Effect of chronic inhibition of nitric oxide synthase on ocular blood flow and glucose metabolism in the rat

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Abstract

***Aims*—To investigate the effects of chronic administration of nitric oxide synthase inhibition on ocular blood flow and metabolic demand in the rat and to compare these effects with changes in the cerebral and peripheral circulation.**

***Methods*—Male Sprague-Dawley rats were injected with the nitric oxide synthase inhibitor L-NAME (75 mg/kg ip), either on a single occasion only or once daily for 10 consecutive days. Controls were injected with saline. Regional blood flow and glucose metabolism were measured from tissue samples, using [^{14}C]-iodoantipyrine and [^{14}C]-2-deoxyglucose respectively, 1 hour after either acute L-NAME injection or 1 hour after the last injection of the chronic treatment protocol.**

***Results*—Mean arterial pressure was significantly increased (+31%) following the acute injection (indicating peripheral vasoconstriction) and this effect was enhanced (+50%) following chronic treatment. In both the ocular and cerebral circulation, blood flow was decreased following acute treatment (−48% and −43% respectively). However, while this response was totally attenuated in the cerebral circulation following chronic L-NAME treatment (−4%), the ocular circulation remained responsive (−57%). Metabolic demand in brain and eye tissue, as reflected in the accumulation of 2-deoxyglucose, was unaffected by either acute or chronic treatment with L-NAME.**

***Conclusion*—Homeostatic mechanisms appear to be activated in the cerebral circulation which re-establish flow metabolism homeostasis, and the effect of L-NAME on cerebral blood flow is attenuated following repeated exposure. This process does not seem to happen in the ocular circulation and, thus, the ocular vasculature appears to behave more like those blood vessels which determine total peripheral resistance than the cerebral circulation. It remains to be seen whether the sustained decrease in blood flow in the eye is sufficient to compromise ocular function and render the eye susceptible to damage from chronic L-NAME induced oligaemia.**

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Experiments using isolated intra- and extraocular arterial vessels indicate that endothelium derived nitric oxide (NO) plays a prominent role in the regulation of vascular tone in the ocular circulation.[1–3] Inhibition of nitric oxide synthase (NOS) by the arginine analogue N^{G}-nitro-L-arginine methyl ester (L-NAME) has been shown to significantly reduce choroidal blood flow following a single intravenous dose.[4] These previous studies have examined short term, acute effects of NOS inhibition, and little is known of the longer term changes in the ophthalmic circulation following chronic repetitive injection of L-NAME. This is of potential interest as vascular endothelial dysfunction may contribute to ocular disease processes. In the present study, therefore, we evaluated the in vivo effects of acute and chronic administration of L-NAME on the ocular circulation in the rat, and contrasted these effects with those in the cerebral and peripheral circulations. Given the coupling that exists between blood flow and metabolic demand in the optic nerve head,[5] a semiquantitative assessment of glucose metabolism was also made in ocular and cerebral tissues from parallel groups of rats similarly treated.

Methods

Male Sprague-Dawley rats (250–275 g at the outset) were injected intraperitoneally (ip) with the nitric oxide synthase inhibitor L-NAME (75 mg/kg; n=10) or saline (n=10) once daily for 10 consecutive days (chronic treatment group). A further group of rats (n=10) was injected with L-NAME only once (acute treatment group). A single ip injection of L-NAME at this dose has been shown to produce a near complete inhibition (93%) of cerebral NOS activity,[6] and to produce vascular effects which last for up to 18 hours after a single injection.[7] Chronic L-NAME treatment, following the protocol described here, produces a level of NOS inhibition (96%) which is only slightly greater than the acute effects.[6]

On the day of the experiment the rats were anaesthetised with halothane in a mixture of oxygen (30%) and nitrous oxide (70%) and cannulae were inserted into both femoral arteries (for the measurement of arterial pressure and sampling of arterial blood), and both femoral veins (for the injection of drugs and radiolabelled tracers). All surgery was

performed by fully trained and experienced personnel and complied with local ethical codes and British Home Office regulations. All experiments adhered to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. A loose fitting plaster cast was applied around the lower abdomen and pelvis, with care being taken to ensure that respiratory movement of the thorax was not compromised. Thus restrained, and supported on blocks, the rats were allowed to recover from anaesthesia for at least 2 hours before further manipulation. All measurements were performed on fully conscious animals. Arterial pressure and rectal temperature were monitored continuously, and blood gases were measured before acute drug treatment and again immediately before the initiation of the measurement procedures for ocular and cerebral blood flow and glucose metabolism.

Blood flow and glucose utilisation in the eye and brain were measured 1 hour after the last injection of the chronic L-NAME treatment, or at the same time after the acute injection, using the [^{14}C]-iodoantipyrine method derived by Sakurada *et al* [8] and a semiquantitative approach derived from the [^{14}C]-2-deoxyglucose method of Sokoloff *et al* [9] respectively, with modifications for tissue sampling as described previously.[10 11]

To measure blood flow, [^{14}C]-iodoantipyrine (50 μCi/rat in 0.6 ml saline) was infused intravenously at a constantly accelerating rate over 45 seconds. During the infusion period, blood was allowed to flow freely from one of the arterial cannulae and samples were collected intermittently onto preweighed filter discs which were reweighed after the experiment to determine sample size. Subsequently, the timing of each sample was determined precisely from an audiocassette recording of the experiment and corrected for the flow characteristics of the cannula tubing. At 45 seconds, the animals were killed by the rapid intravenous injection of sodium pentobarbitone. Both eyes were rapidly dissected intact from the skull, and placed onto filter paper on an ice cooled glass petri dish. The ocular muscles were removed and the eye sliced open at the level of the equator. Tissues from the anterior segment and the vitreous humour were removed, and aqueous matter was absorbed onto the filter paper. All remaining tissues from the posterior ocular segment were placed into preweighed scintillation vials, weighed, digested in tissue solubiliser (Soluene, Packard), and processed for liquid scintillation analysis to measure tissue tracer concentrations. The brains were also dissected intact from the skull and samples of frontoparietal neocortical tissue were processed in the same way as the ocular tissue. All tissues were dissected within 2 to 3 minutes of death, and the weights of both ocular and brain tissue samples were similar (around 20 mg). The filter discs holding the blood samples were placed into scintillation vials and 0.4 ml hydrogen peroxide added to bleach the pigment before liquid scintillation analysis. Blood flow was calculated from [^{14}C] concentrations in blood samples taken during the experiments and from the accumulated tracer in ocular and brain tissue samples measured post mortem. The operational equation for the technique[6] is derived from the Kety–Schmidt[12] modification of the Fick principle for measurement of blood flow in any organ of the body. In these calculations we used a value of 0.8 for the partition coefficient for both ocular and cerebral tissue. Although this value has been derived empirically for brain[6] the small volumes of ocular tissue available make it difficult to do likewise for the eye. However, we have found that with the flow rates found in the eye, the error introduced to the measurement of flow by using an inappropriate partition coefficient in the range of plus or minus 25% in the operational equation is around 3%.

In parallel groups of similarly treated animals, the semiquantitative assessment of glucose metabolism was initiated with a pulse injection of [^{14}C]-2-deoxyglucose (50 μCi/rat in 0.8 ml saline). Over the subsequent 45 minutes, timed arterial samples were collected, immediately centrifuged, and aliquots of plasma taken for the determination of plasma glucose levels and [^{14}C] concentrations by semiautomated glucose oxidase assay (Beckman, UK) and liquid scintillation analysis, respectively. At the end of the measurement period, the rats were sacrificed and the ocular and cerebral tissue prepared as described above. An index of glucose metabolism was derived by dividing the total [^{14}C] measured post mortem in tissue (corrected for sample weight in g) by [^{14}C] in plasma integrated over the experimental period and expressed in arbitrary units per 100 g tissue. As there were no significant differences in plasma glucose levels between groups (see Results), no correction for this factor was included in the calculation.

All data are presented as mean (SEM). Statistical analysis was performed using analysis of variance with post hoc Scheffé test to allow multiple pairwise comparisons. Acceptable levels of significance were set at $p<0.05$ for all statistical analyses.

Table 1 Physiological variables in rats treated with saline or N^G-nitro-L-arginine methyl ester (L-NAME)

	Control	*Acute L-NAME*	*Chronic L-NAME*
Rectal temperature (°C)	37.1 (0.2)	36.4 (1.4)	36.8 (1.0)
Plasma glucose (g/l)	1.64 (0.06)	1.62 (0.08)	1.60 (0.07)
pH	7.43 (0.01)	7.42 (0.05)	7.44 (0.02)
P_{CO_2} (mm Hg)	40.9 (1.1)	41.0 (4.0)	39.1 (1.4)
P_{O_2} (mm Hg)	93.8 (3.0)	91.7 (5.1)	93.4 (3.8)
Heart rate (beats/min)	360 (11)	352 (8)	350 (7)
MAP (mm Hg)	135 (2)	177 (3)*	202 (6)*†

Data are presented as mean (SEM) for each of the three treatment groups (n=10 in each group). *Significantly different from control ($p<0.05$); †Significantly different from acute treatment group.
MAP=mean arterial pressure.

Results

Acute L-NAME treatment induced a marked hypertension in rats previously treated with saline. In these rats, mean arterial pressure (MAP) rose from 130 (SEM 2) mm Hg before the injection of L-NAME (similar to controls at the time of blood flow experiments, Table 1) to 177 (3) mm Hg 1 hour afterwards (Table 1).

Table 2 Blood flow and deoxyglucose uptake in ocular and cerebral tissues of rats treated with either saline or N^G-nitro-L-arginine methyl ester (L-NAME)

	Ocular tissue			*Cerebrocortical tissue*		
	Control	*Acute L-NAME*	*Chronic L-NAME*	*Control*	*Acute L-NAME*	*Chronic L-NAME*
Blood flow (ml/100 g/min)	67 (3)	35 (4)*	29 (3)*	140 (10)	80 (6)*	134 (8)†
Deoxyglucose uptake (per 100 g)	19.9 (3.6)	20.4 (3.8)	20.2 (3.5)	44.8 (2.8)	45.1 (3.4)	46.2 (3.1)
Flow/metabolism ratio	3.367	1.716	1.436	3.125	1.774	2.900

Data are presented as mean (SEM) for each of the three treatment groups (n=5 in each group for both the blood flow and glucose utilisation measurements).
*Significantly different from control ($p<0.005$); †significantly different from appropriate acute treatment group.

In contrast, 1 hour after the last injection in the chronic L-NAME pretreatment protocol, MAP was not only significantly higher than in the control group (+50%) but was also higher than that found 1 hour after an acute injection of L-NAME (+14%) (Table 1). With the exception of MAP, there were no significant differences in any of the other physiological variables, arterial pH, P_{CO_2}, P_{O_2}, plasma glucose, or rectal temperature, either between treatment groups (Table 1), or when analysed within groups before and after acute treatments.

Blood flow in ocular tissue was decreased from 61 (3) ml/100 g/min in the control group to 35 (4) ml/100 g/min when measured 1 hour after a single acute injection of L-NAME, and similar reductions in flow were also found in neocortical brain tissue (reduced from 140 (10) to 80 (6) ml/100 g/min) (Table 2). These immediate vasoconstrictor consequences of a single acute exposure to L-NAME in the eye and brain (decrease in tissue blood flow) were similar to those in the peripheral circulation (as reflected by changes in MAP).

There were also similarities in the effects of chronic treatment in the peripheral and ocular circulations. Blood flow in the eye was reduced 1 hour after the last chronic injection (−57%) (Table 2) indicating a vasoconstrictor response similar to that found in the periphery. Like the effects seen in the peripheral circulation, the 10 day chronic pretreatment with L-NAME resulted in an enhanced ocular response (−17% lower than after an acute injection), although the difference was not significant (Table 2). In marked contrast, the effect of chronic L-NAME treatment upon cerebral blood flow was completely different from that found in either the peripheral or ocular circulations. Neocortical blood flow was not significantly different from control 1 hour after the final injection of L-NAME on day 10, but was significantly higher than after a single acute injection (Table 2).

There were no significant differences in deoxyglucose uptake in cerebral tissue with either acute or chronic L-NAME treatments (Table 2). The ratio of blood flow to metabolic index in brain indicate that, while there is an uncoupling following acute treatment (ratio = 1.774, compared with control ratio of 3.125) with blood flow being less than would normally be predicted on the basis of underlying metabolism, the ratio returns largely to normal in the chronic treatment group (ratio = 2.900). There were, similarly, no significant effects of L-NAME upon metabolic demand in ocular tissue (Table 2), and in both treatment groups the flow metabolism ratios (1.716 and 1.436) were lower than in the control group (3.367) (Table 2).

Discussion

In this study, a single injection of L-NAME in previously naive (saline treated) rats resulted in vasoconstriction in all vascular beds. In the peripheral circulation, the constrictor effect was manifest in a 40% increase in MAP, while in the ocular and cerebral circulations significant vasoconstrictor induced decreases in regional blood flow were measured. Assuming no increase in either intraocular or intracranial pressure, the reduction in blood flow in these two vascular beds in the face of such a marked increase in systemic blood pressure represents a considerable increase in vascular resistance of between 130 and 150%.

The peripheral and cerebrovascular effects of acute L-NAME treatment observed in this study are similar to those reported previously.[13] Moreover, the decrease in ocular blood flow noted here (−48%) following ip L-NAME is of the same order as that found when the constituent parts of the uveal tract were analysed separately in anaesthetised Beagle dogs.[4] In that study, a single intravenous injection of L-NAME resulted in reductions in blood flow of 40, 40, and 48% in the choroid, ciliary body, and iris respectively.[4] More recently, Mann and colleagues, using laser Doppler techniques to measure the short term effects of NOS inhibition on choroidal blood flow in cats, suggest that the choroid contains vasodilatory cholinergic receptors which induce the release of NO from L-arginine.[14] Taken together, the results of these studies suggest that the tonic synthesis and release of nitric oxide provide dilator tone to the vascular bed of the eye and thus play a major role in the determination of resting ocular blood flow in different species.

Repeated exposure to a nitric oxide synthase inhibitor produces incremental decreases in NO production which to a large extent are irreversible.[15] This accounts for the pronounced rise in systemic blood pressure of 50% over baseline observed in this study in rats which had been injected daily with L-NAME for 10 days before the measurements of blood flow (when compared with a 30% increase in mean arterial blood pressure following a single acute injection). Because blood pressure could be measured both before and after the final injection on day 10 in this study, it was evident that a sustained hypertension had been

established and the final injection had no further effect upon MAP. This would be consistent with NOS activity being already maximally inhibited by the chronic treatment. Blood flow in the ocular circulation after chronic treatment was similarly decreased, suggesting a vasoconstrictor response similar to that in the periphery. However, the effects of L-NAME upon cerebral blood flow were almost completely attenuated by the chronic treatment which had returned to near normal baseline values.

The exact mechanisms by which dilator tone is re-established, or the constriction induced by L-NAME is attenuated in the cerebral circulation is not immediately clear. What is clear, however, is that the low levels of blood flow induced by acute L-NAME treatment would not sustain normal function in the brain or, indeed, in the eye. It is possible that the imperative for fuelling the metabolic demands of brain tissue provides, over time, a sufficiently strong stimulus to overcome the constrictor effects of NOS inhibition.

The effects in the brain may be interpreted as the re-establishment (by some mechanism as yet unknown) of flow metabolism homeostasis to protect against the deleterious effects of sustained oligaemia. Similar mechanisms do not appear to be available in the ocular circulation, and the flow metabolism ratio is markedly reduced from control following both acute and chronic L-NAME treatments. Whether this relative oligaemia with respect to tissue metabolism is potentially harmful to ocular tissue remains to be determined. Further work looking at regional blood flow and glucose use with quantitative autoradiography may differentiate between the effects of L-NAME in the choroid and retina and thus help to further elucidate the role of NOS in the ocular circulation and metabolism.

Conclusion

NO is found ubiquitously throughout the cardiovascular system, including the vessels of the eye and particularly in the choroid.[16 17] However, while the acute constrictor effects of NOS inhibition appears to be the same in all vascular beds, the effects of chronic exposure are quite different, despite the fact that the vasculature of the eye and brain share many of the same characteristics. The complexities of NO action which underlie these differences have yet to be determined, but these results caution against the assumption of generalised responses to NOS inhibition between vascular beds. Moreover, should NOS dysfunction underlie the vascular pathology of some ocular disease states, it would appear that homeostatic mechanisms cannot protect the eye against the consequent oligaemia and, as a result, the eye may be particularly vulnerable to damage in a way that even the brain is not.

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