Nitric oxide synthase inhibitors protect rat retina against ischemic injury

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Abstract Elevation of the ocular pressure in the anterior chamber of the rat eye caused major ischemic damage, manifested as changes in retinal morphology. The two most affected structures were the inner plexiform layer, which decreased in thickness by 90%, and the number of ganglion cells, which decreased by 80%. Pretreatment of the animals with N^{ω}-nitro-L-arginine, a nitric oxide (NOS) inhibitor, almost completely abolished the ischemic damage. Administration of aminoguanidine, a NOS inhibitor selective for the inducible enzyme, partially abolished the ischemic damage. Moreover, administration of the NOS inhibitors 1 h after ischemia, also protected the retina from damage, suggesting that similarly acting drugs could be used clinically to limit ischemic injury in humans. We conclude that NOS, and therefore NO, may be involved in the mechanism of ischemic injury to the retina.

Key words: Rat retina; Ischemic injury; Nitric oxide synthase; Neuroprotection

1. Introduction

The involvement of NO in the ethiology of neurotoxicity, particularly that mediated by excitatory amino acids, is controversial. A number of laboratories reported that inhibition of NO formation had protective effects on neural cells in vivo [1–3]. Other reports suggest that inhibition of NOS exacerbates ischemic injury, probably due to the effect of a decrease in NO on vascular beds [4–6]. Despite this controversy (for recent review see [7]), it is generally accepted that excessive generation of NO may contribute, or even directly cause damage to tissues after ischemic insult.

The purpose of the present study was to investigate whether NO has a major role in the ethiology of ischemic damage in the rat retina, a relatively simple and well-established model of neural tissue. Our results suggest that NO is indeed involved in a major way in the mechanism of ischemic damage. Moreover, the ability to partially prevent the damage by post-ischemic treatment of experimental animals suggests that NOS inhibitors may be of use in limiting ischemic injury in humans.

2. Materials and methods

2.1. Induction of retinal ischemia

Adult male albino rats, Sprague–Dawley strain, 300–400 g, were used throughout. The animals were housed at 12/12 h light/dark cycle, under controlled temperature (24°C) and with food and water ad libitum. The animals were anaesthesized with Equithesine (3 ml/kg) [8] and kept on a heated pad for the duration of anaesthesia with frequent monitoring of rectal temperature (37.0–37.2°C). Ischemia was achieved and the animals were treated essentially as described by Takahashi et al. [9]. Briefly, we instilled sterile saline into the anterior chamber of one eye at 140 cm H_2O pressure for 75 min, while the second eye served as non-ischemic control. Following ischemia, the animals were allowed to regain consciousness and kept for 7 days in a standard vivarium environment. On day 7, the animals were sacrificed by an injection of sodium penthotal and eyes were enucleated for histological examination.

2.2. NOS inhibitors administration

N^{ω}-Nitro-L-arginine (NNA) or aminoguanidine (AG) were dissolved in water and titrated with HCl to insure complete dissolution of the drugs. The final pH of the solutions was 7.0–7.5. Animals were injected intraperitoneally with either vehicle alone (vehicle controls) or with 5, 25 or 50 mg/kg body weight of NNA and 25 or 50 mg/kg AG 1 h before the onset or 1 h after the termination of ischemia.

2.3. Histology and morphometric studies

The enucleated eyes were fixed in picric acid 2%, formaldehyde 40% in 95% ethanol for 48 h. Following fixation, the preparations were dehydrated by successive passages in 70–100% ethanol and an additional passage in 100% methanol. The preparation was then treated with 2 and 4% celloidine in 100% methanol, with 100% methyl salicylate and embedded in paraffin (Paraplast Plus, Sigma). The posterior part of the eye was sectioned saggitally at 7 μm thickness through the optic nerve, mounted and stained with hematoxylline/eosine. For the estimation of the thickness of the inner retinal layer (IRL, between the internal limiting membrane and the interface of the outer plexiform and the outer nuclear layer) and the inner plexiform layer (IPL), measurements were performed with a calibrated reticle at ×400 magnification at four locations: adjacent to the optic disk (on both sides) and at the equator (on both sides). To estimate the number of retinal ganglion cells (RGCs), all cells from the equator to the optic disk (on each side) were counted.

2.4. Statistics

Four sections of each eye were used for measurements. 2–6 animals were used in each treatment group. Since there were virtually no differences in the measurements of layer thicknesses in the four locations, the results were pooled from all the animals that were treated by the same protocol. Results are presented as mean ± S.E. where n is the number of measurements performed and N denotes the number of eyes examined for each condition. Student's t-test was used to estimate the significance of the results. Differences between any two conditions were considered significant at $P < 0.05$.

2.5. Materials

NNA and AG were purchased from Sigma. All the other chemicals were of analytical grade.

3. Results

Elevation of the ocular pressure in the anterior chamber of the eye caused ischemic damage to retinal tissue, as could be documented by morphometric evaluation of the histological eye preparations. The representative qualitative picture of the changes induced by ischemia in rat retina and the effects of NOS inhibitors are shown in Fig. 1. A 75 min ischemic episode resulted in a major decrease of the thickness of IRL 7 days after the episode (Fig. 1B). Quantitation of the effects of ischemia revealed that the thickness of IRL decreased from 110 ± 2 μm

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in untreated controls (injected with vehicle alone, $n = 20$, $N = 5$) to 49 ± 5 μm in ischemic eyes of animals injected with vehicle alone (ischemia, $n = 20$, $N = 5$, $P < 0.001$, see Fig. 2A). Most of the damage could be attributed to a dramatic decrease in the thickness of the IPL. In the same experiment, this structure decreased from 49 ± 2 μm ($n = 20$, $N = 5$) in untreated controls to 5 ± 2 μm ($n = 20$, $N = 5$, $P < 0.001$) in ischemic eyes, i.e. by approximately 90% (Fig. 2B). In many cases the IPL virtually disappeared after ischemia (see Fig. 1B). We have also observed ischemic damage to the inner nuclear layer, manifested as disorganization of the cells.

The IPL is a non-cellular structure. We have, therefore, also estimated the number of RGCs, neural cells that do not regenerate after injury in the same experiment. In untreated controls, the number of RGCs in the segment measured was 148 ± 2 ($n = 40$, $N = 5$). Ischemia resulted in a major (80%) decrease in the number of RGCs, to 29 ± 2 ($n = 40$, $N = 5$, $P < 0.001$, Fig. 3).

Pretreatment of the animals with a non-selective NOS inhibitor, NNA, 1 h before ischemia resulted in a significant decrease in both indices of ischemic damage ($P < 0.001$). At the optimal dose of NNA (25 mg/kg), the IRL was 105 ± 7 μm (97% of untreated controls, see Fig. 2A), while the IPL was 43 ± 3 μm (88% of untreated controls, Fig. 2B), both values not significantly different from untreated controls ($P < 0.05$). Similarly, the treatment with NNA protected the RGCs from ischemic

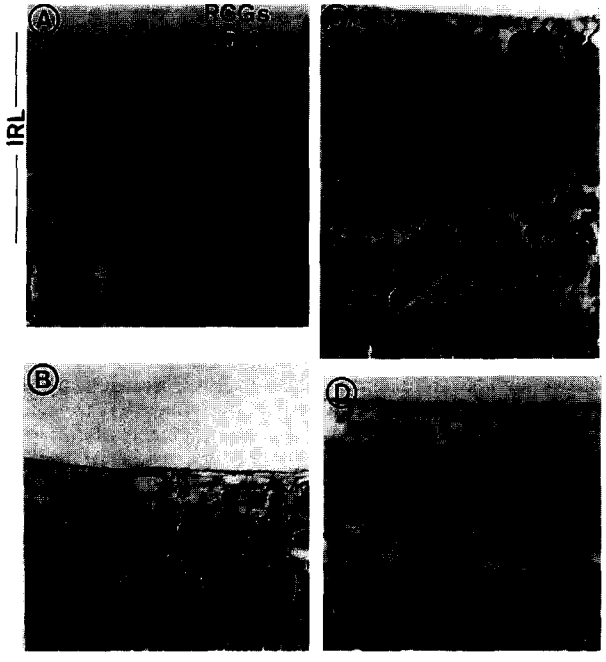

Fig. 1. Morphology of the IRL under different experimental conditions. (A) Micrograph of a section of a rat retina in control eye taken from an animal injected with the vehicle. (B) Ischemic retina from an animal injected with vehicle. Note the almost complete disappearance of the inner retinal layer (IPL), few and atypical ganglion cells (RGCs) and disorganized inner retinal layer (INL). (C) Ischemic retina from an animal injected with 25 mg/kg NNA 1 h before ischemia. Note the increase in the thickness of IPL and of INL. RGCs are clearly preserved. (D) Ischemic retina from an animal injected with 25 mg/kg AG 1 h before ischemia. In this eye, AG partially prevented the damage to IPL and preserved the number of RGCs. Note: the markings denoting the different retinal layers and ganglion cells are shown for control retina (panel A) only.

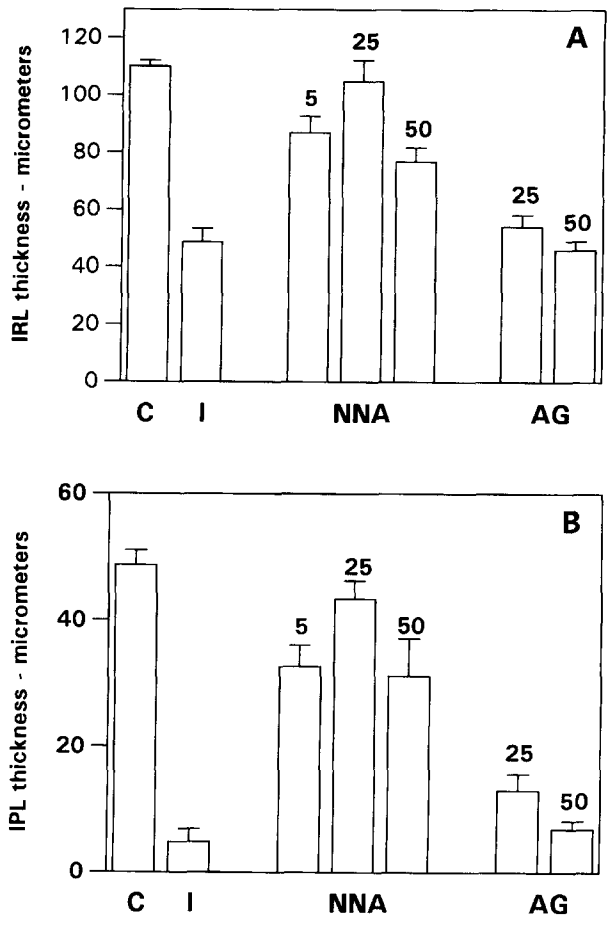

Fig. 2. Prevention of morphological damage to retina by early administration of NNA or AG. Panel A, IRL; panel B, IPL. Drugs were injected 1 h before the induction of ischemia. The dose of each drug (in mg/kg) is given above the columns. C, untreated, vehicle-injected controls ($n = 20$, $N = 5$); I, ischemic eyes in vehicle-injected animals ($n = 20$, $N = 5$). Ischemic eyes from NNA- or AG-injected animals: NNA 5 mg/kg ($n = 8$, $N = 2$); NNA 25 mg/kg ($n = 20$, $N = 5$); NNA 50 mg/kg ($n = 24$, $N = 6$), AG 25 mg/kg ($n = 20$, $N = 5$); AG 50 mg/kg ($n = 16$, $N = 4$).

death, yielding 135 ± 4 cells in the segment that was measured (91% of untreated controls, $P < 0.001$, Fig. 3). A lower (5 mg/kg) or a larger dose (50 mg/kg) of NNA had a reduced neuroprotective effect. In many sections, a thickening of the IPL and INL was observed, suggesting that the combined effect of ischemia and NNA affected the morphology of the IRL (see Fig. 1C).

To assess the role of inducible NOS in ischemic injury, we used the more selective NOS inhibitor, AG [10]. Administration of 25 mg/kg AG resulted in a variable protection of the retina against the ischemic insult. In a number of eyes, a significant prevention of ischemic damage to the IPL could be observed (see Fig. 1D). When the entire experiment was analyzed, a much more modest protection was seen. The thickness of the IRL was 54 ± 4 μm (49% of untreated controls, $n = 16$, $N = 4$, Fig. 2A), which was not statistically different from ischemic eyes ($P > 0.05$, see above). Similarly, the IPL thickness was 13 ± 3 μm ($n = 16$, $N = 4$; 27% of untreated controls, Fig. 2B). This was significantly different from the ischemic injury alone (5 ± 2 μm, $P < 0.02$), suggesting that AG offers a limited pro-

tection against damage to the IPL. The number of RGCs reflected the most significant effect of AG ($P < 0.001$ when compared to ischemic values). We observed 98 ± 4 cells in the measured segment ($n = 32$, $N = 4$), i.e. 66% of untreated controls. A greater dose of AG (50 mg/kg) was even less effective (see Fig. 3).

In order to evaluate the possible therapeutic effects of drugs in clinical situations, it was important to test whether the NOS inhibitors used here were effective when administered after ischemia. We have tested, therefore, the effects of administration of either NNA or AG 1 h after the termination of ischemia. The ischemic damage, both when the morphology of the IRL and the number of RGCs were assessed, was significantly reduced at lower doses of the drugs, especially when 5 mg/kg NNA was tested (Figs. 4AB and Fig. 5). The morphological indices were not different from untreated controls and the number of RGCs (133 ± 4) was significantly ($P < 0.005$) higher than in ischemic eyes.

4. Discussion

Nitric oxide, a novel and ubiquitous messenger, has been implicated in neuronal injury following anoxia, hypoxia, head trauma and a number of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. A vast body of conflicting evidence as to the contribution of NO to neuronal cell death exists in scientific literature [7]. This is further complicated by the existence of constitutive and inducible forms of NOS and the various effects of the enzyme's activation on different tissues. Part of the controversy can be explained in terms of opposing effects of NO on neuronal cells and on the CNS haemodynamics.

In the present report, we investigated the possible role of NO in the ischemic injury to rat retina in vivo, a convenient model of neuronal tissue. The retina may serve as a preferential model of neuronal tissue ischemic injury for several reasons: (1) ischemic injury can be produced locally by a relatively simple procedure; (2) the morphological evaluation of injury is straightforward; (3) although the retina, and the eye in general,

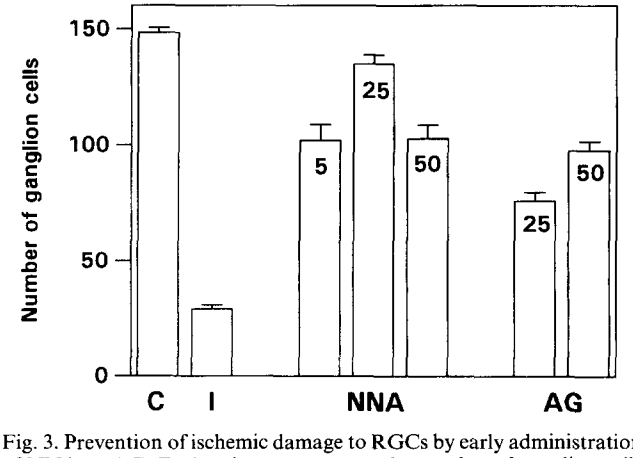

Fig. 3. Prevention of ischemic damage to RGCs by early administration of NNA or AG. Each column represents the number of ganglion cells in a part of retina between the equator and the optic disk. C, untreated, vehicle-injected controls ($n = 40$, $N = 5$); I, ischemic eyes in vehicle-injected animals ($n = 40$, $N = 5$). Ischemic eyes from NNA- or AG-injected animals: NNA 5 mg/kg ($n = 16$, $N = 2$); NNA 25 mg/kg ($n = 40$, $N = 5$); NNA 50 mg/kg ($n = 48$, $N = 6$), AG 25 mg/kg ($n = 40$, $N = 5$); AG 50 mg/kg ($n = 32$, $N = 4$).

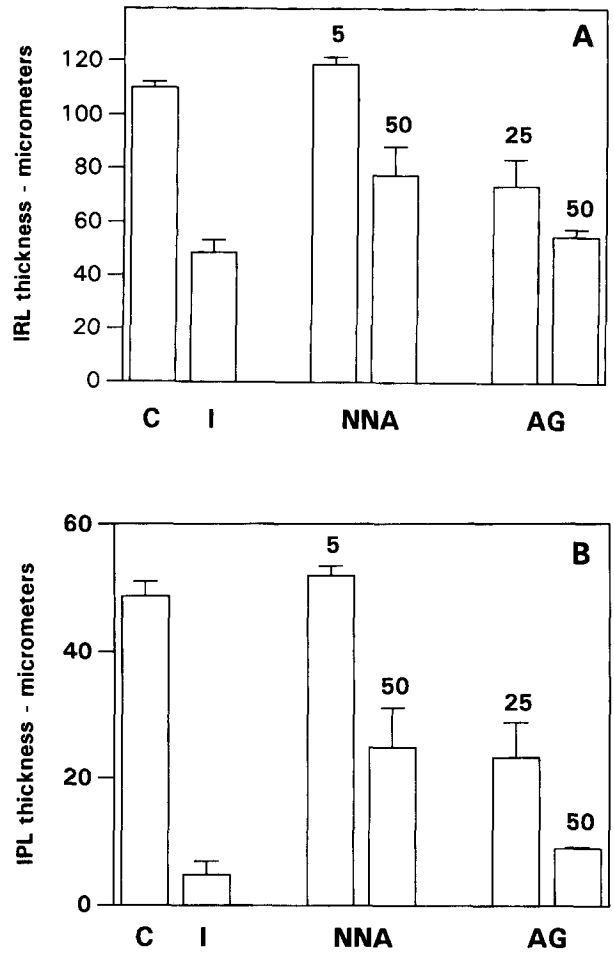

Fig. 4. Protection against morphological retinal damage by late administration of NNA or AG. Panel A, IRL; panel B, IPL. Drugs were injected 1 h post-ischemia. For other experimental conditions and abbreviations, see Fig. 2. For NNA (5 or 50 mg/kg) treatment, $n = 12$, $N = 3$. For AG treatment, $n = 8$, $N = 2$ for 25 mg/kg dose; $n = 20$, $N = 5$ for 50 mg/kg dose.

are not cellularly homogenous and simple structures, their complexity is much less than that of the central nervous system; (4) ischemic injury is implicated in a number of pathological states, such as central retinal artery occlusion, glaucoma, diabetic retinopathy, etc. This makes the study of retinal ischemic injury important from the clinical point of view.

Our results can be summarized as follows: systemic administration of NOS inhibitors significantly protected the retina from ischemic injury. Although the inner nuclear layer displayed morphological signs of damage, the thickness of the non-cellular IPL and the number of RGCs were the most affected. The latter index is particularly significant, since the damage to RGCs is reversible. The protection was most dramatic with relatively low doses of the non-selective NOS inhibitor, NNA. A high dose of NNA (50 mg/kg) was less effective than lower doses, a result which is compatible with numerous reports attesting to the deleterious effects of reduction of NO on neuronal survival, possibly via a decrease in local perfusion [4,5]. Aminoguanidine, a selective inhibitor of the inducible NOS [10], was much less effective. We propose that NO is involved in mediating the ischemic damage in the retina, with the possibility that the constitutive NOS is more important than

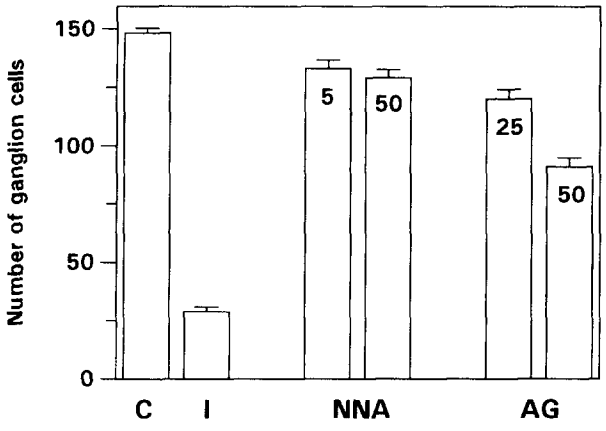

Fig. 5. Protection against ischemic damage to RGCs by late administration of NNA or AG. Drugs were injected 1 h post-ischemia. For other experimental conditions and abbreviations, see Fig. 3. For NNA (5 or 50 mg/kg) treatment, $n = 24$, $N = 3$. For AG treatment, $n = 16$, $N = 2$ for 25 mg/kg dose; $n = 40$, $N = 5$ for 50 mg/kg dose.

the inducible form. Although indirect effects of a systemic administration of NOS inhibitors cannot be excluded, it has been shown that NOS is present in the retina [11] and we have measured significant concentrations of NO in the vitrous (Geyer, unpublished). Furthermore, a recent report on prevention of retinal ischemia by intravitreal administration of NNA [12] suggests that local NO production may be responsible for the ischemic damage.

Despite the impressive prevention of ischemic damage by NNA, there were clear alterations in the morphology of the retina in many preparations. These included an increase in the thickness of the IPL and the INL. Although we have no explanation for these changes, they are compatible with either edema or hypertrophy of the affected structures.

Recently, a number of laboratories published conflicting reports on the function of NO in retinal ischemia in different animal models. Protective effects of NOS inhibitors [12,13], no effect [14] and deleterious effect [15] were reported. It is important, however, to emphasize that these reports utilized electroretinography as an index of ischemic injury, rather than morphological parameters. It is noteworthy that NOS inhibitors partially protected also against light-induced photoreceptors degeneration [16].

Our preliminary finding that NOS inhibitors afforded significant protection against retinal injury when administered post-ischemia suggest that NOS inhibitors, particularly those with low systemic toxicity, may be promising agents to limit injury in acute pathological situations, such as retinal artery occlusion or chronic conditions, such as glaucoma. Indeed, the morphological picture of human retina resulting from these pathological states very closely resembles that of the rat experimental model used here [17]. Following central retinal artery occlusion, there is a dramatic thinning of inner retinal layers and a pronounced loss of ganglion cells. In glaucoma, the predominant feature is a major loss of ganglion cells. Hence, although the present findings are preliminary, they warrant further research, particularly in view of the almost complete protection against injury by low doses of NNA.

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