Role of nitric oxide in exercise hyperaemia during prolonged rhythmic handgripping in humans

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1. We sought to determine whether the vasodilating molecule nitric oxide (NO) contributes to the forearm hyperaemia observed during prolonged rhythmic handgripping in humans.

2. Two bouts of exercise were performed during experimental protocols conducted on separate days. During each protocol the subject performed a 10 min and a 20 min bout of rhythmic (30 min^{-1}) handgripping at 15% of maximum. Two exercise bouts were required to facilitate pharmacological interventions during the second protocol. Blood flow in the exercising forearm was measured every minute with plethysmography during brief pauses in the contractions. During both exercise bouts in the first protocol, forearm blood flow increased 2- to 3-fold above rest after 1 min of handgripping and remained constant at that level throughout the exercise.

3. During the 10 min bout of exercise in the second protocol, acetylcholine was given via a brachial artery catheter at 16 $\mu g\ min^{-1}$ for 3 min to evoke NO release from the vascular endothelium. This caused forearm blood flow to increase above the values observed during exercise alone.

4. During the 20 min trial of handgripping in the second protocol, the NO synthase blocker N^G-monomethyl-L-arginine (L-NMMA) was infused in the exercising forearm via the brachial catheter after 5 min of handgripping. The L-NMMA was infused at 4 mg min^{-1} for 10 min.

5. L-NMMA during exercise caused forearm blood flow to fall to values ~20–30% lower than those observed during exercise alone. When ACh was given during exercise after L-NMMA administration the rise in blood flow was also blunted, indicating blockade of NO synthase. These data suggest NO plays a role in exercise hyperaemia in humans.

Marked vasodilatation occurs in blood vessels that perfuse active skeletal muscles during rhythmic contractions. The mediators contributing to this response remain obscure after more than a century of investigation (Gaskell, 1880; Shepherd, 1983; Laughlin, 1987; Sheriff, Rowell & Scher, 1993). Nitric oxide (NO) is a recently identified vasodilating molecule synthesized from L-arginine in the vascular endothelium. NO release from the endothelium is known to be a key local regulator of basal vascular tone (Vallance, Collier & Moncada, 1989). The contribution of NO to exercise hyperaemia remains unclear.

A number of studies have attempted to evaluate the possible contribution of NO to exercise hyperaemia. Results from these studies have been conflicting. Persson, Gustafsson, Wiklund, Hedqvist & Moncada (1990) showed that hyperaemic responses in rabbit tenuissimus muscle electrically stimulated to contract were unaffected by infusion of the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA). Similarly, Wilson & Kapoor (1993) demonstrated that forearm blood flow responses to graded rhythmic handgripping were unaffected by L-NMMA infusion prior to exercise. By contrast, when NO synthase blockers were administered via the phrenic artery during diaphragmatic contractions in dogs, marked vasoconstriction was observed (Hussain, Stewart, Ludemann & Magder, 1992). In rats during treadmill exercise, NO synthase inhibition had a modest effect on overall muscle blood flow, but markedly blunted vasodilatation in muscles containing a high fraction of slow twitch oxidative fibres (Hirai, Visneski, Kearns, Zelis & Musch, 1994). Along similar lines, O'Leary, Dunlap & Glover (1994) demonstrated that systemic NO synthase blockade in dogs reduced hindlimb blood flow during mild but not heavy exercise on the treadmill. These

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studies suggest that NO synthase blockers may reduce blood flow to fatigue-resistant muscle fibres during exercise.

There are several possible explanations for these conflicting findings. (1) Differences in the experimental model used. (2) Differences in the timing of NO synthase inhibitor administration (i.e. before *vs.* during contractions). For example, the distribution of blood flow in contracting muscles may differ from that of resting muscles, so NO synthase blockers given to muscles at rest may not reach the vessels involved in the hyperaemia (Marshall & Tandon, 1984; Armstrong, Delp, Goljan & Laughlin, 1987). Additionally, the factors that initiate the dilatation may not be those that sustain it (Shepherd, 1983; Sheriff, Rowell & Scher, 1994). (3) Differences in the intensity of the exercise. During more intense exercise when fatiguable muscle fibres are recruited, any intervention that 'blocks' the action of a vasodilating substance may cause a temporary reduction in muscle blood flow. Under such circumstances even a brief change in flow might alter muscle metabolism and cause the release of some other metabolic vasodilating factor and restore blood flow to the level observed prior to blockade of the original substance under study.

Taken together these observations highlight the general problems associated with studying and identifying factors that contribute to exercise hyperaemia and raise specific issues related to determining the role NO plays in exercise hyperaemia. In this study we evaluated the contribution of NO to exercise hyperaemia *during* prolonged, mild, rhythmic handgripping in humans. We administered the NO synthase blocker L-NMMA during exercise since a previous study in humans showed that giving the drug prior to exercise had no effect on the blood flow responses to rhythmic handgripping (Wilson & Kapoor, 1993). We also used mild handgripping so that the involvement of more fatiguable muscle fibre types would be limited (Hirai *et al.* 1994; O'Leary *et al.* 1994). Our results indicate that NO contributes to exercise hyperaemia in humans during mild, rhythmic handgripping.

METHODS

Subjects

Six healthy, non-smoking male subjects of age 18–45 years participated in this study after giving written informed consent. The protocols were approved by the Institutional Human Subjects Committee. Permission to administer L-NMMA was obtained from the US Food and Drug Administration.

Forearm exercise

Subjects performed rhythmic forearm exercise by squeezing a handgrip dynamometer at a rate of 30 min^{-1} at 15% of maximum and matching their force output to a target displayed on an oscilloscope. Maximum voluntary contraction was carefully determined in each subject. Three efforts consisting of a slow build-up of force over 3–5 s were performed until a plateau was observed. At least 2 min of rest was taken between efforts and the efforts typically varied by less than 5%. Additionally, the subjects were instructed to make the contractions with their forearms only, to avoid straining and to breathe normally during the efforts.

Forearm blood flow

Forearm blood flow was measured using venous occlusion plethysmography with mercury-in-Silastic® strain gauges (Greenfield, Whitney & Mowbray, 1963). At rest, forearm blood flow was measured 4 times each minute. During exercise it was measured once each minute during a brief pause (5–8 s) in contractions (Strandell & Shepherd, 1967; Joyner, Nauss, Warner & Warner, 1992).

Arterial blood pressure and heart rate

In studies in which no drugs were administered, arterial pressure and heart rate were measured using a Finapres (Imholz, van Montfrans, Settels, van der Hoeven, Karemaker & Wieling, 1988). This device non-invasively provides an accurate estimate of the arterial waveform on a beat-to-beat basis. During trials in which drugs were administered, arterial blood pressure and heart rate were measured using a 5 cm, 20-gauge Teflon catheter placed in the brachial artery in the non-dominant forearm. It was flushed continuously with heparinized saline at 3 ml h^{-1} and connected to a pressure transducer. Mean arterial pressure was calculated as one-third the pulse pressure plus diastolic pressure. Heart rate was counted using the arterial waveforms.

Drug infusions

Drugs were infused via the brachial artery catheter with a syringe pump. Drug concentrations were formulated so that each drug was administered at 4 ml min^{-1}. Saline was also administered at 4 ml min^{-1} when drugs were not being given to control for the effect of the infusion on forearm blood flow. Acetylcholine (ACh) was infused at 16 μg min^{-1} to stimulate nitric oxide release from the vascular endothelium (Vallance *et al.* 1989). L-NMMA (Cal-Biochem, La Jolla, CA, USA) was infused at 4 mg min^{-1} for 10 min to inhibit NO synthase. This dose was chosen on the basis of our previous experience with this substance (Dietz, Rivera, Warner & Joyner, 1994*a*; Dietz, Rivera, Eggener, Fix, Warner & Joyner, 1994*b*).

Specific protocols

Protocol 1. The purpose of protocol 1 was to determine whether the forearm blood flow, mean arterial pressure and heart rate responses to prolonged rhythmic handgripping were stable. It also provided baseline data for comparison with the drug interventions in protocol 2. Subjects reported to the laboratory at least 3 h after a light meal after having abstained from caffeine for at least 6 h. They were seated in a reclining chair in a quiet room maintained at 22–24 °C. Three maximum voluntary contractions were performed. A target force was then set at 15% of maximum and the subjects were instrumented to measure forearm blood flow, arterial pressure and heart rate (Finapres; Ohmeda, Louisville, CO, USA). Twenty minutes later forearm blood flow at rest was measured for 2 min followed immediately by 10 min of rhythmic handgripping. During the final 5–8 s of each minute of handgripping forearm blood flow was measured. After exercise forearm blood flow was measured for 2 min. The subjects then rested for 15 min. This procedure was then repeated during a 20 min exercise bout.

Protocol 2. Twenty-four hours or more after protocol 1, the subjects participated in protocol 2 at approximately the same time of day. The subject was seated in the laboratory chair, and a brachial arterial catheter was placed in the non-dominant forearm,

which was then instrumented to measure forearm blood flow. The time course of protocol 2 is shown in Fig. 1. A similar time course was used in protocol 1 except no drugs were administered. After 20 min of rest blood flow was measured for 2 min, followed by infusion of ACh for 3 min to establish the baseline vasodilator responses to ACh. After a 15 min break, 2 min of forearm blood flow measurements were made, followed by a 10 min bout of rhythmic forearm handgripping with blood flow measured as in protocol 1. Following the fifth minute of exercise, ACh was administered at 16 μg min^{-1} for 3 min. ACh was administered during exercise since the distribution of blood flow in the exercising muscles may differ from that in resting muscles (Armstrong *et al.* 1987). Exercise continued for 2 min following the cessation of the ACh administration, and 2 min of post-exercise measurements were also made.

After 15 min of rest a 20 min bout (Fig. 1, bout 2) of exercise was then performed. After 5 min of rhythmic handgripping, the NO synthase blocker L-NMMA was infused at 4 mg min^{-1} for 10 min. After L-NMMA infusion, ACh was administered at 16 μg min^{-1} for 3 min to assess the effects of the L-NMMA on ACh-stimulated NO-mediated vasodilatation during exercise. This was followed by an additional 2 min of exercise and 2 min of recovery. Five minutes later forearm blood flow was measured for 2 min and ACh was administered for 3 min. This was done to determine if the L-NMMA blunted the vasodilator responses to ACh in the resting forearm.

Statistics

Data are expressed as means ± S.E.M. Each subject served as his own control. The effects of L-NMMA on forearm blood flow during exercise and other interventions in protocol 2 were compared with similar time points in protocol 1. They were also compared with observations made in protocol 2 prior to administration of L-NMMA. These comparisons were made using either paired t tests or two-way analysis of variance for treatment and time with t tests for repeated means (Bruing & Kintz, 1987). Significance was set at the $P < 0{\cdot}05$ level throughout.

RESULTS

Protocol 1

Forearm blood flow averaged 2·7 ± 0·5 ml (100 ml)$^{-1}$ min^{-1} at rest. With the onset of exercise, blood flow increased to 7·2 ± 1·5 ml (100 ml)$^{-1}$ min^{-1} after the 1st minute ($P < 0{\cdot}05$ *vs.* rest). During the final 9 min of exercise, mean forearm blood flow values for each minute ranged from 7·1 ± 1·2 to 8·3 ± 1·5 ml (100 ml)$^{-1}$ min^{-1}. After exercise, flow returned rapidly toward the values observed at rest. The forearm blood flow responses observed before and during the 20 min bout of rhythmic handgripping were similar to those in the first bout of exercise and stable over time.

Heart rate was 60 ± 2 beats min^{-1} at rest and rose to 64–66 beats min^{-1} during the first bout of exercise. Similar heart rate responses were seen in the second bout of exercise. Mean arterial pressure increased from ~80 mmHg at rest to ~85–90 mmHg during exercise in both trials. The increases in heart rate and blood pressure above the values at rest were observed in all six subjects.

Protocol 2

Effects of ACh on forearm blood flow at rest and during exercise before L-NMMA

At rest, forearm blood flow averaged 2·0 ± 0·3 ml (100 ml)$^{-1}$ min^{-1}. Infusion of ACh at 16 μg min^{-1} caused forearm blood flow to rise to a value of 6·7 ± 1·7 ml (100 ml)$^{-1}$ min^{-1} ($P < 0{\cdot}05$ *vs.* rest; see Fig. 2).

At rest before the 10 min bout of exercise, forearm blood flow averaged 2·2 ± 0·4 ml (100 ml)$^{-1}$ min^{-1}. After 1 min of exercise it rose to 6·2 ± 0·7 ml (100 ml)$^{-1}$ min^{-1} ($P < 0{\cdot}05$ *vs.* rest). Over the next 4 min of exercise, mean

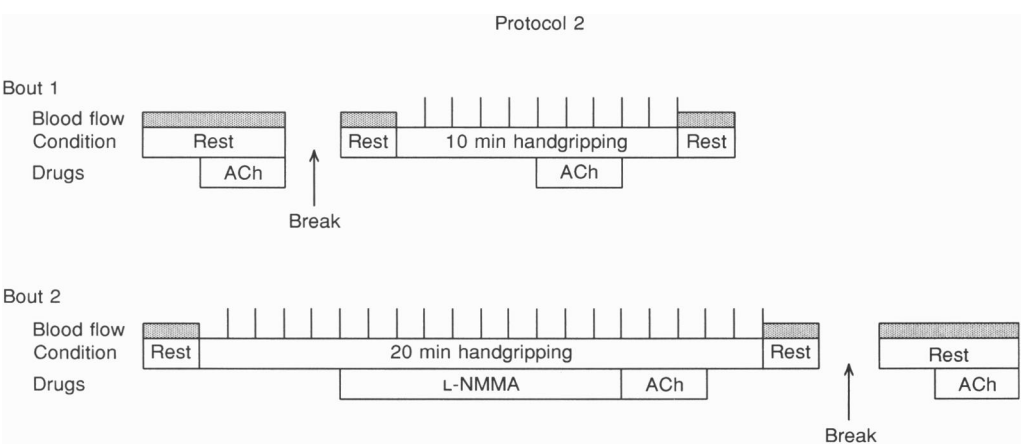

Figure 1. Schematic demonstrating experimental protocol 2

Protocol 1 was similar except no drugs were given. Bout 1 of exercise took place after the subjects were instrumented with a brachial artery catheter to administer drugs and the mercury-in-Silastic® strain gauges to measure forearm blood flow. Forearm blood flow was measured 4 times min^{-1} (shaded area) during 5 min of rest. Acetylcholine (ACh) was given during the last 3 min. Following a 5 min break a 10 min bout of exercise was performed with ACh given during the exercise. Forearm blood flow was measured once each minute (vertical marks). This was followed by 20 min of handgripping (bout 2) where L-NMMA was given to block NO synthase followed by ACh. Studies at rest were again performed after the 20 min of handgripping.

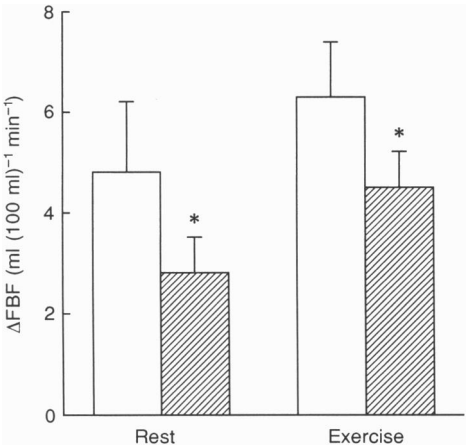

Figure 2. Forearm blood flow (FBF) responses to acetylcholine before and after L-NMMA

Effects of acetylcholine given intra-arterially at 16 μg min^{-1} for 3 min on the changes in forearm blood flow observed before and after the nitric oxide synthase inhibitor L-NMMA was given at 4 mg min^{-1} for 10 min in protocol 2 (see Fig. 1). □, control; ▨, L-NMMA. With the forearm at rest (left bars) there was a marked blunting of the rise in forearm blood flow evoked by ACh administration after L-NMMA treatment. A similar effect was observed when the ACh was administered during exercise before and after L-NMMA administration in protocol 2 (right bars). * $P < 0{\cdot}05$ *versus* control value.

forearm blood flow values for each minute ranged from 6·9 ± 0·7 to 7·5 ± 0·9 ml (100 ml)$^{-1}$ min^{-1}. When ACh was given during exercise, forearm blood flow rose to 13·3 ± 1·4 ml (100 ml)$^{-1}$ min^{-1} ($P < 0{\cdot}05$ *vs.* exercise alone; see Fig. 2). After ACh administration blood flow values returned toward those observed during exercise alone. Following exercise blood flow declined rapidly to pre-exercise levels. The arterial pressure and heart rate responses were similar to those seen during protocol 1.

Effects of L-NMMA on forearm blood flow during exercise

Prior to the 20 min bout of exercise, forearm blood flow averaged 2·3 ± 0·6 ml (100 ml)$^{-1}$ min^{-1}. It rose to 7·7 ± 0·9 ml (100 ml)$^{-1}$ min^{-1} after 1 min of exercise ($P < 0{\cdot}05$ *vs.* rest). Mean values for each minute then stabilized between 7·7 ± 1·5 and 8·7 ± 1·1 ml (100 ml)$^{-1}$ min^{-1} during the next 4 min of handgripping. Over the next 10 min of contractions, L-NMMA was administered at 4 mg min^{-1} and forearm blood flow drifted downward. Figure 3 shows the time course of these responses. Forearm blood flow averaged 6·2 ± 0·8 ml (100 ml)$^{-1}$ min^{-1} after 5 min of L-NMMA. Five of the last six measurements of flow were lower ($P < 0{\cdot}05$) while L-NMMA was being infused in comparison to measurements made at the same time during protocol 1. These values were also lower than the pre-L-NMMA values observed in protocol 2. Figure 4 shows that similar responses were seen in all of the subjects. The arterial pressure and heart rate responses to exercise were similar to those seen previously.

Effects of ACh on forearm blood flow after L-NMMA during exercise

After L-NMMA administration was complete, ACh was administered for 3 min while exercise continued. ACh caused forearm blood flow to rise to 10·0 ± 0·8 ml (100 ml)$^{-1}$ min^{-1} (Fig. 2). This was a significant increase in comparison with the flow values observed during exercise in the presence of L-NMMA. However, the rise in flow with ACh during exercise was also significantly attenuated in comparison with that seen during exercise prior to L-NMMA during bout 1 of protocol 2. After exercise, forearm blood flow fell towards baseline values during the 2 min of recovery.

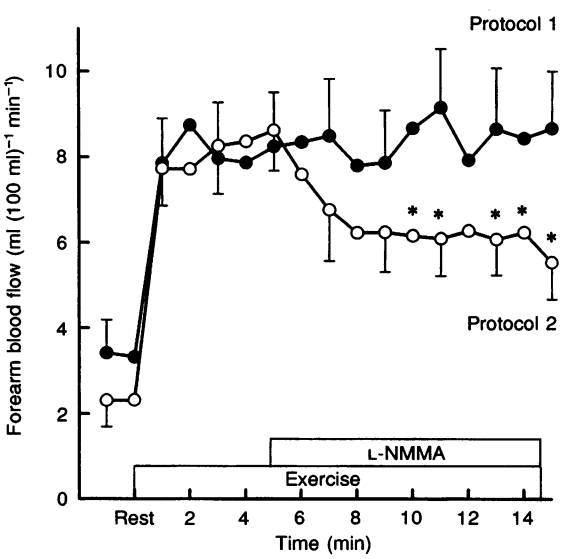

Figure 3. Time course of L-NMMA effects on forearm blood flow responses to exercise

Effects of exercise with and without L-NMMA on forearm blood flow during 15 min of rhythmic handgripping at 15% of maximum. In protocol 1, no drug was infused and the values were steady over 15 min of exercise. In protocol 2, L-NMMA was infused at 4 mg min^{-1} 5 min after the onset of contractions and caused significant reductions in the forearm blood flow responses to the exercise (* $P < 0{\cdot}05$, protocol 2 value *versus* protocol 1 value).

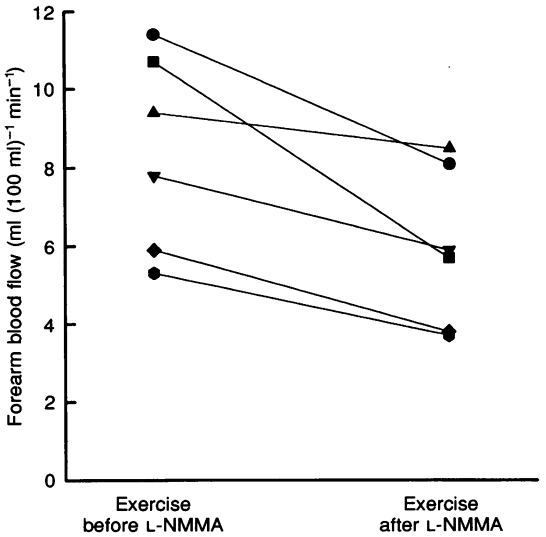

Figure 4. Individual effects of L-NMMA on forearm blood flow during exercise

Forearm blood flow responses for each subject during rhythmic handgripping at 15% of maximum voluntary contraction during protocol 2. Values on the left represent an average during the last three blood flow measurements prior to L-NMMA administration. Values on the right represent an average of those observed during the final 3 min of L-NMMA administration. Forearm blood flow declined in all six subjects.

Effects of ACh on forearm blood flow at rest following L-NMMA administration

Five minutes after the second bout of exercise, forearm blood flow averaged $1{\cdot}9 \pm 0{\cdot}3$ ml (100 ml)$^{-1}$ min^{-1}. Forearm blood flow rose to $4{\cdot}6 \pm 0{\cdot}9$ ml (100 ml)$^{-1}$ min^{-1} when ACh was given at 16 μg min^{-1} for 3 min and this response was blunted (Fig. 2) in comparison with the observations made at rest before L-NMMA ($P < 0{\cdot}05$ *vs.* pre-L-NMMA response).

DISCUSSION

In this study, inhibition of NO synthase during prolonged mild rhythmic handgripping in humans reduced forearm blood flow by ~30% (Fig. 3). The responses were consistent in all subjects studied (Fig. 4). This observation indicates that NO contributes to exercise hyperaemia in humans. Additionally, it means that NO joins a long list of potential mediators of exercise hyperaemia (Shepherd, 1983; Laughlin, 1987; Sheriff *et al.* 1993).

Plethysmography and blood flow to contracting forearm muscles

In the present study venous occlusion plethysmography was used to assess blood flow to contracting forearm muscles. Use of this technique during exercise requires a brief pause in contractions while flow is measured. Blood flow measured during these pauses is thought to reflect that occurring during exercise (Strandell & Shepherd, 1967; Joyner *et al.* 1992). During exercise the rise in forearm blood flow with exercise occurs in the active muscles and not the skin (Strandell & Shepherd, 1967).

Blockade of NO synthase in human forearm

L-NMMA was infused at 4 mg min^{-1} via a brachial artery catheter to block NO synthase in the forearm. This blunted the NO-mediated vasodilatation with ACh by 30–50% at rest and during exercise, indicating substantial inhibition of NO synthase (Fig. 2) (Vallance *et al.* 1989). In our previous studies when L-NMMA has been given at 4 mg min^{-1} for 10 min during rest, we have consistently been able to blunt the forearm vasodilator responses to ACh by 75–80% or more in almost all subjects (Dietz *et al.* 1994*a*, *b*). A likely explanation for the smaller effect of L-NMMA in the present study is that during exercise the L-NMMA was diluted by the greater blood flow in the brachial artery so its concentration was lower when it reached the vascular endothelium. Indeed, even when we tested the effect of L-NMMA on the responses evoked by ACh at rest the final test injection of ACh had been preceded by long periods of exercise. In previous studies by ourselves and others, intra-arterial nitrovasodilators were given before and after L-NMMA, and L-NMMA did not alter the dilator responses to these drugs. This indicates that our findings were not due to some non-specific effect of the L-NMMA on the blood vessels (Vallance *et al.* 1989; Wilson & Kapoor, 1993; Dietz *et al.* 1994*a*, *b*).

Role of NO in exercise hyperaemia

Several previous studies have demonstrated that administration of NO synthase blockers has no effect on blood flow to contracting muscles (Persson *et al.* 1990; Wilson & Kapoor, 1993; see Introduction). By contrast, the present and other studies that have also used NO synthase blockers suggest that NO may contribute to vasodilatation during exercise (Hussain *et al.* 1992; Hirai *et al.* 1994; O'Leary *et al.* 1994). When these studies are evaluated together (see Introduction) it becomes clear that several issues must be considered when examining the role of NO in exercise hyperaemia. Firstly, in studies suggesting that NO does not play a role in exercise hyperaemia, the NO synthase blockers were given prior to contractions (Persson *et al.* 1990; Wilson & Kapoor, 1993). In these studies it is possible that the NO synthase blockers given at rest did not reach the blood vessels involved in the exercise hyperaemia so that when exercise was initiated 'unblocked' vessels were

perfused. In one of the studies in which NO was shown to play a role in exercise hyperaemia, the NO synthase blockers were given during contractions as in the present study (Hussain *et al.* 1992).

It is also possible that other vasodilating substances may have been released at the onset of exercise in a manner that masked the potential role of NO in exercise hyperaemia.

Secondly, in other studies demonstrating that NO contributes to exercise hyperaemia this contribution was observed either in fatigue-resistant muscle fibre types or during mild exercise (Hirai *et al.* 1994; O'Leary *et al.* 1994), as in the present study. In these previous studies, the magnitude of the effect of the NO synthase blockers on muscle blood flow was similar to that observed in the present study. A possible explanation for these findings is that in fatigue resistant fibre types and during mild exercise there is generally 'luxuriant' perfusion of active skeletal muscles, as suggested by low levels of O_2 extraction during these conditions (Strandell & Shepherd, 1967; Joyner *et al.* 1992). Under such circumstances a substantial reduction in blood flow can occur without causing marked changes in muscle metabolism that might cause the build-up of other vasodilating factors in the active skeletal muscles (Joyner *et al.* 1992).

Possible mechanisms of NO release during exercise

A key questions that remains concerns the stimulus for and source of the NO release. There are several possible explanations that deserve consideration. First, NO can be released directly from autonomic nerves (Burnett, Lowenstein, Bredt, Chang & Snyder, 1992). Although intact motor and autonomic nerves are not required to produce exercise hyperaemia (Gaskell, 1880; Shepherd, 1983) their involvement cannot be rejected, primarily because it has not been thoroughly evaluated. Second, it is possible that NO is released via cholinergic nerve stimulation of the vascular endothelium (Broten, Miyashiro, Moncada & Feigl, 1992). This is attractive since there can be cholinergically mediated, NO-linked dilatation in skeletal muscle during mental stress (Dietz *et al.* 1994*b*). However, atropine has little effect on muscle blood flow during exercise (Armstrong & Laughlin, 1980). Third, perhaps NO is released from the contracting muscles themselves. Again, this cannot be discounted completely, because it has not been evaluated systematically. Finally, NO may be released from the vascular endothelium via a flow-induced mechanism (Rubanyi, Romero & Vanhoutte, 1986). The increase in shear stress on the endothelial cells caused by the increase in flow during exercise could stimulate NO release. Under these circumstances the contribution of NO to exercise hyperaemia might then be more easily observable during mild exercise, but less easily seen during heavy exercise when other metabolic dilating factors might be released from the active muscles.

Conclusions

These results demonstrate that when NO synthase blockers are given during mild, rhythmic handgripping the exercise-induced increase in forearm muscle blood flow is attenuated. These observations are consistent with the animal studies demonstrating a role for NO in fatigue-resistant muscle fibres during mild exercise (Hussain *et al.* 1992; Hirai *et al.* 1994; O'Leary *et al.* 1994). The contribution of NO to muscle vasodilatation during heavy exercise in humans remains to be determined.

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