Comparison of forearm vasodilatation to substance P and acetylcholine: contribution of nitric oxide

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1. Forearm blood flow responses to incremental challenges of acetylcholine and substance P, administered via the brachial artery, were measured by venous occlusion plethysmography in eight subjects in the presence of saline, the nitric oxide synthase inhibitor, NG-monomethyl-L-arginine, and a control vasoconstrictor, noradrenaline.

2. Substance P and acetylcholine caused dose-dependent increases in forearm blood flow (P < 0.001). When separated by 30 min saline infusions, repeated responses did not undergo tachyphylaxis.

3. Noradrenaline caused a mean reduction in basal blood flow of 34±4% (P < 0.001), and augmented the percentage increases in blood flow with both substance P (P = 0.05) and acetylcholine (P = 0.03) infusions.

4. NG-Monomethyl-L-arginine caused a mean reduction in basal blood flow of 42–45% (P < 0.001) and significantly inhibited the responses to both substance P (P < 0.001) and acetylcholine (P = 0.05).

5. In comparison with saline responses, NG-monomethyl-L-arginine caused a mean inhibition of 69±8% for substance P-induced vasodilatation and 40±5% for acetylcholine-induced vasodilatation. However, comparing responses with those to the control vasoconstrictor noradrenaline, NG-monomethyl-L-arginine caused a mean inhibition of 81±5% for substance P responses and 58±3% for acetylcholine responses. Inhibition by NG-monomethyl-L-arginine of the response to substance P was significantly greater than inhibition of the response to acetylcholine (P = 0.02).

6. Hence, in healthy men, a greater proportion of the forearm vasodilatation to substance P than to acetylcholine appears to be nitric oxide-mediated. Given its greater stability, substance P may be more suitable as a pharmacological tool in the investigation of stimulated nitric oxide production and endothelial cell function.

INTRODUCTION

Endothelial cells are known to produce a number of vasoactive mediators, including nitric oxide [1], prostacyclin [2] and endothelin-1 [3]. Continuous release of nitric oxide and endothelin-1 by endothelial cells contributes to the maintenance of basal vascular tone [4, 5] and blood pressure [6, 7] in humans. The assessment of endothelial cell function, and of the mechanisms by which the endothelium regulates vascular function, is now recognized to be an important area of research with relevance to the pathogenesis of cardiovascular disease.

Under physiological conditions, nitric oxide is produced within endothelial cells by the action of the constitutive, endothelial-type, nitric oxide synthase enzyme (nitric oxide synthase III). This enzyme releases nitric oxide through conversion of L-arginine to L-citrulline. One method of assessing endothelial cell function has been to measure the vasorelaxation caused by activation of nitric oxide synthase III as an index of the capacity for stimulated nitric oxide production. Historically, acetylcholine has been the favoured endothelial cell stimulant [4, 8] and this agent has been used successfully to demonstrate impaired endothelium-dependent vasodilator responses in various diseased states [9–11]. However, acetylcholine has many drawbacks as a pharmacological tool, including its instability in solution and its rapid degradation in vivo. Indeed, such is the rapidity of its degradation that less than 1% of acetylcholine infused into the brachial artery reaches the hand [12]. Furthermore, the magnitude of the consequent vasodilator response is majorly dependent on basal blood flow and conduit artery length [13]. In addition to releasing nitric oxide, acetylcholine can also stimulate the release of endothelium-derived hyperpolarizing factor [14] and has direct vasoconstrictor actions on vascular smooth muscle [15]. Therefore, there is a need for more stable and nitric oxide-specific, endothelial cell stimulants.

Key words: acetylcholine, NG-monomethyl-L-arginine, noradrenaline, substance P, venous occlusion plethysmography.

Abbreviation: l-NMMA, NG-monomethyl-L-arginine.

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Substance P is an 11-amino acid peptide with a physiological role as a neurotransmitter in the central, peripheral and enteric nervous systems. In addition, administration of substance P intradermally causes an acute inflammatory response [16] and, intra-arterially, an endothelium-dependent vasodilatation [17]. Substance P stimulates neurokinin type 1 receptors [18] on endothelial cells to generate nitric oxide [19]. Increasingly, it is being used to assess endothelial cell function in health and disease in man [11, 20–23]. However, direct comparisons between acetylcholine and substance P are lacking. Moreover, previous assessments of the nitric oxide-dependence of acetylcholine- [4] and substance P- [19] induced vasodilatation, have not controlled for the baseline vasoconstriction produced by nitric oxide synthase inhibitors such as N⁵-monomethyl-L-arginine (l-NMMA).

Therefore, the aim of the present study was to compare the degree of nitric oxide dependence of the vasodilator responses to acetylcholine and substance P using l-NMMA in the forearm of healthy man. Additionally, these responses were assessed in the presence of a control vasoconstrictor, noradrenaline.

METHODS

Subjects

Nine healthy male subjects aged between 20 and 34 years participated in a series of six studies which were undertaken in accordance with the Declaration of Helsinki (1989) of the World Medical Association and with the approval of the Lothian Research Ethics Committee. The written informed consent of each subject was obtained before the study. No subject received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h and from food, caffeine-containing drinks and tobacco for at least 4 h before each study. All studies were performed in a quiet, temperature-controlled room maintained at 23.5–24.5°C.

Drugs

Pharmaceutical-grade substance P [Clinalfa, Calbiochem-Novabiochem (UK) Ltd, Nottingham, U.K.], acetylcholine [Iolab, Bracknell, U.K.], l-NMMA [Clinalfa, Calbiochem-Novabiochem (UK) Ltd] and noradrenaline (Sanofi Winthrop Ltd, Guildford, U.K.) were administered intra-arterially. The compounds were dissolved in physiological saline (0.9% NaCl; Baxter Healthcare Ltd). To prevent its oxidation, noradrenaline was dissolved in saline containing 0.1% ascorbic acid (Evans Medical Ltd, Langhurst, U.K.).

Intra-arterial administration

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, U.K.) attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, Kent, U.K.) under 1% ligno-caine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, Herts, U.K.) local anaesthesia. Patency was maintained by infusion of saline via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, Hants, U.K.). The total rate of intra-arterial infusion was maintained constant throughout all studies at 1 ml/min.

Measurements

Blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges that were applied to the widest part of the forearm [24]. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington, DC, U.S.A.). Upper arm cuffs were inflated intermittently to 40 mmHg pressure for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analog voltage output from an EC-4 strain gauge Plethysmograph (D.E. Hokanson) was processed by a MacLab® analog-to-digital converter and Chart® v3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded on to a Macintosh Classic II computer (Apple Computers Inc, Cupertino, CA, U.S.A.). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751; Takeda Medical Inc, Tokyo, Japan) [25].

Study design (Fig. 1)

Subjects rested recumbent throughout each study. Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm was cannulated. To allow for equilibration, saline was infused
for the first 30 min, with baseline forearm blood flow measurements being made every 10 min. In all of the studies, substance P and acetylcholine were infused for 6 min at each dose. Forearm blood flow measurements were made for the last 3 min of each infusion period.

Eight subjects attended each of 3 study days separated by at least 1 week. On each of the days, the subjects received three incremental doses of substance P at 0.67, 1.35 and 2.71 ng/min (0.5, 1 and 2 pmol/min) followed by two further challenges of the same three incremental doses, each separated by 30 min of saline. The second and third incremental dose challenges were accompanied by co-infusion of one of the following (see Fig. 1): saline (protocol 1); L-NMMA (protocol 2) at 0.75 mg/min (4 µmol/min), or noradrenaline (protocol 3) at 40 ng/min (120 pmol/min) for the second challenge and 80 ng/min (240 pmol/min) for the third.

These three protocols were repeated with substitution for substance P of incremental doses of acetylcholine at 5, 10 and 20 pg/min (27.5, 55 and 110 nmol/min). The three protocols, as well as the substance P and acetylcholine phases were fully randomized. Again, eight subjects completed the 3 study days with seven subjects common to both substance P and acetylcholine phases.

Data analysis and statistics

Plethysmographic data were extracted from the Chart data files, and forearm blood flows were calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel v4.0; Microsoft). Recordings from the first 60 s after wrist-cuff inflation were not used, because of the instability in blood flow that this causes [26]. Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. Basal blood flow was taken to be the final recording of the saline equilibration phase before drug infusion. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point: in effect using the non-infused arm as a contemporaneous control for the infused arm [26].

Data were examined by two-factor analysis of variance with repeated measures and paired Student's t-test using Excel v4.0 (Microsoft) as appropriate. All results are expressed as means±SEM. Statistical significance was taken at the 5% level.

RESULTS

Substance P and acetylcholine responses

Throughout, there were no significant changes in blood pressure, heart rate or forearm blood flow in the non-infused arm. Mean absolute forearm blood flows in the infused arm are shown in Table 1.

Both substance P and acetylcholine caused dose-dependent increases in blood flow (P<0.001 for each). There were no significant differences (two-factor analysis of variance) between the three incremental dose challenges obtained during co-infusion of saline, when separated by a 30 min washout period, with either substance P or acetylcholine (Figs 2 and 3). The magnitude of the responses to acetylcholine was significantly greater than that to substance P (P<0.001).

Both substance P and acetylcholine infusions caused transient and reversible flushing in the infused forearm. Although an inflammatory mediator, substance P infusion did not produce discomfort or pruritis, and there were no signs of oedema or inflammation.

Noradrenaline responses

Noradrenaline caused a mean reduction in baseline blood flow of 38±5% at 40 ng/min and 51±4% at 80 ng/min in the substance P phase (P<0.001 for both compared with baseline), and

<table>
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<tr>
<th></th>
<th>Substance P (ng/min)</th>
<th>Acetylcholine (µg/min)</th>
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<tbody>
<tr>
<td></td>
<td>0.67</td>
<td>1.35</td>
</tr>
<tr>
<td>(a) Saline</td>
<td>5.8±0.9</td>
<td>7.4±1.3</td>
</tr>
<tr>
<td>L-NMMA (0.75 mg/min)</td>
<td>4.6±0.6</td>
<td>4.1±0.6</td>
</tr>
<tr>
<td>Noradrenaline (40 ng/min)</td>
<td>4.8±1.1</td>
<td>5.4±1.0</td>
</tr>
<tr>
<td>(b) Saline</td>
<td>5.6±0.9</td>
<td>6.8±1.0</td>
</tr>
<tr>
<td>L-NMMA (0.75 mg/min)</td>
<td>2.9±0.4</td>
<td>3.1±0.4</td>
</tr>
<tr>
<td>Noradrenaline (80 ng/min)</td>
<td>3.7±0.6</td>
<td>4.3±0.6</td>
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34±6% and 37±7% in the acetylcholine phase (P≤0.002 for both compared with baseline). There were no significant differences in the degree of vasoconstriction induced by noradrenaline between the two protocols, although there was a trend for the 80 ng/min vasoconstriction to be greater in the acetylcholine phase (P = 0.06).

In comparison with saline (protocol 1), the percentage blood flow increases (area under the curve) attained in the presence of noradrenaline were significantly greater for both substance P (P = 0.05) and acetylcholine (P = 0.03) (Figs 2 and 3).

L-NMMA responses

L-NMMA at 0.75 mg/min caused a mean reduction in baseline blood flow of 45±5% and 42±6%
in the substance P and acetylcholine phases, respectively (P<0.001 for both). There were no significant differences in the L-NMMA-induced vasoconstriction between the acetylcholine and substance P phases.

L-NMMA inhibited mean forearm blood flow responses to both substance P (P<0.001) and acetylcholine (P = 0.05) (Figs 2 and 3). In comparison with saline responses (protocol 1), L-NMMA caused a mean inhibition (area under the curve) of 69±8% for substance P responses and 40±5% for acetylcholine responses. However, when comparing responses in the presence of noradrenaline (protocol 3), L-NMMA caused a mean inhibition of 81±5% for substance P responses and 58±3% for acetylcholine responses. The percentage inhibition of substance P responses was significantly greater than that with acetylcholine (P = 0.02).

**DISCUSSION**

When examining endothelial cell physiology, as well as a need for selective antagonists, there is great value in agents that can cause specific and selective induction of a single endothelial cell mediator in a consistent and reproducible manner. Although selective inhibitors of nitric oxide synthase exist, such as L-NMMA, there are currently no endothelial cell stimulants available which selectively cause only nitric oxide release.

Acetylcholine is the predominant endothelial cell stimulant currently used but, as already discussed, it has limitations. In particular, its instability and the dependence of responses on basal blood flow and conduit vessel length are important confounding factors which can independently account for large disparities in responses between subject groups [13]. In contrast, substance P is relatively stable, with a plasma half-life of approximately 100 s in man [27]. Our study using L-NMMA and noradrenaline (as a control vasoconstrictor agent) has shown that vasodilatation caused by substance P is more dependent upon nitric oxide on a direct comparison with acetylcholine using doses commonly applied in other clinical studies [4, 13, 19, 22, 23]. However, a limitation of the study is the finding that the magnitude of the vasodilator responses to acetylcholine was significantly greater than that to substance P. Thus, caution should be applied to the extrapolation of our study findings to higher doses of substance P with equivalent vasodilator responses to those seen with acetylcholine.

Substance P is an inflammatory mediator that can cause an acute oedematous reaction when injected intra-dermally [16]. Therefore, arterial administration of substance P has the potential to cause tissue oedema and plasma protein extravasation. These effects may affect the baseline forearm circumference, but not the rate of forearm expansion with plethysmographic measurements unless the rate of oedema formation was to approach that of forearm blood flow or oedema formation were to raise tissue extracellular fluid pressure above 40 mmHg. However, these effects would be associated with substantial tissue swelling, which would be readily apparent by eye. We have observed no skin oedema or tissue swelling at doses of up to 2 pmol/min of substance P and, therefore, this issue is unlikely to be of relevance in the interpretation of these studies.

Vasodilatation induced by continuous infusions of acetylcholine [28] and substance P [29] undergoes progressive diminution with time, i.e. tachyphylaxis. We have found no significant attenuation in the magnitude of responses to either of these agents using brief incremental infusions which are then repeated after a 30 min saline washout. Thus, the provision of a suitable washout period appears to prevent the development of tachyphylaxis with repeated challenges of these agents and allows their use in comparative studies such as our own.

Comparing the effects of L-NMMA and saline, Vallance et al. [4] have reported a 44% inhibition of the responses to acetylcholine, and Cockcroft et al. [19] have reported a 70% inhibition of the responses to substance P. These results are very similar to our own, which, using comparable doses, have shown that L-NMMA caused a 40% and 69% inhibition of responses to acetylcholine and substance P, respectively. However, we have also shown that noradrenaline, at doses which caused similar vasoconstriction to that with L-NMMA, augmented the percentage increases in blood flow associated with these two vasodilators. This effect may reflect either a reduction in basal blood flow exposing resistance vessels to a higher concentration of the vasodilators [28], or an accentuated response and/or sensitivity to the vasodilators associated with the change in vascular smooth muscle tone and geometry. Moreover, reductions in basal blood flow and resistance vessel diameter will mean that responses to vasodilators will be greater in percentage terms, although absolute increases may be of a similar magnitude. These ‘non-specific’ effects cause an underestimation of the inhibition of the responses by L-NMMA when compared with those to saline, an effect that has been reported previously with bradykinin [30]. Correcting for this effect, we find 58% inhibition with acetylcholine and 81% inhibition with substance P. This interpretation makes the assumption that noradrenaline does not interact with acetylcholine or substance P except through its vasoconstrictor action. However, a direct interaction of noradrenaline with substance P is unlikely given the neutral effect of adrenergic blocking agents on the vasodilator response to substance P [31].

Thus, we have shown that when examining endothelium-dependent vasodilator responses at doses that are relevant to assess endothelial dysfunction [4, 13, 19, 22, 23], substance P is more nitric oxide-specific than acetylcholine. Given its greater stability, substance P may, therefore, be a more
appropriate pharmacological tool to use in the assessment of endothelial cell stimulation of nitric oxide release.

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REFERENCES