Nitric oxide contributes to the rise in forearm blood flow during mental stress in humans

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1. Our aim was to determine whether the vasodilating substance nitric oxide (NO) contributes to the rise in forearm blood flow observed during mental stress in humans. We also determined whether the NO might be released as a result of cholinergic stimulation of the vascular endothelium.

2. Blood flow was measured in both forearms using plethysmography during several 3–5 min bouts of a colour word test. In one forearm the nitric oxide synthase blocker N^G-monomethyl-L-arginine (L-NMMA) and other drugs were infused via a brachial artery catheter. The contralateral forearm served as a control.

3. When L-NMMA was given prior to mental stress it blunted the rise in blood flow in the treated forearm almost completely. The normal blood flow response returned during a second bout of stress conducted after a wash-out period. During a third bout of mental stress, administration of more L-NMMA again blunted the blood flow responses to mental stress.

4. When atropine was given prior to mental stress, the increases in blood flow were reduced in the treated forearm. Subsequent administration of both atropine and L-NMMA caused a somewhat greater reduction in the blood flow responses than those observed with atropine alone.

5. These data demonstrate that NO plays a role in forearm vasodilatation during mental stress in humans. It is likely that most of the NO is released by cholinergic stimulation of the vascular endothelium.

Nitric oxide (NO) is a vasodilating molecule synthesized from L-arginine that may be important in a variety of autonomic responses (Dinerman, Lowenstein & Snyder, 1993). NO from the vascular endothelium is known to be a key local regulator of vascular tone (Vallance, Collier & Moncada, 1989). NO is also released from non-adrenergic, non-cholinergic autonomic nerves. In isolated tissues and anaesthetized animal preparations NO released directly from nerves or by cholinergic stimulation of the vascular endothelium can cause neurally mediated vasodilatation (Toda & Okamura, 1991; Broten, Miyashiro, Moncada & Feigl, 1992; Burnett, Lowenstein, Bredt, Chang & Snyder, 1992; Rajfer, Aronson, Bush, Dorey & Ignarro, 1992). In conscious humans the importance of NO as a mediator of autonomic responses is unknown.

During mental stress in humans, neurogenically mediated vasodilatation causes marked bilateral and symmetrical increases in blood flow to the forearms (Blair, Glover, Greenfield & Roddie, 1959). The mechanisms responsible for the dilatation remain obscure after many years of investigation (Greenfield, 1966; Roddie, 1977). In general, the dilatation is confined to the muscles of the forearm and is absent after local anaesthetic block or surgical section of the sympathetic nerves to the upper extremity (Blair *et al.* 1959). Additionally, it cannot be explained by withdrawal of sympathetic vasoconstrictor tone, nor is it consistently altered by administration of adrenergic or cholinergic blocking drugs (Blair *et al.* 1959; Barcroft, Brod, Hejl, Hirsjärvi & Kitchin, 1960; Anderson, Wallin & Mark, 1987). With this information as a background, we hypothesized that NO contributes to the rise in forearm blood flow during mental stress in humans.

To test this hypothesis a brachial artery catheter was used to administer the nitric oxide synthase blocker N^G-monomethyl-L-arginine (L-NMMA) and other drugs to the non-dominant forearm of thirteen healthy subjects. Forearm blood flow was measured bilaterally using venous occlusion plethysmography with the contralateral forearm

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serving as the control. Blood flow was measured at baseline and throughout successive bouts of mental stress produced by the Stroop colour word test. L-NMMA was given prior to mental stress in some studies. In others atropine was given before L-NMMA in an effort to block any cholinergically mediated NO release from the vascular endothelium.

METHODS

Subjects

Before beginning this study we obtained permission from the United States Food and Drug Administration to administer L-NMMA to humans. The study was approved by the Institutional Review Board. A total of thirteen healthy subjects (6 females and 7 males) between the ages of 18 and 42 years were studied. Each subject gave written informed consent. Prior to the administration of L-NMMA, the female subjects had negative pregnancy test results. It should be noted that methylated arginine compounds such as L-NMMA are normally present in mammalian tissues (including human) and have well-defined clearance pathways (Nakajima, Matsuoka & Kakimoto, 1971; Carnegie, Fellows & Symington, 1977). Additionally, the experience to date with L-NMMA in humans and other species indicates that low doses are non-toxic (Vallance *et al.* 1989; Kilbourn *et al.* 1992).

All subjects were initially brought to the laboratory for a brief orientation and screening study. This was done to familiarize the subjects with the experimental techniques and to insure that the blood flow responses to mental stress were marked and symmetrical in both forearms. Subjects were studied in a reclining chair with the head and chest elevated at a 30 deg angle. Some studies were performed in the morning and others in the afternoon. The subjects were instructed to abstain from caffeine and avoid eating a heavy meal in the 3 h prior to the study. Throughout all studies, the laboratory temperature was maintained between 21 and 23 °C.

Mental stress

Mental stress was produced using 3–5 min bouts of a computerized version of the Stroop colour word test (Stroop, 1935). In pilot studies, it was noted that the blood flow responses to mental stress diminished during successive bouts of the Stroop test. Therefore, the test was altered to have three levels of difficulty; each required the mental tasks to be performed at a faster speed than the previous level. The first (easiest) level was used for subject familiarization and the second or third level was used during the actual experimental trials. In the 30 s prior to the test, the subjects received stereotyped instructions urging them that their best effort was required and were told that their performance on the test was being compared with both a 'laboratory standard' and their previous percentage correct. Additionally, the computer program provided the subjects with auditory distraction and verbal messages expressing concern over the accuracy of their responses during the actual test. These manoeuvres were used to increase the stress experienced during the test.

Forearm blood flow

Forearm blood flow was measured in both forearms using venous occlusion plethysmography with mercury in silastic strain gauges (Greenfield, Whitney & Mowbray, 1963). Forearm blood flow was measured 4–6 times in the 2–3 min prior to mental stress, twice during the verbal instructions, and every 15 s throughout mental stress.

Arterial catheterization

A 20 gauge, 5 cm brachial artery catheter was inserted using aseptic techniques after local anaesthesia with 2 ml of 1% lignocaine. The catheter was connected to a pressure transducer and continuously flushed at 3 ml h^{-1} with saline containing 2 U ml^{-1} heparin. A three-port connector was placed in series with the catheter–transducer system. One port was used to measure arterial pressure and the two other ports were used for drug infusions.

Drug preparation and administration

L-NMMA was obtained from Calbiochem (La Jolla, CA, USA). The purity of the L-NMMA was independently confirmed by the Mayo Clinic Drug Measurement Laboratory using high-performance liquid chromatography. Using strict aseptic techniques, this material was then dissolved in preservative-free, sterile 0·9% saline and filtered through a 0·22 μm filter. The solution was then pyrogen tested. Commercially available pharmaceutical grade acetylcholine (ACh; IOLAB Pharmaceuticals, Claremont, CA, USA), sodium nitroprusside (Elkins-SINN, Inc., Cherry Hill, NJ, USA), and atropine (Gensia Pharmaceuticals, Inc., Irvine, CA, USA) were also used.

The ACh was administered periodically in bolus doses of 64 μg min^{-1} given intra-arterially to stimulate the release of NO from the vascular endothelium. In this way it was used to test the efficacy of the L-NMMA in blocking NO synthase. A marked blunting of the vasodilator response to ACh is consistent with blockade of NO synthase in the vascular endothelium (Vallance *et al.* 1989). Sodium nitroprusside was administered periodically in bolus doses of 10 μg min^{-1} given intra-arterially to demonstrate the continued ability of the forearm vasculature to dilate after L-NMMA administration (Vallance *et al.* 1989). In some studies the forearm was pretreated with 0·2–0·4 mg atropine to block muscarinic cholinergic stimulation of the forearm vasculature (Blair *et al.* 1959). The efficacy of the cholinergic blockade was also tested with bolus doses of ACh.

Subject monitoring

During drug infusion trials arterial pressure and heart rate were monitored using the pressure signal from the arterial catheter.

Protocols

Protocol 1: effects of L-NMMA on forearm vasodilatation during mental stress. Seven subjects (3 females and 4 males) participated in this experiment. First, a brachial artery catheter was placed in the non-dominant arm. Both arms were then instrumented to measure forearm blood flow and resting values were obtained. ACh was then administered and changes in forearm blood flow measured. When blood flow returned to baseline, L-NMMA was administered at 4 mg min^{-1} for 5 min. ACh was then given to confirm a blunting of the vasodilator responses consistent with NO synthase blockade. After additional baseline measurements, the first colour word test was performed and the forearm blood flow responses measured. After a rest period of about 1 h, a dose of ACh was again given to demonstrate the return

of cholinergically mediated forearm vasodilatation. Following baseline measurements, the second Stroop test was then administered. Next, additional L-NMMA at a dose of 2 mg min^{-1} for 10 min was given and the extent of the NO synthase blockade tested with a bolus dose of ACh. After baseline measurements, the third colour word test was performed. At the end of the experiment a dose of sodium nitroprusside was administered to show that the forearm could vasodilate in the presence of L-NMMA.

Protocol 2: effects of atropine followed by L-NMMA on forearm vasodilatation during mental stress. Six subjects (3 females and 3 males) participated in this experiment. The subjects were instrumented as in protocol 1. After baseline forearm blood flow values were obtained, ACh was administered and changes in forearm blood flow were measured. When blood flow returned to normal after the ACh, 0·2 mg atropine was given in the brachial artery to block the muscarinic cholinergic receptors in one forearm. ACh was given again to confirm the absence or a marked (~90%) reduction of the cholinergic vasodilator response. Another 0·2 mg dose of atropine was then administered and this was followed immediately by baseline measurements and a 5 min bout of the colour word test.

The atropine was then allowed to wear off for 30 min and a partial return of the vasodilator response to ACh was documented with a bolus dose of ACh. L-NMMA was then infused at a rate of 4 mg min^{-1} for 5 min, and the blockade of NO synthase was confirmed by a blunting of the vasodilator response to ACh. This was followed by L-NMMA given at 2 mg min^{-1} for 10 min and another 0·2 mg dose of atropine. Following baseline measurements, a second 5 min bout of the Stroop test was administered and the forearm blood flow responses recorded. After the second Stroop test, the blunting of the vasodilator response to ACh was again confirmed. Sodium nitroprusside was then given to confirm that the forearm could vasodilate in the presence of atropine and L-NMMA.

Data analysis

Baseline blood flow, arterial pressure, and heart rate values are the mean values obtained during each of the 2 min that preceded the 'instruction' phase of the stress test. In some subjects there were conspicuous and abrupt increases in forearm blood flow during the instructions, and these values were considered to be part of the responses to stress (Roddie, 1977). In those who showed no change during the instruction, the onset of stress was defined as the point at which the computer program was started. 'Peak' changes in forearm blood flow were determined for each individual trial on the basis of the largest changes seen in the control forearm during the first 2·5 min after the onset of stress. Data from only 2·5 min of each bout of mental stress are presented because several of the subjects were noted to become extremely restless and sigh heavily as the bouts of stress continued. These activities made interpretation of the plethysmographic measurements of forearm blood flow unreliable during the final minutes of mental stress in these subjects. While this approach to the data analysis may seem flexible, it is required because the physiological responses to mental stress, even within the same subject, can be extremely variable (for discussion of these issues, see Roddie, 1977).

Statistics

In both protocols, each subject served as their own control. The responses in the treated forearm were compared with those in the control forearm. Data are expressed as means ± S.E.M. When appropriate, the comparisons were made using Student's paired t tests or two-way analysis of variance (treatment and time). Significance was set at $P < 0·05$ for all protocols.

RESULTS

Protocol 1

In the forearm that received L-NMMA, forearm blood flow (FBF) was 1·9 ± 0·4 ml (100 ml)$^{-1}$ min^{-1} prior to drug infusion and rose to 17·1 ± 3·1 ml (100 ml)$^{-1}$ min^{-1} with ACh. After L-NMMA infusion baseline FBF was reduced to 0·9 ± 0·1 ml (100 ml)$^{-1}$ min^{-1} and rose to only 3·9 ± 1·8 ml (100 ml)$^{-1}$ min^{-1} after ACh administration

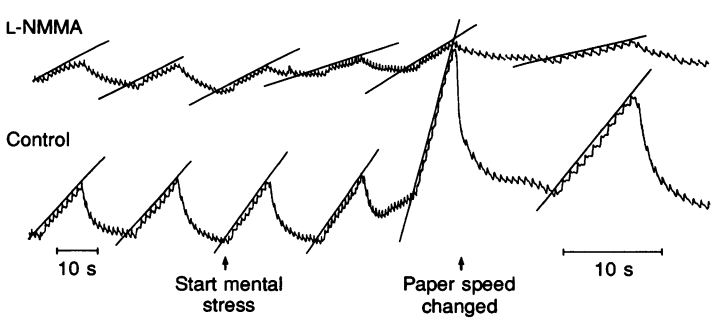

Figure 1. Experimental record of blood flow responses to mental stress
Original plethysmographic record of the forearm blood flow (FBF) responses to mental stress. FBF is proportional to the slope of the plethysmographic tracing. An increase in slope means that FBF rose. The top tracing shows the response in the forearm treated with the NO synthase blocker L-NMMA. The bottom tracing shows the control forearm. Baseline FBF was lower in the forearm treated with L-NMMA. The marked rise in FBF that commenced in the control forearm about 40 s after the onset of mental stress was absent in the forearm treated with L-NMMA. This demonstrates the key role NO can play during neurogenic vasodilatation in the forearm during mental stress. The recorder speed was increased after the dramatic rise in flow to facilitate analysis of the tracing.

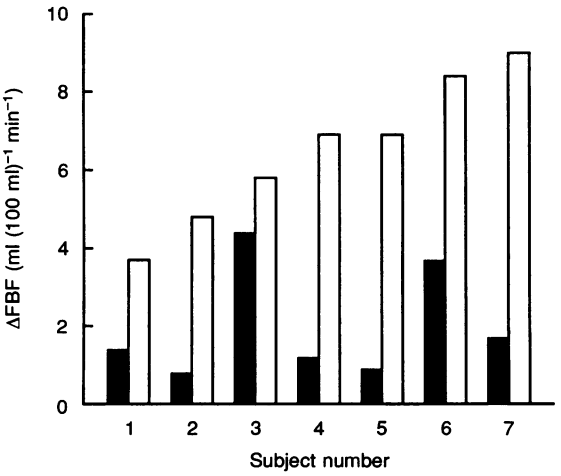

Figure 2. Peak blood flow responses to mental stress
Peak changes in FBF above baseline during the first trial of mental stress in each of the seven subjects. The lower responses seen in the experimental arm of each subject after receiving the NO synthase blocker L-NMMA demonstrate the consistency of the NO-mediated vasodilatory responses to mental stress. ■, experimental forearm; □, control forearm.

(both $P < 0{\cdot}05$ *vs.* pre-L-NMMA values). In the control arm FBF was $1{\cdot}8 \pm 0{\cdot}3$ ml (100 ml)$^{-1}$ min^{-1} before L-NMMA and $1{\cdot}4 \pm 0{\cdot}2$ ml (100 ml)$^{-1}$ min^{-1} after L-NMMA ($P > 0{\cdot}05$). In response to L-NMMA treatment there was a non-significant rise in mean arterial pressure from 80 ± 4 to 83 ± 3 mmHg, confirming that intra-arterial infusion in the forearm did not cause any major systemic haemodynamic changes.

During the first bout of mental stress ($n = 7$) the peak increases in FBF averaged $6{\cdot}5 \pm 0{\cdot}7$ ml (100 ml)$^{-1}$ min^{-1} in the control arm but were only $2{\cdot}0 \pm 0{\cdot}5$ ml (100 ml)$^{-1}$ min^{-1} in the arm treated with L-NMMA ($P < 0{\cdot}05$; Figs 1 and 2). In several subjects blood flow in the control arm rose conspicuously prior to the mental stress test while the subject received verbal instructions about the task. The peak FBF responses were seen at the beginning of the mental stress in some subjects; in others they occurred after a minute or two of stress.

Baseline heart rate was 74 ± 7 beats min^{-1} and rose to 99 ± 5 beats min^{-1} during the first minute of mental stress

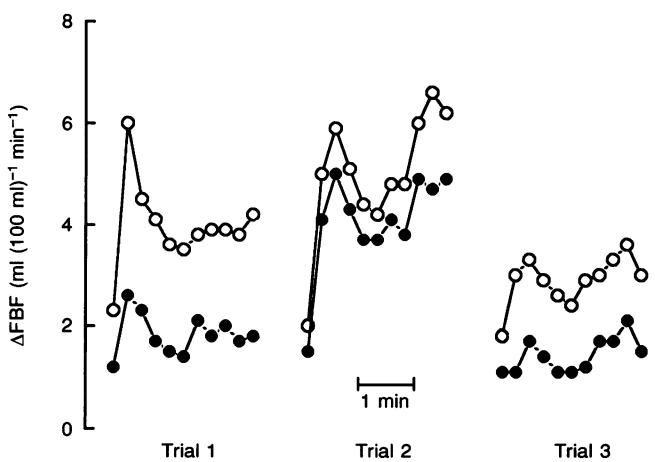

Figure 3. Individual time course of blood flow changes during mental stress
Individual FBF responses during 2·5 min of mental stress in one subject. In the first bout of mental stress the forearm blood flow (FBF) response in the arm that received L-NMMA to block NO synthase was reduced markedly. After the L-NMMA had been allowed to wash out over 1 h, the FBF responses to the second bout of mental stress were similar in both forearms. After more L-NMMA was given in the experimental forearm, the FBF response in the experimental forearm was again blunted during the third bout of mental stress. This data is consistent with the concept that NO can mediate much of the dilatory response to mental stress in humans. ●, experimental forearm; ○, control forearm.

($P < 0{\cdot}05$ *vs.* baseline). It remained at that elevated level throughout the stress and was 96 ± 5 beats min^{-1} during the third minute of mental stress. Baseline mean arterial pressure (MAP) was 89 ± 5 mmHg and increased to 92 ± 6 mmHg ($P > 0{\cdot}05$ *vs.* baseline) during the first minute of stress. During the second and third minutes of stress, MAP averaged 99 ± 4 and 99 ± 5 mmHg, respectively (both $P < 0{\cdot}05$ *vs.* baseline). These modest increases in mean arterial pressure indicate that most of the rise in FBF was caused by vasodilatation.

Before the second trial ($n = 6$) the effects of the L-NMMA were allowed to wear off over an average of 66 ± 8 min. After this recovery period FBF increased from $1{\cdot}0 \pm 0{\cdot}2$ to $12{\cdot}6 \pm 2{\cdot}5$ ml (100 ml)$^{-1}$ min^{-1} in response to ACh, indicating partial recovery of NO synthase function. During the second bout of mental stress the rise in flow in the experimental forearm was greater than that observed in the first bout ($P < 0{\cdot}05$) and approached the increase in flow seen in the control forearm (Figs 3 and 4).

Baseline heart rate was 72 ± 4 beats min^{-1} and mean arterial pressure was 86 ± 7 mmHg prior to the second bout of stress. The time course and the magnitude of heart rate and blood pressure changes were very similar to those seen during the first bout of mental stress.

The third bout of mental stress ($n = 6$) was performed after an additional dose of L-NMMA had been given. After this dose- FBF increased from $0{\cdot}8 \pm 0{\cdot}1$ to $6{\cdot}0 \pm 1{\cdot}7$ ml (100 ml)$^{-1}$ min^{-1} in response to ACh, indicating that NO synthase activity was again attenuated. L-NMMA infusion again selectively blunted the FBF responses to mental stress in the experimental forearm (Figs 3 and 4). Baseline heart rate was 73 ± 5 beats min^{-1} and mean arterial pressure was 90 ± 8 mmHg prior to the third bout of stress. Again, the changes in heart rate and arterial pressure were similar to those in the first bouts of mental stress. Marked (>500%) increases in blood flow were seen in the treated forearm when nitroprusside was administered.

Protocol 2

Prior to the first bout of mental stress, FBF was $1{\cdot}5 \pm 0{\cdot}2$ ml (100 ml)$^{-1}$ min^{-1} and rose to $16{\cdot}8 \pm 2{\cdot}0$ ml (100 ml)$^{-1}$ min^{-1} with ACh. Resting FBF in the experimental arm was $1{\cdot}4 \pm 0{\cdot}2$ ml (100 ml)$^{-1}$ min^{-1} after atropine ($P > 0{\cdot}05$ *vs.* pre-atropine value). When ACh was given after atropine FBF rose to only $2{\cdot}4 \pm 0{\cdot}5$ ml (100 ml)$^{-1}$ min^{-1} ($P < 0{\cdot}05$ *vs.* pre-atropine value). Blood flow was $1{\cdot}4 \pm 0{\cdot}2$ ml (100 ml)$^{-1}$ min^{-1} in the control forearm and did not change with either atropine or ACh administration. Intra-arterial atropine given prior to the first Stroop test in protocol 2 had no effect on heart rate or blood pressure.

During the first bout of mental stress, after treatment with atropine only ($n = 6$), the mean peak increase in FBF was $6{\cdot}6 \pm 1{\cdot}2$ ml (100 ml)$^{-1}$ min^{-1} in the control forearm but only $3{\cdot}4 \pm 0{\cdot}4$ ml (100 ml)$^{-1}$ min^{-1} in the forearm treated with atropine ($P < 0{\cdot}05$; Fig. 5). Following the first trial

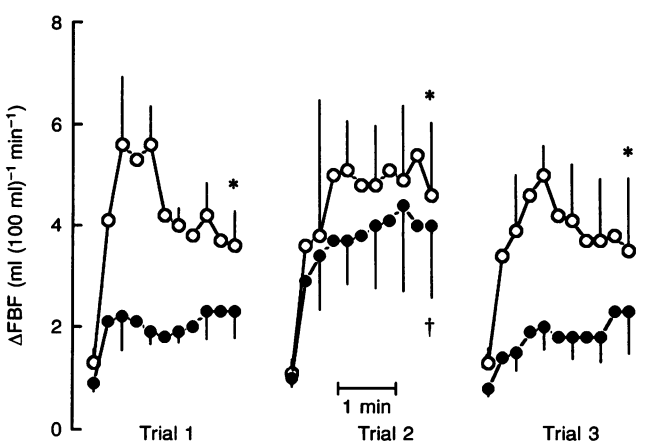

Figure 4. Time course of blood flow responses to mental stress
Mean (± S.E.M.) forearm blood flow (FBF) responses during the three bouts of mental stress in the six subjects who completed all three. In the first bout, the NO synthase blocker L-NMMA was given in one forearm and caused a selective and marked reduction in the FBF responses to mental stress. After allowing the effects of the L-NMMA to wear off, the FBF responses in the experimental arm were restored toward those observed in the control forearm during the second bout of mental stress. Additional L-NMMA in the experimental forearm caused the FBF responses to be again selectively blunted during the third bout of mental stress. These data are consistent with the concept that NO mediates most of the forearm vasodilatation observed during mental stress in conscious humans. ●, experimental forearm; ○, control forearm. Standard error values are shown for alternate data points to enhance clarity. *$P < 0{\cdot}05$ for control *vs.* experimental forearm. †$P < 0{\cdot}05$, response in the experimental forearm in trials 1 or 3 different from the response of the experimental forearm during trial 2.

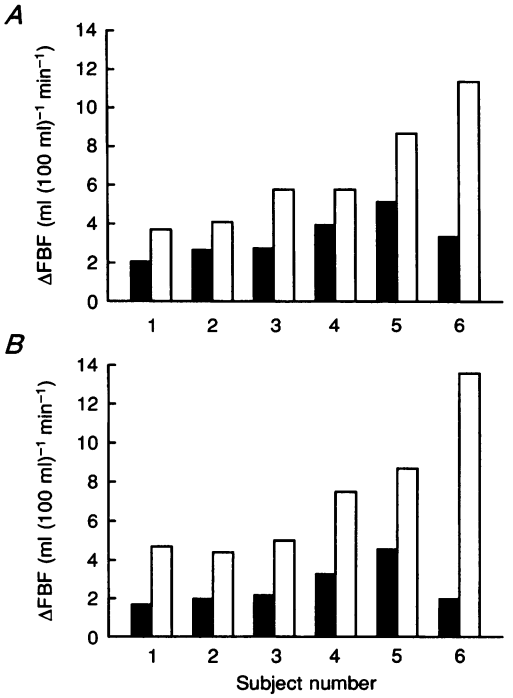

Figure 5. Effects of atropine and L-NMMA on blood flow responses to stress
Individual forearm blood flow responses in the two colour word test trials from protocol 2. *A*, change in FBF in the control forearms (□) compared to the atropine-treated forearms (■) during the first colour word test in each of the six subjects. The atropine-treated forearms had much lower peak blood flows during mental stress than the non-treated forearms. *B*, change in FBF in each subject during the second colour word test. The forearm blood flows in the forearms treated with both L-NMMA and atropine (■) were again significantly less than the blood flows in the control forearms (□). When the peak blood flows in the treated forearms were compared between trials, the flows obtained after treatment with both L-NMMA and atropine were also less than the flows seen when the forearms were treated with atropine alone. This suggests that most of the NO release was cholinergically mediated, but that some NO release might be independent of cholinergic stimulation.

the effects of the atropine were allowed to wear off over 21 ± 1 min. FBF then increased to $7{\cdot}9 \pm 2{\cdot}2$ ml (100 ml)$^{-1}$ min^{-1} in response to ACh, indicating some recovery from the cholinergic blockade.

Before treatment with L-NMMA, FBF in the experimental arm was $1{\cdot}4 \pm 0{\cdot}3$ ml (100 ml)$^{-1}$ min^{-1} and fell significantly to $1{\cdot}0 \pm 0{\cdot}2$ ml (100 ml)$^{-1}$ min^{-1} after the L-NMMA. ACh given after L-NMMA caused FBF to rise to only $3{\cdot}8 \pm 1{\cdot}4$ ml (100 ml)$^{-1}$ min^{-1} ($P < 0{\cdot}05$ *vs.* pre-L-NMMA value). During the second bout of mental stress the peak rise in flow in the control forearm was $7{\cdot}3 \pm 1{\cdot}4$ ml (100 ml)$^{-1}$ min^{-1} and $2{\cdot}6 \pm 0{\cdot}4$ ml (100 ml)$^{-1}$ min^{-1} in the experimental forearm. With atropine alone the flow increase in the treated forearm was $54{\cdot}9 \pm 5{\cdot}0$% of control. With atropine and L-NMMA this increase was only $39{\cdot}6 \pm 5{\cdot}4$% of control ($P < 0{\cdot}05$ atropine *vs.* atropine and L-NMMA). Marked (>500%) increases in FBF were seen in the treated forearm when nitroprusside was administered. The increases in heart rate and mean arterial pressure in protocol 2 were similar to those seen in protocol 1, again confirming that most of the increase in FBF was caused by forearm vasodilatation.

DISCUSSION

The major finding of this study is that NO plays a role in forearm vasodilatation during mental stress in humans. It also appears that most of this NO is released by cholinergic stimulation of the vascular endothelium.

Previous studies have indicated that intact sympathetic innervation of the upper extremity is required to observe forearm vasodilatation during mental stress (Blair *et al.* 1959). In anaesthetized animals sympathetic cholinergic vasodilatation can be observed in skeletal muscle, but marked dilatation is only seen after pharmacological intervention that interrupts adrenergic vasoconstriction (Folkow, Haeger & Uvnäs, 1948). In humans the forearm muscles have no known parasympathetic innervation. Additionally, there is no neurophysiological evidence for vasodilatory fibres to human muscle whose activity might increase during emotional stress in a manner that is independent of baroreceptor control (Wallin & Sundlöf, 1982). Microneurographically measured multiunit sympathetic nerve activity to the upper extremity in humans does not change during mental stress, but noradrenaline spillover from the forearm has been reported to increase suggesting a rise in sympathetic activity (Anderson *et al.* 1987; Lundqvist, Kahan, Melcher & Hjemdahl, 1993). Taken together, these findings suggest that the neural signals involved in forearm vasodilatation during mental stress may differ from recognized patterns of autonomic activity.

Other studies have shown that at least part of the forearm vasodilatation during mental stress can be blocked by atropine but that this response is variable (Blair *et al.* 1959; Barcroft *et al.* 1960). Finally, β-blockers can reduce calf vasodilatation during mental stress, suggesting a possible role for circulating catecholamines (Freyschuss, Hjemdahl, Juhlin-Dannfelt & Linde, 1988). This confusing picture led us to hypothesize that NO might be involved in the forearm dilator response to mental stress in humans. In protocol 1 we injected L-NMMA in the brachial artery at a dose sufficient to reduce markedly the vasodilatory responses to ACh. This

is consistent with the interpretation that NO synthase had been blocked (Vallance *et al.* 1989). After administration of L-NMMA the forearm vasodilation during mental stress was selectively and markedly blunted in the treated forearm while the responses in the control forearm remained normal. The dilatory responses to mental stress recovered when the L-NMMA was allowed to wear off but were again blunted by a second dose of L-NMMA. These observations suggest that NO contributes to the forearm vasodilatation observed during mental stress in humans. The key question remaining then concerned the mechanism of NO release during the mental stress.

Two possible mechanisms were considered. First, the NO could have been released via cholinergic stimulation of the vascular endothelium. In canine coronary arteries the vasodilatation caused by vagal stimulation is almost totally prevented by either atropine or the NO synthase blocker N^G-nitro-L-arginine methyl ester, suggesting that neurally released acetylcholine evokes NO release from the vascular endothelium to cause the vasodilatation (Broten *et al.* 1992). There is also evidence that acetylcholine might be released from some vascular endothelial cells during mechanical stimulation of the blood vessel and stimulate release of NO by neighbouring endothelial cells (Milner *et al.* 1989). Second, the NO may have been released directly from nerves. This mechanism has been demonstrated in the penile and cerebral circulations (Toda & Okamura, 1991; Holmquist, Stief, Jonas & Andersson, 1991; Rajfer *et al.* 1992; Burnett *et al.* 1992).

In an effort to address these two possibilities we conducted protocol 2. In this protocol treatment of the forearm with atropine blunted the rise in FBF during mental stress almost as much as the combination of atropine and L-NMMA. These results are qualitatively similar to those seen in the canine coronary artery and suggest that during mental stress some NO is released via cholinergic stimulation of the vascular endothelium (Milner *et al.* 1989; Broten *et al.* 1992). The additional decrease in blood flow responses after the infusion of L-NMMA suggests that there might be some NO release that is independent of cholinergic mechanisms. One possible explanation is that some NO was released directly from autonomic nerves.

How then can these experimental results be reconciled with previous information concerning forearm vasodilatation during mental stress in humans? It is particularly difficult to reconcile the observation that intact autonomic innervation of the upper extremity is required for this response with the lack of neurophysiological evidence for vasodilatory fibres and the apparent absence of cholinergic innervation of blood vessels in human forearm muscles (Wallin & Sundlöf, 1982). One possible sequence of events might operate in the following way. First, some poorly understood vasodilatory substance is released by the autonomic nerves to the forearm muscles or there is a subtle change in autonomic outflow to the upper extremity that initiates forearm vasodilatation. Second, the subsequent increase in blood flow then stimulates acetylcholine-containing endothelial cells to release their ACh. Third, the ACh then acts on muscarinic receptors on neighbouring endothelial cells and causes NO to be released and augments the vasodilatation. Finally, it is possible that some other vasodilating mechanism initiates the rise in blood flow during mental stress and that the vasodilatation is then reinforced by flow-mediated release of NO.

In summary, these experiments provide evidence that NO plays a key role in the autonomic control of the circulation during mental stress in humans. The observations are consistent with the view that most of the NO release is due to cholinergic stimulation of the vascular endothelium in the forearm muscles. The mechanisms responsible for this stimulation are unknown.

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Acknowledgements

The authors would like to thank the subjects for their participation. The technical assistance of Darrell Loeffler and Kathleen Street, and the secretarial help of Janet Beckman and Cathy Nelson are also greatly appreciated. Peter Shen's work in developing the computerized mental stress test is also appreciated. This work was supported by NIH grant HL-46493 (M. J. J.), NIH training grant GM08288 (N. M. D.), grant RR 585A to the Mayo Clinical Research Center, and the Mayo Foundation.

Received 17 November 1993; accepted 15 March 1994.