Possible role for nitric oxide releasing nerves in the regulation of ocular blood flow in the rat

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Abstract

Aim—To investigate the role of nitrenergic nerves in the regulation of ocular blood flow.

Methods—Conscious, lightly restrained rats were treated with either the neuronal nitric oxide synthase inhibitor 7-nitroindazole (7-NI), or the non-selective inhibitor, NG-nitro-L-arginine methyl ester (L-NAME), and ocular blood flow was measured *ex vivo* from tissue samples, using the fully quantitative [14C]-iodoantipyrine technique.

Results—In the peripheral circulation, L-NAME produced an increase in arterial blood pressure (+22%) while 7-NI had no effect. In contrast, both 7-NI and L-NAME produced significant decreases in ocular blood flow (–31% and –59% respectively). The ocular vascular resistance calculated from ocular blood flow and mean arterial blood pressure increased by 29% following 7-NI, but by 130% following L-NAME.

Conclusions—Nitric oxide releasing neurons may play an important contributory role in regulating ocular blood flow.

(Br J Ophthalmol 1998;82:1199–1202)

There is evidence that ocular blood vessels are innervated by nitric oxide (NO) producing neurons^{1–3} and studies of ocular blood vessels *in vitro* suggest a functional role for these nerves in determining vascular calibre in the eye.^{4–6} Cerebral vessels are similarly endowed^{7–8} and it has become apparent that NO released from neurons innervating small cerebral resistance vessels may regulate blood flow by providing a tonic dilator influence.⁹ There is some *in vivo* evidence that NO releasing nerves can similarly influence blood flow in the avian eye,¹⁰ but these authors have also reported that anaesthesia can fundamentally alter the cardiovascular response to pharmacological inhibition of neuronal nitric oxide synthase (nNOS),¹¹ and others have identified species differences in efficacy and time course of effects.^{12–13} The purpose of the present study was to investigate the effects upon ocular blood flow of a single intraperitoneal injection of the nNOS inhibitor 7-nitroindazole (7-NI), which *in vivo* is relatively selective for the neuronal isoform of the enzyme,¹⁴ and to compare these with the effects of the arginine analogue, NG-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor.

Materials and methods

ANIMAL PREPARATION

A total of 19 male Sprague-Dawley rats (250–300 g) were used in this study. All experiments adhered to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research, and complied with British Home Office regulations. Protocols did not involve any direct manipulation of the eye. On the day of the experiment the rats were anaesthetised and prepared surgically as described in detail previously.⁹ Following surgery, the rats were allowed to recover from the effects of the anaesthesia for at least 2 hours before any further experimental manipulation, and all subsequent measurements were performed on fully conscious animals. Mean arterial blood pressure (MABP) was monitored continuously, and blood gases were measured immediately before the initiation of the measurement procedures for ocular blood flow.

DRUG TREATMENT

7-Nitroindazole (Lancaster Synthesis Ltd, Morecambe, Lancs) was suspended in sesame oil (12.5 mg/ml), taken into solution with mild sonication and kept warm until use. At the end of the recovery period, rats ($n = 5$) were injected with 7-NI at a dose of 25 mg/kg via an indwelling intraperitoneal cannula 30 minutes before the measurement of ocular blood flow. In the rat, maximal nNOS inhibition is manifest within 30 minutes following an intraperitoneal injection of 7-NI, and the $t_{1/2}$ for the response is of the order of 4 hours.¹⁵ Control rats were injected with oil alone ($n = 4$). A parallel group of rats were injected intravenously with either L-NAME (30 mg/kg; $n = 5$; Sigma Chemicals) or an equal volume of saline (0.75 ml; $n = 5$) 20 minutes before the measurement of ocular blood flow.

MEASUREMENT OF OCULAR BLOOD FLOW

Ocular blood flow was measured using the [14C]-iodoantipyrine method derived by Sakurada *et al*¹⁶ and described in detail previously¹⁷ with modifications for tissue sampling.¹⁸ Briefly, [14C]-iodoantipyrine (50 μ Ci/rat in 0.6 ml saline) was injected intravenously over 45 seconds via a femoral cannula, and timed femoral arterial blood samples were collected intermittently. At 45 seconds, the animals were killed by decapitation. Both eyes were rapidly dissected intact from the skull, and placed onto filter paper on an ice cooled dish. The ocular muscles were removed and the eye sliced open at the level of the equator. Tissues from the anterior segments, and the vitreous humour, were removed, and aqueous matter was absorbed onto the filter paper. All

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Accepted for publication
24 March 1998

Table 1 Effects of 7-NI and L-NAME upon physiological measurements

Physiological variables	Treatment			
	Oil	7-NI	Saline	L-NAME
Ocular blood flow (ml/100 g/min)	85 (2)	59 (7)*	84 (8)	35 (4)*
Ocular vascular resistance (mm Hg/ml/100 g/min)	1.54 (0.06)	2.46 (0.28)*	1.50 (0.05)	4.40 (0.31)*
PCO ₂ (mm Hg)	39.6 (3.0)	42.1 (1.5)	42.0 (3.4)	41.2 (2.1)
PO ₂ (mm Hg)	94.1 (3.0)	92.4 (4.1)	96.7 (5.1)	96.9 (5.2)
pH	7.40 (0.03)	7.39 (0.03)	7.43 (0.02)	7.41 (0.04)
MABP (mm Hg)	128 (7)	133 (7)	125 (5)	156 (3)*
Heart rate (beats/min)	442 (43)	317 (18)*	440 (40)	276 (41)*
No	4	5	5	5

All data are presented as mean (SD) *Significantly different from appropriate control group, $p < 0.05$, Scheffé test.

remaining tissues from the posterior ocular segment, together with blood samples, were processed for liquid scintillation analysis to measure tissue and blood tracer concentrations.

Blood flow was calculated from [14C] concentrations in blood samples taken during the experiments and from the accumulated tracer in ocular tissue samples. The operational equation for the technique¹⁶ is derived from the Kety-Schmidt¹⁹ 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 ocular tissue. Although this value has been derived empirically for brain¹⁶ the small volumes of ocular tissue available make it difficult to do likewise for the eye. However, we have found previously²⁰ that within the range of 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% is only around 3%. An index of ocular vascular resistance was calculated for each individual by dividing mean arterial blood pressure by the value derived experimentally for ocular blood flow, and is reported as ml/100 g/min/mm Hg.

STATISTICAL ANALYSIS

Data are presented as mean (SD). Statistical analyses of the results from physiological measurements and ocular blood flow data were performed by analysis of variance followed by a post hoc Scheffé test to allow multiple pairwise comparisons. Acceptable levels of significance were set at $p < 0.05$.

Results

Neither 7-NI nor L-NAME had any significant effect upon rectal temperature, arterial PCO₂, PO₂, pH, or plasma glucose levels when compared with vehicle injected controls (Table 1). L-NAME did, however, increase MABP significantly (+22%), and this was accompanied by a decrease in heart rate (-38%). In keeping with previous reports^{12,13} 7-NI at this dose had no significant effect upon MABP at any time throughout the study, but there was a relatively rapid and significant decrease in heart rate (-28%) which persisted throughout the experimental period (Table 1).

Despite the dissimilarities in the peripheral response, both drugs produced a significant decrease in ocular blood flow, although this was more marked with L-NAME treatment (-58%; ranging from 30 to 39 ml/100 g/min)

than following 7-NI (-31%; ranging from 52 to 67 ml/100 g/min) (Table 1). A very large increase in the calculated ocular vascular resistance following L-NAME (+193%) reflected the greater effect of L-NAME in reducing ocular blood flow in the face of increased perfusion pressure. This was considerably greater than in the 7-NI treated group (+60%) where blood pressure was unaffected by the treatment (Table 1).

Discussion

The peripheral vascular effects of acute L-NAME treatment observed in this study are similar to those reported previously in this animal model.^{21,22} Moreover, the decrease in ocular blood flow noted here (-59%) following L-NAME (intravenously) is both qualitatively and quantitatively similar to our previous data²⁰ where L-NAME was injected intraperitoneally, and is of the same order as that found when the constituent parts of the uveal tract were analysed separately in anaesthetised dogs.²³ 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.²³ More recently, Mann and colleagues, using laser Doppler techniques to measure the short term effects of NOS inhibition on choroidal blood flow in cats, found that the choroid contains vasodilatory cholinergic receptors which, when activated, induce the release of NO from L-arginine.²⁴ Taken together, the results of these studies suggest that the tonic synthesis and release of nitric oxide provides dilator tone to the vascular bed of the eye and thus plays a major role in the determination of resting ocular blood flow in different species.

NG substituted arginine analogues are equally effective in inhibiting all isoforms of NOS, and these previous studies do little to identify the relative importance of endothelial and neuronal sources of NO in the eye. However, a new class of indazole based NOS inhibitors has recently been developed, of which 7-NI is currently the most extensively characterised, which are selective for the neuronal isoform of the enzyme *in vivo*.^{12,13} In the rat, 7-NI inhibits neuronal NOS *in vitro* with a potency (IC₅₀ = 0.9 (SD 0.1) μ M) equal to that of L-NAME (IC₅₀ = 0.9 (0.08) μ M),¹² but there are differences between species in this regard. In the mouse, 7-NI is almost twice as potent as in the rat (IC₅₀ = 0.47 (0.01) μ M) while L-NAME in comparison is equipotent in both species.¹² Moreover, in the mouse the

effect of 7-NI is much shorter lived, and is totally dissipated by 120 minutes following an intraperitoneal injection.¹³

There have to date been no studies on the effects of this drug on human subjects, but 7-NI has been found to attenuate stimulus induced increases in choroid blood flow in the anaesthetised pigeon.¹⁰ From these studies it was concluded that while constitutive, endothelium derived NO is involved in the control of resting ocular vascular tone, NO released from neurons plays a role in rapid blood flow responses associated with stimulus induced activation. This could certainly explain the considerable difference in blood flow responses in the eye which we found between L-NAME and 7-NI with a generally quiescent, but not anaesthetised, ocular system. Following the administration of 7-NI in vivo, neuronal NOS activity is inhibited by between 30% and 40%¹⁴ but the lack of any pressor response, even at doses of up to 50 mg/kg¹³ suggests that 7-NI is indeed selective for the neuronal isoform of the NOS enzyme while having little or no effect upon the endothelial isoform. This is confirmed in the present study where 7-NI produced reductions in ocular blood flow with no effect upon MABP, while in parallel experiments L-NAME induced both peripheral and ocular vasoconstriction. While the decrease in ocular blood flow in response to L-NAME will be the sum of both endothelial and neuronal NOS inhibition in the eye, it is noteworthy that the effect of selective inhibition of neuronal NOS alone is approximately half that of the L-NAME response. Moreover, while ocular vascular resistance was increased by 60% following 7-NI, the effects were considerably more marked in L-NAME treated rats (+193%), reflecting a much greater vasoconstrictor response. Although it might be tempting to speculate on the relative importance of endothelial and neuronal NO production in regulating ocular blood flow, full dose-response curves to both agents would be necessary before such a conclusion would be acceptable.

Recent reports of an endothelium and nitric oxide synthase independent relaxation of vascular smooth muscle in response to 7-NI in vitro,²⁵ question the selectivity of indazole derivatives in inhibiting neuronal NOS. Further in vitro studies have revealed that 7-NI binds to the haem group of the NOS molecule where it competes directly with L-arginine.²⁶ If 7-NI has a wider capacity to interact with a variety of iron dependent systems (as do other NOS inhibitors),²⁷ this could be a possible source of vasoactivity which is independent of NOS inhibition. Whether these effects might also become manifest in vivo remains unclear however. In the majority of studies in which 7-NI has been used no effects upon blood pressure have been observed, suggesting that such effects may only become manifest in very limited physiological conditions, one study clearly identifies an endothelial effect of 7-NI in vivo.¹¹ While this observation may thus be at odds with the rest of the literature, including

work by the same authors,¹⁰ anomalous effects of high doses of 7-NI as used in that study have been reported previously by us,⁹ and the issue remains to be resolved.

Although our studies show that neuronally derived NO provides a tonic dilator influence in the ocular circulation, there is evidence for perivascular innervation arising both from within the eye,^{1,3} and from autonomic ganglia²⁸ and it is not possible to determine the relative contributions of these intrinsic and extrinsic neuronal sources to the phenomenon described here.

In conclusion, the results of this study clearly indicate that the vascular sequelae of 7-NI treatment in the ocular circulation of the conscious rat are qualitatively similar to those observed following NG substituted arginine analogues. These results are consistent with the hypothesis that NO, synthesised and released from neuronal (as opposed to endothelial) sources in the eye, has the potential to regulate ocular blood flow. The source of perivascular NO releasing neurons involved in this process deserves closer scrutiny.

This work was funded by the Wellcome Trust.

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