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EFFECTS OF INHIBITION OF NITRIC OXIDE SYNTHESIS AND OF HYPERCAPNIA ON BLOOD PRESSURE AND BRAIN BLOOD FLOW IN THE TURTLE

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Summary

In the mammalian brain, nitric oxide (NO) is responsible for a vasodilatory tonus as well as the elevation of cerebral blood flow (CBF) induced by hypercapnia. There have been few comparative studies of cerebral vasoregulation in lower vertebrates. Using epi-illumination microscopy *in vivo* to observe CBF velocity on the brain surface (cerebral cortex), we show that turtles (*Trachemys scripta*) exposed to hypercapnia (inspired P_{CO_2}=4.9 kPa) displayed a 62 % increase in CBF velocity, while systemic blood pressure remains constant. Exposing turtles to a P_{CO_2} of 14.9 kPa caused an additional increase in CBF velocity, to 104 % above control values, as well as a 30 % increase in systemic blood pressure. The elevated CBF velocity during hypercapnia could not be blocked by a systemic injection of the NO synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NA). However, L-NA injection caused a temporary stop in CBF as well as a persistent increase in systemic blood pressure, suggesting that there is a NO tonus that is attenuated by the NOS inhibitor and that CBF is strongly dependent on this tonus, although compensatory mechanisms exist. Thus, although the cerebrovascular reaction to hypercapnia appeared to be NO-independent, the results suggest that there is a NO-dependent vasodilatory tonus affecting both cerebral and systemic blood circulation in this species

Key words: *Trachemys scripta*, carbon dioxide, endothelium-derived relaxing factor, N^G-nitro-L-arginine, hypercapnia, nitric oxide, cerebral blood flow, blood pressure, turtle.

Introduction

During recent years, it has become clear that nitric oxide (NO), a small gaseous molecule, is one of the most important vasoregulators in the mammalian brain (for reviews, see Dawson *et al.* 1992; Berdeux, 1993); it is responsible for maintaining a constant vasodilatory tonus (Fernández *et al.* 1993; Macrae *et al.* 1993) and for a coupling between metabolism and cerebral blood flow (CBF) (Dirnagl *et al.* 1993), and it participates in cerebral vasodilation in response to hypercapnia (Iadecola, 1992; Wang *et al.* 1992).

Endogenous NO is synthesized from L-arginine by NO synthase (NOS). Potent and specific inhibitors of this enzyme, such as N^G-nitro-L-arginine (L-NA) and N^G-nitro-L-arginine methyl ester (L-NAME), have been developed. These substances have become tools widely used in the search for NO-mediated vasoregulation (Buxton *et al.* 1993; Ishii *et al.* 1990; Mülsch and Busse, 1990), since an inhibitory effect of a NOS inhibitor on a physiological mechanism provides strong evidence for the involvement of NO.

Very little is known about cerebrovascular regulation in lower vertebrates, especially with regard to the role of NO. However, L-NA was recently found to block the ability of acetylcholine (ACh) to stimulate CBF in turtles, providing the first evidence for the existence of NO-dependent CBF regulation in reptiles (Hylland *et al.* 1996). Moreover, the existence of NOS in neurones in the brains of the turtle *Trachemys scripta* (Brüning *et al.* 1994) and the monitor lizard *Varanus exanthematicus* (Luebke *et al.* 1992) has been demonstrated by NADPH-diaphorase histochemistry and immunohistochemistry. Turtles are of special interest from a comparative perspective since they are representatives of the oldest group of living reptiles. Moreover, freshwater turtles of the genera *Trachemys* and *Chrysemys* have long been of interest in studies of comparative neurobiology owing to the highly unusual anoxia-tolerance displayed by their brains (Lutz and Nilsson, 1994).

Hypercapnia has long been known to cause vasodilation and increased blood flow in the brain of mammals (Siesjö, 1978). More recently, hypercapnia has also been found to cause increased CBF in turtles (Bickler, 1992; Davies, 1991, 1994). In mammals, a P_{CO_2} of 6.7–8.0 kPa causes a three- to sixfold increase in CBF (Iadecola and Zhang, 1994; Sandor *et al.* 1994) and, in turtles, exposure to hypercapnia (P_{CO_2}=6.0 kPa) was found to coincide with a doubling of CBF (Davies, 1991).

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Studies on the mammalian brain have indicated that a major part of the vasodilatory effect of moderate hypercapnia (P_{CO_2}=5.3–10.7 kPa) depends on the production of nitric oxide (NO), since the increase in CBF can be blocked by inhibitors of NOS (Niwa *et al.* 1993; Dirnagl *et al.* 1993; Buchanan and Phillis, 1993; Iadecola, 1992; Wang *et al.* 1992; Pellegrino *et al.* 1993; Iadecola and Zhang, 1994; Fabricius and Lauritzen, 1994). It has been suggested that mechanisms other than NO production are responsible for the elevation of CBF at very high blood P_{CO_2} (>13.3 kPa), because NOS inhibitors are then no longer able to attenuate cerebral vasodilation (Iadecola and Zhang, 1994).

In the present study, we have examined the blood flow velocity in venules on the cortical surface of the freshwater turtle (*T. scripta*) using epi-illumination microscopy *in vivo* in order to study the effect of L-NA injection on the CBF and to investigate whether the increased CBF reported in the hypercapnic turtle is mediated by the formation of NO. Blood pressure was also monitored, since blood flow to the brain is determined by two factors: blood pressure and cerebral vasodilation. Thus, an increase in CBF at a constant blood pressure indicates an underlying vasodilation. The blood pressure data were also used as an indicator of systemic vascular changes in response to NOS inhibition.

Materials and methods

Turtles [*Trachemys scripta* (Schoepff), mass 450–1000 g] were obtained from a turtle farm in Louisiana, USA. The experiments were carried out during November and December. Experimental procedures were approved by the Florida Atlantic University Institutional Animal Care and Use Committee.

CBF velocity and blood pressure were measured as described previously (Hylland *et al.* 1994, 1996). Briefly, the turtles were anaesthetized (50 mg kg^{-1} pentobarbitol intraperitoneally 2 h before experiments) and ventilated with air *via* endotracheal intubation. To allow the CBF observations, a 5 mm×5 mm area of the skull above the telencephalon was removed, and a small opening in the dura was made carefully. The cortical surface was kept moist using a turtle Ringer's solution (125 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 1.3 mmol l^{-1} $CaCl_2$, 1.2 mmol l^{-1} $MgSO_4$, pH 7.4). Experiments were performed at 20±1 °C. Turtles were catheterized for blood pressure measurement in the right subclavian artery. The blood pressure was recorded by connecting the catheter to a Gould Statham P23 Db pressure transducer and amplifying the signal with a Grass 7P1F low-level d.c. amplifier.

A Leitz Ortholux microscope with a video camera, a Leitz Ultropak epi-illuminator and a water-immersion objective (11×) were used to observe and measure CBF velocity (measured as erythrocyte velocity) in venules on the surface of the telencephalon essentially as described by Nilsson *et al.* (1994). Using a stopwatch, the time for single erythrocytes to travel a predetermined length of a blood vessel was recorded. Before each experiment, three venules that allowed observation of the same erythrocytes for at least 1.5 s at basal flow conditions were chosen (if more than three venules fulfilled this criterion, they were chosen randomly). Every 10 or 20 min, the velocity in each vessel was measured five times and an average calculated, hence minimising the effect of short-term variation and measurement error. The diameters of the vessels were measured on the video screen.

The turtles were exposed to two levels of environmental hypercapnia (P_{CO_2}=4.9 or 14.9 kPa) during 20 min periods by switching the air used to ventilate the turtles to gas mixtures containing 5 or 15 % CO_2, 21 % O_2 and 74 or 64 % N_2, respectively.

L-NA was obtained from Sigma Chemical Co. It was dissolved in the turtle Ringer (see above) and 2.5 ml was injected through the subclavian artery catheter (100 µmol kg^{-1}, final dose).

CBF velocities for each venule (µm s^{-1}) and systemic blood pressures were normalised to give percentage values. The average CBF velocity and blood pressure measured in each individual during the initial control period was set to 100 %. Thus, the average flow in the three venules from one individual was considered as one observation. Changes during hypercapnia or drug exposure were evaluated using analysis of variance (ANOVA) for repeated measures (P<0.05 was considered significant) followed by a *post-hoc* Dunnett's test for comparisons against the final pre-treatment value. Student's *t*-tests were used to compare the peak effects of hypercapnia at the two hypercapnia levels as well as to compare the peak effects of hypercapnia in the presence and absence of L-NA. Values are given as means ± S.E.M.

Results

Exposure to the lower level of hypercapnia (5 % CO_2 in the respiratory gas; P_{CO_2}=4.9 kPa) caused the CBF velocity to increase by 61.7±17.9 % (P<0.001), while systemic blood pressure remained at the basal level (Fig. 1). When the turtle was exposed to more severe hypercapnia (15 % CO_2 in the respiratory gas; P_{CO_2}=14.9 kPa), CBF velocity increased by 103.9±16.6 % (P<0.001) and systemic blood pressure increased by 30.2±13.0 % (P<0.05) (Fig. 2). Thus, exposure to a P_{CO_2} of 14.9 kPa appeared to caused an additional increase in CBF velocity compared with exposure to a P_{CO_2} of 4.9 kPa, although this difference was not statistically significant. No change was seen in the diameter of the blood vessels observed.

Injection of L-NA (100 µmol kg^{-1}) into the subclavian artery during ventilation of the turtle with air had a dramatic effect on both basal CBF velocity and blood pressure. There was a complete cessation in CBF for approximately 1 min after the injection, following which normal levels were regained within 15–20 min. The systemic blood pressure increased by 44.2±12.3 % after L-NA injection (mean ± S.E.M. from the experiments shown in Figs 1 and 2), and remained elevated for the remaining 2 h of the experiments. However, L-NA injection did not reduce the increase in CBF caused by hypercapnia (5 or 15 % CO_2 in respiratory air), CBF velocity increased by

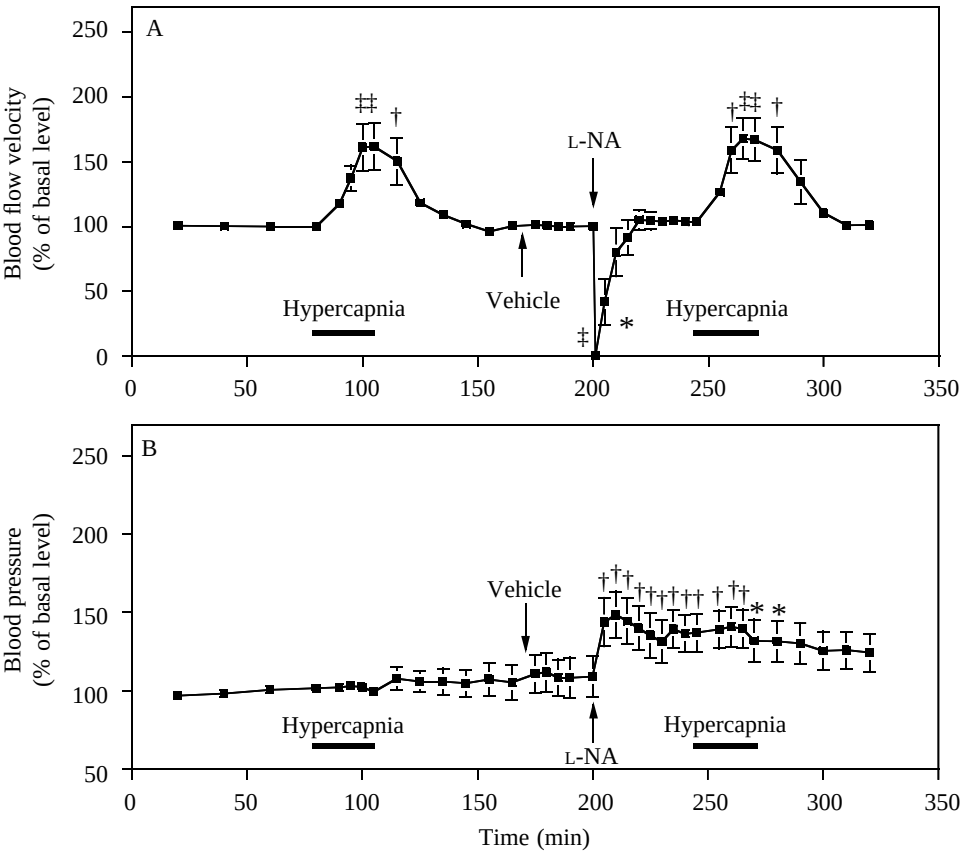

Fig. 1. Effects of hypercapnia (5 % CO_2 in the respiratory gas; P_{CO_2}=4.9 kPa) and injection of L-NA (100 µmol kg^{-1}) on (A) cerebral blood flow velocity measured in the telencephalon and (B) systemic blood pressure (% basal level; 100 %=2.74±0.14 kPa). Periods of hypercapnia are indicated by the horizontal bars. Injections of vehicle (turtle Ringer) or L-NA are indicated by arrows. Values are means ± S.E.M. from five animals. Significant differences from the final pre-treatment value: *P<0.05; †P<0.01; ‡P<0.001.

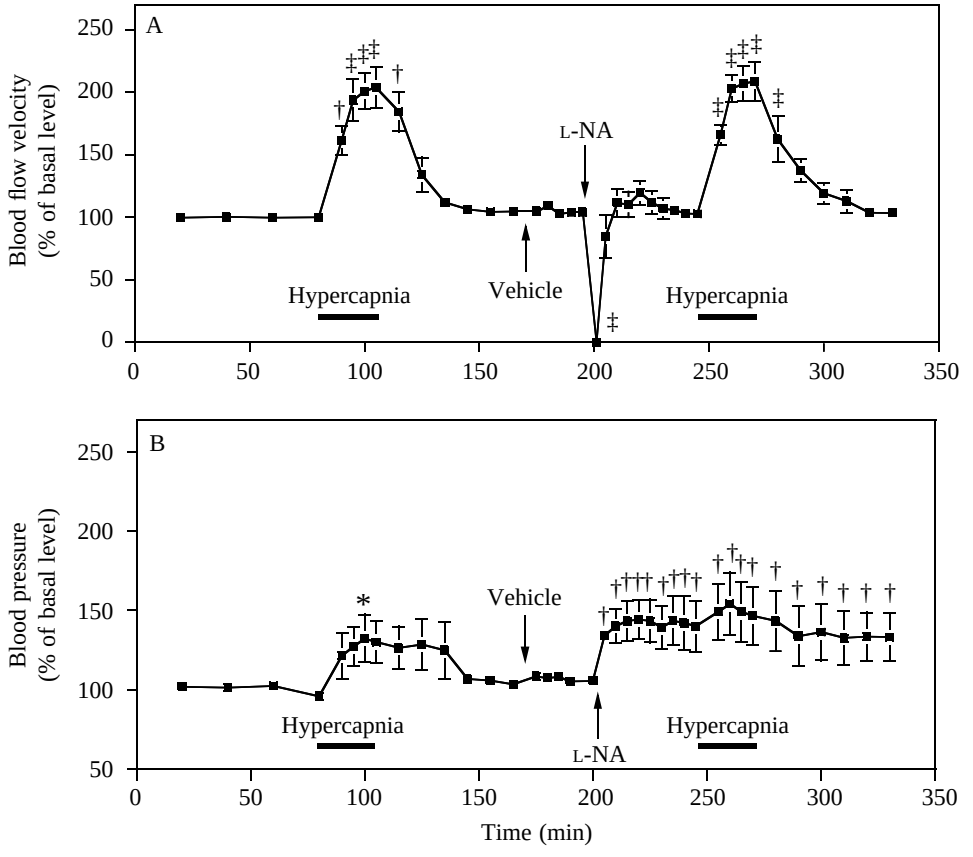

Fig. 2. Effects of hypercapnia (15 % CO_2 in the respiratory gas; P_{CO_2}=14.9 kPa) and injection of L-NA (100 µmol kg^{-1}) on (A) cerebral blood flow velocity measured on the telencephalon and (B) systemic blood pressure (% basal level; 100 %=2.75±0.19 kPa). Periods of hypercapnia are indicated by the horizontal bars. Injections of vehicle (turtle Ringer) or L-NA are indicated by arrows. Values are means ± S.E.M. from five animals. Significant differences from the final pre-treatment value: *P<0.05; †P<0.01; ‡P<0.001.

68.0±16.0 % at 5 % CO_2 (Fig. 1) and by 108.9±15.5 % at 15 % CO_2 (Fig. 2) following L-NA injection, values which were not significantly different from those recorded in the absence of L-NA. After L-NA injection, exposure to 15 % CO_2 caused no further significant increase in systemic blood pressure above the already elevated values (Fig. 2).

Discussion

Nitric oxide tonus

The present experiments indicate that there is a vasodilatory NO tonus that affects the cerebral circulation in the turtle. In this respect, the turtle brain appears to be similar to the mammalian brain, where NO has been found to be responsible for a constant vasodilatory tonus (Fernández *et al.* 1993; Macrae *et al.* 1993). Indeed, because a reduction to zero in turtle CBF was seen immediately after L-NA injection, this NO tonus seems to be profound. However, this circulatory stop was only temporary and CBF recovered completely after 15–20 min; therefore, compensatory mechanisms appear to be able to override the loss of NO tonus in the turtle brain. It is less likely that this reflected a recovery of NOS activity since systemic blood pressure remained elevated for several hours after L-NA injection. Moreover, the effects of L-NA injection persist for several days in mammals (Fernández *et al.* 1993). The persistently elevated systemic blood pressure indicates that there is also a NO tonus in tissues other than the brain and that this tonus is not compensated by non-NO mechanisms after L-NA injection. In mammals, systemic injections of NOS inhibitors also have a profound systemic vasopressor effect (e.g. Macrae *et al.* 1993; Fabricius and Lauritzen, 1994; Iadecola and Zhang, 1994) that has been interpreted as evidence for a NO tonus affecting the systemic circulation.

Role of nitric oxide in hypercapnia

Our results confirm previous studies showing that hypercapnia causes an increased CBF in turtles. At the lower level of hypercapnia used (5 % CO_2; P_{CO_2}=4.9 kPa in inhaled air), a 62 % increase in CBF velocity was measured, although systemic blood pressure remained stable; at the higher level of hypercapnia (15 % CO_2; P_{CO_2}=14.9 kPa), CBF velocity increased by 104 %, i.e. to a greater extent than the systemic blood pressure (30 %). This indicates that the increased flow velocity was caused by cerebral vasodilation.

The increase in CBF velocity measured during exposure to hypercapnia was not blocked by L-NA injection. Preliminary experiments using topical (brain-surface superfusion) administration of L-NA also had no effect on the hypercapnia-induced increase in brain CBF (results not shown). Thus, in contrast to the situation in most mammals (e.g. rodents and cats), the cerebral vasodilation caused by hypercapnia in the turtle apparently does not rely on the formation of NO. However, even in mammals, NO is not always involved in cerebral hypercapnic vasodilation. NO does not appear to be implicated in the vasodilatory effect of hypercapnia in the piglet brain (Meng *et al.* 1995*b*), and there is also a NO-independent component in the CBF response to hypercapnia in rats (Iadecola and Zhang, 1994). The reason why NO does not appear to participate in cerebral hypercapnic vasodilation in turtles is, at present, not clear, although it may be phylogenetic. The involvement of NO in cerebral hypercapnic vasodilation could be a derived amniote feature: we have also been unable to find any effect of NOS inhibitors on CBF during hypercapnia in fish (rainbow trout *Oncorhynchus mykiss*) (V. Söderström and G. E. Nilsson, unpublished results).

In piglets, prostanoids appear to be involved in the increase in CBF that occurs during hypercapnia (Hsu *et al.* 1995; Wagerle *et al.* 1995). Thus, prostanoids are possible candidates for mediating the hyperaemia reported here in the hypercapnic turtle brain.

Methodology

Blood flow rate (volume/time) is determined by two factors: blood vessel diameter and blood velocity. Since no change in vessel diameter was noted, the present measurements of CBF velocity reflected the flow rate in the brain area studied. CBF velocity was only measured at the dorsal surface of the telencephalon, thus, we can not exclude the possibility that regional differences exist in CBF responses to hypercapnia or L-NA injection. For example, redistribution of CBF due to vasodilation in other brain areas could theoretically cause a reduction in CBF velocity in the telencephalon, and *vice versa*. Only veins and venules (no arterial vessels) were seen on the dorsal surface of the telencephalon. Thus, arterial vasodilation/vasoconstriction could not be observed. However, this method has revealed changes in CBF in response to anoxia (Hylland *et al.* 1994) or hypercapnia (present study) that are in agreement with those reported using other methods, such as hydrogen clearance from the blood (Bickler, 1992) or radioactive microspheres (Davies, 1991, 1994). Indeed, in comparision with those methods, *in vivo* microscopy has the advantage of allowing continuous monitoring of CBF velocity.

L-NA administration

In the present study, the NOS inhibitor L-NA was injected into the systemic circulation rather than being applied topically to the brain surface. The latter method was used by Hylland *et al.* (1996), who found that stimulation of CBF velocity by ACh was completely blocked after superfusion of the brain surface with L-NAME. In the present study, injecting L-NA into the circulation caused a temporary stop in cerebral circulation whereas blood pressure increased, indicating a strong vasoconstriction of the cerebral vessels or of the arteries supplying the brain with blood. By contrast, in the previous study (Hylland *et al.* 1996) and in preliminary experiments performed in the present study (results not shown), topical administration (brain surface superfusion) of L-NA (100 μmol l^{-1}, in turtle Ringer) or L-NAME (10 μmol l^{-1}, in turtle Ringer) was without effect on basal CBF velocity. Topical application of 100 μmol l^{-1} L-NA has been found to cause a 93 % inhibition of NOS activity in the cerebral cortex

of piglets (Meng *et al.* 1995*b*). However, in rats, L-NA has been found to lower basal CBF when administered systemically but not when administered topically (Fabricius and Lauritsen, 1994). Other studies on rats have shown that basal CBF can be reduced by topical administration of L-NA (Iadecola and Xu, 1994; Irikura *et al.* 1994*b*).

The dose of L-NA ($100\,\mu mol\,kg^{-1}$) injected in the present study is likely to have produced a profound and long-lasting NOS inhibition, since a persistent increase in blood pressure (indicating widespread systemic vasoconstriction) was measured. In mammals, similar doses of L-NA ($45–136\,\mu mol\,kg^{-1}$ intravenously or intraperitoneally) have been found to reduce significantly the increases in CBF or cerebral levels of cyclic GMP (the ubiquitous second messenger of NO) that occur in response to ACh, neural excitation and hypercapnia (Raszkiewicz *et al.* 1992; Meng *et al.* 1995*a*; Fabricius and Lauritzen, 1994; Irikura *et al.* 1994*a*; Ngai *et al.* 1995).

In conclusion, the drastic temporary drop in CBF and the sustained increase in systemic blood pressure measured in the turtle after a systemic injection of L-NA indicate the presence of a NO-dependent vasodilatory tonus both in the brain and in other parts of the body. However, in contrast to many mammals, the mechanisms underlying the hypercapnic stimulation of CBF in turtle appear to be independent of NO production.

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