Flow-mediated nitric oxide activity in the forearm vasculature of premenopausal women

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Aims Nitric oxide (NO) is involved in acute flow-mediated vasodilatation in various vascular beds. We determined whether acutely increasing flow in the human forearm of premenopausal women increases vascular NO activity.

Methods Forearm blood flow (FBF) was measured by venous occlusion plethysmography. Responses to brachial artery infusion of noradrenaline (a control vasoconstrictor, 20, 50, and 100 ng min⁻¹, each for 5 min) and N^G-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor (200, 400, and 800 µg min⁻¹, each for 5 min), were determined in eight premenopausal women before and following elevation of basal FBF with glyceryl trinitrate (GTN) on two separate occasions.

Results Flow elevation with GTN increased responses to L-NMMA (summary measure 103 ± 12 *vs* 65 ± 12 arbitrary units, *P* < 0.05), but not to noradrenaline (95 ± 35 *vs* 74 ± 12, *P* = 0.50).

Conclusions Acute elevation of FBF in nonpregnant women is associated with enhanced responses to NO synthase inhibition, consistent with flow-mediated increased NO activity.

Keywords: blood flow, L-NMMA, nitric oxide, noradrenaline, shear stress

Introduction

As blood flow through a conduit artery increases, the vessel dilates [1, 2]. Flow-induced vasodilatation appears to serve to maintain vascular homeostasis by keeping physiological shear stress at a relatively constant level. The underlying mechanism is unknown but it has been shown to be mediated by local, non-neurogenic factors [3], which are partly dependent upon an intact endothelium, and involve nitric oxide (NO) [4].

Endothelial production of NO in response to blood flow [5] has been demonstrated in animals *in vivo* [6] and *in vitro* [5–8]. Exposure of human umbilical venous endothelial cells to steady laminar flow results in elevated endothelial NO synthase (eNOS) levels [9]. In the human forearm simultaneous measurements of forearm blood flow (FBF) and brachial artery diameter have been used to

demonstrate flow-induced dilatation [2, 10] but a role for NO in this phenomenon has not been clearly established.

No *in vivo* human studies have investigated the effects of local acute elevation of blood flow on NO activity in the human forearm vasculature. Based on a hypothesis that acute elevation of FBF is associated with flow-mediated increases in vascular NO activity, we sought to determine FBF responses to NO synthase inhibition in premenopausal women following acute flow elevation by brachial artery infusion of glyceryl trinitrate (GTN). We chose to study women because flow-mediated vasodilatation may be an important mechanism of pregnancy-associated NO mediated vasodilatation.

Methods

Subjects

Eight healthy nonpregnant women who were not on hormonal contraception were studied. All subjects were nonsmokers, and refrained from alcohol and caffeine for a minimum of 4 h before each study. Written informed consent was obtained and the studies were approved by the Newcastle Joint Ethics Committee. Constrictor responses

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of these subjects to noradrenaline have been compared with a group of pregnant subjects in a previous study [11].

Study protocol

Studies were carried out in a quiet temperature-controlled laboratory ($25 \pm 1^\circ\text{C}$) with the subjects in the supine position. Forearm blood flow (FBF) was measured simultaneously in both arms by galidinium-in-silastic strain-gauge plethysmography. Drugs or physiological saline were infused at 1 ml min^{-1} continuously into the brachial artery of the nondominant arm through a 27 SWG needle introduced under local anaesthesia. At the start of each study, after inserting the needle, basal blood flow measurements were taken for 30 min (during infusion of saline) to establish resting control values. During the recording periods, circulation through the hands was excluded by inflating wrist cuffs to a pressure of 200 mmHg. The upper arm congesting cuffs were inflated to 40 mmHg for 10 s in five 15 s cycles to occlude venous return. Blood pressure was measured in the control arm every 15 min using a semiautomated oscillometric device.

All drugs were dissolved in sterile physiological saline immediately before infusion.

After resting control FBF measurements during saline infusion had been obtained, basal flow was elevated to about $4 \text{ ml } 100 \text{ ml}^{-1} \text{ min}^{-1}$ by a 10 min infusion of GTN (median dose 10 ng min^{-1} , range $10\text{--}25 \text{ ng min}^{-1}$). Glyceryl trinitrate was then coinfused with three doses of noradrenaline (20, 50, and 100 ng min^{-1} , each for 5 min). Thereafter GTN was re-infused until FBF returned to baseline values before noradrenaline, and then three doses of L-NMMA (200, 400, and $800 \text{ } \mu\text{g min}^{-1}$, each for 5 min) were coinfused with GTN to produce a cumulative dose-response. Blood flow was recorded during the last 2 min of each 5 min drug infusion period. Responses of subjects to noradrenaline and L-NMMA were restudied on a separate occasion but within the same phase of the menstrual cycle without elevation of flow. The order of administration of noradrenaline and L-NMMA was not randomized because of the persisting effects of L-NMMA observed in preliminary studies.

Data capture and statistical analysis

Data were recorded directly onto computer using a MacLab[®] system (AD Instruments Pty Ltd, Castlehill, Australia) with on-line slope analysis to determine FBF. The average of the five slopes for each measurement period was derived to determine FBF. Forearm blood flow was expressed as $\text{ml } 100 \text{ ml}^{-1} \text{ forearm min}^{-1}$. Forearm vascular resistance (FVR) was derived from mean arterial pressure (MAP) and baseline FBF. Differences in baseline

heart rate, blood pressure, and FBF between groups were compared by analysis of variance (ANOVA). Within subject differences in FBF in both control and infused arms were also compared by repeated measures ANOVA.

For comparison of drug responses between study groups, FBF responses were expressed as a percentage of FBF during baseline infusion of GTN (for the first study involving elevation of FBF with GTN) or saline (for the repeat studies where FBF was not elevated). The overall response to each drug in each subject studied was determined by summary measures the general principles of which have been reviewed by Mathews *et al.* [12]: the sum of the percentage constrictor responses for the three doses of the infused drug (arbitrary units), and by the maximal percentage response to each drug. Values were expressed as means (s.e. mean), and differences between paired responses were compared using WInterval (one-sample Wilcoxon confidence interval) nonparametric analysis as data were not uniformly distributed. $P < 0.05$ was considered statistically significant.

Instruments and drugs

Mercury strain gauges and plethysmographs were supplied by D.E. Hokansen Incorporated (Bellevue, Washington, USA) while the twin cuff inflators were obtained from Techno-Medical Services Limited (Surrey, UK). The continuous syringe infusion pumps were obtained from Welmed Limited (Bramley, UK). The 27SWG needles were obtained from Coopers Needleworks Limited (Birmingham, UK). The blood pressure oscillometric device used was the Accutorr 4, obtained from Datascope Corp. (Hoecelaken, California, USA). L-NMMA was obtained from Clinalfa AG (Laufelengen, Switzerland). Noradrenaline (Levophaed) was obtained from Sanofi Winthrop Laboratories (Surrey, UK), and GTN was obtained from David Bull Laboratories (Warwick, UK).

Results

Subject details are shown in Table 1. Systemic blood pressure and FBF in the noninfused control arm did not vary significantly during any study.

The effects of acute flow elevation on constrictor responses are summarized in Figure 1. Forearm blood flow (mean $\pm$ s.e. mean) following 30 min of saline infusion did not differ between studies (1.9 ± 0.2 vs $2.5 \pm 0.3 \text{ ml } 100 \text{ ml}^{-1} \text{ min}^{-1}$, 95% CI for difference $-1.2, 0.06$, $P = 0.16$). Infusion of GTN significantly increased FBF from 2.5 ± 0.3 to $4.2 \pm 0.6 \text{ ml } 100 \text{ ml}^{-1} \text{ min}^{-1}$ (95% CI for difference $-2.9, -0.5$; $P < 0.05$). During the study with FBF elevated by GTN, the basal flow prior to administration of noradrenaline was not different from that prior to infusion of L-NMMA (4.2 ± 0.6 vs 4.9 ± 0.7 ,

respectively, 95% CI for difference $-1.9, 0.3$; $P=0.13$). Similarly, there was no difference in basal flow prior to the infusion of noradrenaline and L-NMMA in the study where FBF was not increased with GTN (1.9 ± 0.2 vs 2.1 ± 0.2 , respectively, 95% CI for difference $-0.5, 0.31$; $P=0.4$). Constrictor responses to L-NMMA were increased following flow elevation; maximal response 29 ± 5 vs $42 \pm 5\%$, 95% CI for difference $1.2, 25$; $P<0.05$, and summary response 65 ± 12 vs 103 ± 12 , 95% CI for difference $14, 62$; $P<0.05$. In contrast, responses to noradrenaline were unaltered by flow elevation; maximal

response 31 ± 4 vs $39 \pm 10\%$, 95% CI for difference $-17, 33$, $P=0.50$, and summary response 74 ± 12 vs 95 ± 30 , 95% CI for difference $-53, 68$; $P=0.14$.

Discussion

This study has demonstrated that in premenopausal women, acute elevation of FBF by local infusion of GTN is associated with enhanced responses to NO synthase inhibition. This is the first human *in vivo* study using local rather than systemic flow elevation to demonstrate increased flow-mediated vascular NO activity.

Although the number of subjects was small, constrictor responses to noradrenaline, employed as a control comparator vasoconstrictor, were unchanged by acute flow elevation. Our observation of augmented vasoconstrictor responses to L-NMMA by acute flow elevation is consistent with a specific flow-mediated increase in forearm vascular NO activity. Similar observations have been made in other studies on the forearm vascular bed. Using a reactive hyperaemia model, Joannides *et al.* [13] found that local infusion of L-NMMA markedly reduced the duration of the hyperaemic response, confirming earlier observations that the delayed phase of hyperaemia may be partly the consequence of NO-mediated, flow-induced

Table 1 Subject details.

<i>n</i>	8
Age (years)	23 (19–34)
Weight (kg)	60 (3.0)
Forearm volume (ml)	710 (31)
SBP (mmHg)	126 (3)
DBP (mmHg)	69 (2)
Heart rate	85 (2)
FVR (mmHg ml ⁻¹ min ⁻¹ 100 ml ⁻¹)	
Normal basal flow	37.6 (3)
Basal flow with GTN	24.6 (3)

Values are means (s.e. mean) or median (range).

Figure 1 Forearm blood flow responses (mean $\pm$ s.e. mean) to noradrenaline and L-NMMA in eight healthy female subjects with basal flow elevated with GTN (●) vs normal basal flow (○). Basal flow denotes FBF during saline infusion only (open circles) or FBF following flow elevation by GTN infusion (filled circles). Responses to L-NMMA were increased following flow elevation ($P<0.01$) whereas responses to noradrenaline were unaltered.

vasodilatation [14]. However myogenic, neural, and local factors such as adenosine, probably play a critical role in modulation of reactive hyperaemic responses to transient interruption of blood flow [15], and may contribute to the release of NO in these experiments rather than flow. Calver *et al.* [16] reported enhanced responses to L-NMMA following acute systemic saline infusion. However, the responses to noradrenaline were also enhanced suggesting a nonspecific effect possibly related to alterations in vascular myogenic tone, systemic baroreflex function, or changes in sympathetic activity [17].

We chose to elevate flow locally by intrarterial administration of subsystemic doses of GTN, an endothelium-independent nitrovasodilator, in order to obviate any confounding systemic effects on drug responses. It is possible that the infusion of a NO donor may have a feedback effect on endothelial NO synthesis [18]. However, if such phenomenon occurs, it would be expected to lead to attenuated NO synthesis, and blunted responses to NO synthase inhibition, rather than the enhanced responses observed in these studies.

An advantage of our *in vivo* study model is that it enables investigation of vascular NO activity in response to exposure to a physiologic flow medium. It does not allow uncoupling of the two-directional effects of flow force on the vessel wall; perpendicular (transmural pressure) and tangential (shear stress) to flow direction. This can only be achieved *in vitro* by fixing flow and varying shear stress through varying medium viscosity [19]. Such studies indicate that both shear and nonshear components are involved in endothelial NO release [19]. Sensing mechanisms that involve stimulation of mechanoreceptors and endothelial cytoskeletal rearrangement are followed by response mechanisms mediated by several signalling pathways. These signalling mechanisms involve multiple second messengers that appear to result in both acute and chronic effects [20–22]. Nitric oxide has been implicated in both acute and chronic responses [23–27].

The mechanism of the vascular NO response to acute increases in blood flow is unclear but is likely to involve channel-mediated nongenomic signalling processes.

In native endothelial cells, shear stress-induced NO production consists of an initial peak followed by a sustained plateau phase which is maintained as long as shear stress is applied [8]. The initial component of this response is likely to mediate the responses to acute flow that we have observed. It has been shown to be calcium-dependent [28], requiring calcium-activated potassium channels [7], and may be mediated through ATP binding to P_{2y}-purinoreceptors [29]. It is also possible that acute elevation of flow stimulates the endothelium to release a vasoconstrictor substance, such as endothelin [30], whose effects are normally masked by endothelial-derived NO. Indeed, chronic increases in blood flow have been shown

to up regulate endothelin-B receptors in arterial smooth muscle [31]. However the rapid time course of observations in the present study argues against a role for endothelin whose *de novo* production *in vitro* requires 2–4 h [30].

There is increasing evidence that genomic mechanisms, probably mediated by oestrogen, contribute to the endothelial NO response to flow increases in women. Isolated small mesenteric arteries from prepubertal female rats only relaxed to flow when pretreated with 17 β -oestradiol, suggesting that estradiol may facilitate flow-mediated vasodilatation [32], and may contribute to our observations in premenopausal women. Further studies of men and postmenopausal women would be of interest in determining whether gender or age effects play any role in flow-mediated vasodilatation.

In conclusion, local acute elevation of forearm blood flow in premenopausal women is associated with enhanced responses to NO synthase inhibition, consistent with flow-mediated increased NO activity.

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