Influence of nitric oxide synthase inhibition on pulmonary O_2 uptake kinetics during supra-maximal exercise in humans

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We have recently reported that inhibition of nitric oxide synthase (NOS) with N^G-nitro-L-arginine methyl ester (L-NAME) accelerates the 'phase II' pulmonary O_2 uptake ($\dot{V}_{O_2}$) kinetics following the onset of moderate and heavy intensity submaximal exercise in humans. These data suggest that the influence of nitric oxide (NO) on mitochondrial function is an important factor in the inertia to aerobic respiration that is evident in the transition from a lower to a higher metabolic rate. The purpose of the present study was to investigate the influence of L-NAME on pulmonary $\dot{V}_{O_2}$ kinetics following the onset of supra-maximal exercise, where it has been suggested that O_2 availability represents an additional limitation to $\dot{V}_{O_2}$ kinetics. Seven healthy young men volunteered to participate in this study. Following an incremental cycle ergometer test for the determination of $\dot{V}_{O_2max}$, the subjects returned on two occasions to perform a 'step' exercise test from a baseline of unloaded cycling to a work rate calculated to require 105% $\dot{V}_{O_2max}$, preceded either by systemic infusion of L-NAME (4 mg kg^{-1} in 50 ml saline) or 50 ml saline as a control (Con). Pulmonary gas exchange was measured on a breath-by-breath basis throughout the exercise tests. The duration of 'phase I' was greater with L-NAME (Con: 14.0 ± 2.1 *versus* L-NAME: 16.0 ± 1.6 s; $P = 0.03$), suggestive of a slower cardiovascular adaptation following the onset of exercise. However, the phase II $\dot{V}_{O_2}$ time constant was reduced by 44% with L-NAME (Con: 36.3 ± 17.3 *versus* L-NAME: 20.4 ± 8.3 s; $P = 0.01$). The accumulation of blood lactate during exercise was also reduced with L-NAME (Con: 4.0 ± 1.1 *versus* L-NAME: 2.7 ± 2.1 mM; $P = 0.04$). These data indicate that skeletal muscle NO production represents an important limitation to the acceleration of oxidative metabolism following the onset of supra-maximal exercise in humans.

Upon the abrupt transition from rest to exercise, there is an initial lag in the contribution of oxidative phosphorylation to the ATP turnover rate required for muscle force generation, with the deficit being made up by an acceleration of substrate-level phosphorylation (i.e. muscle phosphocreatine hydrolysis and, for higher work rates above the so-called lactate threshold (LT), anaerobic glycolysis) (Krogh & Lindhard, 1920; Whipp & Wasserman, 1972). At submaximal work rates below the LT, muscle O_2 consumption, and pulmonary oxygen uptake ($\dot{V}_{O_2}$), rise with an exponential time course to reach a steady-state within 2–3 min in young healthy subjects (Whipp & Wasserman, 1972). At higher sub-maximal work rates (i.e. above the LT), however, a steady

state in $\dot{V}_{O_2}$ is either delayed (if the work rate is below the critical power, CP; Monod & Scherrer, 1965) or is not attained at all (if the work rate is above the CP), due to the emergence of a 'slow component' of $\dot{V}_{O_2}$ that causes $\dot{V}_{O_2}$ to rise above the expected steady-state value (Whipp & Wasserman, 1972; Linnarsson, 1974). At supra-maximal work rates (i.e. work rates with an energy equivalent above the $\dot{V}_{O_2max}$), it is by definition impossible for a steady-state $\dot{V}_{O_2}$ to be attained. Whether or not a $\dot{V}_{O_2}$ slow component can be reliably discerned at these work rates probably depends on the exercise intensity and the time for which exercise can be sustained (Grassi *et al.* 2000; Scheuermann & Barstow, 2003). However, at supra-maximal work rates requiring > ~105–110% $\dot{V}_{O_2max}$, where time to exhaustion

is ≤ 2 min, the available evidence indicates that $\dot{V}_{O_2}$ rises exponentially until $\dot{V}_{O_2max}$ is achieved or the exercise is terminated by fatigue (Özyener *et al.* 2001; Hill *et al.* 2002; Scheuermann & Barstow, 2003; Wilkerson *et al.* 2004*a*).

The factor(s) that limit(s) the rise in $\dot{V}_{O_2}$ in the transition from a lower to a higher metabolic rate is/are obscure. For submaximal cycle exercise < LT in young healthy subjects, there is compelling evidence that 'metabolic inertia' (e.g. the activation kinetics of key oxidative enzymes) represents the principal limitation to muscle O_2 consumption following the onset of exercise (e.g. Whipp & Mahler, 1980; Grassi *et al.* 1998; Rossiter *et al.* 1999; Burnley *et al.* 2000; Koga *et al.* 2001); the limitations to the rate of rise of muscle oxygen consumption > LT are, however, more controversial (Hughson *et al.* 2001). Relatively few studies have examined $\dot{V}_{O_2}$ kinetics during supra-maximal exercise. However, at maximal and supra-maximal work rates, there is some evidence that muscle O_2 availability provides an additional limitation to $\dot{V}_{O_2}$ dynamics. For example, Grassi *et al.* (2000) demonstrated that $\dot{V}_{O_2}$ kinetics were ~25% faster when muscle contractions at an intensity requiring 100% $\dot{V}_{O_2max}$ were initiated with blood flow set at the 'steady-state' requirement across the rest-to-exercise transition in an isolated *in situ* canine muscle preparation. However, in contrast, the performance of prior high-intensity exercise, which is known to result in increased muscle blood flow (Krustrup *et al.* 2001), did not alter phase II pulmonary $\dot{V}_{O_2}$ kinetics during supra-maximal exercise in humans (Wilkerson *et al.* 2004*a*). The potential for O_2 availability to modulate $\dot{V}_{O_2}$ kinetics during maximal intensity exercise therefore remains unclear.

Nitric oxide (NO) has been implicated in a multitude of physiological functions including the control of skeletal muscle vasodilatation and oxidative metabolism (Moncada *et al.* 1991; Stamler & Meissner, 2001). Specifically, it has been proposed that NO is one of several vaso-active substances that are released at exercise onset and which interact to regulate vascular conductance and muscle blood flow (e.g. Moncada *et al.* 1991; Shepherd & Katusic, 1991; Sander *et al.* 1995). It has also been demonstrated, *in vitro,* that NO inhibits a number of key oxidative enzymes and competes with O_2 for the binding site at cytochrome oxidase (e.g. Shen *et al.* 1994; Zhang & Snyder, 1995; Cassina & Radi, 1996; Brown, 1999, 2000). It is therefore possible that NO represents at least one source of the metabolic inertia following the onset of exercise. Indeed, inhibition of nitric oxide synthase (NOS, the enzyme responsible for the synthesis of NO) with the L-arginine analogue N^G-nitro-L-arginine methyl ester (L-NAME) has been shown to result in a speeding of the phase II pulmonary $\dot{V}_{O_2}$ kinetics during both moderate (< LT) and heavy (> LT) exercise both in the horse (Kindig *et al.* 2001, 2002) and in humans (Jones *et al.* 2003, 2004*a*).

These results are important because they demonstrate that $\dot{V}_{O_2}$ kinetics can be speeded by NOS inhibition despite a possible reduction in bulk muscle blood flow during exercise.

The purpose of the present study was to extend our previous investigations (Jones *et al.* 2003, 2004*a*) by examining the effect of L-NAME on $\dot{V}_{O_2}$ kinetics during supra-maximal exercise. As mentioned above, there is a stronger possibility that $\dot{V}_{O_2}$ kinetics are limited by muscle O_2 availability during supra-maximal compared to submaximal exercise (Grassi *et al.* 1998, 2000). Infusion of L-NAME therefore potentially permits an exploration of the interaction of intrinsic (metabolic inertia) and extrinsic (muscle O_2 delivery) factors on pulmonary $\dot{V}_{O_2}$ kinetics during supra-maximal exercise. It would be expected that sluggish cardiac output (and muscle blood flow) kinetics with L-NAME would be reflected in an increased circulatory transit delay (as evidenced by an increased phase I duration) before the exponential increase in $\dot{V}_{O_2}$ (phase II) towards the steady state (Barstow & Molé, 1987; Barstow *et al.* 1990). If this is the case, and O_2 availability is an important determinant of $\dot{V}_{O_2}$ kinetics during supra-maximal exercise, then the anticipated speeding of the phase II $\dot{V}_{O_2}$ kinetics with L-NAME (Kindig *et al.* 2001, 2002; Jones *et al.* 2003, 2004*a*) should be attenuated or eliminated. Our first hypothesis was that L-NAME would significantly increase the duration of the phase I pulmonary $\dot{V}_{O_2}$ response to exercise, consistent with a reduction in skeletal muscle blood flow in this condition. Our second hypothesis was that the phase II pulmonary $\dot{V}_{O_2}$ kinetics would be faster with L-NAME, despite the possibility of a reduction in skeletal muscle blood flow in this condition.

Methods

Subjects

Seven healthy males (mean ± s.d.: age 25 ± 4 years, body mass 78.5 ± 9.2 kg) volunteered to participate in this study. All subjects were informed of the experimental procedures, the potential risks and discomfort, and that they could withdraw from the study at any time without prejudice. All subjects gave their written informed consent. The experiments were approved by the Manchester Metropolitan University and South Cheshire Local Research Ethics Committees.

Procedures

The subjects were required to visit the laboratory on three occasions. On the first visit, they completed an incremental exercise test to exhaustion on an electronically braked cycle ergometer that maintained work rate independent of pedal rate within the range 30–120 rev min^{-1} (Jaeger Ergoline E800, Mindjhaart, The Netherlands). Following

3 min of 'unloaded' cycling, work rate was increased by 5 W every 10 s (i.e. 30 W min^{-1}) until the subject was unable to continue. The subjects cycled at a self-selected pedal rate (60–90 rev min^{-1}) and this pedal rate, and the saddle and handlebar heights and configurations were recorded and reproduced in subsequent tests. Pulmonary gas exchange was measured on a breath-by-breath basis (see below). The gas exchange threshold (GET) was determined from a cluster of measurements including: (1) the first disproportionate increase in $\dot{V}_{CO_2}$ from visual inspection of individual plots of $\dot{V}_{CO_2}$ *versus* $\dot{V}_{O_2}$ (*V*-slope method; Beaver *et al.* 1986); (2) the first increase in the ventilatory equivalent for O$_2$ with no increase in the ventilatory equivalent for CO$_2$; and (3) an increase in end-tidal O$_2$ tension with no fall in end-tidal CO$_2$ tension. The $\dot{V}_{O_2max}$ was determined as the highest value recorded in any 30 s period before the subject's volitional termination of the test. The work rate that would require 105% of the $\dot{V}_{O_2max}$ was calculated as 1.05 times the maximal work rate attained in the incremental test minus 20 W (to account for the 'lag' in the $\dot{V}_{O_2}$ response during the incremental test; Whipp *et al.* 1981).

On the second and third visits to the laboratory, the subjects performed a 'step' bout of exercise at the work rate corresponding to 105% $\dot{V}_{O_2max}$ following infusion of either L-NAME or saline. The conditions were presented in a counter-balanced design with the subjects blinded to the nature of the infusate. For each subject, the conditions were separated by 7 days. 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)$^{-1}$ 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 a 67% reduction in NOS activity in skeletal muscle with this procedure. Throughout the infusions, blood pressure and heart rate (HR) were monitored with an automated sphygmomanometer and a HR monitor (Polar Electro, Finland), respectively. The mean arterial blood pressure (MAP) was calculated as the diastolic pressure plus one-third of the pulse pressure.

Following 60 min rest, the subjects performed a supra-maximal exercise bout for as long as possible. The exercise protocol began with 3 min of baseline pedalling at 20 W (the lowest available work rate on the cycle ergometer), followed by an abrupt transition to the 105% $\dot{V}_{O_2max}$ work rate. The subjects received strong verbal encouragement to continue to exhaustion in each condition. Pulmonary gas exchange was measured breath-by-breath and HR was recorded at 5 s intervals throughout all 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). The system was calibrated prior to each test using gases of known concentration and a precision 3 l calibration syringe. A fingertip blood sample was collected into a capillary tube immediately before and after exercise in each condition and subsequently analysed for blood [lactate] (YSI 1500 Sport Lactate Analyzer, Yellow Springs Instruments, OH, USA). Blood pressure was measured within the first 30 s of recovery from exercise.

Analysis of $\dot{V}_{O_2}$ kinetics

The breath-by-breath $\dot{V}_{O_2}$ data for each transition were initially scrutinized to identify the phase I–phase II transition point (i.e. respiratory exchange ratio (RER) and end-tidal O$_2$ partial pressure (P_{ET,O_2}) starting to fall and end-tidal CO$_2$ partial pressure (P_{ET,CO_2}) starting to increase; Whipp *et al.* 1982). The breath-by-breath data were subsequently interpolated to give second-by-second values and the time course of $\dot{V}_{O_2}$ after the onset of exercise was initially described for each subject using a mathematical model which featured two exponential terms:

$$\dot{V}_{O_2}(t) = \dot{V}_{O_2B} + A_p\big(1 - e^{-(t-TD_p)/\tau_p}\big) + A_s\big(1 - e^{-(t-TD_s)/\tau_s}\big)$$

where $\dot{V}_{O_2B}$ is the baseline $\dot{V}_{O_2}$, and A_p and A_s are the response amplitudes; TD_p and TD_s are the time delays, and τ_p and τ_s are the time constants for the primary (p) and slow (s) components of the response, respectively. The first 20 s of data (containing the phase I response, see above) were not included in the model fit. Model parameters were determined by using a least-squares non-linear regression in which minimizing the sum of squared errors was the criterion for convergence. The amplitude of the $\dot{V}_{O_2}$ slow component was described as the increase in $\dot{V}_{O_2}$ from TD_s to the end of exercise (A_s').

Owing to concerns over the accuracy with which a slow component phase can be discerned in a single transition to supra-maximal exercise, we also described the overall $\dot{V}_{O_2}$ response to exercise (i.e. with no attempt to separate the response into its constituent primary and slow component phases), with a single exponential function following the removal of the first 20 s of data after the onset of exercise:

$$\dot{V}_{O_2}(t) = \dot{V}_{O_2B} + A\big(1 - e^{-(t-TD)/\tau}\big)$$

where A is the amplitude of the $\dot{V}_{O_2}$ response above baseline, TD represents a time delay and τ is the 'effective' time constant describing the kinetics of the change in $\dot{V}_{O_2}$ over the exercise bout.

Table 1. Pulmonary oxygen uptake kinetics during supramaximal exercise in the control (C) and L-NAME (L) conditions

	$\dot{V}_{O_2B}$ (ml min^{-1})		A_p' (ml min^{-1})		TD$_p$ (s)		τ_p (s)		A_s' (ml min^{-1})		TD$_s$ (s)		$\dot{V}_{O_2EE}$ (ml min^{-1})	
Subj.	C	L	C	L	C	L	C	L	C	L	C	L	C	L
1	654	606	2521	2438	21.0	29.8	31.3	15.0	275	368	114	65	2796	2806
2	831	708	3308	3193	16.9	19.7	46.7	34.1	474	475	133	122	3782	3668
3	640	664	2708	2585	12.1	13.3	21.8	18.2	—	—	—	—	2708	2585
4	738	783	2050	1853	14.8	23.6	45.5	25.2	—	344	—	120	2050	2197
5	886	913	3763	3325	15.6	20.7	65.2	22.7	—	—	—	—	3763	3325
6	782	650	2276	2126	13.2	17.6	30.0	20.0	1154	703	121	86	3430	2829
7	748	687	2755	2395	20.8	25.1	13.9	7.7	306	878	80	78	3061	3273
Mean	754	716	2769	2559	16.3	21.4	36.3	20.4	552	606	112	88	3084	2955
S.D.	89	103	592	535	3.5	5.4	17.3	8.3	411	229	25	24	627	499
P		0.21		0.007		0.003		0.01		0.81		0.11		0.29

$\dot{V}_{O_2B}$, baseline $\dot{V}_{O_2}$; A_p', amplitude of phase II $\dot{V}_{O_2}$ response; TD$_p$, time delay before phase II $\dot{V}_{O_2}$ response; τ_p, time constant of phase II $\dot{V}_{O_2}$ response; A_s', amplitude of slow component at the end of exercise; TD$_s$, time delay before onset of slow component; $\dot{V}_{O_2EE}$, end-exercise $\dot{V}_{O_2}$; Subj., subject.

Statistics

Paired *t* tests were used to test for significant differences in the $\dot{V}_{O_2}$ kinetic parameters between the control and L-NAME conditions. Pearson product moment correlation coefficients were used to examine the relationship between variables. Statistical significance was accepted when $P < 0.05$. Results are reported as mean ± S.D.
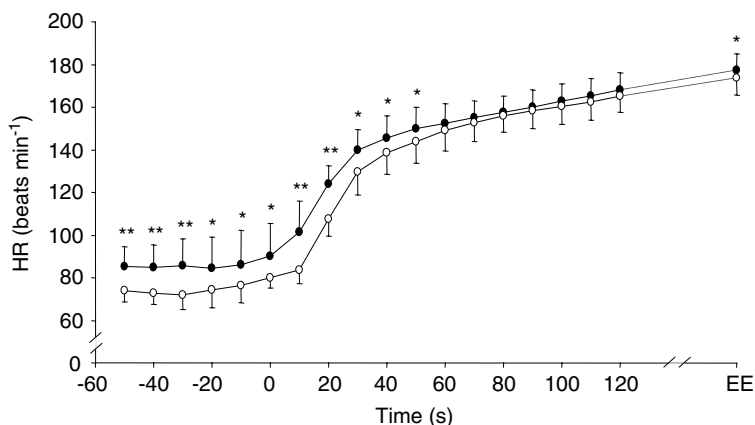
Results

The subjects' mean (± S.D.) $\dot{V}_{O_2max}$ was 52.3 ± 7.5 ml kg^{-1} min^{-1} with GET occurring at 50 ± 8% $\dot{V}_{O_2max}$. The increase in work rate above baseline cycling (20 W) to 105% $\dot{V}_{O_2max}$ was 360 ± 44 W. The time to exhaustion was not significantly different between the conditions (Con: 169 ± 35 *versus* L-NAME: 161 ± 34 s; $P = 0.08$).

As was expected (Sander *et al.* 1999), L-NAME infusion resulted in an elevation of MAP and a reduction in HR. At the end of the unloaded cycling period, MAP was significantly higher (Con: 96 ± 12 *versus* L-NAME: 116 ± 7 mmHg; $P = 0.04$) and heart rate was significantly lower (Con: 86 ± 14 *versus* L-NAME: 75 ± 8 beats min^{-1}; $P = 0.04$) following L-NAME infusion. In the immediate recovery period following exercise, MAP remained elevated (Con: 107 ± 18 *versus* L-NAME: 123 ± 22 mmHg; $P = 0.07$). HR was significantly lower in the L-NAME condition for the first 50 s of exercise, and at exhaustion (Fig. 1).

The $\dot{V}_{O_2}$ kinetic response data are presented in Table 1, and example responses are shown in Figs 2 and 3. L-NAME infusion resulted in a significant lengthening of the duration of phase I (Con: 14.0 ± 2.1 *versus* L-NAME: 16.0 ± 1.6 s; $P = 0.03$; Fig. 2). The O$_2$ pulse (i.e. $\dot{V}_{O_2}$/HR) was not significantly different between the conditions at 5 s of exercise (Con: 11.1 ± 2.7 *versus* L-NAME: 12.4 ± 2.2 ml beat^{-1}; $P = 0.23$) but was significantly greater with L-NAME at 10 s (Con: 11.5 ± 2.8 *versus* L-NAME: 14.2 ± 2.3 ml beat^{-1}; $P < 0.01$) and 15 s (Con: 12.2 ± 1.9 *versus* L-NAME: 13.8 ± 2.3 ml beat^{-1}; $P < 0.01$) of exercise. L-NAME infusion resulted in a

Figure 1

Mean (± S.D.) heart rate response to supra-maximal exercise in the placebo condition (●) and following the infusion of L-NAME (○). *$P < 0.05$; **$P < 0.01$. Time ○ represents onset of supra-maximal exercise.

significant reduction in the time constant describing the phase II $\dot{V}_{O_2}$ response (Con: 36.3 ± 17.3 versus L-NAME: 20.4 ± 8.3 s; $P = 0.01$; Fig. 3). The 95% confidence interval for the estimation of the phase II time constant was 6–7 s, on average. The increased duration of phase I and the reduction of the phase II time constant with L-NAME were not significantly correlated ($r = 0.08$; $P = 0.87$). The individual changes in the duration of phase I and in the phase II $\dot{V}_{O_2}$ time constant with L-NAME are illustrated in Fig. 4. The speeding of the phase II time constant with L-NAME was significantly correlated with the control value for the phase II time constant ($r = 0.90$; $P = 0.006$; Fig. 5), indicating that those individuals with the slowest phase II $\dot{V}_{O_2}$ kinetics in the control condition evidenced a greater speeding of these kinetics following the infusion of L-NAME. In addition to effects on the dynamic adjustment of $\dot{V}_{O_2}$ following the onset of exercise, the infusion of L-NAME was also associated with a reduction in the amplitude of the phase II $\dot{V}_{O_2}$ response (Table 1).

A $\dot{V}_{O_2}$ slow component was evident in 4/7 subjects in the control condition and in 5/7 subjects in the L-NAME condition. The existence or otherwise of the slow component was discerned from the goodness of fit of the single and double exponential models to each data set. On average, the mean squared error was reduced by 20% in those data sets in which a double exponential model provided a better description of the data than a single exponential model. There was no significant difference in the amplitude of the $\dot{V}_{O_2}$ slow component between conditions, but there was a tendency for the slow component to emerge earlier with L-NAME (Table 1). There was no significant difference in the end-exercise $\dot{V}_{O_2}$ between the conditions.

When the $\dot{V}_{O_2}$ data were described with a mono-exponential model with time delay following the removal of the phase I response, the principal findings were essentially unchanged. Specifically, the effective time constant was significantly shorter (Con: 50.5 ± 22.8 versus 34.1 ± 12.6 s; $P = 0.04$) following the infusion of L-NAME.

Immediately before the onset of exercise, blood [lactate] was significantly higher in the L-NAME condition compared to control (Con: 1.1 ± 0.2 versus L-NAME: 1.6 ± 0.3 mM; $P = 0.01$). There was no significant difference between the conditions at the end of exercise (Con: 5.1 ± 1.2 versus L-NAME: 4.3 ± 2.0 mM; $P = 0.10$) but blood lactate accumulation (i.e. Δ blood [lactate]) was significantly reduced with L-NAME (Con: 4.0 ± 1.1 versus L-NAME: 2.7 ± 2.1 mM; $P = 0.04$).

Discussion

In support of our experimental hypotheses, L-NAME resulted in a small but significant increase in the duration of phase I and a significant reduction in the time constant describing the phase II increase in $\dot{V}_{O_2}$. The significant increase in the duration of phase I suggests that NOS inhibition reduced cardiac output early in the transition from light to supra-maximal cycle exercise. However, the phase II time constant was reduced by 44% with L-NAME suggesting that the inhibition of the NO production reduced some of the metabolic inertia to the increase in $\dot{V}_{O_2}$ following the onset of exercise. The present study therefore suggests that any reduction in muscle blood flow, and therefore O_2 availability, was insufficient to fully cancel out the speeding of $\dot{V}_{O_2}$ kinetics brought about by the reduction in metabolic inertia with NOS inhibition.

Phase I

We found that the duration of phase I was slightly but significantly longer in the L-NAME condition compared to the control condition (i.e. ~14 versus 16 s; Fig. 2). These data imply that muscle blood flow was at least transiently reduced following NOS inhibition. This interpretation is supported by the significant increase in the O_2 pulse with L-NAME at 10 and 15 s of exercise. Other situations in which the duration of phase I is increased include subjects with chronic heart failure and in heart transplant recipients where the cardiovascular response to exercise is sluggish (Sietsema et al. 1986, 1994; Mettauer et al. 2000; Borrelli et al. 2003). In our study, the precise cause of the increased duration of phase I with L-NAME is somewhat unclear, but it was associated with a reduced HR both at rest and throughout exercise with the differences being significant over the first 50 s of exercise and at exhaustion. It has been proposed that the reduced vascular conductance with NOS inhibition causes a reflex reduction in HR as the result of a baroreceptor-mediated withdrawal of sympathetic outflow (Sheriff et al. 2000; Joyner & Tschakovsky, 2003). It is possible therefore that L-NAME reduces cardiac output and bulk muscle blood flow during exercise. However, it is also possible that the higher MAP with L-NAME arises from vasoconstriction in the vascular beds of other organs including non-active skeletal muscle, with blood flow to contracting skeletal muscle being well preserved (Frandsen et al. 2001). It has also been suggested that the reduced vascular conductance at rest with NOS inhibition could reduce the effectiveness of the muscle pump at exercise onset (Shoemaker & Hughson, 1999) which would also impact upon the duration of phase I. Interestingly, there was no appreciable difference in the duration of phase I with L-NAME in our two previous studies which were conducted at lower intensities (Jones et al. 2003, 2004a), suggesting that NO might be relatively more important in the regulation of muscle blood flow at the onset of high compared to low intensity cycle exercise.

In humans, NO is important in the regulation of vascular tone at rest and during recovery from exercise, but its role in the regulation of skeletal muscle blood flow during exercise is more controversial. For example, it has been recently reported that (partial) NOS inhibition does not significantly influence muscle blood flow during submaximal or maximal exercise with small muscle groups (Shoemaker *et al.* 1997; Radegran & Saltin, 1999; Frandsen *et al.* 2001). However, simultaneous inhibition of NOS and either prostaglandins (Boushel *et al.* 2002) or endothelial-derived hyperpolarizing factors (Hillig *et al.* 2003) does result in a reduction of skeletal muscle blood flow. It should be borne in mind that in the study of Frandsen *et al.* (2001) in which no effect of L-NAME on muscle hyperaemia was reported, discrete measurements of muscle blood flow were made at 10 and 20 min of submaximal exercise and at an unspecified time point during maximal exercise. The present study suggests, however, that NO might be important in the regulation of muscle blood flow early in the transition to exercise, i.e. over (at least) the first ~15 s following the onset of supra-maximal exercise.

Phase II

Despite the possibility that muscle blood flow was reduced, at least transiently, the phase II $\dot{V}_{O_2}$ kinetics were substantially faster (τ reduced from ~36 to ~20 s) in the L-NAME condition. These results corroborate our earlier work which demonstrated faster phase II $\dot{V}_{O_2}$ kinetics with L-NAME during moderate and heavy submaximal exercise (Jones *et al.* 2003, 2004*a*) and extend them to show that NO represents an important component of the metabolic inertia to the $\dot{V}_{O_2}$ dynamics during supra-maximal exercise also. The precise mechanism by which NO contributes to the metabolic inertia at exercise onset is unclear but, *in vitro*, it has been demonstrated that NO inhibits a number of key tricarboxylic acid and electron transport chain enzymes and also competitively inhibits mitochondrial respiration by competing with O_2 for the binding site at cytochrome oxidase (e.g. Shen *et al.* 1994; Zhang & Snyder, 1995; Cassina & Radi, 1996; Brown, 1999, 2000).

Interestingly, the magnitude of the speeding of the phase II $\dot{V}_{O_2}$ kinetics in the present study (44%) was greater than we have previously observed during exercise
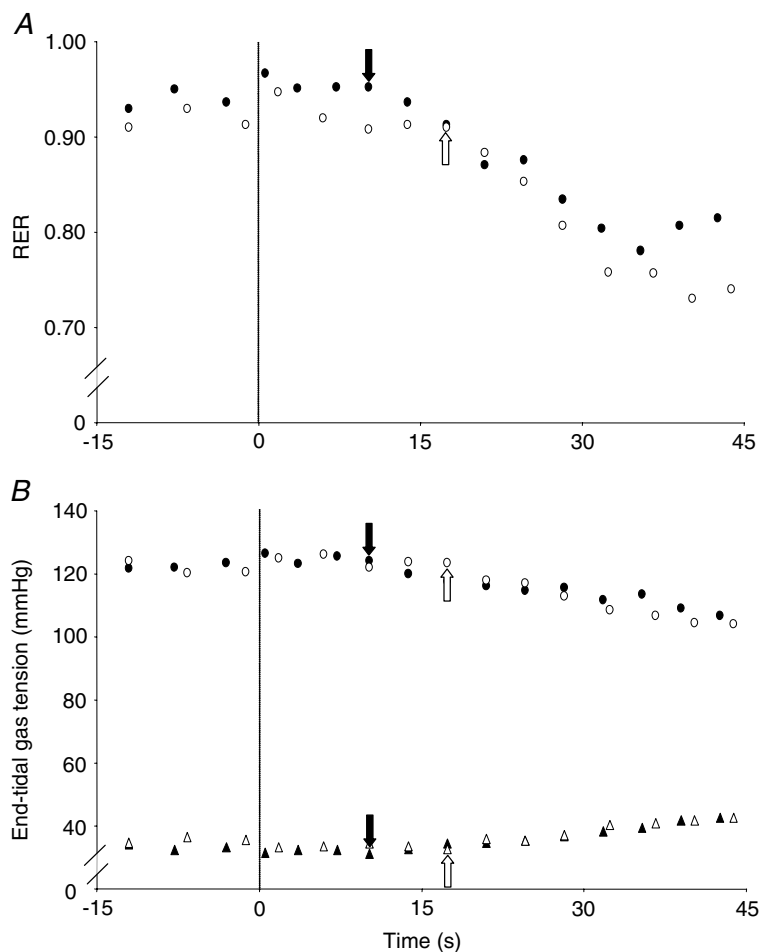

Figure 2
Respiratory exchange ratio (RER; *A*) and end-tidal gas profiles (*B*) following the transition from unloaded cycling to a supra-maximal work rate in the placebo (●) and L-NAME (○) conditions in a typical subject. Notice the delay in the L-NAME condition before RER (*A*) and P_{ET,O_2} (*B*, top) begin to fall and P_{ET,CO_2} begins to rise. These data suggest a more sluggish cardiac output response in the L-NAME condition.

at $\sim$45% $\dot{V}_{O_2max}$ (19% speeding; Jones *et al.* 2003) and $\sim$70% $\dot{V}_{O_2max}$ (13% speeding; Jones *et al.* 2004*a*). The possible limitations to $\dot{V}_{O_2}$ kinetics are complex and depend, in part, upon the intensity domain in which the exercise is performed (Whipp & Ward, 1990; Tschakovsky & Hughson, 1999; Özyener *et al.* 2001). It is generally reported that the phase II time constant is longer at higher work rates and this has been attributed to an increasing O_2 supply limitation (Hughson & Morrissey, 1982; Hughson *et al.* 2001) as well as to differences in the characteristics of the population of muscle fibres contributing to force production (Brittain *et al.* 2001; Jones *et al.* 2002; Koppo *et al.* 2004). In our three studies to date which have investigated the influence of L-NAME on $\dot{V}_{O_2}$ kinetics during constant work rate exercise (Jones *et al.* 2003, 2004*a*; present study) in essentially the same population of young, healthy, moderately trained males,

the phase II time constant in the control condition was $\sim$22 s (moderate exercise), $\sim$25 s (heavy exercise) and $\sim$36 s (supra-maximal exercise). Following the infusion of L-NAME, the phase II time constant was reduced to $\sim$18 s, $\sim$22 s and $\sim$20 s, respectively, in these three studies (Fig. 6). These data might be interpreted to suggest that the longer phase II time constant at higher work rates is related to a greater pernicious influence of NO on oxidative metabolism at higher work rates, and not to an O_2 delivery limitation. That the speeding of the $\dot{V}_{O_2}$ kinetics following NOS inhibition was significantly correlated with the absolute value of the phase II time constant in the control condition (Fig. 5) is consistent with this notion. Certainly, the faster phase II $\dot{V}_{O_2}$ kinetics during supra-maximal exercise following L-NAME administration cannot be ascribed to an enhanced muscle O_2 availability; on the contrary, as
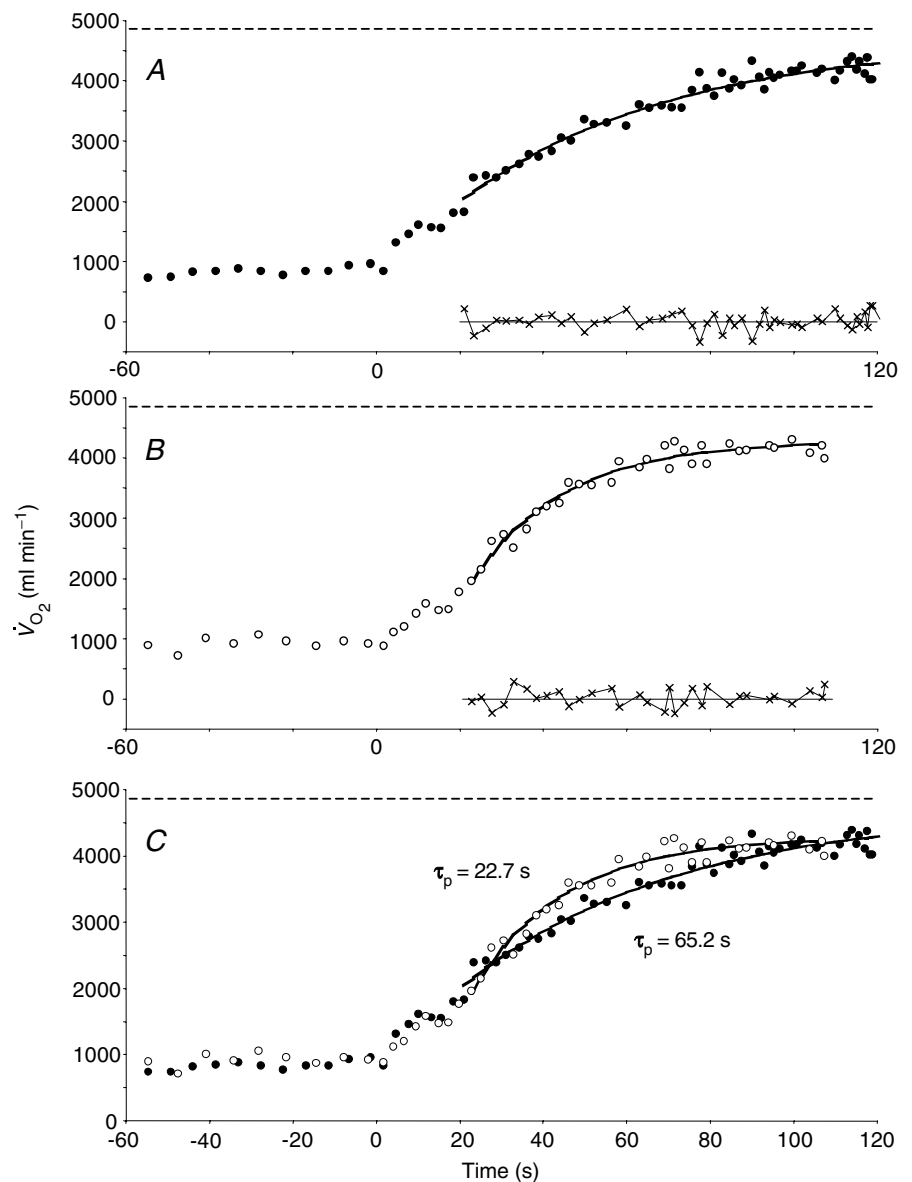

Figure 3

Pulmonary O_2 uptake response to supra-maximal exercise in the placebo condition (*A*) and following the infusion of L-NAME in one subject (*B*). The residuals at the bottom of each plot indicate the goodness of the model fit. In *C*, the O_2 uptake responses to exercise in the placebo (●) and L-NAME (○) conditions are overlaid. Note the dramatic speeding of the phase II $\dot{V}_{O_2}$ kinetics in this subject. The horizontal dashed line in all three plots represents the subject's $\dot{V}_{O_2max}$ value as determined from the preliminary incremental exercise test.

discussed above, the longer duration of phase I suggests that cardiac output (and muscle blood flow) might be at least transiently reduced with NOS inhibition. Although it is generally accepted that muscle O_2 delivery ultimately limits the highest attainable $\dot{V}_{O_2}$ amplitude (i.e. the $\dot{V}_{O_2 max}$), it does not necessarily follow that the rate at which $\dot{V}_{O_2}$ adjusts following the onset of supra-maximal exercise is similarly limited (Whipp, 1994). Consistent with this interpretation, we have recently reported that the performance of prior high intensity exercise, which has been shown to result in an increased muscle blood flow (Krustrup *et al.* 2001), does not significantly alter the phase II time constant during subsequent supra-maximal exercise, although it does increase the $\dot{V}_{O_2}$ attained at end-exercise (Wilkerson *et al.* 2004*a*).

Despite the dramatic effect of NOS inhibition on the phase II time constant, it should be emphasized that most of the metabolic (or other) limitation to $\dot{V}_{O_2}$ kinetics following the onset of exercise remains unexplained. For example, even when the phase II $\dot{V}_{O_2}$ kinetics become faster as the result of an intervention, the time constant has rarely been reported to be less than ~17–21 s either in
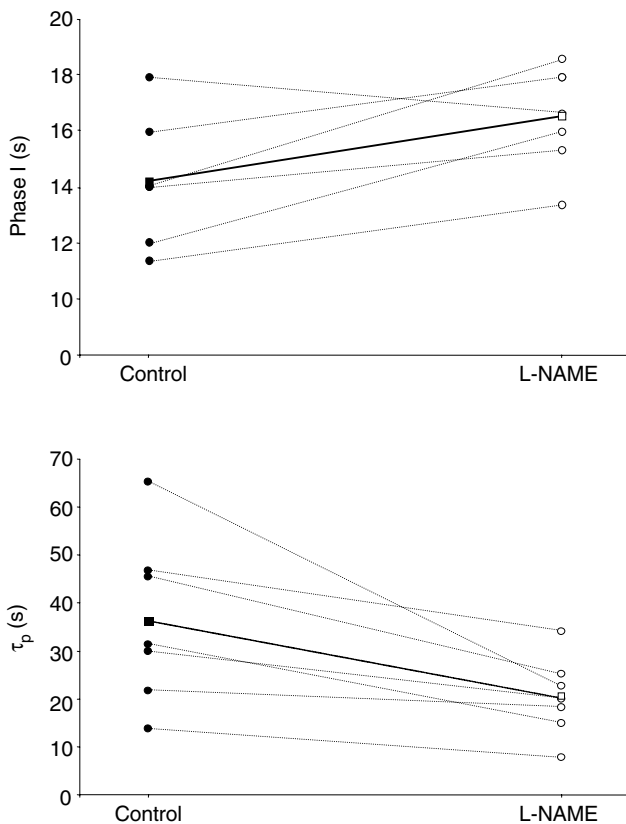

Figure 5
Relationship between the time constant of the phase II $\dot{V}_{O_2}$ response (τ_p) in the placebo condition and the speeding of the phase II $\dot{V}_{O_2}$ kinetics resulting from the infusion of L-NAME.

isolated muscle preparations (Grassi *et al.* 1998, 2000) or in the exercising human (Phillips *et al.* 1995; Scheuermann *et al.* 2002; Jones *et al.* 2003, 2004*a*; Tordi *et al.* 2003), although it can be as fast as 9–12 s in equine (Langsetmo *et al.* 1997; Kindig *et al.* 2002) and human (Koppo *et al.* 2004) athletes. Similar minimum values for the time constant for the fall in P_{O_2} or the rise in $\dot{V}_{O_2}$ of ~10–20 s have been reported in single isolated fibre studies (Hogan, 2001; Kindig *et al.* 2003), suggesting that this is an inherent (metabolic) limitation to oxidative respiration that is essentially independent of O_2 availability. There is compelling evidence that the phase II pulmonary $\dot{V}_{O_2}$ response following the onset of exercise is functionally

Figure 4
Individual changes in the duration of phase I and the time constant (τ_p) for the phase II $\dot{V}_{O_2}$ response in the placebo condition (●) and following the infusion of L-NAME (○). The bold lines and square symbols represent the group mean responses.

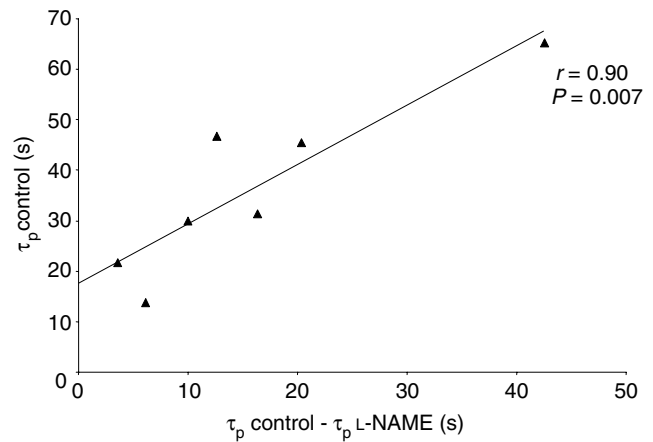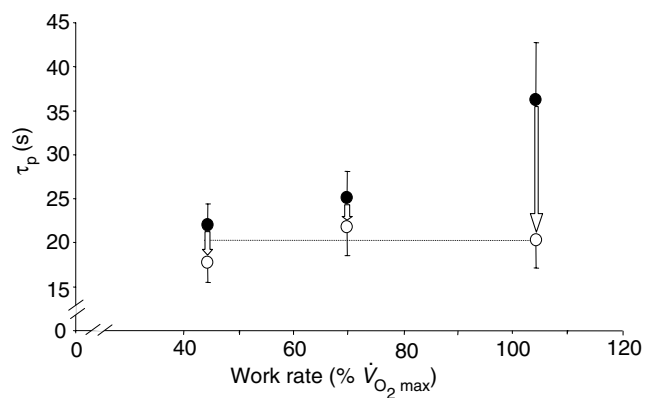**Figure 6**
Mean (± S.D.) values for the time constant of the phase II $\dot{V}_{O_2}$ response (τ_p) in the placebo condition (●) and following the infusion of L-NAME (○) for moderate exercise (Jones *et al.* 2003), heavy exercise (Jones *et al.* 2004*a*), and supra-maximal exercise (present study). The dotted line represents the mean τ_p (~20 s) following the infusion of L-NAME in the three studies. These data suggest that the lengthening of τ_p at higher work rates in the control condition might be related to a nitric oxide-mediated metabolic inertia. See text for further discussion.

linked to the rate of intramuscular phosphocreatine hydrolysis (Whipp & Mahler, 1980; Rossiter *et al.* 1999; Roman *et al.* 2002). It is likely therefore that the 'remaining' limitation to the phase II $\dot{V}_{O_2}$ kinetics following the infusion of L-NAME is linked to a feedback mechanism involving one or more of the products of high-energy phosphate hydrolysis, to the kinetics of creatine kinase activation, or possibly to mitochondrial Ca^{2+} dynamics.

An additional novel feature of the present study was the significant 8% reduction in the amplitude of the phase II $\dot{V}_{O_2}$ response in the L-NAME condition compared to control, although there was no significant difference in the $\dot{V}_{O_2}$ attained at the end of exercise in the two conditions. The 'gain' of the phase II $\dot{V}_{O_2}$ response (i.e. $\Delta\dot{V}_{O_2}/\Delta$ work rate) was reduced from ∼7.7 ml min^{-1} W^{-1} in the control condition to ∼7.1 ml min^{-1} W^{-1} in the L-NAME condition. This lower-than-expected phase II $\dot{V}_{O_2}$ gain during peri-maximal exercise in the control condition (Fig. 3) has been previously described (Jones *et al.* 2002; Pringle *et al.* 2003*a*; Scheuermann & Barstow, 2003; Wilkerson *et al.* 2004*a,b*) and might be attributed to a number of factors including the influence of pH on mitochondrial function (Conley *et al.* 2001) and the recruitment of type II muscle fibres in which blood flow and microvascular P_{O_2} might be sufficiently low during high intensity exercise to limit the increase in $\dot{V}_{O_2}$ (Behnke *et al.* 2003). We have recently observed that prior high-intensity exercise increases the phase II $\dot{V}_{O_2}$ gain during subsequent peri-maximal exercise (Wilkerson *et al.* 2004*a*), suggesting that muscle blood flow and/or its distribution might limit the amplitude to which $\dot{V}_{O_2}$ can rise following the onset of peri-maximal exercise in the 'control' condition. The cause of the reduction in the phase II $\dot{V}_{O_2}$ gain with L-NAME is unclear, although it is known that this parameter is sensitive to a number of factors including the performance of prior exercise, muscle fibre type and fibre recruitment patterns, other pharmacological and nutritional interventions, and O_2 availability (Koga *et al.* 1999; Burnley *et al.* 2000; Jones *et al.* 2002; Pringle *et al.* 2003*a,b*; Rossiter *et al.* 2003). For example, a lower phase II $\dot{V}_{O_2}$ gain during heavy cycle exercise has been reported in subjects with a high percentage of type II fibre distribution in the working muscles (Barstow *et al.* 1996; Pringle *et al.* 2003*a*) and at high compared to low pedal rates, where type II fibre recruitment is likely to be enhanced (Pringle *et al.* 2003*b*). However, it is difficult to see how L-NAME administration could alter muscle fibre recruitment patterns during exercise, although this should not, of course, be ruled out.

A lower phase II $\dot{V}_{O_2}$ gain has also been reported during supine compared to upright cycle exercise, where there is a reduced pressure head for muscle blood flow (Koga *et al.* 1999). One possibility for the lower phase II $\dot{V}_{O_2}$ gain

with L-NAME, therefore, is that NOS inhibition partially reduced muscle blood flow during supra-maximal exercise (see earlier discussion). This postulate is strengthened when one considers that the phase II $\dot{V}_{O_2}$ gain is not altered by L-NAME during moderate or heavy submaximal exercise (Jones *et al.* 2003, 2004*a*) and that $\dot{V}_{O_2max}$ is reduced during maximal intensity treadmill running in the Thoroughbred horse (Kindig *et al.* 2000). In a recent study (Jones *et al.* 2004*b*), we observed that L-NAME infusion resulted in a small (∼6%) but significant reduction in $\dot{V}_{O_2max}$ during incremental cycle exercise in humans, which was associated with a significant reduction in maximal HR (and presumably maximal cardiac output). Assuming that there was indeed a similar reduction in $\dot{V}_{O_2max}$ with L-NAME in the present study, then the same absolute work rate imposed in both conditions would have represented a slightly higher relative exercise intensity in the L-NAME condition. However, because phase II $\dot{V}_{O_2}$ kinetics tend to become slower at higher relative exercise intensities, the reduction of the phase II time constant with L-NAME might be considered to be even more impressive. It should also be pointed out that the time to fatigue was not significantly different in the two conditions implying either that the relative exercise intensity was not appreciably different with and without L-NAME, or that L-NAME infusion preserved exercise tolerance during constant work rate supra-maximal exercise despite a reduction in $\dot{V}_{O_2max}$ resulting from a reduced muscle blood flow.

It has also been suggested that NO might extend the zone of effective tissue respiration by increasing the O_2 gradient away from the blood vessel (Thomas *et al.* 2001). One other consideration therefore is that NOS inhibition might have reduced the gradient for O_2 diffusion away from the capillaries thereby altering the distribution of perfusion to metabolic demand ($\dot{Q}/\dot{V}_{O_2}$, where $\dot{Q}$ is blood flow; Richardson *et al.* 2002). However, it is important to reiterate here that if muscle blood flow or O_2 distribution were indeed reduced with L-NAME, then this did not significantly impact on the acceleration of $\dot{V}_{O_2}$ kinetics caused by removal of the inhibitory effect of NO on mitochondrial function.

Slow component

A $\dot{V}_{O_2}$ slow component was observed in 4 of the 7 subjects in the control condition and in 5 of the 7 subjects in the L-NAME condition (Table 1). The existence of a $\dot{V}_{O_2}$ slow component during supra-maximal exercise is somewhat controversial since in some (Özyener *et al.* 2001; Wilkerson *et al.* 2004*a*), though not all (Hughson *et al.* 2000), studies it has been reported that the $\dot{V}_{O_2}$ response is adequately described with a mono-exponential function during such exercise. Whether or not a $\dot{V}_{O_2}$ slow component can be discerned during supra-maximal exercise possibly depends on the sustainable exercise duration since the slow

component typically emerges at ∼120 s into high-intensity exercise (e.g. Burnley *et al.* 2000; Pringle *et al.* 2003*a*; Koppo *et al.* 2004). For example, in a recent study in which the time to exhaustion at a supra-maximal work rate was ∼110–150 s, we reported that the $\dot{V}_{O_2}$ data were adequately described with a mono-exponential function (Wilkerson *et al.* 2004*a*). However, in the present study (time to exhaustion ∼160–170 s, on average), the $\dot{V}_{O_2}$ response of some subjects was better described with a bi-exponential model. The subjects in whom a $\dot{V}_{O_2}$ slow component could not be discerned had the shortest times to exhaustion (∼120 s).

We have previously reported that L-NAME resulted in a significantly greater amplitude of the $\dot{V}_{O_2}$ slow component during heavy exercise at 40% 'Δ' (∼70% $\dot{V}_{O_2 max}$) with no difference in the time at which the slow component emerged (Jones *et al.* 2004*a*). In the present study, there was no significant difference in the slow component amplitude in the four subjects who demonstrated a slow component response in both conditions but there was a tendency for the slow component to emerge earlier in the L-NAME condition (Table 1). Kindig *et al.* (2001) have previously reported that the $\dot{V}_{O_2}$ slow component emerged earlier (125 *versus* 65 s, on average) during high-intensity treadmill running in the Thoroughbred horse following the administration of L-NAME. The mechanism by which NOS inhibition might cause an earlier onset of the slow component during supra-LT exercise is not immediately apparent, although it has been suggested that alterations in muscle O_2 availability and/or muscle fibre recruitment might be responsible (Kindig *et al.* 2001).

Blood [lactate] and exercise tolerance

The resting blood [lactate] was significantly greater in the L-NAME compared to the control condition. We have noted a non-significant tendency for this same effect in our previous studies with L-NAME (Jones *et al.* 2003, 2004*a*). It is possible that this effect is related to the reduced vascular conductance at rest that occurs with NOS inhibition (Radegran & Saltin, 1999; Frandsen *et al.* 2001). However, the accumulation of blood lactate from the start to the end of exercise (i.e. Δ blood [lactate]) was significantly reduced with L-NAME compared to control. This is consistent with a reduction in the muscle O_2 deficit resulting from a speeding of the $\dot{V}_{O_2}$ kinetics. However, this reduced blood lactate accumulation with exercise in the L-NAME condition did not facilitate exercise tolerance because the time to exhaustion was not significantly different between the two conditions. It is possible that the enhanced mitochondrial respiration resulting from an alleviation of some of the metabolic inertia following NOS inhibition was counterbalanced by a reduced blood flow and maximal attainable $\dot{V}_{O_2}$ during maximal intensity exercise (Kindig *et al.* 2000; Jones *et al.* 2004*b*).

Modelling

The most appropriate procedure for describing the pulmonary $\dot{V}_{O_2}$ response to supra-maximal exercise has been debated (Hughson *et al.* 2000; Scheuermann & Barstow, 2003). In the present study, we chose to use standard exponential curve-fitting procedures since this approach makes no assumption with regard to the amplitude to which $\dot{V}_{O_2}$ should theoretically project (Hughson *et al.* 2000; Scheuermann & Barstow, 2003). One possible limitation was that our subjects only completed one transition to exercise in each condition. Breath-by-breath respiratory gas exchange data is inherently variable but the signal-to-noise ratio can be enhanced by averaging the response to several repeat transitions and/or maximizing the $\dot{V}_{O_2}$ response amplitude (Lamarra *et al.* 1987). Although our subjects only completed one transition, the fact that they were healthy physically active males performing supra-maximal exercise meant that the response amplitude was appreciable (i.e. ∼3 l min^{-1} above baseline). That time to exhaustion was ∼3 min also meant that sufficient data were collected for adequate curve fitting (see Fig. 3 for an example of the fidelity of the data collected). The 95% confidence interval surrounding the estimation of the phase II time constant was 6–7 s, on average, which, although larger than the ideal, was much smaller than the difference between the two conditions. In several data sets, the goodness of fit was significantly improved by the inclusion of a second exponential term to describe the slow component phase of the response (in these cases, the mean squared error was reduced by 20%, on average). It should be pointed out, however, that the inclusion of additional exponential terms in the modelling procedure has the potential to reduce statistical confidence in any of the parameters so derived including the primary variable of interest, the phase II time constant. To be certain that this did not inadvertently influence our results or interpretation, we also performed additional analysis in which the first 20 s of data following the onset of exercise was omitted and a mono-exponential model was applied to the remaining data. However, this did not appreciably affect our principal results or conclusions (i.e. the 'effective' time constant was significantly reduced from 50.5 ± 22.8 to 34.1 ± 12.6 s with L-NAME).

The increased duration of phase I that we have reported here suggests that muscle blood flow was at least transiently reduced following the onset of supra-maximal cycle exercise with NOS inhibition. As we have noted previously (Jones *et al.* 2003, 2004*a*), changes in cardio-vascular dynamics can confound simple interpretation of the pulmonary $\dot{V}_{O_2}$ dynamics during exercise (Barstow & Molé, 1987; Barstow *et al.* 1990). Specifically, in a mathematical modelling study, Barstow *et al.* (1990)

estimated that a slowing of cardiac output kinetics (with constant muscle O_2 consumption kinetics) would result in an increased duration of phase I and a reduced phase II time constant, as indeed we found in the present study. This might suggest that muscle $\dot{V}_{O_2}$ kinetics were unaltered in this and previous studies which have reported faster phase II pulmonary $\dot{V}_{O_2}$ kinetics with L-NAME (Kindig *et al.* 2001, 2002; Jones *et al.* 2003, 2004*a*). However, in our two previous studies (Jones *et al.* 2003, 2004*a*), we were unable to determine any significant effect of L-NAME on the duration of phase I but we still found a significant speeding of the phase II $\dot{V}_{O_2}$ kinetics. Furthermore, in the present study, there was no significant correlation between the increase in the duration of phase I and the speeding of the phase II $\dot{V}_{O_2}$ kinetics with L-NAME, suggesting that the reduced phase II time constant was not dependent on the altered cardiovascular response to exercise. Finally, the significant 33% reduction in Δ blood [lactate] that we observed with L-NAME suggests that the muscle O_2 deficit was reduced in this condition. For these reasons, we believe that the speeding of the phase II $\dot{V}_{O_2}$ kinetics that we report here resulted primarily from a removal of the inhibitory effect of NO on mitochondrial respiration rather than from an 'artefactual' effect caused by substantially altered cardiovascular dynamics. However, further studies involving 'direct' measurements of muscle $\dot{V}_{O_2}$ kinetics are necessary to confirm this.

In summary, we have shown that the inhibition of NOS with L-NAME causes a small but significant increase in the duration of phase I (of ∼2 s), and a significant reduction in the phase II $\dot{V}_{O_2}$ time constant (of ∼16 s) following the onset of exhaustive constant-load cycle exercise at a work rate calculated to require 105% $\dot{V}_{O_2\,max}$. These results demonstrate that phase II $\dot{V}_{O_2}$ kinetics can be speeded with NOS inhibition even during supra-maximal exercise, despite the possibility that cardiac output is simultaneously reduced. That NOS inhibition can significantly reduce the phase II $\dot{V}_{O_2}$ time constant during supra-maximal exercise to values that are typically reported for moderate intensity exercise (i.e. ∼20 s), presumably by alleviating the pernicious effects of NO on mitochondrial respiration, suggests that an NO-dependent metabolic inertia represents an important limitation to $\dot{V}_{O_2}$ kinetics following the onset of high-intensity cycle exercise.

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