Limited role for nitric oxide in mediating cerebrovascular control of newborn piglets

J Patel, O Pryds, I Roberts, D Harris, A D Edwards

Abstract

Aims—To investigate the effects of the nitric oxide (NO) synthase inhibitor L-nitro-arginine methyl ester (L-NAME) on cerebral blood flow, and its response to alterations in arterial carbon dioxide tension (CBF-CO₂ reactivity).

Methods—Cerebral blood flow was measured six times at varying arterial carbon dioxide tension (PaCO₂) using the intravenous ¹³³Xenon clearance technique in eight mechanically ventilated piglets of less than 24 hours postnatal age. After the third measurement L-NAME was administered as a bolus (20 mg/kg) and subsequently infused (10 mg/kg/hour).

Results—PaCO₂ ranged between 2.7–8.9 kPa. Cerebral blood flow decreased by 14.0% (95% confidence interval 1.9–27.4) after L-NAME. CBF-CO₂ reactivity was 18.4% per kPa (95% CI 14.1–22.2) before L-NAME and 15.2%/kPa (95% CI 11.1–19.3) afterwards; the difference between the CBF-CO₂ reactivities was 3.2%/kPa (95% CI -0.4–6.8): these were not significantly different.

Conclusions—Inhibition of nitric oxide synthesis reduces cerebral blood flow no more than a 0.5–1.0 kPa fall in PaCO₂. Nitric oxide is not an important mediator of CBF-CO₂ reactivity.

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Keywords: L-nitro-arginine methyl ester, cerebral blood flow, carbon dioxide reactivity, ¹³³Xenon clearance.

Disruption of cerebral blood flow is implicated in the development of ischaemic and haemorrhagic brain injury in the perinatal period, leading to long term neurodevelopmental sequelae.^{1,2} Therefore, further information on its control is required if therapeutic interventions to improve cerebral injury are to be developed.

Nitric oxide (NO) is an important mediator in the cerebrovascular control of adult animals,^{3,4} and has been strongly implicated in the mechanism of CBF-CO₂ reactivity.^{4,6} In addition to its physiological properties, excessive NO generation following cerebral ischaemia may contribute significantly to neuronal injury.^{7–9} However, comparatively few data are available on its role in the cerebral circulation during the perinatal period. Important age dependent differences may be present, or other mediators such as prostaglandins may have a greater role in controlling cerebrovascular responses in the newborn.^{10,11}

In a previous study inhibition of NO synthesis by competitive inhibition of the enzyme nitric oxide synthase (NOS) with the L-arginine analogue, L-NAME, caused cerebral vasoconstriction, as shown by a small fall in cerebral blood volume (CBV).¹² However, L-NAME had no effect on the response of CBV to changes in PaCO₂, also suggesting significant differences between neonatal and adult cerebrovascular control. In that study it was not possible to determine whether the vasoconstriction induced by L-NAME affected resistance vessels, and thus cerebral perfusion, or whether the fall in CBV was due to alterations in venous capacitance vessels. Equally, studies of the adult brain have used CBF-CO₂ as a measure of the cerebrovascular response to changes in PaCO₂, which have made it difficult to compare directly the results of neonatal and adult studies.

The aim of the present study was to quantify the effects of NOS inhibition on cerebral blood flow and CBF-CO₂ reactivity in newborn piglets using the intravenous ¹³³Xenon technique. We tested the null hypothesis that these parameters remain unaffected following the administration of L-NAME.

Methods

Eight Large White piglets born at 113–117 (median 115) days after conception and weighing 1.30–2.00 (median 1.60) kg were studied on the first day of life. After premedication with intramuscular midazolam (0.20 mg/kg) anaesthesia was induced and maintained with inhaled isoflurane (0.7–1.2%). Core body temperature was maintained by heating lamps at between 38 and 39°C, within the normal range for newborn piglets. Subjects were given a tracheotomy and mechanically ventilated using a pressure limited ventilator (SLE 2000, Surrey, UK) with an inspiratory time of 0.5 seconds, a peak inspiratory pressure of 1.3–1.8 kPa, an initial ventilatory rate of 30 breaths/minute, and an inspired gas mixture of oxygen, air, and isoflurane that was sufficient to maintain light anaesthesia and permit manipulation of arterial blood gases.

An ear vein was cannulated and glucose 10% was infused at 5–7 ml/hour to provide blood glucose concentrations of ≥ 4 mmol/l (BM-stix, Boehringer Mannheim, Germany). An umbilical venous catheter (5.0F) was placed for central venous access and infused with 10% dextrose containing 1 IU of heparin/ml at 1 ml/hour. An umbilical arterial catheter (3.5F) was sited for arterial access and infused with 0.9% saline containing 1 IU of heparin/ml at 1 ml/hour.

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Table 1 Physiological variables before each measurement of cerebral blood flow

Parameter	Before L-NAME			After L-NAME		
	1	2	3	1	2	3
Arterial pressure (mm Hg)	58.0 (6.5)	60.8 (7.9)	65.0 (8.9)	75.4 (8.8)*	75.5 (10.5)*	73.6 (9.6)*
Core temperature (°C)	38.0 (0.9)	38.0 (0.9)	38.3 (0.9)	38.3 (0.8)	38.4 (0.8)	38.1 (0.7)
Arterial pH	7.49 (0.05)	7.49 (0.10)	7.53 (0.16)	7.47 (0.17)	7.45 (0.10)	7.40 (0.10)
PaO ₂ (kPa)	17.2 (4.6)	19.4 (6.0)	16.9 (6.4)	19.5 (6.1)	21.6 (9.1)	19.9 (7.7)
PaCO ₂ (kPa)	5.2 (1.2)	5.3 (1.8)	4.8 (1.6)	4.9 (2.2)	5.1 (1.4)	5.8 (2.1)
Base excess (mmol/l)	3.0 (1.9)	5.7 (2.9)	4.2 (4.9)	2.7 (2.3)	1.7 (3.0)	2.6 (2.2)
Haemoglobin (g/dl)	8.8 (1.3)	9.3 (1.4)	9.3 (1.5)	10.0 (1.7)	10.2 (1.7)	9.9 (1.8)
Blood glucose (mmol/l)	5.6 (2.3)	6.3 (2.5)	6.4 (2.1)	7.0 (2.1)	6.8 (1.9)	7.0 (2.1)

Values are mean (SD). For all values n=8 animals. Cerebral blood flow measured on six occasions; three measurements before the administration of L-NAME and three afterwards.

* Arterial pressure was significantly different after administration of L-NAME ($P < 0.005$).

hour. Mean arterial blood pressure (MAP) was recorded directly from the umbilical arterial catheter using a pressure transducer (Statham, California, USA). End-tidal partial pressure of carbon dioxide (PCO₂) was monitored throughout the study (Capnomac II, Datex, Helsinki, Finland) only to confirm that PaCO₂ remained stable at the set concentration; frequent blood gas analysis was performed on umbilical arterial blood samples (ABL 3, Radiometer, Copenhagen, Denmark) to obtain absolute values for PaCO₂.

MEASUREMENT OF CEREBRAL BLOOD FLOW

Cerebral blood flow was determined using the intravenous ¹³³Xenon technique, as described before.¹³ ¹³³Xenon (40–60 mBq/kg) was injected into the umbilical venous catheter, and the activity recorded by scintillators (Simonsen Medical A/S, Randers, Denmark) placed over the left parieto-temporal region and the thorax. Before each of the cerebral blood flow measurements, the background activity was measured for 0.5 or 5 minutes and accounted for in the subsequent calculation.

Cerebral ¹³³Xenon clearance was determined for 11 minutes, starting from the time when the activity in the lung had decreased to 20% of the peak activity. This level was used to reduce the effect of scattering from the airways. CBF was calculated using the Obrist 2 compartment analysis, and adjusted for the haemoglobin concentration.¹⁴ The blood-brain partition coefficients for grey and white matter, derived from studies on adult human brain, were assumed to be 0.8 and 1.5, respectively.¹⁴ CBF represents the weighted mean of cerebral grey and white matter blood flow,¹⁵ is considered to represent global cerebral blood flow, and is presented as ml/100 g/minute. The test-retest variation in cerebral blood flow measurements is 10.7%.¹⁶

EXPERIMENTAL PROTOCOL

A period of stabilisation lasting at least 75 minutes was allowed following subject preparation. A total of six measurements of cerebral blood flow were then performed in each animal at different concentrations of PaCO₂. PaCO₂ was manipulated within or close to the physiological range, at random, by changing the ventilatory rate or by introducing an extra dead space, as appropriate. A stabilisation period of 20 minutes was allowed following each attempted manipulation of PaCO₂. The ventilation was

not manipulated between the third and fourth measurements. FIO₂ was adjusted, aiming to keep arterial oxygen tension (PaO₂) at ≥ 13.0 kPa. After the third measurement of cerebral blood flow L-NAME was administered as a bolus (20 mg/kg) and was subsequently infused in 10% glucose at a rate of 10 mg/kg/hour through the peripheral venous cannula. This dosage of L-NAME has been found to have near maximal effect on the cerebral vasculature of newborn piglets.¹² Cerebral blood flow measurements were restarted 20 minutes after administration of the L-NAME bolus. Finally, L-arginine was administered as an intravenous bolus (1g) to reverse NOS inhibition, and the effects on MAP were observed.

At the end of the experiment, subjects were sacrificed by an overdose of pentobarbital. Experiments were carried out under license from the Home Office and according to United Kingdom guidelines.

STATISTICAL ANALYSIS

The null hypotheses tested were that cerebral blood flow and CBF-CO₂ reactivity remain constant after administration of L-NAME. A calculation of study power using previous data, which estimated the intrasubject coefficient of variation as between 8% and 17%, revealed that a minimum of eight piglets should be investigated to detect an effect of L-NAME on the cerebral blood flow of at least 15% ($\alpha=0.05$ and $\beta \geq 0.90$). Inspection of data distributions showed that cerebral blood flow values were positively skewed and the data were therefore transformed logarithmically to obtain homogeneity of variance, thus assuming a log-linear relation between cerebral blood flow and PaCO₂. The regression analysis was performed

Table 2 Arterial carbon dioxide tension for individual piglets before each measurement of cerebral blood flow

Piglets	PaCO ₂ (kPa)					
	Before L-NAME			After L-NAME		
	1	2	3	1	2	3
1	4.5	6.5	2.9	2.7	4.9	5.7
2	4.4	4.8	6.1	4.1	5.4	5.9
3	5.3	3.6	5.2	6.3	4.6	3.7
4	4.4	3.3	7.5	8.2	4.8	3.5
5	4.9	3.6	5.8	7.6	3.3	4.5
6	5.3	6.6	3.4	3.3	5.0	8.8
7	4.5	8.5	3.8	3.9	8.3	5.7
8	7.9	5.2	3.9	3.2	4.5	8.9

using dummy variables which accounted for interindividual variation in cerebral blood flow. The estimated regression coefficient thus depended only on the intra-individual variation in cerebral blood flow and was used to estimate the relation between it and PaCO_2 . The log-linear relation implies that the calculated cerebral blood flow response is percentual (the antilogarithm of the regression coefficient is equal to the cerebral blood flow reactivity). The correlation between cerebral blood flow and MAP was also tested this way.

In addition, analysis of variance and the Student-Newman-Keuls test were used to ascertain if there was a systematic difference in PaCO_2 before and after L-NAME, and whether there were differences in other physiological parameters between measurements. The SPSSPC statistical programme (SPSS Inc., Chicago, Illinois, USA) was used for calculations, and the level of significance set at 0.05.

Results

The values recorded during cerebral blood flow measurements, before and after the administration of L-NAME, are given in table 1. There was no significant difference in the mean values of core temperature, arterial blood gas analyses, base excess, haemoglobin or glucose between measurements. The PaCO_2 of individual subjects during each of the six measurements is shown in table 2. End-tidal PCO_2 fluctuation during cerebral blood flow measurements was median 0.2 kPa (range 0–0.3). Following PaCO_2 manipulation MAP changed median + 3 mm Hg (range 2–5). After L-NAME, MAP increased from 65 mm Hg (SD 8) to 75 mm Hg (SD 9), mean change 10 mm Hg (95% CI 5 to 11) ($P < 0.05$). This rise was maintained until the administration of L-arginine, when MAP decreased from 75 mm Hg (SD 8) to 55 mm Hg (SD 6), mean change -18 mm Hg (95% CI -10 to -25) ($P < 0.05$).

The mean (SD) values of cerebral blood flow were 49.4 (16.5) ml/100 g/minute before and 46.1 (21.5) ml/100 g/minute after administration of L-NAME, and were not significantly different. However, when values were adjusted for differences in PaCO_2 (see below), L-NAME resulted in a significant decrease in cerebral blood flow of 14.0% (95% CI 1.9–27.4) ($P < 0.05$).

The relation between cerebral blood flow and PaCO_2 for all measurements for each animal before and after the administration of L-NAME is shown in figs 1 and 2. The overall CBF- CO_2 reactivity for all six measurements was 16.2%/kPa (95% CI 12.0–20.5). CBF- CO_2 reactivity was 18.4%/kPa (95% CI 14.1–22.2) before L-NAME and 15.2%/kPa (95% CI 11.1–19.3) after L-NAME; the difference between the CBF- CO_2 reactivities was 3.2%/kPa (95% CI -0.4–6.8); these values were not significantly different from each other.

The relation between cerebral blood flow and MAP was 0.8% mm Hg (95% CI -0.5–2.1) before, and 0.7% mm Hg (95% CI -0.4–1.7)

Figure 1 Relation between cerebral blood flow and PaCO_2 for all measurements for each animal. Data points before and after L-NAME are shown. Natural log transformed cerebral blood flow values (Ln cerebral blood flow) are shown. CBF- CO_2 reactivity represented by the slope of the regression line before L-NAME (solid line) was not significantly different after L-NAME (dotted line).

after administration of L-NAME, which were not significantly different from zero.

Discussion

Cerebral blood flow measurement using the $^{133}\text{Xenon}$ clearance technique has been validated against other techniques in experimental animals and in newborn infants.^{17–19} The technique assumes that cerebral blood flow remains constant during a measurement; in this study this was believed to be the case as end-tidal PCO_2 and FIO_2 remained stable during measurements. A cerebral blood flow greater than 120 ml/100 g/minute may prevent complete equilibration of $^{133}\text{Xenon}$ between blood and brain,²⁰ but such values were not seen in the study. Calculation of absolute cerebral blood flow values requires knowledge about the blood to brain partition coefficients. As these values are unknown for newborn piglets, the values derived from adult humans were used. Nevertheless, although the absolute values need to be interpreted with caution, the direction and proportion of any change should be accurate. The cerebral blood flow values in our study are comparable with those obtained using other techniques.^{21 22}

L-NAME was administered in concentrations shown previously to have close to a maximal effect on MAP and CBV. Administration of L-arginine completely reversed the effects on MAP, demonstrating the specificity of the observed effect. There was no significant relation between cerebral blood flow and MAP during the study. Even though cerebral blood flow pressure autoregulation was not specifically investigated, our data do not indicate a role for NO in cerebral blood flow pressure autoregulation during acute rises in MAP.

Administration of L-NAME caused a small decrease in cerebral blood flow. These results are consistent with our previous experiments in newborn piglets, and in other studies in mature animals, in showing that a tonic release of NO participates in the maintenance of basal CBF.^{12 23 24} The proportional change in cere-

Figure 2 Relation between cerebral blood flow and PaCO_2 for all measurements for each animal. Each animal is represented by a different symbol. Measurements before (solid line) and after (dotted line) L-NAME are shown. Natural log transformed cerebral blood flow values (Ln cerebral blood flow) are shown.

bral blood flow is not dissimilar to the change previously noted in CBV, and suggests that tonic NO secretion has some effect on the calibre of resistance vessels, modulating rate, and distribution of cerebral perfusion in newborn animals. However, the fall in cerebral blood flow induced by L-NAME is no greater than would normally occur with a 0.5–1.0 kPa fall in PaCO_2 . This is consistent with suggestions that although constitutive NO secretion may control the distribution of flow, perhaps matching perfusion to metabolism, it is not an important mediator of the generalised alterations in vascular tone which determine bulk flow.²⁵

Most reports have shown that NO participates in CBF- CO_2 reactivity in adult rats, cats, dogs and rabbits, and that inhibition of this effect by L-arginine analogues is stereospecific, dose related, and reversed by co-application of L-arginine.^{26–29} Although a few studies offer conflicting results,^{30–32} the weight of evidence suggests that in adult animals NO is a mediator of CBF- CO_2 reactivity. In contrast to these studies, but consistent with our previous experiments in newborn piglets, we found no evidence to suggest that NO was involved in CBF- CO_2 reactivity in the newborn piglet.

This may be a specific maturational effect. Armstead *et al* have shown that the vascular responses of piglets change with age: acetylcholine, which elicits NO mediated vasodilation, produced NO dependent pial arteriolar vasodilation in juvenile pigs but not in newborn pigs.³³ In newborn pigs acetylcholine produced pial arterial vasoconstriction dependent on stimulation of the prostaglandin $\text{H}_2/\text{thromboxane A}_2$ receptor.¹⁰ Greenberg *et al* suggested that in 1 to 2 week old piglets NOS inhibition led to greater declines in cerebral blood flow.²² Wyatt *et al* have also shown that cerebral reactivity to carbon dioxide increases during 26–40 weeks of gestation, demonstrating differences related to brain development.³⁴

The influence of midazolam and isoflurane need to be considered as they have effects on the cerebral circulation.^{35–36} Following premedication with midazolam, at least 90 min-

utes elapsed before the experiments were performed, thus reducing the possibility of drug related effects. Isoflurane was used as it is a clinically relevant anaesthetic and its use is associated with haemodynamic stability. Both drugs have also been shown to maintain CBF- CO_2 reactivity.^{35–36}

These data suggest the testable hypothesis: that participation of the L-arginine NO system in the generalised and co-ordinated control of cerebral blood flow required to modulate CBF- CO_2 reactivity, develops after birth. Similar postnatal developments in NO responsiveness in the pulmonary vasculature have been described in piglets.^{37–39}

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