mean age 26 ± 2 years) gave informed consent to participate in each study. Some subjects participated in more than one study, in which case studies were performed on different days separated by at least 1 week. Each vasodilator was studied on a separate occasion. Studies took place in a quiet, temperature-controlled room ($24 \pm 1^{\circ}$C) and subjects lay quietly on a bed for 30 min before infusions or measurements were made. A 27 gauge needle (Cooper's Needleworks, U.K.) was inserted into the left brachial artery under local anaesthesia (1% lidocaine <1 ml) and isotonic saline $\pm$ drugs was infused at 1 ml min^{-1} by a constant rate infusion pump (Braun, Germany). Forearm blood flow was measured simultaneously in both arms by venous occlusion plethysmography (Whitney, 1953). Temperature-compensated electrically calibrated strain gauges were placed around the point of maximum forearm circumference (Hokanson *et al*., 1975; Walker *et al*., 2001). Pneumatic cuffs were positioned around the wrists and upper arms. During measurement of forearm blood flow the wrist cuffs were inflated to suprasystolic pressure (180 mmHg), to exclude the hands from the circulation. Upper arm cuffs were rapidly inflated to 40 mmHg for 10 s in every 15 s. During periods of high flow the deflation period was extended to ensure complete emptying of the arm veins. Cumulative doses of vasodilator drugs were infused stepwise, each dose for 6 min, according to protocols described below, and blood flow measured during the final 3 min of each infusion period. The mean of the last five measurements was used for analysis. Blood pressure was measured by sphygmomanometry in the right arm.

Drugs were obtained from CIBA Vision Opthalmics, U.K. (acetylcholine chloride); Allen and Hanbury's, U.K. (albuterol sulphate); Merck, Sharpe, and Dohme, U.K. (indometacin sodium trihydrate); Antigen Pharmaceuticals, Ireland (lidocaine hydrochloride 1% w v^{-1}); Clinalfa, Switzerland (N^G-monomethyl-L-arginine hydrochloride); David Bull Laboratories, U.K. (sodium nitroprusside dihydrate).

Protocol 1: Effect of L-NMMA on basal flow

After cannulating the brachial artery, saline was infused for 15 min and basal forearm blood flow measured. Cumulative doubling doses of L-NMMA were then infused from 1 to 64 μmol min^{-1} ($n=12$). Each dose was infused for 6 min and blood flow was measured during the final 3 min. The mean of the final five blood flows for each dose of L-NMMA was used for analysis.

Protocol 2: Effect of L-NMMA or norepinephrine on vasodilators

Saline was infused for 15 min and basal forearm blood flow measured. Four increasing doses of agonist were infused, each for 6 min, followed by saline for 15 min during which blood flow returned to basal values. L-NMMA (16 μmol min^{-1}) was then infused alone for 6 min. This dose of L-NMMA was maintained for the next 24 min, co-infused with four increasing doses of the agonist. Doses of the agonists were: acetylcholine (11, 33, 110, 330 nmol min^{-1}, $n=8$); albuterol (0.33, 1.0, 3.3, 10 nmol min^{-1}, $n=8$); and nitroprusside (1.7, 3.3, 10, 20 nmol min^{-1}, $n=4$). In control experiments norepinephrine (240 pmol min^{-1}) was infused in place of L-NMMA before and during coinfusion of acetylcholine ($n=4$) and albuterol ($n=4$) as above.

Protocol 3: Effect of high dose L-NMMA on responses to acetylcholine and albuterol

Saline was infused for 15 min and baseline forearm blood flow measured. Acetylcholine (330 nmol min^{-1}, $n=8$) or albuterol (10 nmol min^{-1}, $n=8$) was infused for 6 min and blood flow measured for the final 3 min. Saline was infused for 15 min to allow blood flow to return to baseline followed by L-NMMA (64 μmol min^{-1}) alone for 6 min, during the last 3 min of which blood flow was measured. L-NMMA infusion was maintained for a further 6 min and the same vasodilator as before was co-infused. Blood flow was measured during the last 3 min.

Protocol 4: Effect of indometacin $\pm$ L-NMMA on high dose acetylcholine

On separate occasions ($n=6$) baseline forearm blood flow was measured and then acetylcholine (330 nmol min^{-1} for 6 min) was infused and response measured as before. Saline was infused for 15 min, baseline blood flow was re-established, followed by indometacin (0.34 μmol min^{-1} for 20 min) as described (Wilson & Kapoor, 1993). This dose of indometacin was chosen as it abolishes forearm release of prostaglandins. A second infusion of acetylcholine was then administered as before. In further separate studies the protocol was repeated ($n=8$) with an infusion of L-NMMA (64 μmol min^{-1} for 12 min) following indometacin.

Statistics

Data are summarized as mean $\pm$ s.e.mean. Vasoconstrictor responses to L-NMMA were calculated as percentage decrease of blood flow ratio of the infused to non-infused arm (Benjamin *et al*., 1989; Petrie *et al*., 1998). Vasodilator responses were calculated as increases of forearm blood flow above the immediately preceding baseline (ml min^{-1} 100 ml^{-1} forearm volume; Benjamin *et al*., 1995; Walker *et al*., 2001). Data from each protocol were analysed separately by repeated measures analysis of variance (ANOVA). In studies of effects of L-NMMA on vasodilator agonists the primary analysis compared responses to (agonist + saline vehicle) against (agonist + L-NMMA). Differences were considered significant when $P<0.05$. If differences were significant, a secondary analysis was undertaken to assess effects of L-NMMA on individual doses of agonist by Student's paired *t*-test. Student's paired *t*-test was used to compare forearm responses in protocols involving one dose of agonist only.

Results

Neither blood pressure nor blood flow in the non-infused arm changed significantly during any protocol.

Protocol 1

L-NMMA reduced blood flow in the infused arm ($P<0.0001$, ANOVA). Figure 1 shows the percentage reduction in flow caused by increasing doses fitted by a rectangular hyperbola with a maximum of $53\pm2\%$ reduction and IC_{50} of 1.5 μmol min^{-1}. A dose of 16 μmol min^{-1} produced a fall in blood flow of $49\pm3\%$, and this dose was used in protocol 2.

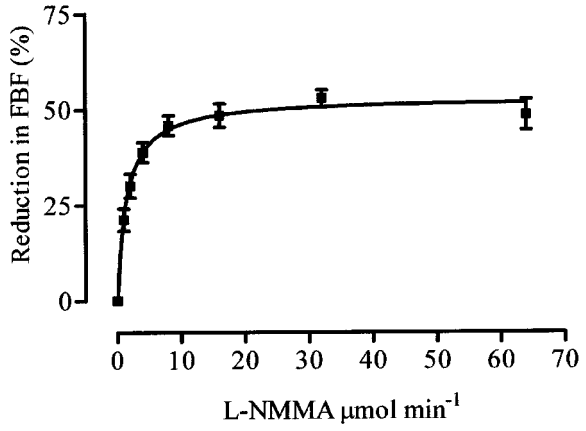

Figure 1 Effect of a cumulative, doubling dose, intrabrachial artery infusion of L-NMMA expressed as percentage reduction in basal forearm blood flow (FBF) relative to flow in the non-infused arm ($n=12$, mean$\pm$s.e.mean).

Protocol 2

Each agonist produced dose-dependent increases in forearm blood flow and the highest dose of each produced an approximate 4 fold increase. Figure 2 shows responses to acetylcholine and albuterol infused alone and in the presence of L-NMMA or norepinephrine. L-NMMA had no significant effect on responses to nitroprusside (1.7, 3.3, 10, and 20 nmol min^{-1}, $P=0.7$) increases above baseline 3.5 ± 0.7, 4.6 ± 0.7, 9.0 ± 0.8, 12.3 ± 1.0 ml min^{-1} 100 ml^{-1} with saline versus 2.4 ± 0.1, 4.3 ± 0.5, 8.4 ± 0.8, 12.1 ± 1.0 ml min^{-1} 100 ml^{-1} with L-NMMA, respectively. L-NMMA significantly antagonized acetylcholine and albuterol ($P<0.001$, $P<0.0001$, respectively). The lowest dose of acetylcholine (11 nmol min^{-1}) was inhibited $73\pm7\%$ by L-NMMA and inhibition fell progressively with increasing doses of acetylcholine ($P<0.005$ for the trend). The highest dose of acetylcholine (330 nmol min^{-1}) was not significantly affected by L-NMMA ($P=0.5$). L-NMMA had a significant inhibitory effect on forearm responses to all doses of albuterol. Norepinephrine (240 pmol min^{-1}) caused a $29\pm5\%$ reduction in basal flow ($P<0.001$), but had no significant effect on responses to acetylcholine or albuterol (Figure 2).

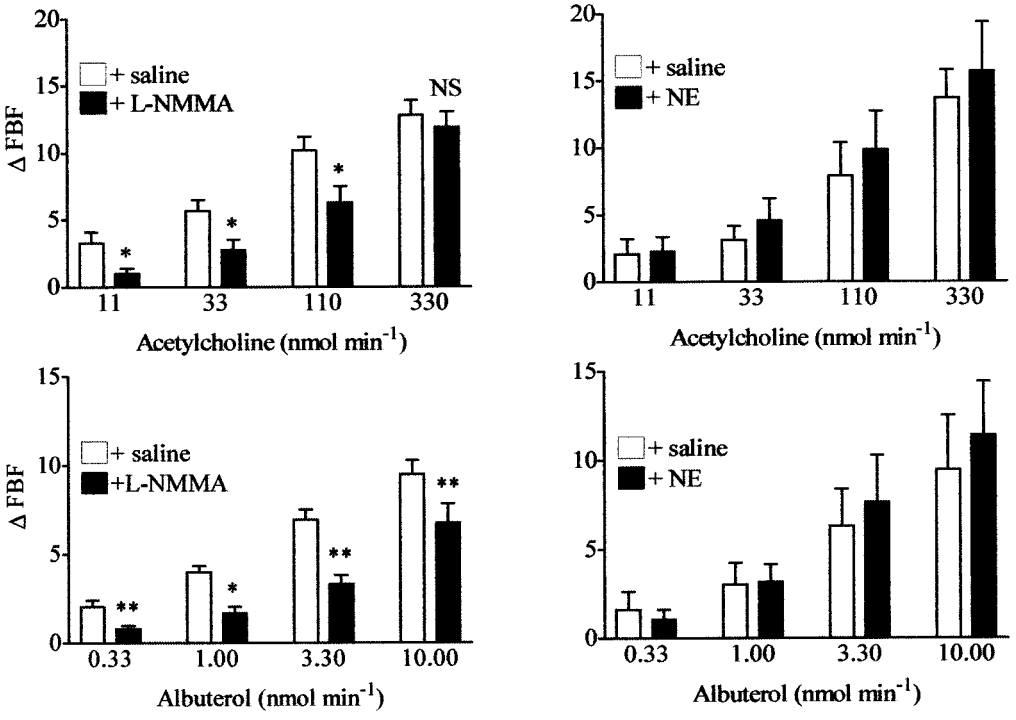

Figure 2 Increases in forearm blood flow above baseline (Δ FBF, ml min^{-1} 100 ml forearm^{-1}, mean$\pm$s.e.mean) for increasing dose, cumulative infusion of vasodilator (acetylcholine or albuterol) with saline vehicle (open bars) and with coinfusion of L-NMMA (16 μmol min^{-1}) or norepinephrine (NE, 240 pmol min^{-1}) (closed bars). $P<0.001$ for interaction with L-NMMA for both agonists, repeated measures analysis of variance; $n=8$. *Post hoc* analysis for each individual response$\pm$L-NMMA (Student's paired *t*-test, $^*P<0.01$, $^{**}P<0.001$). $P=$NS for interaction with NE for both agonists; $n=4$.

Protocol 3

Acetylcholine (330 nmol min^{-1}) increased forearm blood flow by 21.4 ± 3.0 ml min^{-1} 100 ml^{-1} when infused with vehicle (saline) and by 22.9 ± 4.2 ml min^{-1} 100 ml^{-1} when infused with L-NMMA (64 μmol min^{-1}; $P=0.5$), see Figure 3. Albuterol (10 nmol min^{-1}) increased forearm blood flow by 9.4 ± 1.6 ml min^{-1} 100 ml^{-1} when infused with vehicle and by 4.3 ± 1.1 ml min^{-1} 100 ml^{-1} when infused with L-NMMA ($55 \pm 5\%$ reduction, $P<0.01$).

Protocol 4

The findings are summarized in Figure 3. Indometacin reduced basal forearm blood flow by $17 \pm 5\%$ from 4.0 ± 0.6 ml min^{-1} 100 ml^{-1} to 3.4 ± 0.6 ml min^{-1} 100 ml^{-1} ($P<0.05$). When infused alone, indometacin had no significant effect on responses to acetylcholine (330 nmol min^{-1}): acetylcholine increased forearm blood flow by 12.9 ± 1.6 ml min^{-1} 100 ml^{-1} versus 14.3 ± 1.2 ml min^{-1} 100 ml^{-1} after indometacin. The combination of indometacin + L-NMMA reduced baseline blood flow by $29 \pm 4\%$ (from 2.6 ± 0.5 ml min^{-1} 100 ml^{-1} to 1.8 ± 0.2 ml min^{-1} 100 ml^{-1}). The combination reduced responses to acetylcholine by $42 \pm 19\%$ ($P<0.05$). Acetylcholine increased blood flow by 12.1 ± 1.5 ml min^{-1} 100 ml^{-1} (control) compared to 6.2 ± 1.8 ml min^{-1} 100 ml^{-1} with indometacin + L-NMMA.

Discussion

It has previously been found that brachial artery administration of L-NMMA reduces basal forearm blood flow and reduces, but does not abolish, increases in blood flow caused by acetylcholine (Vallance *et al*., 1989; Chowienczyk *et al*., 1993), and albuterol (Dawes *et al*., 1997). The present observations confirm these studies and extends them to higher doses of L-NMMA than have been reported previously. The reduction in basal blood flow caused by L-NMMA 4 μmol min^{-1} (used in several previous studies) was $39 \pm 3\%$. Other studies have demonstrated greater reduction in basal blood flow at similar doses (Newby *et al*., 1997) possibly due to subject variability such as differences in basal flow. The response to L-NMMA (4 μmol min^{-1}) observed in the present study is significantly ($P<0.0001$) less than the reduction caused by 16 μmol min^{-1}, which was $49 \pm 3\%$. Doses higher than 16 μmol min^{-1} did not have significantly greater effects on basal flow, and a dose of 16 μmol min^{-1} was used to study inhibition of blood flow responses to vasodilator agonists.

L-NMMA did not inhibit responses to the endothelium-independent agonist nitroprusside, in agreement with earlier observations (Dawes *et al*., 1997; Cardillo *et al*., 1997). Each of the endothelium-dependent agonists was significantly inhibited by L-NMMA. Tachyphylaxis is unlikely to be responsible for the forearm responses to albuterol as we have previously shown a lack of tolerance to two consecutive, cumulative infusions of albuterol in this vascular bed (Dawes *et al*., 1997). It is also unlikely that functional antagonism is responsible for the effects of L-NMMA on acetylcholine and albuterol as a control vasoconstrictor, norepinephrine, had no significant effect on responses to either of these agonists.

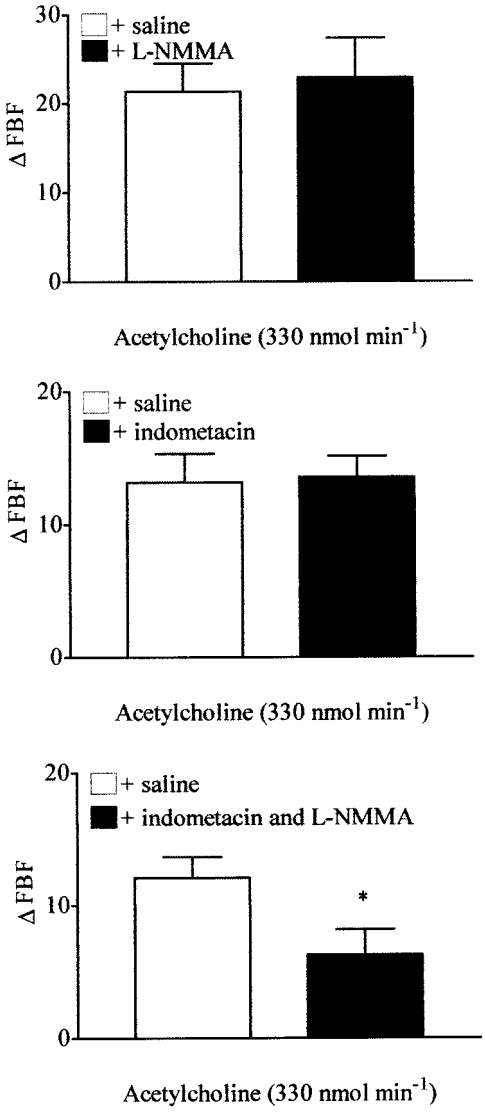

Figure 3 Increases in forearm blood flow above baseline (Δ FBF, ml min^{-1} 100 ml $forearm^{-1}$, mean ± s.e.mean) for single dose infusion of acetylcholine with saline vehicle (open bars) and with coinfusion of L-NMMA (64 μmol min^{-1}); or indometacin (0.34 μmol min^{-1} for 20 min); or indometacin and L-NMMA (closed bars). P = NS for interaction of acetylcholine with L-NMMA ($n=8$) and of acetylcholine with indometacin ($n=6$). $^*P<0.05$ for interaction of acetylcholine with L-NMMA and indometacin ($n=8$); analysis by Student's paired t-test.

Inhibition of acetylcholine by L-NMMA (16 μmol min^{-1}) was strongly influenced by acetylcholine dose ($73 \pm 7\%$ inhibition at 11 nmol min^{-1}, $P<0.01$; $4 \pm 11\%$ inhibition at 330 nmol min^{-1}, P = NS). This is in keeping with a previous report (Bruning *et al*., 1996). In contrast, significant inhibition of albuterol was observed at all doses. Consequently, the dose-dependency of inhibition of acetylcholine does not appear to be due to a non-specific phenomenon such as dilution of L-NMMA in the increased blood flow caused by high doses of vasodilators. Effects of L-NMMA in this vascular bed persist after infusion is discontinued (Vallance *et al*., 1989), which also argues against this explanation. We studied the effect of a 4 fold higher dose of L-NMMA (64 μmol min^{-1}) on the response to high dose acetylcholine

(330 nmol min^{-1}) to exclude the possibility that L-NMMA is less effective at inhibiting eNOS under stimulated rather than basal conditions. Responses to high dose acetylcholine are resistant to inhibition by this higher dose of L-NMMA in contrast to albuterol (10 nmol min^{-1}) responses which are inhibited by $55 \pm 5\%$. These findings therefore argue that at higher doses of acetylcholine, L-NMMA is a less effective inhibitor. This can be explained if activation of the L-arginine/NO pathway is less important at high doses whereas responses to low doses of acetylcholine are mediated mainly by the L-arginine/NO mechanism.

L-NMMA-insensitive responses to acetylcholine are not sensitive to inhibition by indometacin alone. However, combined COX and NO inhibition attenuate responses to high dose acetylcholine by $42 \pm 19\%$ suggesting a compensatory increase in activity of prostaglandins or NO to high dose acetylcholine-induced vasodilatation when one or other pathway is inhibited. There still remains a component of the response to high dose acetylcholine that is resistant to inhibition by indometacin and L-NMMA suggesting mechanisms other than vasodilator prostaglandins and NO. Possible mechanisms include EDHF (Chen *et al*., 1988; Taylor & Weston, 1988; Garland *et al*., 1995) or a non-endothelium dependent process, e.g. *via* muscarinic receptors on vascular smooth muscle (Brayden & Bevan, 1985; Jaiswal *et al*., 1991; Neild *et al*., 1990) or pre-junctional inhibition of adrenergic nerves (Vanhoutte, 1974) although this latter possibility appears less likely in the forearm vasculature (Linder *et al*., 1990). It is difficult to study directly the role of EDHF in mediating forearm responses to high dose acetylcholine in humans because of the toxicity of K^+ channel antagonists, such as charybdotoxin and apamin, which are used *in vitro* to inhibit vascular responses to EDHF.

The dose dependence of the inhibition could be related to the instability of acetylcholine in blood (Benjamin *et al*., 1995; Duff *et al*., 1953). Thus methacholine, a stable muscarinic agonist, is less susceptible to inhibition by L-NMMA than is acetylcholine (Chowienczyk *et al*., 1993) and local inhibition of acetylcholinesterase by edrophonium increases sensitivity of forearm vasculature to acetylcholine and renders acetylcholine responses insensitive to inhibition by L-NMMA (Chowienczyk *et al*., 1995).

However, whatever the mechanism, the observation that high doses of acetylcholine are resistant to inhibition by L-NMMA is important in interpreting studies of patients with vascular risk factors, e.g. essential hypertension, that use acetylcholine to probe NO function. Some studies in hypertensive patients show attenuated forearm responses to acetylcholine predominantly at high doses (approximately 220 to 880 nmol min^{-1}, (Linder *et al*., 1990; Taddei *et al*., 1993)). There is conflicting data using lower acetylcholine doses showing either no impairment of forearm vascular responses (40 to 80 nmol min^{-1} (Cockcroft *et al*., 1994)) or attenuated responses throughout the lower acetylcholine dose range (40 to 160 nmol min^{-1} (Panza *et al*., 1990)).

The observation that responses to high dose acetylcholine are resistant to inhibition by L-NMMA and indometacin when the antagonists are administered separately whereas there is partial inhibition when they are administered together, suggests that, in healthy men, there is a compensatory increase in the contribution of prostaglandins or NO to high dose acetylcholine-induced dilatation when one or other pathway is inhibited. This has not been demonstrated previously, but accords with studies in patients with types I diabetes mellitus (Meeking *et al*., 2000). It will be important to take account of the possibility that distinct mediators can compensate when one or other pathway is inhibited in interpreting studies in which acetylcholine is used to investigate endothelial function.

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