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Comparative effects of L-NOARG and L-NAME on basal blood flow and ACh-induced vasodilatation in rat diaphragmatic microcirculation

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1 The effects of N^{ω}-nitro-L-arginine (L-NOARG) and N^{ω}-nitro-L-arginine methyl ester (L-NAME) on diaphragmatic microcirculation in male Sprague-Dawley rats were assessed under basal conditions and after acetylcholine (ACh) stimulation. In addition, L-arginine (L-arg) was used with the aim of preventing L-NOARG and L-NAME from inhibiting ACh-induced vasodilatation, in order to explore the possibility that L-NOARG is not only a nitric oxide (NO) synthase inhibitor but also a muscarinic receptor antagonist.

2 Male Sprague-Dawley rats were anaesthetized with urethane and mechanically ventilated. The left hemi-diaphragm of each rat was prepared and microvascular blood flow was recorded during continuous superfusion with bicarbonate-buffered prewarmed Ringer solution by using laser-Doppler flowmetry. The drugs were topically applied to the surface of the hemi-diaphragm.

3 Baseline microvascular blood flow was unaffected after 15 min superfusion with any one of the following agents: L-NOARG (0.1 mM), L-NAME (0.1 mM), L-arg (10 mM).

4 ACh (0.03 mM, 0.1 mM and 0.3 mM) elicited a significant increase of microvascular blood flow ($171 \pm 16\%$, $214 \pm 55\%$, and $323 \pm 68\%$ of baseline values, respectively), via interaction with the muscarinic receptor, for the vasodilator response was severely inhibited by 15 min superfusion with atropine (0.3 mM).

5 Following 15 min superfusion with either of the L-arg analogues (0.1 mM), the ACh-induced vasodilator response was significantly inhibited. Pretreatment with L-arg (10 mM) for 5 min, followed by co-administration of L-arg (10 mM) and L-NOARG (0.1 mM) for another 15 min significantly prevented the inhibitory effect of L-NOARG on ACh-induced vasodilatation. However, a similar pretreatment schedule with L-arg failed to prevent L-NAME from exerting its inhibitory effect.

6 Neither of the L-arg analogues potentiated sodium nitroprusside (10 μM and 30 μM)-induced vasodilatation. However, adenosine (0.1 mM)-induced vasodilatation was slightly but significantly attenuated by either L-NOARG (0.1 mM) or L-NAME (0.1 mM), an effect which was prevented by L-arg (10 mM).

7 In conclusion, an increase in endothelium-dependent blood flow stimulated by ACh may occur in diaphragmatic microcirculation of anaesthetized rats independently of low baseline NO activity. The results also suggest that L-NAME has muscarinic receptor antagonist action in addition to its ability to inhibit NO synthase. Thus, we suggest that L-NAME should not be used as a specific NO synthase inhibitor in the rat diaphragm in situations in which there is potential for muscarinic receptors to be stimulated.

Keywords: EDRF; endothelium; nitric oxide; N^{ω}-nitro-L-arginine; N^{ω}-nitro-L-arginine methyl ester; sodium nitroprusside; adenosine; laser-Doppler flowmetry

Introduction

The role of endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) in modulating blood flow in the diaphragmatic vascular bed is a topic of considerable interest, since the diaphragm is the principal respiratory muscle. It has been shown that basal diaphragmatic vascular tone in the isolated left hemi-diaphragms of dogs is partially regulated by NO (Hussain *et al*., 1992). Furthermore, enhanced NO release contributes to active hyperaemia and the matching of diaphragmatic O_2 consumption to changes in metabolic demands (Hussain *et al*., 1992; Chang *et al*., 1993). By contrast, intravenous administration of N^{ω}-nitro-L-arginine (L-NOARG) in chronically instrumented conscious dogs does not affect either baseline vascular resistance or tissue blood flow, as measured by the radioactive microspheres method in resting diaphragms. However, L-NOARG significantly increases diaphragmatic vascular resistance by 80% during exercise (Shen *et al*., 1994).

The diaphragmatic microcirculation determines whether diaphragm perfusion is adequate. Topical applications of L-NOARG have been shown by intravital microscopy to dose-dependently cause a significant reduction in A_2 arteriolar diameters ranging from 3% to 15.7% of control values in rat diaphragms (Boczkowski *et al*., 1994). Nevertheless, our previous study involving laser-Doppler flowmetry (LDF) indicated that baseline NO activity in the diaphragmatic microvascular bed of resting rats was quite low, in that N^{ω}-nitro-L-arginine methyl ester (L-NAME) did not affect basal microcirculatory blood flow (Chang *et al*., 1994; 1995a). In addition, it was shown that L-NAME could nearly abolish acetylcholine (ACh)-induced vasodilatation in rat diaphragms, whereas pretreatment with L-arginine (L-arg) failed to prevent L-NAME from exerting its inhibitory effect (Chang *et al*., 1994). Since L-NAME is more soluble and easier to use than L-NOARG in *in vivo* experiments, the issue of whether L-NAME and L-NOARG possess different pharmacological characteristics in diaphragmatic microcirculation needs to be explored.

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Further evidence of the need to determine these characteristics comes from the observation that alkyl esters of L-arg have been demonstrated, by receptor binding analysis, to be muscarinic antagonists as well as NO synthase inhibitors in rabbit coronary arteries and in canine isolated colonic smooth muscle (Buxton *et al*., 1993).

The initial aim of the present study was, therefore, to compare the effects of L-NOARG with those of L-NAME on the regulation of diaphragmatic microcirculation under basal conditions and after ACh stimulation. Rat diaphragms were prepared to allow a continuous recording of microcirculatory blood flow by laser-Doppler flow meters (LDF). The second aim was to determine whether L-NOARG is also a muscarinic receptor antagonist. For this purpose, the ability of L-arg to overcome the inhibition of ACh-induced vasodilatation produced by either L-NOARG or L-NAME was assessed.

Methods

Animal preparation

Male Sprague-Dawley rats (8–10 weeks old; weight 300–350 g) were housed at the Laboratory Animal Centre of the College of Medicine at National Cheng Kung University. All animals were acclimatized to a 12 h light : 12 h dark cycle and were maintained on Purina rat chow and tap water *ad libitum*. The animals were fasted overnight but allowed free access to water the day before the experiment.

The animals were initially anaesthetized with intraperitoneal injections of sodium pentobarbitone (30 mg kg^{-1}) followed by intravenous injection of 50% w/v urethane (1.2–1.5 g kg^{-1}) and placed in a supine position on a rodent operating table (Harvard Apparatus, South Natick, MA). After a tracheostomy with PE-240 tubing, a muscle relaxant (gallamine triethiodide; 60 mg kg^{-1}) was administered and the rats were artificially ventilated at tidal volumes between 6–7 ml kg^{-1} at a rate of 70–80 breaths min^{-1} (model 683, Harvard Apparatus). Adequacy of ventilation was monitored with a microcapnometer (model JS-02262, Polaris, Jerusalem, Israel) through a T-shaped connection to the tracheostomy tube to keep end-tidal P_{CO_2} at 35–40 mmHg. Supplementary O_2 was applied to the inspiratory port at a fractional concentration of 40% (balanced air). Mean systemic blood pressure (BP_{sys}) was measured with a polyethylene catheter (PE-50) inserted via the right carotid artery and connected to a pressure transducer (model P23 XL, Viggo-Spectramed, Oxnard, CA). The system was filled with heparin/saline (10 iu ml^{-1}). A cardiotachometer (model 13-4615-66, Gould Inc., Cleveland, OH) triggered by the pressure signal was used to monitor heart rate. Another catheter (PE-10) was inserted into the left external jugular vein for administration of fluid and anaesthetic. Normal saline (3–5 ml $h^{-1}{\cdot}100g^{-1}$) was infused throughout the experiment via a peristaltic pump (model MS-Reglo, Ismatec, Glattbrugg, Switzerland). Depth of anaesthesia was evaluated hourly by applying pressure to a paw and observing changes in heart rate or blood pressure. When either one changed by more than 10% of baseline values, supplementary doses of urethane (50 mg) were administered intravenously. Rectal temperature was continuously monitored with a thermistor and maintained at 36–38°C by a heating lamp and a temperature-regulated bed (model 50-7129, Harvard Apparatus). Arterial blood gas and a haematocrit (Hct_{sys}) were determined in 200 μl and 80 μl blood samples, respectively.

Diaphragm preparation

The techniques of diaphragm preparation have been described in detail elsewhere (Chang *et al*., 1995b). Briefly, a thoracotomy was performed in the right fifth and sixth intercostal spaces, and a 1 cm long segment of the right sixth rib was removed. The diaphragm was separated from the lungs and the mediastinal tissues. An ovoid-shaped stainless steel plate coated with white glossy acrylic was slipped behind the diaphragm to hold the left hemi-diaphragm flat. The plate was fixed to the operation table via a miniature ball-jointed clamp system (model 50-4373, Harvard Apparatus).

Midline and transverse abdominal incisions were made and the ligament between the liver and the central tendon was severed. With the animal in the Trendelenberg position, the upper abdominal wall was folded back and retracted. Abdominal viscera were immobilized by wrapping them with a plastic cast. Superfusion of the abdominal side of the left hemi-diaphragm at a flow rate of 5 ml min^{-1} via a peristaltic pump (model MC-MS/CA/4/8, Ismatec) was begun immediately after exposure, with bicarbonate-buffered Ringer solution. The solution was equilibrated with 5% CO_2-95% N_2. The temperature of the superfusing fluid was maintained at 37°C as monitored by a thermistor thermometer at the outlet (model 8110-20, Cole-Palmer Instruments, Chicago, IL). A side port connected to a syringe pump (model 55-3333, Harvard Apparatus) was set up for administration of drugs. The infusion rate was set at 1% of the superfusing fluid rate, to standardize the final concentration of test agents.

Laser-Doppler flowmetry

A commercially available laser-Doppler flowmeter (Laserflo BPM^2, Vasamedics, St. Paul, MN) equipped with a small calibre probe (model P 443-3) was used to measure microvascular flow rates. The signal from the LDF was obtained as a direct current (Q_{LDF}, updated 8 times s^{-1}). The time constant was set at 1 s.

In all experiments, continuous LDF signals were recorded. The probe, held in an MM-3 micromanipulator (Narishigi Instruments, Tokyo, Japan), was placed perpendicular to the surface of the diaphragm, with the probe tip just touching the water film of the suffusate on the surface of the diaphragm. A site on the left costal diaphragm without visible large vessels was chosen and confirmed by visualization with a long-working-distance stereoscopic zoom microscope (SMZ-1, Nikon, Tokyo, Japan). After stable readings had been obtained, the probe was kept in the same fixed position for the duration of the experiments. An average reading time of 30 s was required to provide a stable signal which was independent of vasomotion. At the end of the experiments, the animals were killed by an intravenous injection of saturated potassium chloride. The *postmortem* LDF signal was considered as biological zero and subtracted from the LDF values recorded *in vivo*.

Experimental protocols

Experiments were initiated after a 30 to 45 min stabilization period. Arterial blood gases and haematocrit were determined. The animals used in this study met the following criteria during the stabilization period: (1) $BP_{sys} > 80$ mmHg (2) pH 7.35–7.45, $P_{O_2} > 100$ mmHg, (3) $Hct_{sys} > 40\%$, (4) $Q_{LDF} >$ two fold increase compared to baseline values after topical application of adenosine at 0.1 mM, and (5) no obvious haemorrhage in the muscle tissue under investigation. Indomethacin (10 mg kg^{-1}) was given intravenously to all animals at least 30 min before the start of the experiments. This dose is adequate for blocking prostaglandin synthesis *in vivo* (Beck *et al*., 1993). Eight series of experiments were performed. After the period of stabilization, in Series 1, six rats received topical saline at 1% of the flow rate of the superfusion fluid for 15 min (the time-control group). In nine rats in Series 2 and six rats in Series 3, either L-NOARG or L-NAME at 0.1 mM was applied to the preparation for 15 min by continuous suffusion, respectively. In six rats in Series 4 and another six rats in Series 5, after pretreatment with L-arg at 10 mM for 5 min, L-arg (10 mM) was co-administrated with either L-NOARG (0.1 mM) or L-NAME (0.1 mM), respectively, for another 15 min. In seven rats in Series 6, four rats in Series 7, and another four rats in Series 8, either L-arg 10 mM and vehicle of L-NOARG, or atropine 0.3 mM was suffused for 15 min. After

these procedures, non-cumulative concentration-response curves to topical applications of ACh 0.03 mM, 0.1 mM and 0.3 mM were obtained for all animals in the eight series. Each concentration was given continuously until a stable Q_{LDF} was recorded, and a 5 min rest period elapsed before a higher dose was applied. Applications of higher doses were separated by a 5 to 10 min rest period in order to allow the Q_{LDF} to return to baseline values, and the vasodilator effects of topical SNP 10 μM and 30 μM were evaluated. Finally, after another 5 to 10 min rest period, adenosine 0.1 mM was applied topically to all the preparations.

Drugs

Urethane, gallamine, triethiodide, dextran 70, indomethacin, L-arginine hydrochloride, L-NOARG, L-NAME, ACh, sodium nitroprusside (SNP), adenosine, and atropine sulphate were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). All compounds except urethane, indomethacin and L-NOARG were prepared fresh daily in saline and stored on ice during the experiments. Urethane was prepared in saline at a 50% w/v concentration. Indomethacin was dissolved in 50 mM sodium carbonate. L-NOARG was dissolved in a saline solution containing dimethyl sulphoxide (DMSO, final concentration 0.1%) and NaOH (final concentration 0.9 mN) to obtain a final concentration of 10 mM.

Data acquisition and statistical analysis

BP_{sys} and Q_{LDF} were fed into a chart recorder (Gould RS 3200 polygraphy) for continuous recording. The output of pressure signals, heart rate, and the analogue output from the LDF were directed into a multichannel analogue interphase unit, where the data were sampled at 10 Hz with a 12-bit analogue-to-digital converter (AT codas, Dataq Instrument, Akron, OH) and stored in a personal computer. Recording periods complicated by artefacts were excluded before data analysis. The average LDF signal during a recording time of 30 s was defined as one measurement.

Results are expressed as means $\pm$ s.e.mean. Responses to ACh, SNP and adenosine were measured as a stable increase in Q_{LDF} and expressed as a percentage of baseline values. Baseline Q_{LDF} immediately before the start of suffusion with ACh, SNP or adenosine represented 100% for calculation of percentages of the maximal change of Q_{LDF}. Differences in mean values between groups were analysed for statistical significance by use of repeated measure analysis of variance, followed by Student's *t* test with Bonferroni correction if necessary. When appropriate, Student's *t* test for paired data was also used. A probability value of $P<0.05$ was considered statistically significant. The number of observations (measurements) is denoted by *n*.

Results

Systemic and microcirculatory variables

The results are based on experiments carried out on 48 rats that met the inclusion criteria for BP_{sys}, arterial blood gases and Hct_{sys}. Mean BP_{sys} was 108 ± 2 mmHg; heart rate 440 ± 6 min^{-1}; arterial pH 7.41 ± 0.01, arterial PO_2 152.3 ± 4.8 mmHg, arterial PCO_2 32.8 ± 0.6 mmHg and Hct_{sys} 47 ± 1%. The resting rate for Q_{LDF} was 340 ± 16 mV. There were no significant differences in baseline P_{SYS}, heart rate and Q_{LDF} between the animals of the eight experimental groups, as shown in Table 1 ($P=0.263$, 0.449, and 0.265, respectively). After topical administration of adenosine 0.1 mM, Q_{LDF} increased to 367 ± 28% of its baseline value.

Responses of basal blood flow

Topical applications of L-NOARG or L-NAME both at 0.1 mM for 15 min did not affect basal Q_{LDF} ($P=0.207$ and 0.391, respectively). Also, superfusion with L-arg 10 mM did not induce a significant effect on basal Q_{LDF} ($P=0.139$) (Table 2). Pretreatment with L-arg 10 mM for 5 min, followed by co-administration of L-arg 10 mM and either L-NOARG or L-NAME 0.1 mM for another 15 min, did not affect basal Q_{LDF} (data not shown).

Responses to ACh

Figures 1 and 2 illustrate that the vasodilator response, induced by incremental concentrations of ACh (0.03 mM – 0.3 mM), could be significantly abolished by L-NOARG 0.1 mM (15 min pretreatment) ($P=0.01$, compared to saline group). Also, pretreatment with L-arg 10 mM, followed by co-administration of L-arg (10 mM) with L-NOARG (0.1 mM), prevented L-NOARG from exerting this inhibitory effect on ACh-induced vasodilatation ($P=0.276$, (compared to saline group). The vasodilator response to non-cumulative concentrations of ACh (0.03 mM – 0.3 mM) in the L-NAME group was significantly attenuated in comparison with those of the saline group (Figures 1 and 3, $P=0.01$). However, pretreatment with L-arg (10 mM), followed by co-administration of L-arg (10 mM) and L-NAME (0.1 mM), did not restore the ACh-induced vasodilatation (Figures 1 and 3, $P=0.696$, compared to L-NAME group). The vasodilator response to cumulative concentrations of ACh in the vehicle group was not significantly different from that in the saline group (Figure 3, $P=0.435$). L-Arg 10 mM alone did not affect the vasodilator response to ACh (Figure 3, $P=0.276$, compared to saline group). Topical application of atropine 0.3 mM for 15 min significantly inhibited the ACh-induced vasodilatation (97 ± 1%, 110 ± 1%, and 105 ± 2%; $P=0.01$, compared to saline group).

Table 1 Baseline mean systemic arterial blood pressure (BP_{Sys}), heart rate (HR) min^{-1} and diaphragmatic microvascular blood flow recorded by laser-Doppler flowmetry (Q_{LDF}) of the animals in the eight experimental groups

	BP_{Sys} (mmHg)	HR (beats min^{-1})	Q_{LDF} (mV)
Saline ($n=6$)	100 ± 4	428 ± 12	365 ± 34
L-NOARG ($n=9$)	105 ± 7	464 ± 23	322 ± 23
L-NAME ($n=6$)	104 ± 7	438 ± 12	355 ± 28
L-Arg + L-NOARG ($n=6$)	113 ± 4	450 ± 6	352 ± 31
L-Arg + L-NAME ($n=6$)	109 ± 2	439 ± 8	344 ± 21
L-Arg ($n=7$)	116 ± 3	463 ± 16	332 ± 34
Vehicle ($n=4$)	114 ± 5	427 ± 28	320 ± 23
Atropine ($n=4$)	112 ± 4	463 ± 18	312 ± 39

Values are mean $\pm$ s.e.mean. L-NOARG, N^{ω}-nitro-L-arginine (0.1 mM); L-NAME, N^{ω}-nitro-L-arginine methyl ester (0.1 mM); L-arg + L-NOARG, L-arginine (10 mM) co-administered with L-NOARG (0.1 mM); L-arg + L-NAME, L-arginine (10 mM) co-administered with L-NAME (0.1 mM); L-arg (10 mM); vehicle of L-NOARG; Atropine (0.3 mM).

Table 2 Changes of diaphragmatic microvascular blood flow recorded by laser-Doppler flowmetry (Q_{LDF}) after a 15 min superfusion of test agents

	Before	*After*
Saline ($n=6$)	354 ± 52	342 ± 73
L-NOARG ($n=9$)	339 ± 32	351 ± 44
L-NAME ($n=6$)	316 ± 31	338 ± 44
L-Arg ($n=9$)	341 ± 55	376 ± 52
Vehicle ($n=4$)	316 ± 36	328 ± 28

Values are mean $\pm$ s.e.mean. Abbreviations as shown in Table 1.

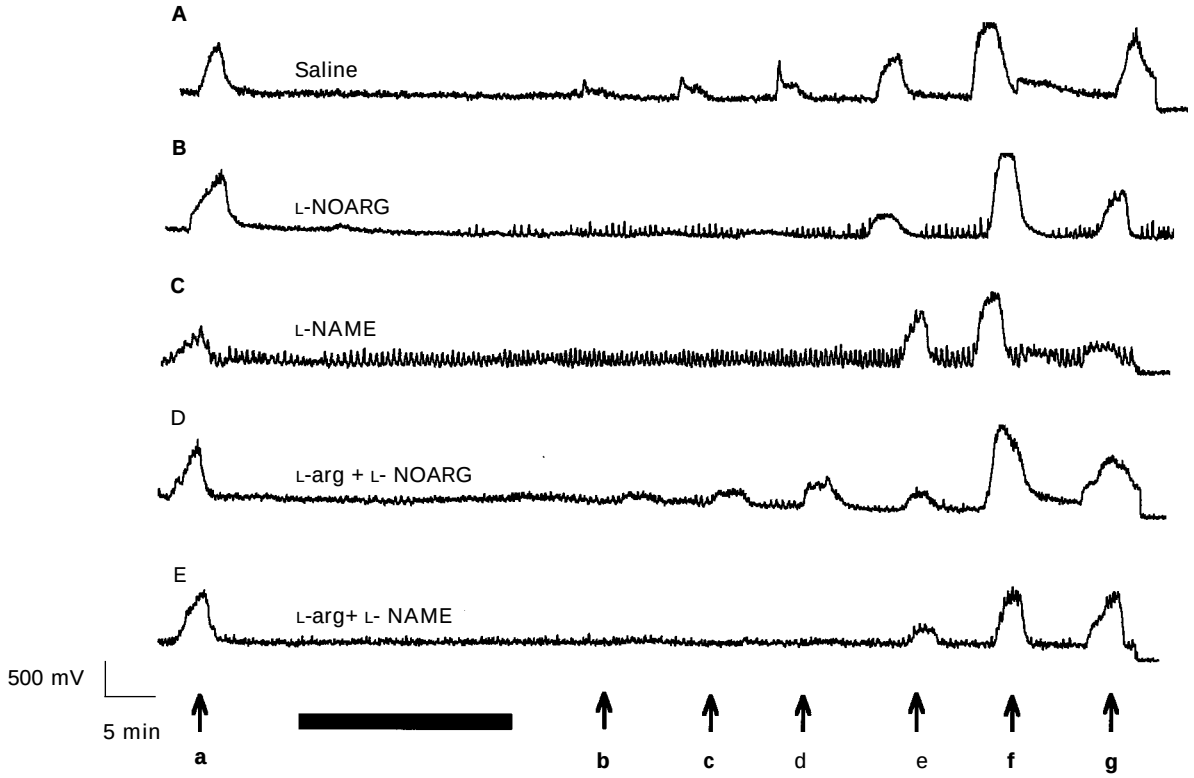

Figure 1 Five representative tracings showing the effects of a 15 min superfusion of (A) saline, (B) L-NOARG (N^{ω}-nitro-L-arginine, 0.1 mM), (C) L-NAME (N^{ω}-nitro-L-arginine methyl ester, 0.1 mM), (D) L-arg + L-NOARG (L-arginine at 10 mM co-administered with L-NOARG 0.1 mM), and (E) L-arg + L-NAME (L-arginine at 10 mM co-administered with L-NAME 0.1 mM) on basal blood flow and agonist-induced vasodilator responses, as measured by laser-Doppler flowmetry in rat diaphragms. Solid bar indicates the duration of application of test agents. Arrows indicate the time of administration of (a) adenosine (0.1 mM); acetylcholine (b) 0.03 mM (c) 0.1 mM, (d) 0.3 mM; sodium nitroprusside (e) 10 μM (f) 30 μM); (g) adenosine (0.1 mM).

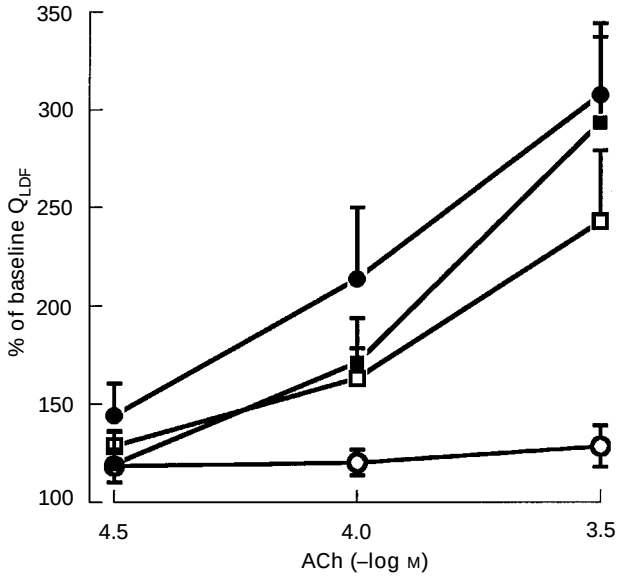

Figure 2 Dose-response curves of diaphragmatic microcirculatory blood flow measured by laser-Doppler flowmetry (Q_{LDF}) to increasing doses of topical application of acetylcholine (ACh) in animals from the saline group (●, $n=6$), vehicle group (■, $n=4$), L-arginine (10 mM) co-administered with N^{ω}-nitro-L-arginine (0.1 mM) group (□, $n=6$), and N^{ω}-nitro-L-arginine (0.1 mM) group (○, $n=9$). Responses, shown as a percentage increase of baseline Q_{LDF}, are given as the means from the number of animals shown in parentheses; vertical lines show s.e.mean.

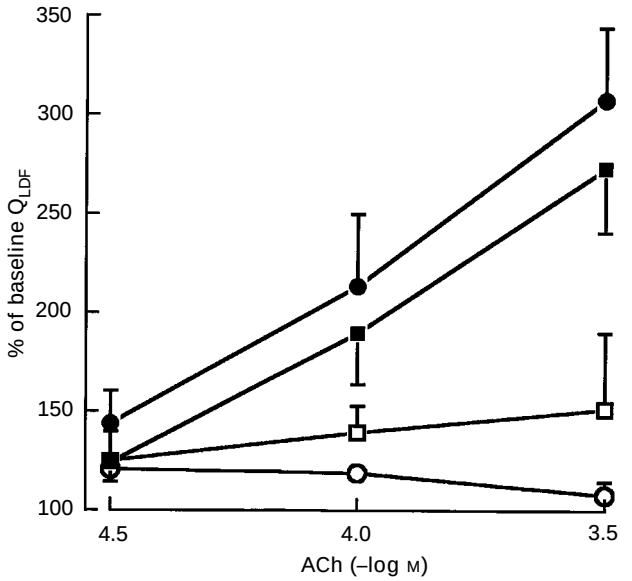

Figure 3 Dose-response curves of diaphragmatic microcirculatory blood flow measured by laser-Doppler flowmetry (Q_{LDF}) to increasing doses of topical application of acetylcholine (ACh) in animals from the saline group (●, $n=6$), L-arginine (10 mM) group (■, $n=7$), of L-arginine (10 mM) co-administered with N^{ω}-nitro-L-arginine methyl ester (0.1 mM) group (□, $n=6$), and N^{ω}-nitro-L-arginine methyl ester (0.1 mM) group (○, $n=6$). Responses, shown as a percentage increase of baseline Q_{LDF}, are given as the means from the number of animals shown in parentheses; vertical lines show s.e.mean.

Responses to SNP and adenosine

Figure 4 shows that there were no significant differences in the vasodilator response to SNP (10 μM and 30 μM) between the animals of the eight experimental groups ($P=0.753$ and 0.594, respectively). Also there was no significant difference in the vasodilatation induced by adenosine 0.1 mM, except in the L-NOARG and L-NAME groups where the vasodilator responses were slightly depressed ($261\pm42\%$ and $267\pm36\%$; $P=0.046$ and 0.045, compared to saline group, respectively).

Discussion

The results of this study show that: (1) ACh, an endothelium-dependent agonist, elicited a significant increase of the diaphragmatic microcirculatory blood flow in a concentration-dependent manner; (2) blockade of NO synthesis either by L-NOARG or L-NAME did not significantly affect the basal diaphragmatic microcirculatory blood flow; (3) the vasodilator effect of ACh on the diaphragmatic microcirculation was almost abolished by either L-NOARG or L-NAME; (4) pretreatment with L-arg significantly prevented L-NOARG from exerting its inhibitory effect on ACh-induced vasodilatation. Conversely, pretreatment with L-arg in a similar manner failed to prevent the inhibitory effect of L-NAME.

Effects of L-NOARG and L-NAME on basal microcirculatory blood flow

In this study, diaphragmatic microcirculation under basal conditions was not affected by local application of either one of the L-arg analogues. This data suggests that basal NO activity of the diaphragmatic microcirculation was quite low, and NO probably does not play a significant role in modulating the resting diaphragmatic microcirculation of the anaesthetized rats. It could be argued that in our diaphragm preparations NO synthase in the endothelial cells of the microvasculature was less effective due to a shortage of the supply of its substrate, since tissue metabolites including L-arg were washed away by the superfusing fluid. However, this argument can be refuted by the finding that L-arg (10 mM) alone did not affect basal diaphragmatic microcirculatory blood flow.

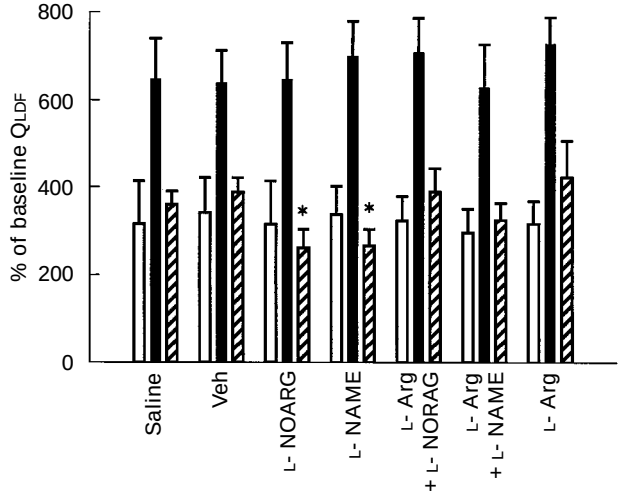

Figure 4 The increase of diaphragmatic microcirculatory blood flow, measured by laser-Doppler flowmetry (Q_{LDF}), after topical applications of sodium nitroprusside 10 μM (open columns) and 30 μM (solid columns) and adenosine 0.1 mM (hatched columns) in animals from the saline group ($n=6$), the vehicle group ($n=4$), N^{ω}-nitro-L-arginine group (L-NOARG, 0.1 mM, $n=9$), N^{ω}-nitro-L-arginine methyl ester group (L-NAME, 0.1 mM, $n=6$), L-arginine (10 mM) co-administered with L-NOARG (0.1 mM) group (L-arg + L-NOARG, $n=6$), L-arginine (10 mM) co-administered with L-NAME (0.1 mM) group (L-arg + L-NAME, $n=6$), and L-arginine group (L-arg, 10 mM, $n=7$). Responses, shown as a percentage increase of baseline Q_{LDF}, are given as the means $\pm$ s.e.mean from the number of animals shown in parentheses. $^*P<0.05$ compared with the saline group.

The importance of the availability of extracellular L-arg as a rate-limiting factor in NO synthesis *in vivo* varies depending on the vasculature and the species under study (Persson *et al*., 1990; Rosenblum *et al*., 1990; Hoffend *et al*., 1993; Schuschke *et al*., 1994). Consistent with our data, L-arg 6 mM, directly infused into canine phrenic circulation, did not significantly alter vascular resistance (Ward & Hussain, 1994). Furthermore, topical applications of L-arg 30 mM for 15 min do not modify rat diaphragmatic arteriolar tone (Boczkowski *et al*., 1994). Thus, the low baseline NO activity in our current findings cannot be attributed to the rate-limiting step of L-arg availability.

However, basal diaphragmatic blood flow has been shown to decline to 56 and 78% of control values when L-NOARG 0.6 mM was directly infused into the phrenic artery of the canine diaphragm, (Chang *et al*., 1993; Ward & Hussain, 1994 respectively). There are three possible explanations for the difference between these and the present results. Firstly, a different species was used. Nevertheless, in another study on chronically instrumented conscious dogs, intravenous administration of L-NOARG did not affect baseline blood flow in the resting diaphragm (Shen *et al*., 1994). This finding raises the possibility that different methods of diaphragm preparation over and above any difference between species may result in different rates of oxygen consumption, and that this could be a more important factor in determining the involvement of NO (Chang *et al*., 1993).

A second explanation for the difference may be that gallamine triethiodide was used in the present study to keep the diaphragm at rest, so avoiding changes in metabolic rates. In dogs, preparation of the vascularized isolated hemi-diaphragm takes a protracted amount of time and involves extensive surgery (Hussain *et al*., 1989). Therefore, muscle fasciculation which contributes to an increase in metabolic need and an accompanying increased blood flow may be present in such preparations. In fact, one of the important stimuli for the basal release of NO is related to the hydromechanical force associated with pulsatile blood flow (Pohl *et al*., 1986). Moreover, it has been shown that when diaphragmatic metabolic demand is increased during acute exercise, the increase in phrenic vascular resistance induced by L-NOARG is greater, changing from an insignificant increase during spontaneous breathing at rest to an 80% increase during increased metabolic demand (Shen *et al*., 1994).

The third possible explanation for the difference between the present and previous results relates to the method and technique used to measure blood flow. In the canine diaphragmatic preparation blood flow was measured in the phrenic artery, so reflecting global blood flow, whereas in the rat diaphragm preparation, LDF was used to measure flow at the microcirculatory level. It is possible that the overall microvascular response might not be well predicted from the vasomotor response of a single site recorded by the LDF because of the wide spatial variety in the rat diaphragmatic microvascular bed (Chang *et al*., 1995b). Further work, either by computer-aided scanning of LDF, or by integrating the LDF probe to receive light from multiple scattering volumes simultaneously, will be necessary to clarify this point (Salerud & Nilsson, 1986).

In contrast with our present findings, topical applications of L-NOARG by intravital microscopy have been shown to cause dose-dependent reductions in A_2 arteriolar diameters, ranging from 3% to 15.7% of control values in resting rat diaphragms (Boczkowski *et al*., 1994). This finding seems at first to contradict the results of this study. However, based on morphological measurements of changes in vessel diameters it seems that NO release induces a heterogeneous distribution of microvascular responses (Komaru *et al*., 1991; Habazettl *et al*.,

1994). Changes in the diameters of arterioles either upstream or downstream could modify diaphragmatic microcirculatory perfusion despite a reduction in the diameter of A_2 arterioles, as observed by Boczkowski *et al*. (1994). Further investigation, by simultaneously integrating *in vivo* microscopes with LDF to observe the changes in diaphragmatic arterioles of various sizes and of microvascular perfusion, will be necessary to settle this argument.

Effects of L-NOARG and L-NAME on ACh-induced microcirculatory blood flow change

In the present study, ACh elicited an increase in diaphragmatic microcirculatory blood flow in a dose-dependent manner. This vasodilator response resulted from a specific pharmacological interaction with the muscarinic receptor, for it was inhibited by atropine. After application of either of the arginine analogues, the microcirculatory change stimulated by ACh was nearly abolished. Consistent with our results, phrenic vascular resistance has been shown to decline progressively on exposure to increasing concentrations of ACh, and this effect could be reversed completely by L-argininosuccinic acid (Hussain *et al*., 1992). In contrast, ACh has been shown to produce a dose-dependent increase in arteriolar diameter in the diaphragm that was not significantly altered by the application of L-NOARG (Boczkowski *et al*., 1994). There is no obvious explanation for this discrepancy apart from the use of different anaesthetics and the different methods employed to measure microcirculation.

Thus, in the diaphragmatic microcirculation of rats, both L-NOARG and L-NAME prevented ACh-induced vasodilator responses despite low baseline NO activity. Similarly, N^G-monomethyl-L-arginine (L-NMMA) has been shown to block the response to ACh without affecting the basal tone of arterioles in rat mesenteric circulation (Ebeigbe *et al*., 1990). It has been suggested that tonically released NO is primarily newly synthesized NO, while stored NO is primarily released by agonists, so contributing to the separation of the effect of NO synthesis inhibitors on the baseline vascular tone from its response to agonists (Bellan *et al*., 1993).

In this experiment, pretreatment of L-arg in 100 fold excess, followed by co-administration of L-arg with L-NOARG, prevented ACh-induced vasodilatation from being abolished by L-NOARG. However, similar administration of L-arg failed to prevent L-NAME from exerting its inhibitory effects on ACh-induced vasodilatation. A similar phenomenon had also been observed in the rabbit isolated, buffered-perfused ear preparation, and several possible mechanisms including differences in the mode of action of L-NAME at the level of NO synthase or at the level of L-arg uptake, and heterogeneity in the synthetic mechanisms for NO have been suggested (Randall & Griffith, 1991). In studies of rabbit coronary arteries and canine isolated colonic smooth muscle, alkyl esters of L-arginine have been shown, by receptor binding analysis, to be muscarinic antagonists as well as NO synthase inhibitors. Further support for this comes from the observation that the antagonism of muscarinic receptors by L-NAME could not be overcome by L-arg, so leading to the suggestion that alkyl esters of L-arg are poor choices as specific NO synthase inhibitors in situations in which there is the potential for muscarinic receptors to be stimulated (Buxton *et al*., 1993).

Despite these findings, a similar inhibitory effect of L-NOARG and L-NAME on vasodilator responses to ACh has been noted in the mesenteric as well as in the pulmonary vascular bed, which should not occur if the methyl ester has significant muscarinic receptor blocking activity (Cheng *et al*., 1994; Santiago *et al*., 1994). Similarly, L-NAME was shown to have no effect on the decrease in heart rates evoked by efferent vagal stimulation, a muscarinic receptor-mediated response (Cheng *et al*., 1994). It has also been found that L-NAME is rapidly converted to L-NOARG in canine blood and plasma (Krejcy *et al*., 1993). These data suggest that the relative effects of the alkyl ester of L-NOARG on muscarinic receptor function and on NO synthase may be dependent on species, vascular bed studied or route of administration.

Although L-NOARG and L-NAME had similar inhibitory effects on ACh-induced vasodilatation, L-arg failed to prevent L-NAME from having its inhibitory effects on ACh-induced vasodilatation in our study, which suggests that L-NAME does have a muscarinic receptor blocking action as well as an NO synthase inhibitory effect in rat diaphragmatic microcirculation. In agreement with this suggestion, treatment with L-NAME in canine hearts can abolish the protective effects of ACh-induced ischaemic pre-conditioning that may be mediated via myocardial muscarinic receptors, whereas treatment with L-NMMA does not affect ACh-induced infarct size reduction (Yao & Gross, 1993).

After local applications of either of the arginine analogues, the vasodilator response to SNP was still maintained, which rules out the possibility that the abolition of ACh-induced vasodilatation by these arginine analogues was due to a general, nonspecific suppression of vasodilator responses. Enhanced vasodilator responses to SNP and other nitroso compounds have been found in the presence of L-arg analogues, and this has been related to an increased sensitivity of guanylate cyclase in vascular smooth muscle to exogenous NO when endogenous NO production is reduced (Moncada *et al*., 1991; Gardiner *et al*., 1991). This phenomenon did not occur in the diaphragmatic microcirculation of anaesthetized rats in our study. We speculate that because of the low basal NO activity, the sensitivity of guanylate cyclase in the vascular smooth muscle of the diaphragm to exogenous NO remains unchanged despite the removal of endogenous NO.

In the present study, it was shown that following administration of either one of the arginine analogues, the vasodilator response induced by adenosine could be slightly but significantly suppressed. Moreover, pretreatment with L-arg prevented both arginine analogues from exerting this inhibitory effect, suggesting that NO is partially involved in the adenosine-induced vasodilator response in rat diaphragmatic microcirculation. It has been suggested that luminal adenosine receptors cause vasodilatation by endothelial-dependent mechanisms including NO and that abluminal adenosine receptors cause dilatation by another mechanism (Baker & Sutton, 1993). We speculate that some of the abluminally applied adenosine transgresses the vascular wall in the diaphragmatic microvascular bed and acts on luminal adenosine receptors. As adenosine A_2 receptors are expressed in endothelial cells (Iwamoto *et al*., 1994), luminal adenosine may amplify NO release by increasing endothelial adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) (Graier *et al*., 1992). It is also possible that luminal adenosine potentiates flow-induced NO release via the opening of endothelial K_{ATP} channels (Kuo & Chancellor, 1995).

In summary, our results indicate that the increase in microcirculatory blood flow mediated by NO may occur irrespective of low baseline NO activity in the diaphragm of anaesthetized rats. They also indicate that special caution should be taken when using L-NAME as a specific NO synthesis inhibitor, if the responses under test include those mediated by muscarinic receptors, because of its potential for muscarinic receptor antagonism.

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