

# Nitric oxide synthase inhibition with L-NAME reduces maximal oxygen uptake but not gas exchange threshold during incremental cycle exercise in man

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We hypothesized that the effective inhibition of nitric oxide synthase (NOS), achieved via systemic infusion of *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), would reduce the gas exchange threshold (GET) and the maximal oxygen uptake ( $\dot{V}_{O_{2,max}}$ ) during incremental cycle exercise in man if NO is important in the regulation of muscle vasodilatation. Seven healthy males, aged 18–34 years, volunteered to participate in this ethically approved study. On two occasions, the subjects completed an incremental exercise test to exhaustion on an electrically braked cycle ergometer following the infusion of either L-NAME (4 mg kg<sup>-1</sup> in 50 ml saline) or placebo (50 ml saline, CON). At rest, the infusion of L-NAME resulted in a significant increase in mean arterial pressure (MAP; CON vs. L-NAME, 89 ± 8 vs. 103 ± 11 mmHg (mean ± s.d.; *P* < 0.05)) and a significant reduction in heart rate (HR; CON vs. L-NAME, 60 ± 12 vs. 51 ± 8 beats min<sup>-1</sup>; *P* < 0.01). At submaximal work rates, there was no significant difference in  $\dot{V}_{O_2}$  between the conditions and no difference in the GET (CON vs. L-NAME, 1.94 ± 0.47 vs. 2.01 ± 0.41 l min<sup>-1</sup>). However, at higher work rates, differences in  $\dot{V}_{O_2}$  between the conditions became more pronounced such that  $\dot{V}_{O_{2,max}}$  was significantly lower with L-NAME (CON vs. L-NAME, 4.02 ± 0.41 vs. 3.80 ± 0.34 l min<sup>-1</sup>; *P* < 0.05). The reduction in  $\dot{V}_{O_{2,max}}$  was associated with a reduction in HR<sub>max</sub> (CON vs. L-NAME, 186 ± 10 vs. 178 ± 7 beats min<sup>-1</sup>; *P* < 0.01). These results demonstrate that NOS inhibition with L-NAME has no effect on GET but reduces  $\dot{V}_{O_{2,max}}$  during large muscle group exercise in man, presumably by direct or indirect effects on cardiac output and muscle blood flow.

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Nitric oxide (NO) is known to play an important role in a wide array of physiological processes (Moncada *et al.* 1991; Stamler & Meissner, 2001). Nitric oxide synthase (NOS), the enzyme responsible for the synthesis of NO, is present both within the vascular endothelium (known as eNOS) and within myocytes (known as nNOS). NO production increases during muscle contraction (Balon & Nadler, 1994) and it has been suggested that NO might be one of the factors contributing to skeletal muscle vasodilatation at the onset of exercise either by direct effects on the vascular endothelium (Moncada *et al.* 1991; Shepherd & Katusic, 1991) or by blunting sympathetically mediated vasoconstriction (Sander *et al.* 1995; Thomas & Victor, 1998). However, although it has been clearly demonstrated that NO contributes to the regulation of muscle blood flow at rest and following recovery from exercise (Dyke *et al.* 1995; Shoemaker *et al.* 1997; Radegran & Saltin, 1999; Frandsen *et al.* 2001), its importance during dynamic exercise is

more controversial. Indeed, recent studies utilizing the one-legged knee extensor exercise model suggest that NO might not be essential for the regulation of muscle blood flow during steady-state exercise in man (Radegran & Saltin, 1999; Frandsen *et al.* 2001).

NOS activity can be inhibited with L-arginine analogues such as *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA) and the more potent *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME). L-NAME effectively inhibits both eNOS and nNOS (Sander *et al.* 1999; Frandsen *et al.* 2001). NOS inhibition results in a reduction in vascular conductance, an increase in mean arterial pressure (MAP), and a reduction in heart rate (HR) both before and during exercise (Sander *et al.* 1999; Frandsen *et al.* 2001). It has been suggested that the reduced HR with NOS inhibition results from baroreceptor-mediated sympathetic withdrawal (Sheriff *et al.* 2000; Joyner & Tschakovsky, 2003), although direct effects on cardiac function have also been reported

(Sherman *et al.* 1997). Indeed, Kindig *et al.* (2000) reported that infusion of L-NAME caused a significant reduction in cardiac output ( $\dot{Q}$ ) during both submaximal and maximal treadmill running in the Thoroughbred horse. At running speeds corresponding to 50 and 80%  $\dot{V}_{O_2\max}$ , the reduction in muscle  $O_2$  delivery was compensated for by an increase in muscle  $O_2$  extraction such that pulmonary  $\dot{V}_{O_2}$  was unchanged. However, at a running speed corresponding to 100%  $\dot{V}_{O_2\max}$ ,  $O_2$  extraction was unable to increase further, leading to a significant reduction in  $\dot{V}_{O_2\max}$  (and exercise tolerance) in the L-NAME compared to the control condition. In contrast, in one of the few studies to investigate the influence of NOS inhibition on cardio-respiratory responses to exercise in man, Frandsen *et al.* (2001) reported that L-NAME had no significant effect on leg blood flow or  $\dot{V}_{O_2}$  during either submaximal or maximal one-legged knee extension exercise. It is unclear whether the different results of Kindig *et al.* (2000) and Frandsen *et al.* (2001) are related to differences in the species studied (i.e. horse *versus* man), to the dose of L-NAME administered (20 mg kg<sup>-1</sup> in the horse *versus* 4 mg kg<sup>-1</sup> in man), or to differences in exercise modality (i.e. 'whole-body' *versus* small isolated muscle mass exercise). However, it is possible that NOS inhibition might impair muscle blood flow during maximal intensity exercise using large muscle groups (where maximal  $\dot{Q}$  is attained and the degree of sympatholysis has to be carefully regulated) but not during small muscle mass exercise (where the maximal  $\dot{Q}$  is not approached and muscle perfusion is very high).

To date, no studies have examined the influence of NOS inhibition on the  $\dot{V}_{O_2}$  response to incremental exercise using large muscle groups in man. During such exercise, it is known that both the gas exchange threshold (GET) and  $\dot{V}_{O_2\max}$  are sensitive to conditions which influence muscle  $O_2$  availability (e.g. Hogan *et al.* 1983; Koike *et al.* 1990; Saltin & Strange, 1992; Koga *et al.* 1999; Richardson *et al.* 1999; Wagner, 2000). If NO is important in the regulation of skeletal muscle blood flow during large muscle group exercise in man, then NOS inhibition would be expected to reduce both GET and  $\dot{V}_{O_2\max}$  during incremental exercise. The purpose of this study therefore was to investigate the effect of NOS inhibition with L-NAME on the cardio-respiratory response to incremental cycle exercise in man. We hypothesized that, relative to the control condition, NOS inhibition would significantly reduce both GET and  $\dot{V}_{O_2\max}$ .

## Methods

### Subjects

Seven healthy, recreationally active but not highly trained, male subjects (mean  $\pm$  s.d. age 25  $\pm$  4 years, body mass 78.5  $\pm$  9.2 kg) volunteered to participate in this study

which had been approved by the Manchester Metropolitan University Research Ethics Committee. The subjects provided written informed consent after the possible benefits and risks of participation had been explained to them. All subjects had extensive prior experience of performing incremental exercise tests to fatigue in our laboratory. All procedures conformed to the standards set by the Declaration of Helsinki.

### Procedures

The subjects attended the laboratory to perform an incremental exercise test to volitional exhaustion on an electrically braked cycle ergometer (Jaeger Ergoline E800, Germany) on two occasions separated by 7 days. They were instructed to arrive at the laboratory rested and fully hydrated, having avoided the consumption of food and caffeine in the preceding 2 h. For each subject, the two exercise tests were conducted in the morning at approximately the same time of day ( $\pm$  1 h).

Following arrival at the laboratory, the subjects lay supine and a cannula was placed in a hand vein. The subjects then rested for 20 min before either L-NAME (Merck Biosciences AG, Nottingham, UK; 4 mg (kg body mass)<sup>-1</sup> in 50 ml saline) or an equivalent volume of saline (as a placebo) was infused over 60 min. Our protocol for the L-NAME infusion was based on that described by Frandsen *et al.* (2001), who demonstrated that this resulted in a 67% reduction in NOS activity in skeletal muscle. Throughout the infusions, blood pressure was measured with an automated sphygmomanometer and heart rate was monitored continuously by telemetry (Polar Electro, Finland). The subjects were blind to the nature of the infusate. However, because of the profound effects of L-NAME on heart rate and blood pressure (Sander *et al.* 1999; see Results), it was not possible to blind the principal investigators to whether subjects had received L-NAME or a placebo infusion. Four subjects received L-NAME on their first visit to the laboratory and the placebo infusion on their second visit to the laboratory. This order was reversed in the other three subjects. Following the L-NAME or saline infusion, the subjects rested for 60 min before performing an incremental cycle ergometer test to exhaustion (see below).

After mounting the cycle ergometer, the subjects accelerated the flywheel and performed 3 min of baseline pedalling at the lowest work rate available on the ergometer (20 W). Subsequently, the work rate was then increased by 5 W every 10 s (i.e. 30 W min<sup>-1</sup>) until the subject was unable to continue. The subjects cycled at the same self-selected pedal rate throughout the test (this varied between 70 and 90 r.p.m. across the subjects) and the pedal rate, along with the saddle and handlebar height and configuration, were recorded for each subject and reproduced in the second test. During the test, the

subjects were verbally encouraged to continue for as long as possible. The subjects voluntarily terminated the test when their pedal rate fell more than 5 r.p.m. below their selected pedal rate. In all cases, this fall was precipitous. The exact time to volitional exhaustion from the onset of the ramp test was measured with a stopwatch and recorded to the nearest second.

Pulmonary gas exchange was measured breath-by-breath and HR was recorded every 5 s throughout the exercise tests. The subjects wore a nose-clip and breathed through a low dead space, low resistance mouthpiece and volume sensor assembly. Pulmonary gas exchange was measured with a mass spectrometer and volume turbine system (Morgan EX670, Morgan Medical Limited, Gillingham, Kent, UK). The system was calibrated prior to each test using gases of known concentration and a precision 3 l calibration syringe. Blood pressure was measured immediately before the onset of exercise and within 30 s of the termination of exercise. Mean arterial pressure was calculated as the diastolic pressure plus one-third of the pulse pressure.

The breath-by-breath  $\dot{V}_{O_2}$  data for all tests were interpolated to give second-by-second values and then averaged over consecutive 10 s periods. The 'baseline'  $O_2$  cost of cycling in the incremental tests was taken as the average  $\dot{V}_{O_2}$  measured during the final minute of the 3 min baseline period. The gas exchange threshold (GET) was determined from a cluster of gas exchange indices as the  $\dot{V}_{O_2}$  at which there was: (i) a disproportionate increase in  $\dot{V}_{CO_2}$  from visual inspection of individual plots of  $\dot{V}_{CO_2}$  versus  $\dot{V}_{O_2}$  (Beaver *et al.* 1986); (ii) an increase in  $\dot{V}_E/\dot{V}_{O_2}$  ( $\dot{V}_E$ , expiratory ventilation) with no increase in  $\dot{V}_E/\dot{V}_{CO_2}$ ; and (iii) an increase in end-tidal  $O_2$  tension with no fall in end-tidal  $CO_2$  tension (Wasserman *et al.* 1999). We also examined the respiratory exchange ratio (RER) profiles to ensure that our identification of the GET was not confounded by the existence of 'false positives' (cf. Ozcelik *et al.* 1999).  $\dot{V}_{O_2\max}$  was determined as the average value recorded over the final 30 s of each of the tests. The  $O_2$  pulse for each work rate and at exhaustion was calculated by dividing  $\dot{V}_{O_2}$  by HR ( $\dot{V}_{O_2}/HR = \text{stroke volume} \times \text{arterial-venous } O_2 \text{ content difference}$ ).

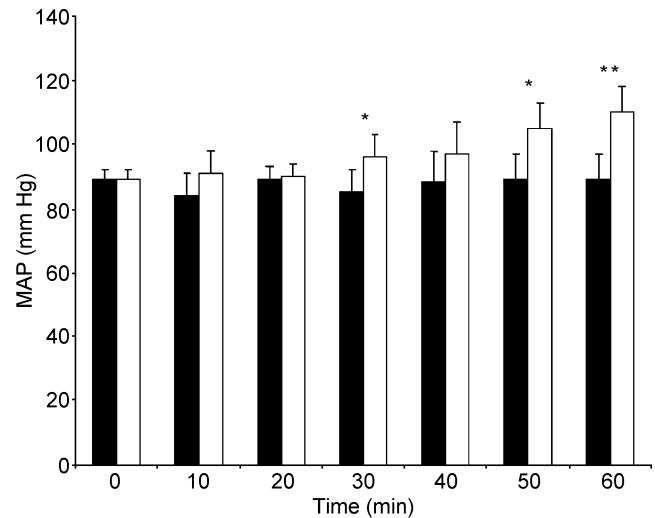
### Statistical analysis

The results are presented as mean  $\pm$  s.d. Paired *t* tests were used to determine the significance of any differences between the physiological responses at rest and during exercise following the L-NAME and placebo infusions.

## Results

### Efficacy of NOS inhibition

Infusion of L-NAME resulted in a significant increase in MAP (Fig. 1) and a significant reduction in HR

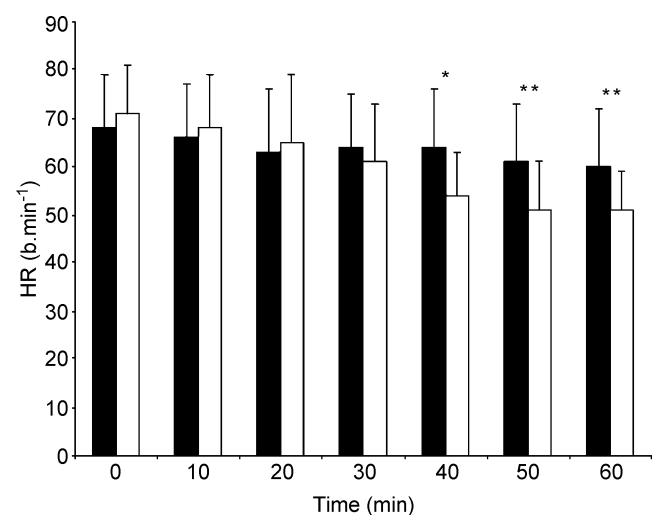


**Figure 1.** Influence of the infusion of saline (control) and of a NOS-inhibitor (L-NAME) on mean arterial blood pressure over 60 min

Filled bars, control; open bars, L-NAME. \* $P < 0.05$ ; \*\* $P < 0.01$ .

(Fig. 2) beyond 30–40 min when compared to the control condition, consistent with successful inhibition of NOS. At the onset of exercise, MAP remained significantly elevated (CON vs. L-NAME,  $97 \pm 8$  vs.  $108 \pm 10$  mmHg;  $P < 0.05$ ) and HR remained reduced (CON vs. L-NAME,  $84 \pm 10$  vs.  $78 \pm 8$  beats  $\text{min}^{-1}$ ;  $P = 0.09$ ) compared to the control condition.

Although our subjects experienced no serious side-effects of receiving L-NAME, most were subdued and felt sleepy and lethargic in the afternoon and evening following L-NAME treatment.



**Figure 2.** Influence of the infusion of saline (control) and of a NOS-inhibitor (L-NAME) on heart rate over 60 min

Filled bars, control; open bars, L-NAME. \* $P < 0.05$ ; \*\* $P < 0.01$ .

### Submaximal responses

HR tended to be lower throughout the ramp test in the L-NAME compared to the control condition and this difference was significant at 20, 50, 320 and 350 W (Fig. 3).  $\dot{V}_{O_2}$  also tended to be lower in the L-NAME compared to control condition and this attained significance at 260, 290 and 320 W (Fig. 4). There was no significant difference in  $O_2$  pulse between the conditions. GET was not significantly different between conditions (CON vs. L-NAME,  $1.94 \pm 0.47$  vs.  $2.01 \pm 0.41$  l min<sup>-1</sup>; Table 1 and Fig. 5).

### Maximal responses

The time to exhaustion was reduced with L-NAME (CON vs. L-NAME,  $12.3 \pm 1.3$  vs.  $11.9 \pm 1.1$  min;  $P = 0.06$ ). Maximal HR attained was significantly different between the two conditions (CON vs. L-NAME,  $186 \pm 10$  vs.  $178 \pm 7$  beats min<sup>-1</sup>;  $P < 0.01$ ; Fig. 3) and MAP measured within 30 s of the termination of exercise tended to be greater with L-NAME (CON vs. L-NAME,  $105 \pm 15$  vs.  $122 \pm 12$  mmHg;  $P = 0.08$ ).  $\dot{V}_{O_{2max}}$  was significantly lower in the L-NAME condition (CON vs. L-NAME,  $4.02 \pm 0.41$  vs.  $3.80 \pm 0.34$  l min<sup>-1</sup>;  $P < 0.05$ ; Fig. 4 and Table 1). There was no significant difference in the maximum  $O_2$  pulse between the conditions (CON vs. L-NAME,  $22.0 \pm 2.0$  vs.  $22.4 \pm 2.1$  ml  $O_2$  beat<sup>-1</sup>). The  $\dot{V}_{O_2}$  response to incremental exercise with and without NOS inhibition is shown for a typical subject in Fig. 6.

There was no significant difference between the conditions for  $\dot{V}_{CO_{2max}}$  (CON vs. L-NAME,  $4.67 \pm 0.23$  vs.  $4.53 \pm 0.33$  l min<sup>-1</sup>;  $P = 0.07$ ),  $\dot{V}_{E_{max}}$  (CON vs. L-NAME,  $160 \pm 30$  vs.  $150 \pm 36$  l min<sup>-1</sup>),  $\dot{V}_E/\dot{V}_{O_{2max}}$  (CON vs.

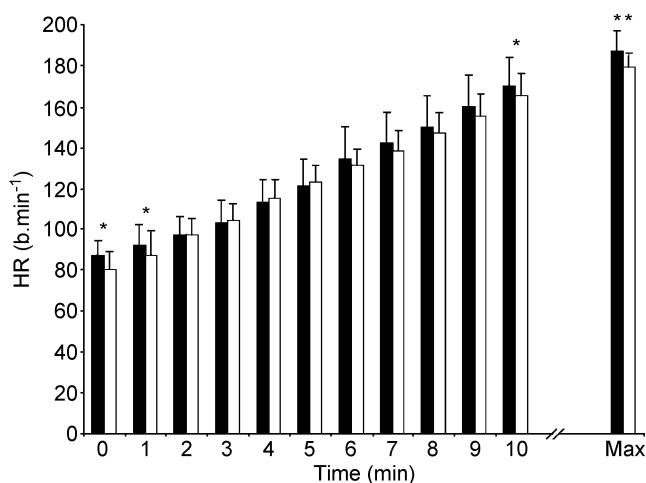
L-NAME,  $39.8 \pm 7.8$  vs.  $39.5 \pm 7.3$ ),  $\dot{V}_E/\dot{V}_{CO_{2max}}$  (CON vs. L-NAME,  $34.3 \pm 5.8$  vs.  $33.1 \pm 8.5$ ), or  $RER_{max}$  (CON vs. L-NAME,  $1.17 \pm 0.08$  vs.  $1.20 \pm 0.06$ ).

### Discussion

To our knowledge, this is the first study to investigate the effect of NOS inhibition (achieved via systemic infusion of L-NAME) on the cardiovascular and respiratory responses to incremental large muscle group exercise in man. The principal novel finding was the significant reduction in  $\dot{V}_{O_{2max}}$ , with no change in submaximal  $\dot{V}_{O_2}$  or GET, following NOS inhibition. These results suggest that muscle  $O_2$  delivery was well maintained during submaximal cycle exercise following NOS inhibition. However, the reduction in  $\dot{V}_{O_{2max}}$ , which was associated with a significant reduction in  $HR_{max}$ , strongly suggests that it was not possible to fully maintain blood flow to the contracting skeletal muscle mass during maximal intensity cycle exercise following NOS inhibition.

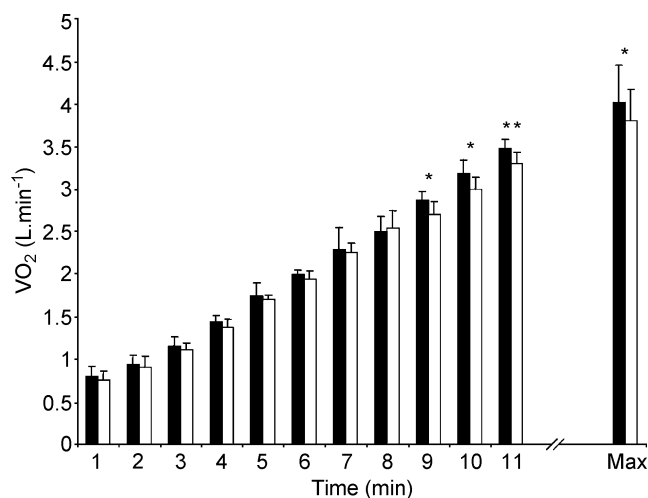
### Comparison with previous studies

Our results are, in part, consistent with those of Kindig *et al.* (2000) who examined the effect of L-NAME on the cardiovascular and respiratory responses to incremental treadmill running in the Thoroughbred horse. These authors reported that L-NAME caused a significant increase in MAP and a significant reduction in HR and  $\dot{Q}$  throughout exercise compared to the control condition. During submaximal exercise at treadmill speeds requiring 50 and 80% of previously determined  $\dot{V}_{O_{2max}}$



**Figure 3. Heart rate response to incremental exercise in the control and L-NAME conditions**

Filled bars, control; open bars, L-NAME. Values are reported at the onset of the test (time 0), for every minute of the test up to minute 10, and at exhaustion (Max). \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Figure 4. Pulmonary oxygen uptake response to incremental exercise in the control and L-NAME conditions**

Filled bars, control; open bars, L-NAME. Values are reported at the onset of the test (time 0), for every minute of the test up to minute 10, and at exhaustion (Max). \* $P < 0.05$ ; \*\* $P < 0.01$ .

**Table 1. Individual values for gas exchange threshold (GET) and maximal oxygen uptake ( $\dot{V}_{O_2\max}$ ) in the L-NAME and control conditions**

Subject	GET (ml kg <sup>-1</sup> min <sup>-1</sup> )		$\dot{V}_{O_2\max}$ (ml kg <sup>-1</sup> min <sup>-1</sup> )	
	Control	L-NAME	Control	L-NAME
1	16.7	19.1	44.5	43.6
2	24.6	25.0	50.0	50.9
3	31.8	32.5	65.2	61.9
4	19.7	19.3	45.0	42.0
5	23.5	24.5	55.5	48.5
6	32.7	30.7	48.4	47.1
7	25.7	29.2	53.7	48.4
Mean	25.0	25.8	51.8	48.9*
S.D.	5.9	5.3	7.2	6.5

\* Significantly different ( $P < 0.05$ ).

they found that muscle  $O_2$  extraction increased to compensate for the reduction in muscle  $O_2$  delivery such that pulmonary  $\dot{V}_{O_2}$  was not significantly different between the L-NAME and control conditions. However, during maximal intensity exercise, increased  $O_2$  extraction could no longer compensate for the reduced muscle  $O_2$  delivery, and thus the  $\dot{V}_{O_2\max}$  in the L-NAME condition was significantly reduced compared to control. The results of our study are similar in that HR was reduced with L-NAME both during submaximal exercise (with no change in  $\dot{V}_{O_2}$ ) and during maximal exercise (where there was also a significant reduction in  $\dot{V}_{O_2\max}$ ). However, the magnitude of the reduction in  $\dot{V}_{O_2\max}$  (~11%) in the study of Kindig *et al.* (2000) was greater than the magnitude of the reduction in  $\dot{V}_{O_2\max}$  that we observed (~6%). One possible explanation for this discrepancy is the difference in the dose of L-NAME administered (20 mg kg<sup>-1</sup> in their study and 4 mg kg<sup>-1</sup> in ours). This dose of 4 mg (kg body mass)<sup>-1</sup> has been shown previously to result in a 67% reduction in nNOS activity (Frandsen *et al.* 2001). It is possible that complete NOS blockade with higher doses of L-NAME would have resulted in an even greater reduction in  $\dot{V}_{O_2\max}$ , but it is not known whether higher doses pose significant health risks to human subjects.

Only one other study has given L-NAME systemically and examined the effect on  $\dot{V}_{O_2}$  during incremental exercise in man. In this study, Frandsen *et al.* (2001) measured limb blood flow and oxygen extraction, and therefore  $\dot{V}_{O_2}$ , during incremental one-legged knee extension exercise in five subjects. They reported a significant reduction in leg vascular conductance and HR during submaximal exercise with L-NAME, but no significant differences in leg blood flow,  $O_2$  extraction or  $\dot{V}_{O_2}$ . These data imply that  $\dot{Q}$  was reduced but the perfusion of working muscle was maintained with NOS inhibition. At rest and during recovery from exercise in man, there is clear evidence that NOS inhibition reduces

tissue blood flow, with  $\dot{V}_{O_2}$  being maintained by an increased  $O_2$  extraction (Dyke *et al.* 1995; Shoemaker *et al.* 1997; Radegran & Saltin, 1999; Frandsen *et al.* 2001). However, several studies have shown that NO is not obligatory for normal muscle vasodilatation during exercise, at least in small muscle groups, indicating the existence of some redundancy in the system (Shoemaker *et al.* 1997; Radegran & Saltin, 1999; Boushel *et al.* 2002; Hillig *et al.* 2003). It seems unlikely that the maintained skeletal muscle blood flow with L-NAME despite evidence for a reduced vascular conductance in the study of Frandsen *et al.* (2001) can be fully explained by the greater perfusion pressure. Rather, it appears that the reduced vascular conductance arises from vasoconstriction in the vascular beds of other organs including non-active skeletal muscle (Hirai *et al.* 1994), with blood flow to contracting skeletal muscle being well preserved. Local factors which might facilitate vasodilatation in the face of NOS inhibition include adenosine, potassium, prostaglandins (PG) and endothelial-derived hyperpolarizing factors (EDHF), although it is likely that the integration of a number of factors that interact determines blood flow (Frandsen *et al.* 2000; Boushel *et al.* 2002; Hillig *et al.* 2003). For example, Boushel *et al.* (2002) have recently reported that the combined inhibition of NOS and PG resulted in a ~30% reduction in skeletal muscle blood flow during exercise. Furthermore, Hillig *et al.* (2003) have reported that combined inhibition of NOS and cytochrome P450 (to block EDHF formation) resulted in a ~16% reduction in muscle blood flow. These results suggest a synergy between NO, PG and EDHF in the regulation of muscle vasodilatation and exercise hyperaemia. While there is apparently no compensatory increase in interstitial adenosine, potassium or prostacyclin following NOS inhibition (Frandsen *et al.* 2000), it is presently unknown whether NOS inhibition alters the EDHF or bradykinin responses to exercise. However, irrespective of the mechanism involved, our results, in concert with those of Frandsen *et al.* (2001), demonstrate that during exercise where only a relatively small amount of muscle is recruited (for example, during single leg knee extension exercise and submaximal cycle exercise), the system is able to maintain a close matching of blood flow to metabolic rate (i.e.  $\dot{Q}/\dot{V}_{O_2}$ ) in working muscle, even when NO production is markedly attenuated.

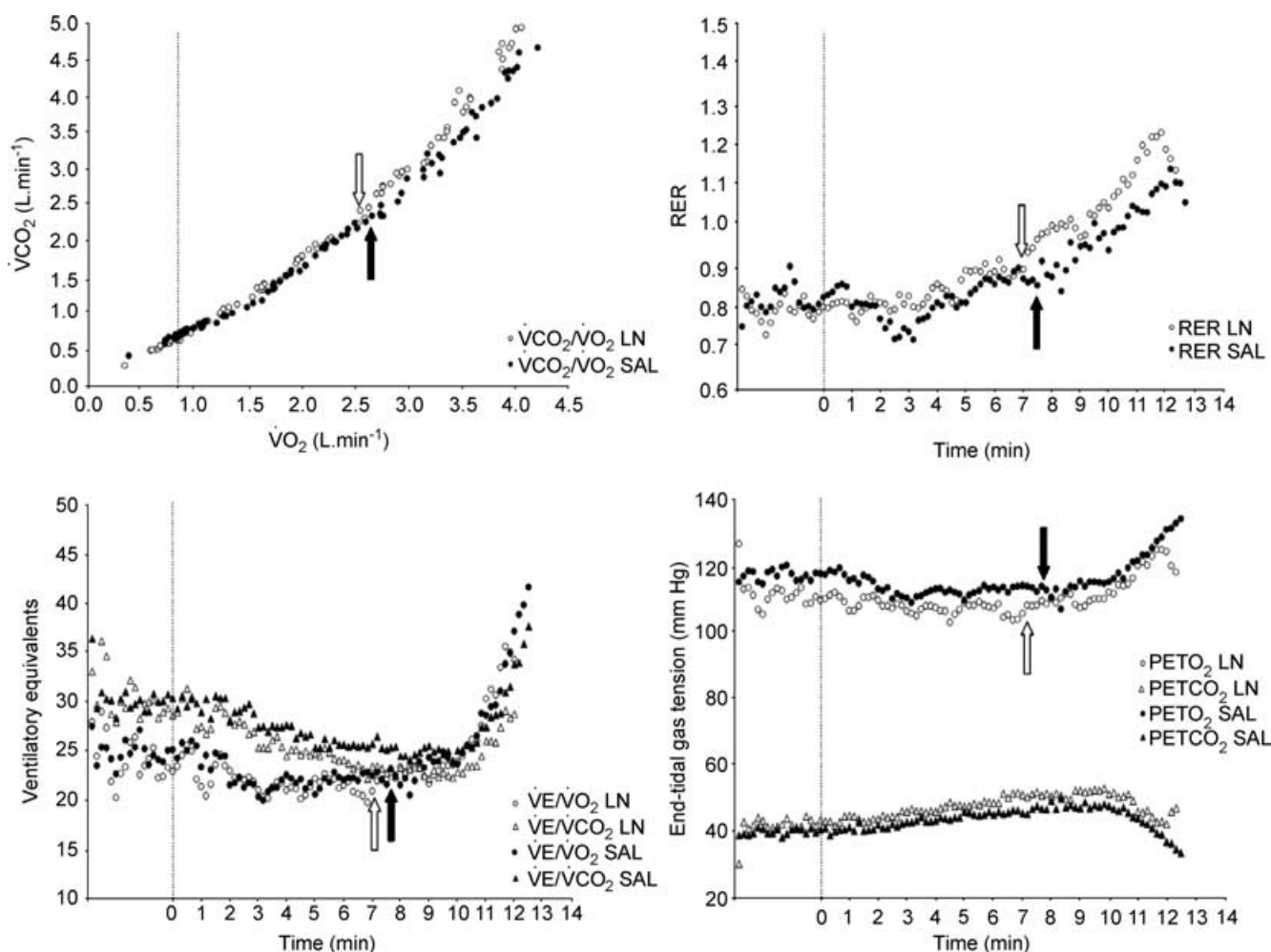
### Influence of NOS inhibition on the gas exchange threshold and maximal oxygen uptake

To our knowledge, ours is the first study to assess the influence of NOS inhibition on GET. The physiological mechanisms responsible for GET (and the simultaneous increase in blood [lactate] which typically accompanies it) have been extensively debated (cf. Jones & Ehrsam,

1982; Walsh & Banister, 1988; Wasserman *et al.* 1999). However, it is known that GET is sensitive to reductions in  $O_2$  availability. For example, interventions which might be expected to reduce muscle  $O_2$  availability such as the inspiration of hypoxic gas (Hogan *et al.* 1983; Hughson & Kowalchuk, 1995; Walsh & Banister, 1995) or gas with increased carbon monoxide content (Koike *et al.* 1990), or the performance of supine exercise (Koga *et al.* 1999) all result in a greater accumulation of blood lactate and therefore an earlier appearance of GET. Furthermore, GET is lower in patients with diseases of the central or peripheral circulation (Wasserman *et al.* 1999). The fact that GET was not significantly altered with L-NAME in our study therefore further suggests that  $\dot{Q}/\dot{V}_{O_2}$  of active skeletal muscle was preserved despite effective NOS inhibition

during submaximal cycle exercise (at least up to  $\sim 50\%$  of whole-body  $\dot{V}_{O_{2max}}$ ), consistent with previous findings (Frandsen *et al.* 2001). However, unlike in the study of Frandsen *et al.* we found that  $\dot{V}_{O_{2max}}$  was significantly reduced following NOS inhibition.

One important difference between our study and that of Frandsen *et al.* (2001) is the exercise modality (upright two-legged cycling *versus* one-legged knee extension exercise). Although exercise performance during knee extension exercise is sensitive to  $O_2$  availability (Richardson *et al.* 1999), the volume of muscle recruited to perform exercise of this type is so small that fatigue is believed to result principally from peripheral rather than central factors. Indeed, relative to cycle ergometry (where peak muscle blood flow is  $\sim 150 \text{ ml kg}^{-1} \text{ min}^{-1}$ ),



**Figure 5.** Determination of the gas exchange threshold (GET) using a 'cluster' of gas exchange indices

Upper left panel illustrates the metabolic rate at which there is a disproportionate increase in  $CO_2$  output ('V-slope' method; Beaver *et al.* 1986); lower left panel shows the ventilatory equivalents for  $O_2$  and  $CO_2$ ; upper right panel shows the respiratory exchange ratio; and lower right panel shows the end-tidal gas tension responses to exercise following the inhibition of nitric oxide synthase (O) and following a placebo infusion ( $\bullet$ ) in a typical subject. See text for further information. GET in each condition is shown by the open and filled arrows, respectively. There was no significant difference in the group mean GET between conditions.

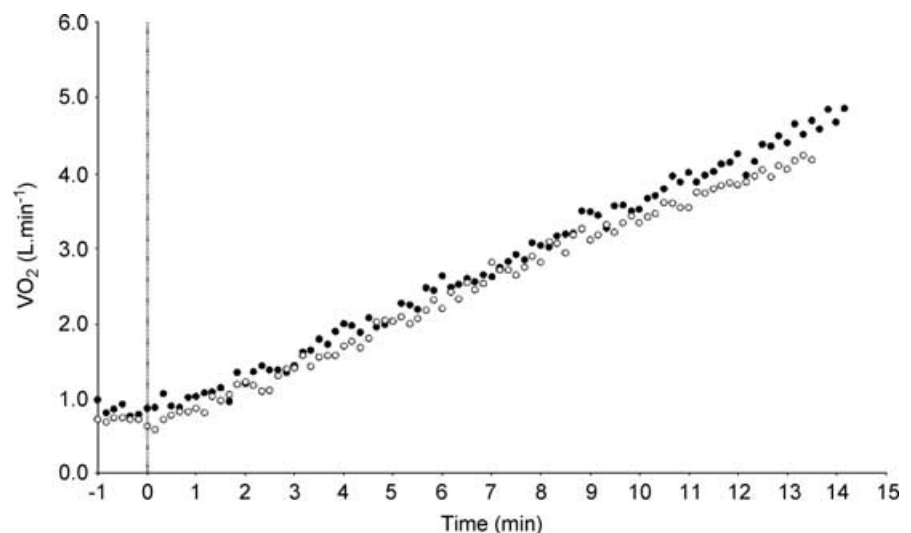
there is a much higher blood flow per unit muscle mass during knee extension exercise (peak muscle blood flow of  $\sim 400 \text{ ml kg}^{-1} \text{ min}^{-1}$ ) (Knight *et al.* 1992; Richardson *et al.* 1993). Indeed, in the control condition in the study of Frandsen *et al.* (2001), subjects attained maximal HRs of  $\sim 155 \text{ beats min}^{-1}$  and  $\dot{V}_{O_{2\max}}$  values of  $\sim 0.85 \text{ l min}^{-1}$ , on average, at exhaustion (appreciably less than the corresponding values in the present study). These values indicate that subjects became fatigued before maximal  $\dot{Q}$  was attained. As mentioned above, the similar leg blood flow and  $\dot{V}_{O_2}$ , despite a significant reduction in  $\text{HR}_{\max}$  to  $\sim 125 \text{ beats min}^{-1}$  with L-NAME in the study of Frandsen *et al.* (2001) could presumably have resulted from a compensatory increase in stroke volume and/or a redistribution of blood flow away from other vascular beds.

In contrast to one-legged knee extension exercise, incremental two-legged cycle exercise involves the recruitment of a substantially greater muscle mass and provides a greater challenge to the cardiovascular system such that it is generally accepted that  $\dot{V}_{O_{2\max}}$  is limited by a failure of  $O_2$  supply to meet muscle  $O_2$  demand (Knight *et al.* 1992; Saltin & Strange, 1992; Wagner, 2000; but see also Noakes, 1997 for an alternative perspective). In our study, it is very unlikely that an increase in stroke volume could have compensated for the reduction of maximal HR with L-NAME (from 186 to 178  $\text{beats min}^{-1}$ , on average) because stroke volume reaches a maximum at around 40–50%  $\dot{V}_{O_{2\max}}$  in normal healthy subjects (Rowell, 1974; Stringer *et al.* 1997). It is also unlikely that muscle blood flow could be maintained by a redistribution of blood flow away from other organs when the exercising muscles already receive a very high fraction of the maximal  $\dot{Q}$  during high-intensity exercise with large muscle groups (Knight *et al.* 1992). Although muscle  $O_2$  extraction could be increased to compensate for a reduced  $O_2$  delivery, this might be insufficient to prevent a reduction in

$\dot{V}_{O_{2\max}}$  during maximal intensity cycle exercise where the arterial–venous  $O_2$  difference might be approaching a ceiling (Knight *et al.* 1992; Kindig *et al.* 2000).

### Mechanisms

For the reasons given above, we believe that a likely explanation for the significantly lower  $\dot{V}_{O_{2\max}}$  we observed with L-NAME is the significant reduction in HR (and therefore, presumably,  $\dot{Q}$  and muscle blood flow) at maximal work rates. In support of this, it is interesting to note that the magnitude of the reduction in  $\text{HR}_{\max}$  and  $\dot{V}_{O_{2\max}}$  with L-NAME were similar (4.3 versus 5.5%, respectively). Indeed, the maximum  $O_2$  pulse was not significantly different between the conditions ( $\sim 22 \text{ ml } O_2 \text{ beat}^{-1}$ ), suggesting that the reduction in  $\dot{V}_{O_{2\max}}$  with L-NAME was proportional to the reduction in  $\text{HR}_{\max}$ . It has been suggested that the reduced HR with L-NAME might be mediated by a baroreflex caused by the elevated blood pressure which reduces sympathetic efferent activity to the heart (Hirai *et al.* 1994). It should be mentioned here that this inhibition of sympathetic outflow with L-NAME might also increase vascular conductance and mask any reduction in muscle blood flow during submaximal exercise that would otherwise have resulted from a local reduction in NO-mediated vasodilatation (Sheriff *et al.* 2000). It is interesting to note, however, that MAP was not significantly different between the L-NAME and control conditions early in the recovery period following the termination of exercise. This suggests that the lower maximal HR with L-NAME might, at least in part, result from central effects of NOS inhibition on the heart. In addition to the direct or indirect effects on HR, it is also possible that L-NAME reduces stroke volume during exercise. For example, the reduced vascular conductance with L-NAME will increase myocardial afterload and reduce venous return. Indeed, Kindig *et al.*



**Figure 6.** Oxygen uptake response to incremental exercise following the inhibition of nitric oxide synthase (○) and following a placebo infusion (●) in a typical subject

Note the appreciable reduction in maximal oxygen uptake following nitric oxide synthase inhibition.

(2000) found that while the reduced  $\dot{Q}$  they observed at a work rate equivalent to 50%  $\dot{V}_{O_2\max}$  could be attributed equally to reductions in HR and stroke volume, the lower  $\dot{Q}$  at work rates equivalent to 80 and 100%  $\dot{V}_{O_2\max}$  was principally caused by a reduced stroke volume. In this context, it is interesting to note that Sherman *et al.* (1997) have reported that intracoronary infusion of L-NAME in intact dogs reduced segment shortening and myocardial  $O_2$  consumption. A reduction in cardiac myocyte contractility with L-NAME might therefore also have the potential to reduce  $\dot{Q}$  during exercise, although direct measurements have not been made in humans.

As exercise intensity (and therefore the mass of recruited muscle and the supporting  $\dot{Q}$ ) increases during incremental exercise, there is an increase in sympathetic activity in order to maintain blood pressure (Rowell, 1993). Our results might therefore also be interpreted to suggest that locally mediated vasodilatation is unable to completely overcome the sympathetically mediated increase in vascular tone during near-maximal intensity cycle exercise following NOS inhibition such that muscle blood flow is reduced compared to the control condition. In this case, the reduced HR we observed would be a consequence rather than a cause of the reduced muscle blood flow and  $\dot{V}_{O_2}$ . If true, this implies that NO might be important in the regulation of muscle blood flow during very high intensity exercise in man either through local effects on the vascular endothelium or by opposing sympathetic vasoconstriction (Moncada *et al.* 1991; Shepherd & Katusic, 1991; Sander *et al.* 1995; Thomas & Victor, 1998). One other possibility for the reduction in  $\dot{V}_{O_2\max}$  with L-NAME is the development of a (more pronounced)  $\dot{Q}/\dot{V}_{O_2}$  mismatch at maximal work rates. Again, it might be expected that any effect of NOS inhibition on regional  $\dot{Q}/\dot{V}_{O_2}$  would be greater during exercise requiring a large compared to a small muscle mass due to the differences in muscle perfusion noted earlier. Consequently, a more pronounced regional perfusion heterogeneity invoked by NOS inhibition might impact upon  $\dot{V}_{O_2\max}$  measured during 'whole body' exercise (present study) but not during isolated small muscle group exercise (Frandsen *et al.* 2001).

It should be pointed out that the reduction in  $\dot{V}_{O_2\max}$  that we observed cannot be attributed to an impairment of muscle contraction or mitochondrial function. On the contrary, *in vitro* studies indicate that NO might also play an important role in the regulation of muscle contraction and/or energy turnover. For example, NO has been shown (i) to reduce sarcoplasmic reticulum  $Ca^{2+}$  release,  $Ca^{2+}$  sensitivity and cross-bridge cycling kinetics, muscle force and actomyosin ATPase activity (Perkins *et al.* 1997; Heunks *et al.* 2001a,b); (ii) to inhibit key metabolic enzymes such as creatine kinase, aconitase, oxoglutarate dehydrogenase,

glyceraldehydes-3-phosphate dehydrogenase, and respiratory complexes I–IV (Zhang & Snyder, 1995; Cassina & Radi, 1996; Andersson *et al.* 1998; Brown, 1999; Kaasik *et al.* 1999); and (iii) to modulate the respiratory rate by competing with  $O_2$  for the binding site at cytochrome oxidase (Shen *et al.* 1994; Giulivi, 1998; Brown, 2000; Elfering *et al.* 2002). Therefore, NOS inhibition theoretically has the potential to enhance mitochondrial respiration. Indeed, several recent studies have shown that NOS inhibition with L-NAME accelerates the adaptation of  $\dot{V}_{O_2}$  towards the steady-state  $\dot{V}_{O_2}$  requirement upon the sudden transition to a moderate ( $\sim 40\text{--}50\%$   $\dot{V}_{O_2\max}$ ) or heavy ( $\sim 70\text{--}80\%$   $\dot{V}_{O_2\max}$ ) work rate both in the Thoroughbred horse (Kindig *et al.* 2001, 2002) and in humans (Jones *et al.* 2003, 2004). The similar absolute  $\dot{V}_{O_2}$  for the same work rate between the L-NAME and control conditions during submaximal exercise that we observed in the present study is consistent with the similar 'steady-state'  $\dot{V}_{O_2}$  attained during submaximal constant work rate exercise in these previous studies (Kindig *et al.* 2001, 2002; Jones *et al.* 2003, 2004). It is pertinent to emphasize therefore that the lower  $\dot{V}_{O_2\max}$  attained with L-NAME in the present study occurred despite a probable enhancement of mitochondrial and contractile function.

It is also important to stress that our results cannot be explained by a reduced effort on the part of the subjects in the incremental test following L-NAME infusion. The subjects were blind to the nature of the infusate, were highly motivated, and had significant experience of performing exercise tests involving maximal effort in our laboratory. The actual  $\dot{V}_{O_2\max}$  attained during incremental exercise is, of course, effort dependent; however, in our study,  $\dot{V}_{O_2}$  was also significantly reduced at the same absolute high-intensity (but submaximal) work rates in the L-NAME condition (Fig. 4).  $HR_{\max}$  is frequently used to judge the extent to which a maximal effort is given during incremental exercise tests. Although the  $HR_{\max}$  was significantly lower in the L-NAME condition compared to control, this clearly resulted from the direct or indirect action of the drug, as evidenced by the lower HR for the same work rate throughout exercise (Fig. 3). Interestingly, although  $\dot{V}_{O_2\max}$  was reduced with L-NAME, there was no significant reduction in the maximal values of  $\dot{V}_{CO_2}$  and  $\dot{V}_E$  between the experimental and control conditions (although there was a statistical tendency for these to be reduced also;  $P = 0.07\text{--}0.15$ ). This might be explained by a relatively greater contribution of anaerobic glycolysis to energy metabolism following L-NAME administration, with the greater  $\dot{V}_{CO_2}$  and  $\dot{V}_E$  (relative to  $\dot{V}_{O_2}$ ) resulting from increased bicarbonate buffering of the lactic acidosis. The necessity for an increased anaerobic ATP production to maintain ATP turnover during near-maximal exercise might also explain the reduction in exercise tolerance



with L-NAME in comparison to the control condition. Unfortunately, we did not measure blood [lactate] in the present study and so are unable to confirm this possibility.

### Limitations

A limitation to our study is the lack of direct measurements of muscle blood flow during exercise. Such measurements are technically challenging during upright cycle ergometer exercise. However, it has been shown that there is a strong linear relationship between muscle and pulmonary  $\dot{V}_{O_2}$  during incremental cycle exercise, with muscle  $O_2$  consumption contributing  $\sim 83\%$  to the pulmonary  $\dot{V}_{O_2}$  at maximal work rates (Knight *et al.* 1992). The remaining  $\sim 17\%$  has been attributed principally to the  $O_2$  cost associated with the work of breathing (Aaron *et al.* 1992). Because  $\dot{V}_{E\max}$  was not significantly different between the L-NAME and control conditions in the present study, the reduced pulmonary  $\dot{V}_{O_2}$  at near-maximal work rates was almost certainly a direct reflection of a reduced rate of  $O_2$  consumption in the leg muscles. Further studies with direct measurements of muscle blood flow are required to clarify the mechanisms underpinning the reduction in  $\dot{V}_{O_2\max}$  during whole-body exercise following NOS inhibition.

In conclusion, the systemic infusion of L-NAME (which caused a significant increase in MAP and a significant reduction in HR) did not significantly alter GET or  $\dot{V}_{O_2}$  at submaximal work rates. However, our results demonstrate for the first time that NOS inhibition causes a significant (albeit relatively small, i.e.  $\sim 6\%$ ) reduction in  $\dot{V}_{O_2\max}$  during whole body exercise in humans. These findings suggest that muscle blood flow was well preserved during submaximal exercise following NOS inhibition, indicating multifactorial regulation of vascular tone to ensure precise matching of  $\dot{Q}/\dot{V}_{O_2}$ . However, at near-maximal work rates, there was a significant reduction in  $\dot{V}_{O_2}$  following NOS inhibition which was associated with a significantly lower HR. Because NOS inhibition theoretically has the potential to enhance mitochondrial respiration by several mechanisms, our results strongly suggest that the reduction in  $\dot{V}_{O_2\max}$  was caused by a reduced blood flow to the working muscles.

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