Nitric oxide and exercise in the horse

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1. The effects of exercise on the production rate of nitric oxide (NO) in exhaled air ($\dot{V}_{NO}$) and the effects of inhaled NO (80 p.p.m.) on cardiovascular and respiratory parameters were investigated in five Thoroughbred horses.

2. The concentration of NO ([NO]) in exhaled air collected from within the nasal opening was lower when collected at a high flow rate of 80 l min^{-1} than at a low flow rate of 20 l min^{-1}: when trotting at 3·7 m s^{-1} the values were 0·78 ± 0·15 and 1·23 ± 9·14 p.p.b., respectively, and when cantering at 9 m s^{-1} the values were 1·69 ± 0·31 and 2·25 ± 0·32 p.p.b., respectively.

3. Nebulized methoxamine (40 mg ml^{-1} for 60 s), an α_1-adrenergic agonist, further reduced [NO] during the 9 m s^{-1} canter to 1·05 ± 0·14 and 1·99 ± 0·41 p.p.b. when collected at 80 and 20 l min^{-1}, respectively, and induced cyclical changes in the breathing pattern.

4. Exercise induced a linear increase in $\dot{V}_{NO}$ with work intensity to a maximum (428·1 ± 31·6 pmol min^{-1} kg^{-1}) which coincided with the maximal oxygen uptake for the horses (138·3 ± 11·7 ml min^{-1} kg^{-1}), although a further increase in $\dot{V}_{NO}$ (779·3 ± 38·4 pmol min^{-1} kg^{-1}) occurred immediately after exercise. The changes in $\dot{V}_{NO}$ correlated well with the tidal volume ($r = 0·968$; $P < 0·01$) and the haematocrit ($r = 0·855$; $P < 0·01$).

5. In the first 2 min of high intensity exercise, inhaled NO (80 p.p.m.) significantly ($P < 0·05$) reduced the pulmonary artery pressure: during the first minute, pulmonary artery pressure was 83·1 ± 7·6 mmHg compared with a control value of 94·4 ± 6·3 mmHg, and during the second minute, 84·2 ± 7·1 mmHg compared with a control value of 98·4 ± 4·7 mmHg. There were no other significant changes in cardiovascular or respiratory indices, including cardiac output, measured during exercise between control and inhaled NO tests.

6. The results show that exhaled NO is released from the airways of the horse and may contribute to the regulation of pulmonary vascular tone during exercise.

Furchgott & Zawadski (1980) first described an endothelium-derived relaxing factor (EDRF) which was subsequently shown to be nitric oxide (NO; Palmer, Ferridge & Moncada, 1987). Since these observations, it has become evident that NO has a range of physiological and pathological functions in the body, including host defence, ciliary function, neurotransmission, inhibition of platelet activity and bronchodilatation (reviewed by Moncada, Palmer & Higgs, 1991).

NO is produced from L-arginine and molecular oxygen (O_2) by one of three isoforms of the enzyme nitric oxide synthase (NOS). Most cells can produce NO but there are certain types of cells which generally contain one or more isoforms of NOS (Nathan & Xie, 1994). Two NOS isoforms, vascular endothelial (ecNOS or NOS III) and neuronal (ncNOS or NOS I) are dependent on calcium and calmodulin. An inducible isoform of NOS (iNOS or NOS II) is expressed in most cells types and its expression is enhanced by endotoxin and some cytokines (Nathan & Xie, 1994).

NO regulates vascular tone in both the systemic and pulmonary circulations (Moncada *et al.* 1991). It is one of the major vasodilators released from vascular endothelial cells (Vane, Angaard & Botting, 1990). A deficiency in the activity of ecNOS may be responsible, at least in part, for pulmonary hypertension in man (Higenbottam, Pepke-Zaba, Scott & Wallwork, 1988) while inhaled NO in low concentrations (up to 80 p.p.m.) reduces pulmonary hypertension in man (Pepke-Zaba, Higenbottam, Dinh-Xuan, Stone & Wallwork, 1991). Inhaled NO is also used therapeutically in human patients with severe lung diseases, such as adult respiratory distress syndrome (ARDS), to improve selectively the vascular perfusion of ventilated regions of the lung, thereby reducing intrapulmonary shunt

and minimizing O_2 toxicity (Bigatello, Hurford, Kacmarek, Roberts & Zapol, 1994; Zapol & Hurford, 1994).

Exercise will increase pulmonary artery pressure (PAP), despite inducing a reduction in the pulmonary vascular resistance (PVR; West *et al.* 1993; Manohar, 1994; Kane, Tesauro, Koizumi, Gupta & Newman, 1994; West & Mathieu-Costello, 1995). The contribution of endogenous NO to the modulation of PVR during exercise is uncertain. In sheep, inhaled NO does not appear to affect pulmonary vascular tone, although N^{ω}-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NOS, induced vasoconstriction and an increase in PAP both at rest and during exercise (Kane *et al.* 1994; Koizumi, Gupta, Bannerjee & Newman, 1994).

Several studies have reported an increase in the production rate of NO ($\dot{V}_{NO}$) in exhaled air during exercise (Persson, Wiklund & Gustafsson, 1993; Bauer, Wald, Doran & Soda, 1994; Iwamoto, Pendergast, Suzuki & Krasney, 1994; Trolin, Anden & Hedenstierna, 1994). Exhaled NO is formed by many different cell types but the exact contribution of each to the exhaled concentration is uncertain. Persson *et al.* (1993) suggested that most of the exhaled NO originated from the airways, possibly the terminal and respiratory bronchioles. This was contested by Borland, Cox & Higenbottam (1993) as exhaled NO and carbon dioxide appeared to be cleared from the same pulmonary site. Cremona, Higenbottam, Takao, Hall & Bower (1995) showed that the pulmonary endothelium of resistance arteries contributed to exhaled NO in isolated pig lungs. In contrast, several reports have suggested that exhaled NO in man originates primarily from the nasal or paranasal regions (Gerlach, Rossaint, Pappert, Knorr & Falke, 1994; Lundberg *et al.* 1995). During exercise it is known that tracheobronchial blood flow increases in man (Gilbert, Fouke & McFadden, 1987) and the horse (Manohar 1990), which appears to be a locally mediated response (Baile, Dahlby, Wiggs & Pare, 1985; Gilbert *et al.* 1987). Also, the effect of changes in airway surface liquid tonicity which induces a change in bronchial mucosal blood flow is blocked by NOS inhibitors suggesting an important role for NO in regulating airway blood flow (Smith, Prazma, Coleman, Henry, Drake & Boucher, 1993). Increased NO in exhaled air during exercise could therefore represent a contribution of NO to the regulation of tracheobronchial mucosal blood flow.

The exercise-induced increase in PAP in the horse (>80 mmHg; Erickson, Erickson & Coffman, 1990; Manohar, 1994), is significantly higher than in other species, including man (approximately 30 mmHg; West *et al.* 1993). The incidence of exercise-induced pulmonary haemorrhage (EIPH), a major problem in the racehorse (West *et al.* 1993; Manohar, 1995), has been associated with a sudden and marked increase in PAP (Erickson *et al.* 1990; Manohar, 1994; West & Mathieu-Costello, 1995). Little is known about the role of NO in the control of vascular tone in the horse, although Manohar (1995) reported a reduction in pulmonary vascular pressures in the resting horse after administration of glyceryl trinitrate, a NO donor (Moncada *et al.* 1991).

In the present study, the objective was to investigate the contribution of NO to regulation of PAP in the horse during exercise. Endogenous NO was measured in exhaled air during graded exercise and the source of NO production considered. Finally, the cardiovascular and respiratory responses of the horse to inhaled NO, a selective pulmonary vasodilator, were investigated.

METHODS

Animals

Five clinically healthy Thoroughbred horses (2 geldings, 3 mares), aged between 5 and 9 years and weighing 427–550 kg, underwent treadmill training for at least 8 weeks prior to commencement of the study. The horses were trained 6 days a week on the treadmill by trotting (4 m s^{-1} for 20 min) or cantering (9 m s^{-1} for 10 min) on alternate days with the treadmill set at a 5 deg incline. Heart rate monitors (described below) were attached to the horses at various stages during training to assess the level of fitness. The experimental protocols were approved by the Ethics Committee of the Animal Health Trust (Newmarket, UK).

Exercise protocol

Study 1. Determination of the source of endogenous NO production in exhaled air. Four horses were exercised on the treadmill set at 3 deg, according to the following protocol: walk (1·7 m s^{-1}) for 8 min, trot (3·7 m s^{-1}) for 5 min, canter (9 m s^{-1}) for 3 min, walk (1·7 m s^{-1}) for 5 min, trot (3·7 m s^{-1}) for 5 min and canter (9 m s^{-1}) for 3 min, then walk (1·7 m s^{-1}) for 5 min (recovery). Exhaled air was collected for 1·0 min by suction (described below) after 4·0 min of trot and 1·5 min of each canter. Each 1·0 min collection consisted of consecutive 30 s collections at two flow rates: 20 or 80 l min^{-1}, in a randomized order. Immediately after the first canter, methoxamine (40 mg ml^{-1}), an α_1-adrenergic agonist, was administered for 60 s to both nasal openings via a Y-shaped plastic tube from a Portaneb 50 nebulizer (Medic-Aid, Pagham, UK).

Study 2. Effect of exercise on the respiratory production rate of NO ($\dot{V}_{NO}$). Five horses were exercised on the treadmill set at 5 deg, according to the following protocol: walk (1·7 m s^{-1}) for 8 min, trot (3·7 m s^{-1}) for 8 min, gallop (11 m s^{-1}) for 2 min and walk (1·7 m s^{-1}) for 5 min (recovery). The treadmill was lowered to 0 deg for the recovery phase of the test. Exhaled air was collected by suction at 20 l min^{-1} for 1·0 min at the following times: (1) at rest, (2) after 5 min walk, (3) after 5 min trot, (4) from 0 to 1 min of gallop, (5) from 1 to 2 min of gallop, (6) from 0 to 1 min of recovery, and (7) from 4 to 5 min of recovery.

Study 3. Effect of inhaled NO on cardiovascular and respiratory parameters. Five horses completed the exercise protocol used in study 2 twice, with successive treadmill studies separated by 1 day. During the second treadmill study, NO was administered continuously from the mid-point (4·0 min) of the trot until the completion of exercise. The NO (1000 p.p.m. in nitrogen, BOC Special Gases, Guildford, UK) was delivered to the opening of the left nostril of the horse via inert Teflon tubing attached to a mask (described below) and mixed freely with the inhaled air. The

administered dose of NO for each horse was regulated by adjusting the output from stored NO by a flowmeter (model no. 846024, BOC Special Gases) based on the minute ventilation calculated in the first (control) treadmill study. The aim was to maintain a mean concentration of NO in inhaled air of approximately 80 p.p.m. NO is relatively stable under the described conditions with minimal production of nitrogen dioxide (NO_2) during the short exposure to the atmosphere (Foubert *et al.* 1992; E. Demoncheaux, personal communication).

A large fan (model no. PBB4-630-32K, Solar and Palau, Barcelona, Spain) at the front of the horse forced air over the horse at a speed equivalent to the treadmill speed, simulating airflow expected during field exercise.

Cardiovascular and respiratory measurements

A lightweight fibreglass mask, which housed two flow tubes, one per nostril, was fastened over the external nares of the horse. Ultrasonic flow transducers (BRDL Ltd, Birmingham, UK) within the flow tubes measured flow velocity while a respiratory mass spectrometer (MGA 2000, Case, Biggen Hill, UK) monitored instantaneous respiratory gas concentrations via a flexible capillary positioned in the left flow tube (Butler *et al.* 1993). The ultrasonic flow transducers were calibrated before each exercise protocol by a flowmeter (model 2100, KDG Flowmeters, Burgess Hill, UK), as described by Butler *et al.* (1993). Instantaneous respiratory airflow through both flow tubes and respiratory O_2 and CO_2 concentrations were recorded on a six-channel recorder with rectilinear co-ordinates (Gould) and 1 min blocks of data at selected points were sampled by a DAS 1600 A/D converter (Keithley Metrabyte, Reading, UK). Acquisition and analysis were performed using software written in ASYST (v. 3.1, MacMillan Software Co, New York). The following variables were determined as described by Butler *et al.* (1993): respiratory minute volume ($\dot{V}_E$), tidal volume ($\dot{V}_T$), rate of O_2 consumption ($\dot{V}_{O_2}$), rate of CO_2 production ($\dot{V}_{CO_2}$) and respiratory frequency (f_R). Values for $\dot{V}_E$ and $\dot{V}_T$ were corrected to body temperature, atmospheric pressure and saturated with water vapour, and $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were corrected to standard temperature and pressure and dry. Cardiac output ($\dot{Q}$) was calculated from $\dot{V}_{O_2}$ and the arteriovenous difference in oxygen content (C_{O_2}) using the Fick equation.

Heart rate (f_C) was recorded telemetrically (PEH 100 Hippocard, Isler Bioengineering, Zurich, Switzerland) from electrodes on the mid- and lateral thorax.

A catheter introducer (8 Fr, Arrow, Reading, PA, USA) was placed into the left jugular vein using local anaesthesia (xylocaine, 2%, Astra Pharmaceutical, Kings Langley, UK) before studies 2 and 3. A thermodilution catheter (7 Fr Criticath, Spectramed Inc., Minneapolis, MN, USA) was advanced into the pulmonary artery to measure central body temperature and to withdraw mixed venous blood samples. Correct placement was verified by following pressure traces obtained with a strain-gauge sensor (T-150-AD, Viggo-Spectramed, Swindon, UK) and displayed on a monitor (Minamon 7132A, Kontron Instruments, Watford, UK). Arterial blood was withdrawn from a catheter (20 G × 32 mm, Intraflon, Vygon, Ecouen, France) placed in either the left or right transverse facial artery under local anaesthesia. A second catheter introducer was also placed in the left jugular vein for study 3 and a transducer-tipped catheter (6 Fr, Millar Mikro-tip, Millar Instruments, Houston, TX, USA) was advanced until situated in the pulmonary artery, approximately 10 cm past the pulmonary valve, and was used to record mean pulmonary artery pressure (PAP). The transducer-tipped catheter was calibrated both before and after each exercise protocol using a mercury manometer. The haematocrit (Hct) and haemoglobin content were measured using a Coulter STKR haematology analyser (Coulter Electronics Ltd, Luton, UK) from jugular venous blood that was collected via the side-port of the catheter introducer. The venous blood (5 ml) was placed into tubes containing ethylenediamine tetraacetic acid (EDTA), stored on ice and analysed within 1 h of collection. Heparinized saline (10 i.u. ml^{-1}) was used to flush catheters and extension tubes.

Blood gas samples (arterial and mixed venous blood) were collected into 1 ml pre-heparinized syringes (QS50, Radiometer, Copenhagen, Denmark). Blood gas and pH analysis were performed within 1 h of collection on a blood gas analyser (ABL 330, Radiometer) with an automated temperature correction facility based on the characteristics of human blood, which were also accurate for the horse (Butler *et al.* 1993). The blood gas and pH measurements were corrected to the temperature recorded in the pulmonary artery at the time the sample was collected.

Cardiovascular and respiratory measurements were recorded at the following times during study 2: (1) at rest, (2) from 3 to 4 min of walk, (3) from 3 to 4 min of trot, (4) from 0 to 1 min of gallop, (5) from 1 to 2 min of gallop, (6) from 0 to 1 min of recovery, and (7) from 4 to 5 min of recovery; and at the following times during study 3: (1) at rest, (2) from 3 to 4 min of walk, (3) from 7 to 8 min of walk, (4) from 3 to 4 min of trot, (5) from 7 to 8 min of trot, (6) from 0 to 1 min of gallop, (7) from 1 to 2 min of gallop, and (8) from 0 to 1 min of recovery.

Collection of exhaled air and measurement of NO

A vacuum pump (model E2M5, Edwards High Vacuum Int., Crawley, UK) was used to collect exhaled air through a polypropylene tube (2 m × 12 mm i.d.) attached to the mask on the horse. The suction was regulated by a valve which had been calibrated for two flow rates: $20{\cdot}0 \pm 0{\cdot}3$ and $80{\cdot}0 \pm 0{\cdot}5$ l min^{-1}. The end of the collection tube protruded into the opening of the left nostril during study 1 and was fixed perpendicular to the airflow of the left nostril during study 2. To account for a dilution effect of inhaled air, NO concentrations measured in exhaled air collected during study 2 were doubled. An additional sample of exhaled air was also collected at rest in study 2 using a one-way valve apparatus attached to the mask while the horse was standing in stocks. A one-way valve system was not feasible during exercise because this induced unacceptable resistance to respiration in the horse.

The exhaled air was collected into evacuated 50 l polypropylene Douglas bags (PK Morgan Ltd, Rainham, UK) and the [NO] in each collection was measured within 10 min using a sensitive chemiluminescent analyser (model 42, Thermoelectron, Warrington, UK) with a sampling rate of 700 ml min^{-1}, a response time of 20 s and a detection limit of 0·25 p.p.b. The analyser was calibrated before each experiment by using a gas mixture of NO in N_2 (130 p.p.b.; Spectraseal, BOC), which was verified by reference to a national standard (Department of Trade and Industry, Warren Spring Laboratory, Stevenage, UK). NO-free gas was produced by passing the gas mixture through activated charcoal and potassium permanganate on alum (Purafil, Thermoelectron). The linearity of the analyser was tested by calibrating it against a katharometer (PK Morgan, Rainham, UK) and an infrared absorption carbon monoxide (CO) analyser (PK Morgan). A gas mixture containing NO, CO and helium was passed through the three gas analysers before and after serial dilution with N_2 (Cremona *et al.* 1995). Dilution curves of the ratios of the three gases were linear in the

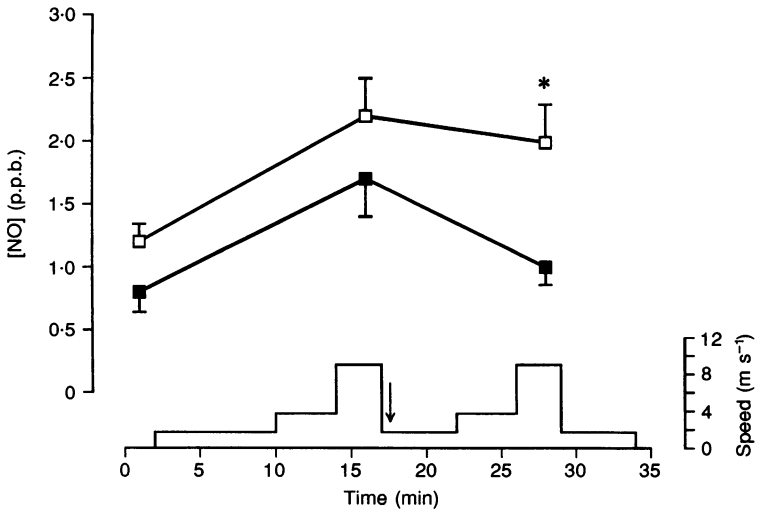

Figure 1
Study 1. NO concentration (means ± S.E.M.) in exhaled air (p.p.b.) from horses ($n = 4$) at rest, during canter (11 m s^{-1}) and during a second canter (11 m s^{-1}) after aerosol administration of methoxamine (40 mg ml^{-1}; ↓) for 60 s immediately after the first canter. Here and in subsequent figures the exercise protocol is indicated beneath the main records (right-hand ordinate). Exhaled air was collected by suction from inside the nostril at 20 (□) and 80 l min^{-1} (■); * $P < 0{\cdot}05$.

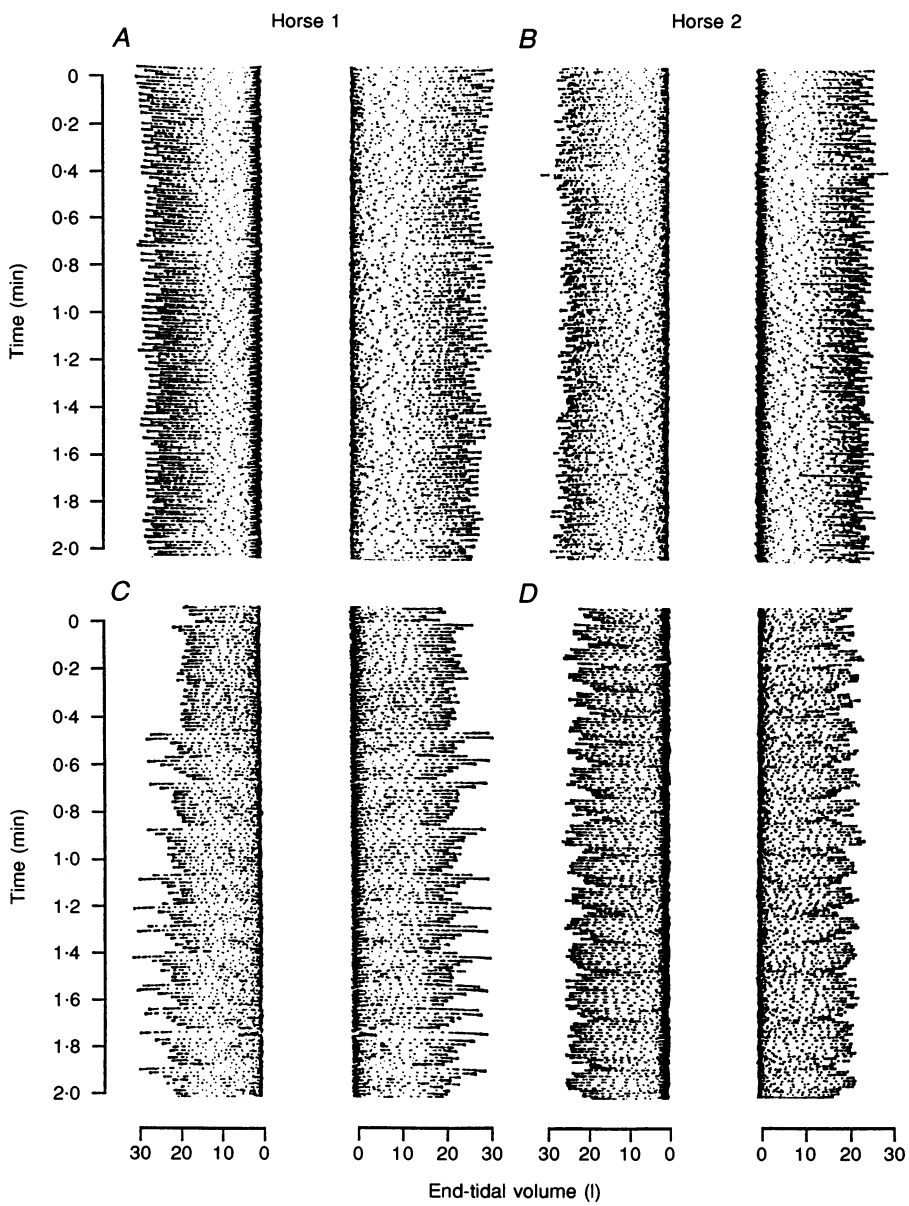

Figure 2
The changes in end-tidal volume, reflecting the breathing pattern, of horses during normal cantering (*A* and *B*) and after aerosol administration of methoxamine (40 mg ml^{-1}; *C* and *D*) for 60 s.

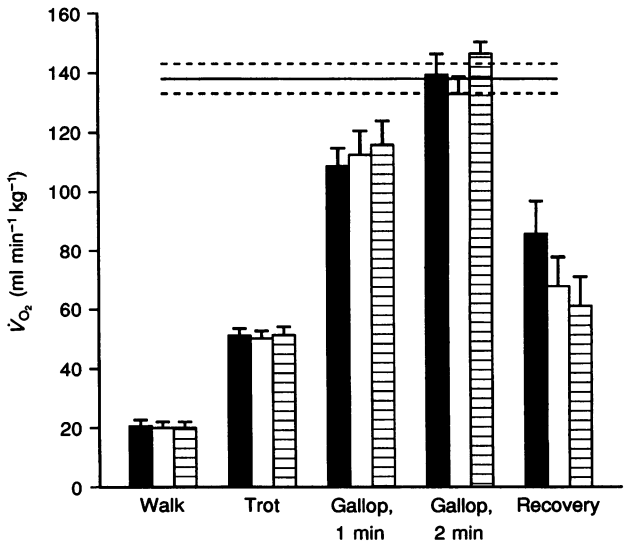

Figure 3

$\dot{V}_{O_2}$ (ml min^{-1} kg^{-1}) from horses ($n = 5$) at different stages of exercise during study 2 (■) and study 3. Control (□) and inhaled NO (▤), compared with $\dot{V}_{O_2,max}$ (mean, continuous line; ± S.E.M., dotted lines).

range detected by the NO analyser. The NO analyser was located beside the treadmill during the study and the ambient [NO] during each collection was noted and later subtracted from the average [NO] for the corresponding sample. $\dot{V}_{NO}$ (in pmol min^{-1} kg^{-1}) was calculated by the formula:

$$\dot{V}_{NO} = [NO] \times \dot{V}_E,$$

where [NO] was measured in parts per million and $\dot{V}_E$ in millilitres per minute per kilogram.

Maximum oxygen uptake ($\dot{V}_{O_2,max}$)

The $\dot{V}_{O_2,max}$ for each horse was calculated by measuring $\dot{V}_{O_2}$ during a stepwise incremental exercise test. This consisted of walking (1·7 m s^{-1}) for 10 min, cantering (6 m s^{-1}) for 1 min, then speed increments of 1 m s^{-1} each minute from 8 m s^{-1} until fatigue (approximately 13 m s^{-1}) on the treadmill at a 5 deg incline.

Statistical analysis

Data are presented as means ± S.E.M. Significant differences in [NO] and $\dot{V}_{NO}$ during the exercise protocol were calculated using one-way analysis of variance and significant differences between control and NO administration tests on cardiovascular and respiratory measurements were calculated using two-way analysis of variance. The Student's t test was applied as a *post hoc* test and differences were considered significant when $P < 0·05$.

RESULTS

Effect of collection flow rate and methoxamine on [NO] in exhaled air

The [NO] measured in exhaled air collected at 20 l min^{-1} was 1·23 ± 0·14 p.p.b. during trot and 2·25 ± 0·32 p.p.b. during canter. At the higher flow rate of 80 l min^{-1} the [NO] of the exhaled air was reduced to 0·78 ± 0·15 p.p.b. during trot and 1·69 ± 0·31 p.p.b. at canter (see Fig. 1). Nebulized methoxamine further reduced [NO] in exhaled air during the second canter, compared with the first canter ($P < 0·05$), to 1·99 ± 0·41 p.p.b. at 20 l min^{-1} and 1·05 ± 0·14 p.p.b. at 80 l min^{-1}. Methoxamine also induced cyclical changes in $\dot{V}_T$ over time that were not apparent during the first canter (Fig. 2).

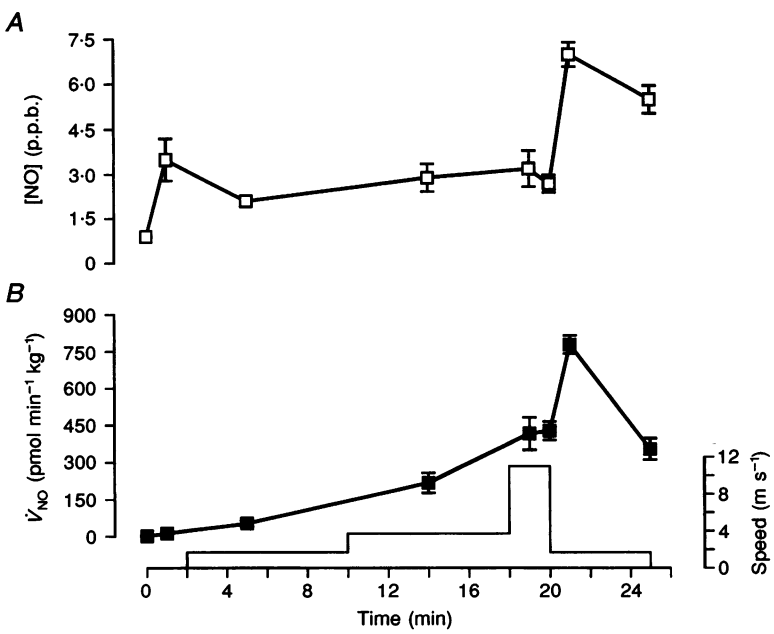

Figure 4

Study 2. [NO] in exhaled air (*A*) and NO production rate ($\dot{V}_{NO}$; *B*) in the horse at different exercise intensities.

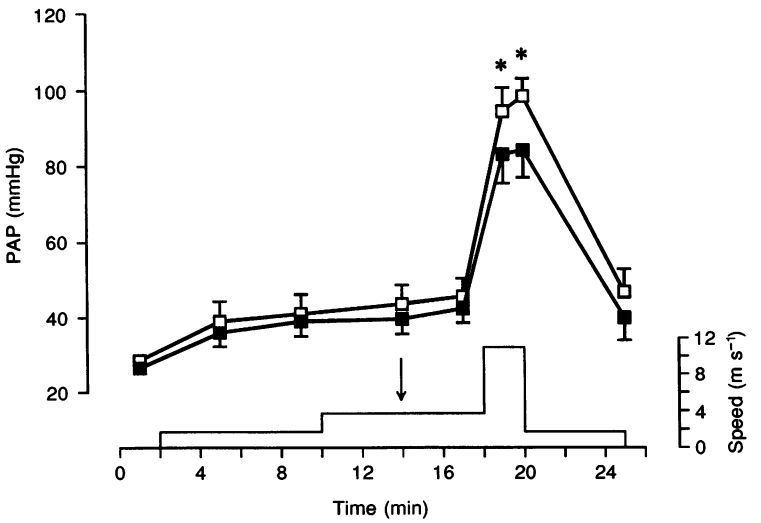

Figure 5
Study 3. Pulmonary artery pressure (PAP) in horses ($n = 5$) during control exercise (□) and during inhalation of NO (■). NO administration (80 p.p.m.) commenced 13 min (↓) into the exercise protocol. * $P < 0{\cdot}05$.

Exercise intensity

A comparison of $\dot{V}_{O_2}$ at each stage of exercise revealed that the horses approached their $\dot{V}_{O_2,max}$ ($138{\cdot}3 \pm 11{\cdot}7$ ml min^{-1} kg^{-1}) during studies 2 and 3, reaching a peak of $139{\cdot}3 \pm 16{\cdot}2$ ml min^{-1} kg^{-1} in study 2, $132{\cdot}7 \pm 5{\cdot}9$ ml min^{-1} kg^{-1} in the first (control) test and $144{\cdot}9 \pm 5{\cdot}3$ ml min^{-1} kg^{-1} in the second (inhaled NO) test of study 3 during the second minute of galloping (Fig. 3).

NO in exhaled air during graded exercise

In study 2, the [NO] was relatively constant during exercise, compared with rest ($0{\cdot}89 \pm 0{\cdot}3$ p.p.b.), although a significant increase ($7{\cdot}0 \pm 0{\cdot}4$ p.p.b.; $P < 0{\cdot}05$) was measured during the first minute of recovery (Fig. 4). [NO] at rest was higher when measured on the treadmill ($3{\cdot}1 \pm 0{\cdot}7$ p.p.b.) than when standing in the stocks ($P < 0{\cdot}05$), although $\dot{V}_{NO}$ values were not significantly different for each rest measurement. $\dot{V}_{NO}$ showed a significant and linear increase with exercise intensity from rest ($2{\cdot}5 \pm 0{\cdot}9$ pmol min^{-1} kg^{-1}) until during the second minute of gallop ($428{\cdot}1 \pm 31{\cdot}6$ pmol min^{-1} kg^{-1}; Fig. 4). However, a further increase in $\dot{V}_{NO}$ ($779{\cdot}3 \pm 38{\cdot}4$ pmol min^{-1} kg^{-1}) was found during the first minute of recovery.

Effect of inhaled NO on PAP during exercise

In study 3, NO (80 p.p.m.) induced a significant ($P < 0{\cdot}05$) decrease in PAP during the first ($83{\cdot}1 \pm 7{\cdot}6$ mmHg) and second ($84{\cdot}2 \pm 7{\cdot}1$ mmHg) minutes of the gallop, compared with control of $94{\cdot}4 \pm 6{\cdot}3$ and $98{\cdot}4 \pm 4{\cdot}7$ mmHg, respectively (Fig. 5). There were no significant differences between control and inhaled NO at other stages of exercise.

Cardiovascular and respiratory measurements

Study 2. Cardiovascular (f_C and Hct; Fig. 6) and respiratory parameters ($\dot{V}_T$, $\dot{V}_E$, $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and f_R; Figs 7 and 8) generally followed the changes in exercise intensity during the protocol. However, maximum $\dot{V}_T$ occurred during the

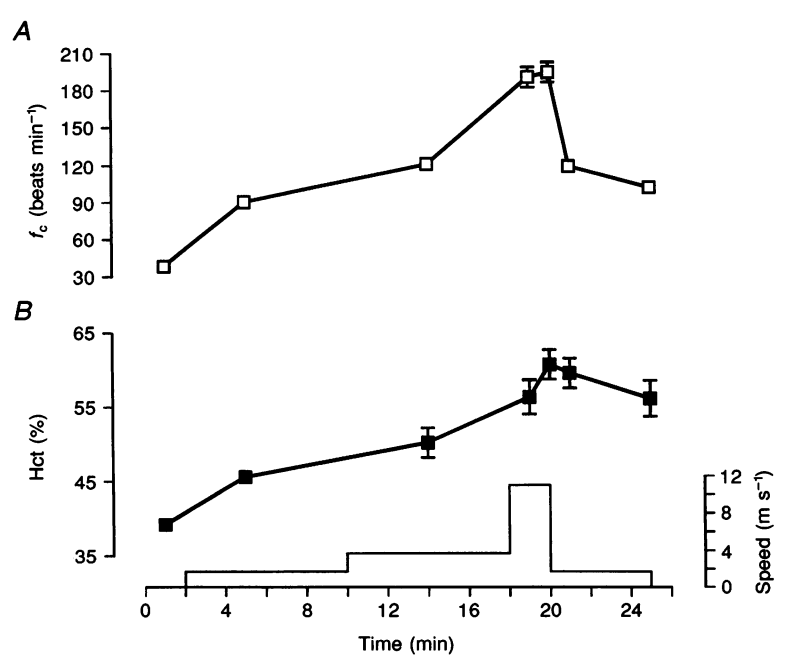

Figure 6
Cardiovascular parameters in the horse ($n = 5$) during exercise: heart rate (f_C; *A*) and haematocrit (Hct; *B*) from study 2.

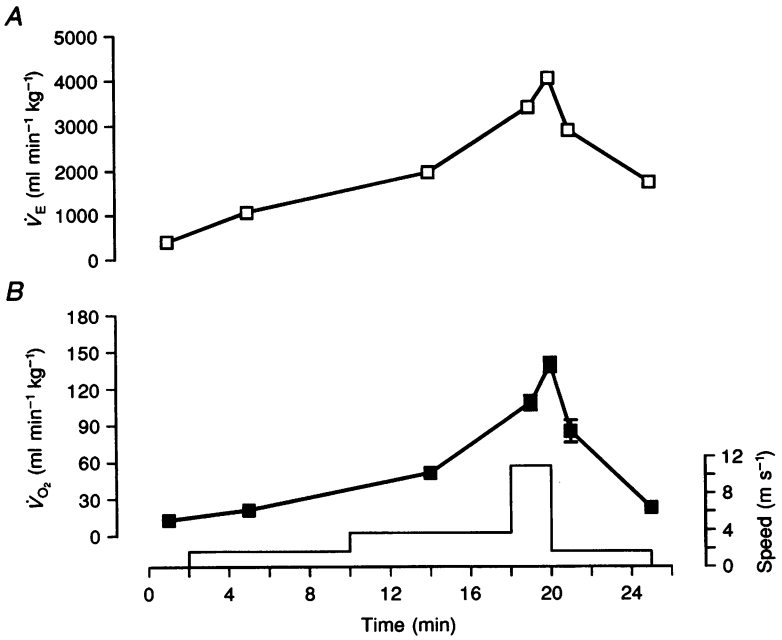

Figure 7
Respiratory parameters in the horse ($n = 5$) during exercise: minute ventilation ($\dot{V}_E$; *A*) and oxygen uptake ($\dot{V}_{O_2}$; *B*) from study 2.

1 min recovery after the gallop. Regression analysis of $\dot{V}_{NO}$ with $\dot{V}_T$ and Hct revealed good correlation ($r = 0{\cdot}968$ and $0{\cdot}855$, respectively; $P < 0{\cdot}01$).

Study 3. There were no significant differences between control and inhaled NO exercise tests at different stages of exercise (Table 1).

DISCUSSION

We have demonstrated that NO is present in the exhaled air of horses. Gustafsson, Leone, Persson, Wiklund & Moncada (1991) measured NO in exhaled air from the rabbit, guinea-pig and man, although [NO] at rest in the horse ($3{\cdot}25 \pm 0{\cdot}75$ p.p.b.; Fig. 4*A*) appears to be lower than that reported in other species (Table 2). However, the wide range of reported [NO] across the species (3·25–26·3 p.p.b.) suggests that variation in experimental protocol could influence the measurement of [NO]. A higher resting [NO] was measured in the horse when exhaled air was collected by suction pump than by one-way valve and probably reflects differences in the collection apparatus and the excitation state of the animal.

Exhaled air was collected for endogenous NO measurement at a point either (1) perpendicular to the nasal airflow, or (2) directly over the nasal mucosa, at different suction rates to investigate the source of exhaled NO (discussed later). It was not feasible to collect exhaled air from the horse during exercise using a one-way valve system because of the

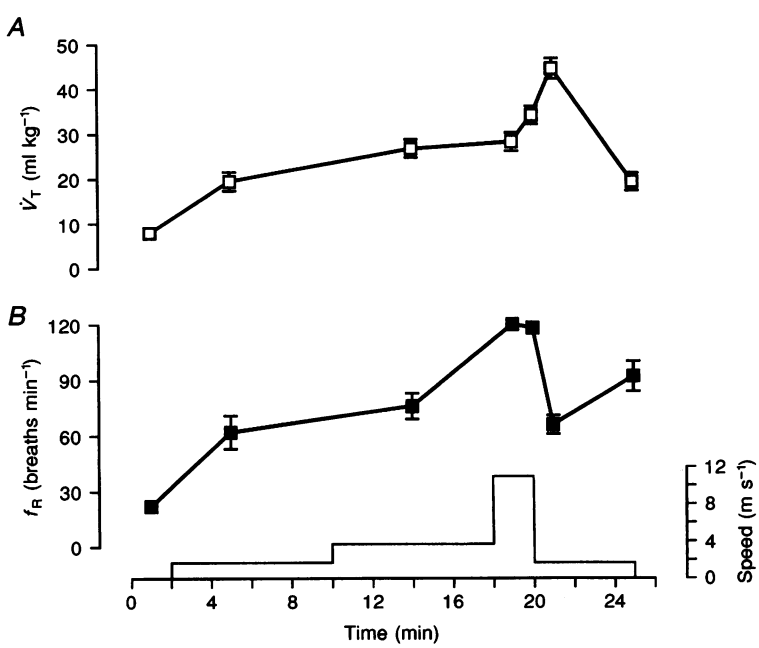

Figure 8
Respiratory parameters in the horse ($n = 5$) during exercise: tidal volume ($\dot{V}_T$; *A*) and respiratory frequency (f_R; *B*) from study 2.

Table 1. A comparison of the effect of inhaled NO (80 p.p.m.; study 3) on cardiovascular and respiratory parameters in five Thoroughbred horses (means ± S.E.M.) collected at 7–8 min of walk (1·7 m s^{-1}), 7–8 min of trot (3·7 m s^{-1}), 0–1 min of gallop (11 m s^{-1}), 1–2 min of gallop (11 m s^{-1}) and 0–1 min of recovery (1·7 m s^{-1})

		Stage of exercise				
Parameter	Test	Walk	Trot	Gallop 1	Gallop 2	Recovery
PAP (mmHg)	Control	41·2 ± 5·2	45·6 ± 4·8	94·4 ± 6·3	98·4 ± 4·7	46·8 ± 6·0
	NO	39·3 ± 3·9	42·4 ± 3·7	83·1 ± 7·6*	84·2 ± 7·1*	40·0 ± 6·3
$\dot{V}_T$ (ml kg^{-1})	Control	21·6 ± 2·6	25·7 ± 1·7	28·9 ± 1·6	33·1 ± 1·9	46·5 ± 2·1
	NO	21·1 ± 2·8	28·0 ± 2·4	31·5 ± 1·5	37·6 ± 3·3	47·2 ± 2·2
$\dot{V}_E$ (ml min^{-1} kg^{-1})	Control	1044 ± 69	2053 ± 138	3500 ± 187	3813 ± 173	2901 ± 140
	NO	1204 ± 114	1919 ± 160	3413 ± 321	3820 ± 356	2980 ± 165
$\dot{V}_{O_2}$ (ml min^{-1} kg^{-1})	Control	24·6 ± 1·9	52·4 ± 2·1	112·5 ± 6·5	132·7 ± 5·9	80·5 ± 3·2
	NO	25·0 ± 3·3	52·9 ± 1·7	121·6 ± 8·5	144·9 ± 5·3	86·2 ± 3·7
$\dot{V}_{CO_2}$ (ml min^{-1} kg^{-1})	Control	24·9 ± 2·1	52·9 ± 1·7	115·9 ± 6·9	146·3 ± 7·0	109·1 ± 4·6
	NO	24·4 ± 3·9	50·8 ± 3·0	119·9 ± 9·7	155·6 ± 4·1	115·6 ± 4·8
P_{a,O_2} (mmHg)	Control	102·3 ± 3·6	105·3 ± 2·8	63·8 ± 3·8	60·4 ± 3·1	120·9 ± 5·6
	NO	110·9 ± 3·1	102·5 ± 2·5	70·9 ± 5·4	65·6 ± 3·7	118·9 ± 5·5
P_{a,CO_2} (mmHg)	Control	45·9 ± 2·1	44·5 ± 2·1	60·3 ± 3·5	66·7 ± 4·6	36·2 ± 2·3
	NO	44·1 ± 3·2	45·9 ± 1·9	58·9 ± 2·6	61·3 ± 2·1	35·4 ± 3·8
P_{v,O_2} (mmHg)	Control	29·3 ± 1·2	22·7 ± 1·1	15·2 ± 1·0	13·4 ± 0·8	36·6 ± 5·6
	NO	31·7 ± 1·6	23·9 ± 0·7	19·1 ± 1·0	15·1 ± 0·7	40·5 ± 2·2
P_{v,CO_2} (mmHg)	Control	60·3 ± 1·8	64·1 ± 2·2	110·7 ± 5·5	136·2 ± 9·1	68·4 ± 5·6
	NO	56·9 ± 4·9	65·1 ± 2·4	100·7 ± 4·9	125·9 ± 9·2	54·1 ± 2·6
f_R (breaths min^{-1})	Control	51·7 ± 3·1	82·3 ± 8·3	121·4 ± 0·6	118·1 ± 1·3	68·5 ± 2·6
	NO	61·6 ± 5·6	75·2 ± 10·9	119·6 ± 0·9	118·5 ± 0·8	66·2 ± 3·2
f_C (beats min^{-1})	Control	89·2 ± 5·1	125·8 ± 4·5	193·0 ± 5·9	196·5 ± 6·3	121·8 ± 2·9
	NO	89·0 ± 4·7	127·7 ± 5·9	193·7 ± 5·9	195·3 ± 5·4	119·8 ± 3·1
$\dot{Q}$ (ml min^{-1} kg^{-1})	Control	142·0 ± 10·3	194·5 ± 12·1	271·5 ± 18·4	285·5 ± 21·8	89·7 ± 7·5
	NO	157·6 ± 23·4	199·1 ± 10·8	293·9 ± 16·8	306·5 ± 19·6	91·2 ± 6·3

* $P < 0·05$.

unacceptably high resistance at high flow rates. The lightweight mask, incorporating ultrasonic flow transducers, was specifically developed to create negligible resistance which is imperative in the horse when performing near-maximal exercise (Butler *et al.* 1993). We collected exhaled air using a polypropylene tube placed over the left nostril and perpendicular to air flow. [NO] was therefore doubled to account for dilution from inhaled air while assuming that inhaled and exhaled volumes will be approximately equal, even allowing for differences in temperature and humidity. NO is stable in air for the conditions described (Foubert, *et al.* 1992; E. Demoncheaux, personal communication) while the fan at the front of the horse rapidly removed residual exhaled air.

Exercise increased $\dot{V}_{NO}$ in man (Persson *et al.* 1993; Bauer *et al.* 1994; Trolin *et al.* 1994) and in the horses in the present study. Persson *et al.* (1993) found that [NO] actually decreased during exercise but $\dot{V}_{NO}$, which accounts for changes in $\dot{V}_E$, correlated with exercise intensity. Similarly, Bauer *et al.* (1994) and Trolin *et al.* (1994) reported an increase in $\dot{V}_{NO}$ during exercise in man, although Iwamoto *et al.* (1994) found that $\dot{V}_{NO}$ increased in most, but not all subjects during exercise. We measured a linear increase in $\dot{V}_{NO}$ with exercise intensity. $\dot{V}_{NO}$ in the horse (428·1 ± 31·6 pmol min^{-1} kg^{-1} during the second minute of the gallop) is substantially higher than that reported in man (e.g. 135·6 pmol min^{-1} kg^{-1}; Bauer *et al.* 1994). This species difference may be expected if, indeed, NO contributes to the regulation of pulmonary vascular tone in the horse because pulmonary vascular pressures in this species are significantly higher during exercise than other species, including man (Erickson *et al.* 1990; West *et al.* 1993; West & Mathieu-Costello, 1995).

The source of NO in exhaled air is uncertain. Lundberg *et al.* (1995) suggested that the majority of nasally exhaled NO in man originated from iNOS in the paranasal sinuses where it functions in host defence (Gerlach *et al.* 1994). For example, Kartgener's syndrome in man, characterized by sinusitis,

Table 2. [NO] measured at rest in different species

Species	Physiological model	[NO] (p.p.b. ± S.D.)	Reference
Man	Single full exhalation	3·4 ± 0·04	Borland *et al.* (1993)
	Spontaneous breathing	14·7 ± 1·34	Borland *et al.* (1993)
Pig	Isolated perfused lung	5·8 ± 1·8	Cremona *et al.* (1995)
Man	Exhaled air over one min	26·3 ± 6·7	Iwamoto *et al.* (1994)
Man	Spontaneous breathing	8·6 ± 0·7	Trolin *et al.* (1994)
Rabbit	Anaesthetized	15·0 ± 0·8	Gustafsson *et al.* (1991)
Man	Spontaneous breathing	8·0 ± 0·8	Gustafsson *et al.* (1991)
Man	Single exhalation	3·25 ± 0·75	Persson *et al.* (1993)
Man	Spontaneous breathing	12·9 ± 2·4	Bauer *et al.* (1994)

bronchiectasis and sinus inversus, has been associated with a deficiency of NO in the paranasal sinuses (Lundberg *et al.* 1995). Furthermore, nitrate-forming bacteria in the upper airways could also contribute to exhaled NO (Gerlach *et al.* 1994). The horse is an obligate nasal breather and if NO is a prominent component of the nasal and paranasal defences in this species, an increased mucosal blood flow during exercise would be expected to enhance $\dot{V}_{NO}$. However, there were no significant differences at trot or canter in [NO] when collected from within the nostril at a high (80 l min^{-1}) or low (20 l min^{-1}) flow rate suggesting that the equine nasal mucosa may not be a major physiological source of NO. In a preliminary study, we have also found no significant difference in exhaled [NO] from anaesthetized horses with or without an endotracheal tube in place (authors' unpublished results). Three recent studies appear to confirm a more prominent role of the lower airways in the production of NO found in exhaled air. Hodgson *et al.* (1993) found that respiratory heat loss, and presumably mucosal blood flow, decreased during high-intensity exercise in the horse, thereby reducing the likelihood of nasal mucosa being primarily responsible for $\dot{V}_{NO}$ increasing with exercise intensity. Maroun, Mehta, Turcotte, Cosio & Hussain (1995) reported a linear increase in $\dot{V}_{NO}$ with increasing $\dot{V}_{O_2}$ in men breathing entirely through the mouth, thereby avoiding autoinhalation of NO from the nasal cavity. They concluded that the NO in exhaled air could originate from the lower airways and may act to improve performance by counteracting hypoxic pulmonary vascoconstriction (HPV) and, possibly, by bronchodilatation (Maroun *et al.* 1995). More importantly, the linear increase in $\dot{V}_{NO}$ was only found in athletic, and not sedentary, humans. Since the athletic ability of the horse is substantially greater than that of man (Wagner *et al.* 1989), the relatively higher $\dot{V}_{NO}$ during exercise that we found in the horse may have been expected. Finally, a study by Imada, Iwamoto, Nonaka, Kobayashi & Uhno (1996) demonstrated that $\dot{V}_{NO}$ from human nasal epithelium will actually decrease during exercise as part of the mechanism regulating nasal resistance. It would therefore appear unlikely that the nasal epithelium would contribute substantially to the increase in $\dot{V}_{NO}$ during exercise in the horse.

Several studies have suggested that NO in exhaled air could originate from the lower airways, particularly the terminal and respiratory bronchioles (Gustafsson *et al.* 1991; Persson *et al.* 1993; Cremona *et al.* 1995). Persson *et al.* (1993) found a correlation between [NO] and peak CO_2 levels during a single exhalation in man and suggested that the vasculature lining the respiratory and terminal bronchioles contributed to NO in exhaled air. The vascular endothelium is in close apposition to the abluminal surface of the alveoli and a proportion of endogenous NO will escape from the vasculature and can be detected in the exhaled air (Moncada *et al.* 1991; Cremona *et al.* 1995). The vascular endothelial cells have a high capacity for NO production and can respond promptly to changes in blood flow (Dawson, Linehan & Bronikowski, 1989). Increased shear stress and vascular flow, such as will occur during exercise, have been reported to enhance NO release from vascular endothelial cells (Pohl, Wagner & DeWitt, 1993). Since a reasonable correlation was found between $\dot{V}_{NO}$ and Hct in our study, increased shear stress and vascular flow could also explain the enhanced $\dot{V}_{NO}$ in the horses during exercise.

A stronger correlation was measured between $\dot{V}_{NO}$ and $\dot{V}_T$, particularly when considering the period immediately after near-maximal exercise. Similarly, Persson *et al.* (1993) reported that hyperventilation in man acutely increased [NO]. These results suggest that the release of endogenous NO could be influenced by respiratory effort. Both Persson, Lonnqvist & Gustafsson (1995) and Dainty, McGrath, Spedding & Templeton (1990) reported that activation of stretch receptors in the lung by positive end-expiratory pressure ventilation and increased vagal tone could enhance NO release. However, isolated lung preparations and cultured endothelial cells will release NO in response to a variety of chemical stimuli without innervation (Cremona *et al.* 1995).

Variation in the tracheobronchial circulation is an alternative explanation for the effect of hyperventilation and increased respiratory effort on $\dot{V}_{NO}$. The tracheobronchial blood flow is an intrinsic part of the thermoregulatory mechanism of respiratory heat exchange (Baile *et al.* 1985; Gilbert *et al.* 1987) and is known to increase with exercise in man (Gilbert *et al.* 1987) and the horse (Manohar, 1990). The physiological

control of tracheobronchial blood flow is locally mediated in response to a reduction in the humidity of inhaled air, such as will occur during hyperventilation or the increased $\dot{V}_T$ during exercise, both of which can overwhelm the conditioning effect of the mucosal lining of the upper respiratory tract (Baile *et al.* 1985; Gilbert *et al.* 1987). Although the local mediator of tracheobronchial flow is uncertain (Baile *et al.* 1985), NO is a major vasodilator in many tissues (Moncada *et al.* 1991). NO regulates tracheobronchial blood flow in response to airway surface liquid tonicity (Smith, 1993). The increased tracheal mucosal blood flow immediately after exercise would probably contribute to an increased $\dot{V}_{NO}$.

Methoxamine exerts a pressor response on airway vasculature (Cabanes *et al.* 1992) and significantly reduces [NO] in exhaled air of exercising horses. Methoxamine is known to constrict tracheobronchial vasculature (Dinh-Xuan, Chaussain, Regnard & Lockhart, 1989). It will also affect airway resistance, both directly, by inducing constriction of bronchial smooth muscle, and indirectly, by opposing hyperaemia and mucosal airway oedema (Dinh-Xuan *et al.* 1989; Cabanes *et al.* 1992). The cyclical changes in the breathing pattern of the horses were probably induced by variation in airway resistance after inhalation of methoxamine. In some human patients, inhaled methoxamine can induce bronchoconstriction (Dinh-Xuan *et al.* 1989) but in most asthmatics it blocks exercise-induced asthma (EIA). Airway obstruction from engorged tracheobronchial mucosal blood vessels and the associated oedema is reported to be the mechanism responsible for EIA which occurs immediately after exercise in susceptible humans (Baile *et al.* 1985; Gilbert *et al.* 1987). Perhaps in horses, as a result of the greater rise in $\dot{V}_E$ with exercise, the exercise-induced increase in bronchial blood flow is more important to water and heat exchange. If NO does indeed regulate tracheobronchial blood flow, manipulation of NO expression could be used therapeutically to alleviate both EIA in man and similar conditions in the horse.

A deficiency of ecNOS activity has been implicated in pulmonary hypertension (Higenbottam *et al.* 1988; Pepke-Zaba *et al.* 1991). Exercise will increase pulmonary vascular tone (West & Mathieu-Costello, 1995), which is compensated for by a rapid decrease in pulmonary vascular resistance via recruitment of the microvasculature (Johnson & Hsia, 1994; Kane *et al.* 1994). This functional recruitment of pulmonary capillaries is probably mediated by vasodilatation of previously low-flowing capillaries and not by inducing flow in 'dormant' capillaries (Johnson & Hsia, 1994). Since NO is one of the major vasodilators released from vascular endothelial cells to maintain basal dilator tone and to oppose pressor substances in pulmonary vasculature (Moncada *et al.* 1991; Pohl *et al.* 1993), it could also contribute to the reduction of PVR during exercise.

Low doses of inhaled NO have been shown to reverse an increased PAP and improve $\dot{V}_a/\dot{Q}$ in the neonatal pig (Nelin, Moshin, Thomas, Sasidharan & Dawson, 1994) and during severe pulmonary diseases of man (Bigatello *et al.* 1994; Zapol & Hurford, 1994). Inhaled NO (80 p.p.m.) induced a small but significant decrease in PAP during exercise in the horses of this study. Manohar (1995) recently reported a decrease in pulmonary vascular pressures in the resting horse after the infusion of glyceryl trinitrate, a NO donor (Moncada *et al.* 1991). However, systemic hypotension is a disadvantage of commonly used anti-hypertensive agents, such as glyceryl trinitrate (Roos, Rich, Uncles, Daugherty & Frank, 1994). The effects of inhaled NO will be restricted to the pulmonary circulation because its affinity for oxyhaemoglobin is so high as to remove excess NO in the alveolus by the formation of methaemoglobin and nitrite (Wennmalm, Benthin & Petersson, 1992).

Inhaled NO had no apparent effect on PAP in sheep, despite a significant increase in PAP at rest and during exercise after the administration of L-NAME (Kane *et al.* 1994; Koizumi *et al.* 1994), an inhibitor of ecNOS (Moncada *et al.* 1991). From their results, Kane *et al.* (1994) and Koizumi *et al.* (1994) suggested that NO has a basal vasodilator function that persists, but is not enhanced, during exercise to oppose α-mediated vasoconstriction. However, there are species differences in the activity of NO which preclude the extrapolation of effects between species. For example, L-NAME will increase pulmonary vascular resistance (PVR) in pigs, sheep and man, but not the dog (Cremona, Wood, Hall, Bower & Higenbottam, 1994). Furthermore, in athletic species the lung is adapted to high-intensity exercise by an eightfold increase in pulmonary vascular flow and a thin pulmonary blood–gas barrier to permit rapid gas exchange (Wagner *et al.* 1989; West & Mathieu-Costello, 1995). The superior $\dot{V}_{O_2,max}$ of the horse requires a comparatively higher cardiac output and subsequently, a higher PAP than other species, which will also predispose the horse to stress failure and subsequent rupture of pulmonary capillaries during near-maximal exercise (Wagner *et al.* 1989; West *et al.* 1993; West & Mathieu-Costello, 1995).

Stress failure of pulmonary capillaries, resulting from excessive pulmonary vascular pressure during high-intensity exercise has been implicated in the pathogenesis of EIPH (West *et al.* 1993; West & Mathieu-Costello, 1995), a major problem in the horse during racing (Erickson *et al.* 1990; West *et al.* 1993). The severity of pulmonary capillary damage directly correlates with increasing PAP (West & Mathieu-Costello, 1995) and blood volume (Wagner *et al.* 1989). Maximal changes in PAP are induced by the sudden onset of high intensity exercise whether on the racecourse (Erickson *et al.* 1990; Manohar, 1994) or in an exercise protocol such as used in the present study. Therefore, while NO may only contribute to vasodilatation of pulmonary capillaries (Gryglewski, Palmer & Moncada, 1986) and oppose α-mediated vasocontriction during exercise (Kane *et al.* 1994; Koizumi *et al.* 1994), any reduction in the basal activity of NO during peak exercise may contribute to a marginal but significant increase in PAP.

The synthesis of NO requires normoxia (Cremona *et al.* 1995; Grimminger, Spriestersbach, Weissman, Walmrath &

Seeger, 1995) while its effect in pulmonary arteries is selectively impaired by even moderate hypoxia (Johns, Linden & Peach, 1989). Hypoxic inhibition of NO production has been suggested as one of the mechanisms of HPV (Archer, Tolins, Raij & Weir, 1989) because acute (Johns *et al.* 1989) hypoxia will depress endothelium-dependent relaxation of the vasculature. Inhaled NO will reverse HPV in a dose-dependent manner (Frostell, Fratacci, Wain, Jones & Zapol, 1991) and reduced the rise in PAP in the horses of this study. Alveolar hypoxia has been reported in the horse during high-intensity exercise (Wagner *et al.* 1989; Butler *et al.* 1993), which could reduce NO-dependent relaxation of pulmonary vasculature. Arterial hypoxaemia has also been reported in the horse during near-maximal exercise (Wagner *et al.* 1989; Butler *et al.* 1993). The affinity of haemoglobin for NO is inversely proportional to its oxygen saturation (Iwamoto & Morin, 1993) and arterial hypoxaemia will enhance the inactivation of NO. Any reduction in NO activity may contribute to the incidence of EIPH in the horse during near-maximal exercise. Pelletier, Robinson, Kaiser & Derkson (1995) recently demonstrated a difference in the regional blood flow in the equine lung and that blood flow to the caudodorsal aspect of the lung is endothelium dependent. Interestingly, EIPH is usually restricted to the caudodorsal aspect of the lung (Erickson *et al.* 1990).

In summary, NO was detected in the exhaled air of the horse and $\dot{V}_{NO}$ increased linearly with exercise intensity. One possible source of NO in exhaled air is from the tracheobronchial circulation. Inhaled NO significantly reduced PAP in the horse at near-maximal exercise. The incidence of EIPH in the horse could be related to a small but significant reduction in NO activity during near-maximal exercise.

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