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Short communication

Inhibition of NMDA receptors and nitric oxide synthase reduces ischemic injury of the retina

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Abstract

This study was performed to examine the roles of body temperature, NMDA receptors and nitric oxide (NO) synthase in post-ischemic retinal injury in rats. Cell loss in the ganglion cell layer and thinning of the inner plexiform layer were observed 7 days after ischemia. Cell loss in the ganglion cell layer but not thinning of the inner plexiform layer was reduced by hypothermia during ischemia. Intravenous injection of dizocilpine (MK-801) or N^{ω}-nitro-L-arginine methyl ester (L-NAME) prior to ischemia ameliorated retinal injury. These results suggest that activation of NO synthase following NMDA receptor stimulation is involved in ischemia-induced retinal injury.

Keywords: Ischemia; Neuronal death; Nitric oxide (NO) synthase; *N*-Methyl-D-aspartate (NMDA); Retina

1. Introduction

Glutamate has been postulated to play an important role in the neurodegeneration observed in hypoxic-ischemic injury in the central nervous system (Choi, 1988; Meldrum and Garthwaite, 1990). The observation that activation of NMDA receptors generates nitric oxide (NO) raised the possibility of the involvement of NO in glutamate neurotoxicity (Garthwaite et al., 1988). This hypothesis was supported by evidence that inhibition of NO synthesis prevented glutamate neurotoxicity in cultured neurons (Dawson et al., 1992; Tamura et al., 1992; Lipton et al., 1994) and reduced ischemia-induced brain damage in vivo, although some of the early in vivo studies were controversial (Dalkara and Moskowitz, 1994). This hypothesis is further supported by the reduced post-ischemic brain damage of neuronal NO synthase knockout mice (Huang et al., 1994).

Glutamate also acts as a major excitatory neurotransmitter in the vertebrate retina and has long been known to exert a neurotoxic effect on different populations of neurons in the inner retina (Lucas and Newhouse, 1957). We have previously demonstrated that NO is a key factor leading to neuronal death triggered by NMDA receptor stimulation by glutamate in cultured rat retinal neurons (Kashii et al., 1994; Kashii et al., 1996). Moreover, our previous study demonstrated that N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase, protected retinal neurons against NMDA-induced neurotoxicity in vivo (Morizane et al., 1997). The involvement of NO in ischemia-induced retinal damage has been suggested by Geyer et al. (1995). They demonstrated that inhibition of NO synthase by N^{ω}-nitro-L-arginine almost completely abolished the ischemic damage of the rat retina. These initial observations led us to examine the effects of NMDA receptor inhibitor, MK-801, and L-NAME since a large number of studies have attempted to elucidate the role of NO in ischemic brain damage using NO synthase inhibitors. We also examined the effects of hypothermia on ischemia-induced damage of the retina in parallel to that of MK-801 under body temperature maintenance at 37°C, as hypothermia is known to counteract the neurodegeneration induced by brain ischemia and has been suggested to be

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involved in the neuroprotective action of low-dose MK-801 (for reviews, Ginsberg, 1993; Barone et al., 1997).

2. Materials and methods

2.1. Animals

Male albino Sprague–Dawley rats weighing 170–230 g were used. The animals were housed under a 12 h light/dark cycle with free access to food and water.

2.2. Induction of transient retinal ischemia

Under sodium pentobarbital anesthesia (50 mg/kg i.p.; Nembutal, Abbott Laboratories, North Chicago, USA), the animals' rectal temperature was maintained at 37°C, or as otherwise noted, using an electrical heating pad with a temperature probe (Fine Science Tools, Vancouver, Canada).

The anterior chamber of the right eye, of which the pupil had been dilated with 1% atropine sulfate (Nacalai Tesque, Kyoto, Japan), was cannulated with a 27-gauge needle connected to a bottle of irrigating solution for ophthalmic operation (BSS PLUS dilution buffer, Alcon, Fort Worth, USA). Retinal ischemia was induced by raising intraocular pressure to 130 mmHg for 45 min, or as otherwise noted, by lifting the container. After ischemic insult, the rectal temperature was kept at 37°C in all the cases using a thermostat until animals recovered from anesthesia. The left eyes were kept intact.

Drugs used were as follows: MK-801 (RBI, Natick, USA), L-NAME (Sigma, St. Louis, USA) and N^{ω}-nitro-D-arginine methyl ester (D-NAME; Sigma). The drugs dissolved in saline were intravenously administered 30 min prior to retinal ischemia.

2.3. Histological evaluation

The animals were killed and both eyes were enucleated 7 days after ischemic insult. Horizontal sections through

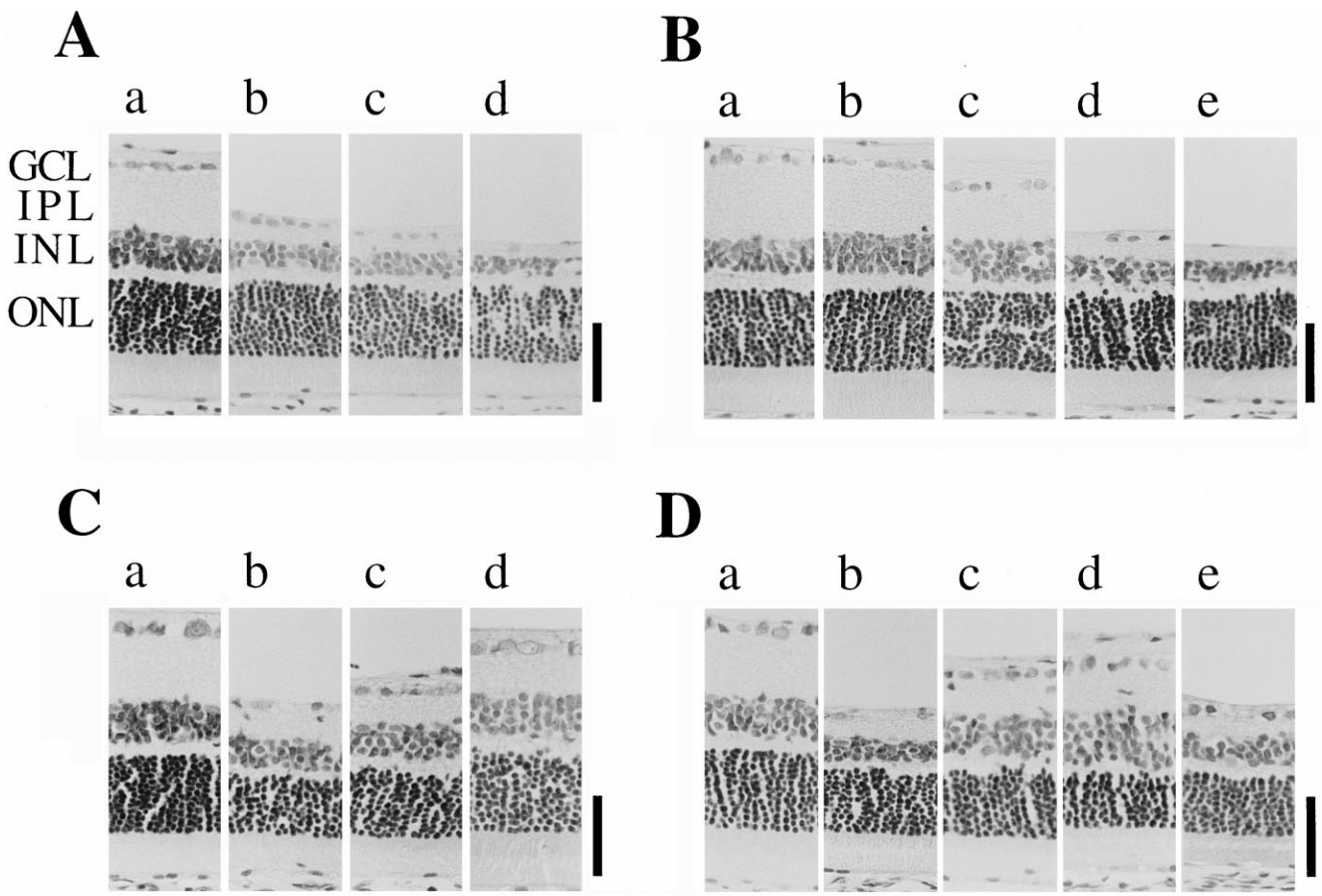

Fig. 1. Microscopic appearances of transverse sections of rat retinas 7 days after ischemia. (A) An intact retina (a), those following 60 min of ischemia with rectal temperature at 33°C (b), 35°C (c) and 37°C (d). (B) Retinas after ischemia for various durations with rectal temperature at 37°C. Control (a), 15, 30, 45 and 60 min (b, c, d and e, respectively). (C,D) Effects of intravenous drug treatments 30 min before ischemia upon retinal degeneration after 45-min ischemia at 37°C. Intact retinas (a) and those after ischemia (b) from vehicle-treated animals. Retinas after ischemia from animals treated with MK-801 (3 and 10 mg/kg; Cc and Cd, respectively), L-NAME (10 and 30 mg/kg; Dc and Dd, respectively) and D-NAME (30 mg/kg; De). GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer. Scale bar = 50 μm.

the optic disk of the eye (paraffin sections 5 μm thick, stained with hematoxylin and eosin) were subjected to morphometry as described elsewhere (Morizane et al., 1997). Briefly, the cell density (cells/mm) in the ganglion cell layer within 1 mm of the optic disk, thicknesses of the inner plexiform layer (in μm), the inner nuclear layer (in μm) and the outer nuclear layer (in μm) about 0.5 mm from the optic disk were measured for each eye. These parameters for the right eyes were normalized respectively to those of the intact left eyes and are shown as percentages.

The data are expressed as means ± S.E.M. Due to unequal S.D. values among the groups, we employed nonparametric tests: Mann–Whitney's *U*-test for comparisons between the effects of enantiomers and Yonckheere trend test for examining time- or dose-dependent shifts of the mean values.

3. Results

3.1. Relationship between body temperature during retinal ischemia and subsequent histological damage

Fig. 1A shows an example of the dependence of ischemia-induced retinal damage on body temperature. The

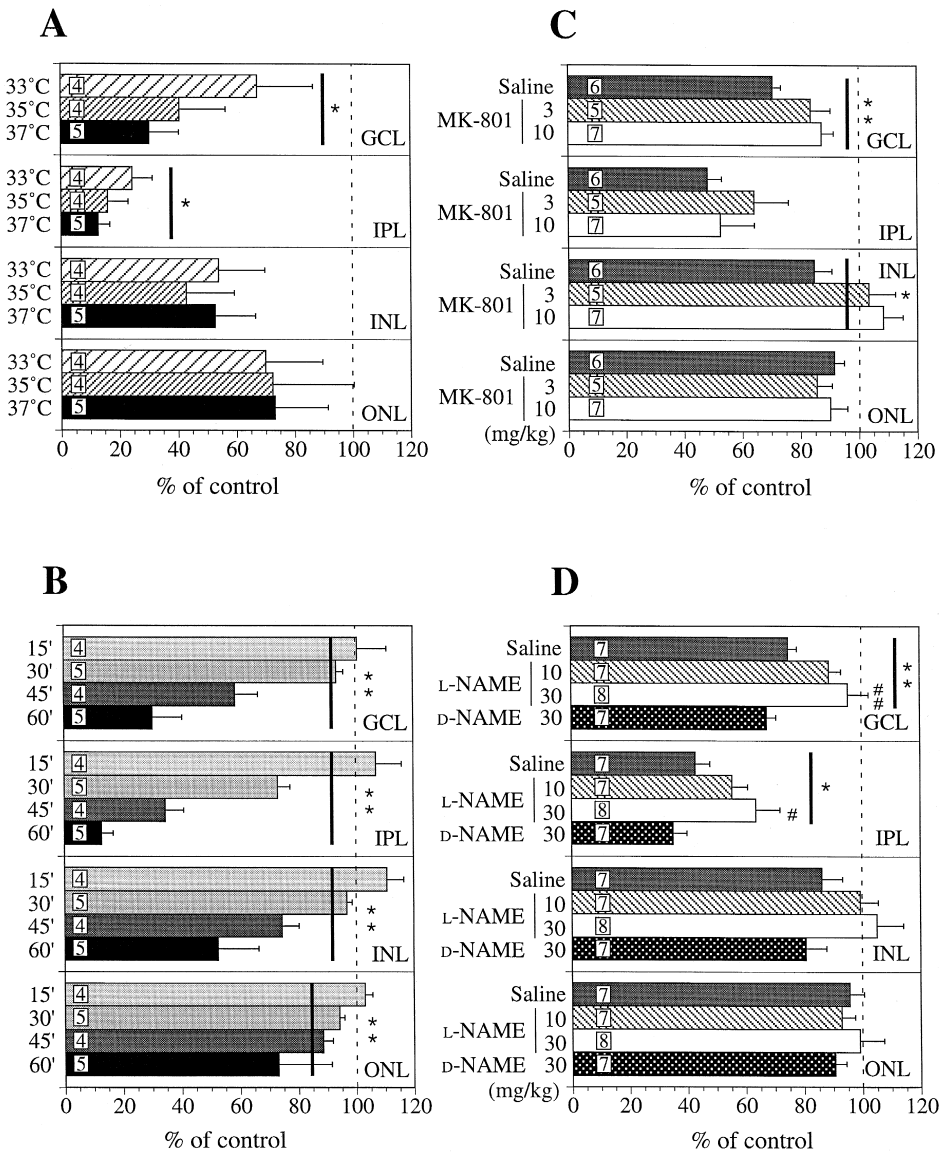

Fig. 2. Morphometric evaluation of the retina 7 days after ischemia. The following 4 parameters for the right eye, which underwent ischemia, were normalized to those for the left, intact eye and are shown as percentages: linear cell density (cells/mm) in the GCL (ganglion cell layer); thicknesses of the IPL (inner plexiform layer), the INL (inner nuclear layer) and the ONL (outer nuclear layer). The ciphers in bars show the numbers of animals used. (A) shows the effect of hypothermia on ischemia-induced retinal damage. The rectal temperatures during ischemia are shown for each bar. (B) shows injury of the retina after ischemia for various durations at 37°C. 15 min–60 min: duration of ischemia. (C,D) show the effects of MK-801 and L-NAME, respectively, on retinal morphology after 45 min of ischemia at 37°C. $*P < 0.05$, $**P < 0.01$, among the groups indicated (Yonckheere test). $\#P < 0.05$, $\#\#P < 0.01$ vs. D-NAME (Mann–Whitney's *U*-test).

duration of the retinal ischemia was 60 min, and the rectal temperature was 33°C, 35°C or 37°C during ischemia. Marked reduction of the cell density of the ganglion cell layer was observed when the body temperature was maintained at 35 and 37°C but not at 33°C. In contrast, marked reduction of the thickness of the inner plexiform layer was observed at all body temperatures examined. The degree of reduction of the thickness of the inner nuclear layer and the outer nuclear layer was less than that of the inner plexiform layer. As shown in Fig. 2A, reduction of the cell density of the ganglion cell layer and thickness of the inner plexiform layer was dependent on the body temperature during ischemia.

Fig. 1B and Fig. 2B show the dependency of retinal damage on the length of the period of ischemia. There were no obvious changes in cell density of the ganglion cell layer, thickness of the inner plexiform layer, inner nuclear layer or outer nuclear layer after 15-min ischemia. Significant and time-dependent damage of the retina was observed after ischemia for longer than 30 min. Marked reductions in the cell density of the ganglion cell layer, and thickness of the inner plexiform layer and inner nuclear layer were induced by ischemia for longer than 45 min. No reduction of the thickness of the outer nuclear layer was observed when the period of ischemia did not exceed 45 min. As damage of the outer nuclear layer was elicited by the ischemia for longer than 60 min, effects of the drugs were evaluated with 45-min ischemia in the following studies.

3.2. Effects of MK-801 and L-NAME on ischemia-induced retinal damage

Fig. 1C shows an example of the effect of MK-801 at 3 and 10 mg/kg on retinal damage 7 days after 45-min ischemia. The ischemia-induced damage of the inner retina was reduced by intravenous injection of MK-801 prior to ischemic insult. As summarized in Fig. 2C, significant reductions of ischemia-induced damage were observed with decreasing cell density of the ganglion cell layer and thickness of the inner nuclear layer, although no significant effect on thickness of the inner plexiform layer was detected. MK-801 did not affect thickness of the outer nuclear layer of control and ischemic retinas.

Fig. 1D and Fig. 2D show the effects of L-NAME (10 and 30 mg/kg) and D-NAME (30 mg/kg), the inactive enantiomer, on ischemia-induced retinal injury. L-NAME significantly inhibited reduction of cell density of the ganglion cell layer and thickness of the inner plexiform layer in a dose-dependent manner. Significant recovery of the ischemic damage was observed with L-NAME at 30 mg/kg. L-NAME ameliorated reduction of thickness of the inner nuclear layer, although the recovery was not statistically significant. L-NAME did not affect the thickness of the outer nuclear layer of control or ischemic retinas. In contrast to L-NAME, D-NAME at 30 mg/kg did not affect ischemia-induced retinal damage.

4. Discussion

The present study demonstrated that delayed neuronal injury of the retina following transient ischemia was reduced by the inhibition of either NMDA receptors or NO synthase. Ischemia-induced cell loss in the ganglion cell layer was markedly reduced by lowering body temperature to 33°C, suggesting that the process of neurodegeneration after retinal ischemia is dependent on temperature. The rescue of the ganglion cell layer cells by hypothermia is consistent with the observations in brain ischemia (for reviews, Ginsberg, 1993; Barone et al., 1997). We have also examined the relationship between the length of ischemia and retinal damage. The results indicated that 60-min ischemia caused not only neurodegeneration in the inner retinal layer but also damage to photoreceptor cells in the outer nuclear layer while ischemia for less than 45 min mainly caused neurodegeneration in the inner retinal layer. Clinical electroretinographical studies (for reviews, Berninger and Arden, 1988; Kothe et al., 1989) and autopsy reports (e.g., Foos, 1976) suggested that the inner retinal neurons are more vulnerable than the outer retinal cells such as photoreceptor cells in ischemic diseases of the retina. Thus, we employed 45-min ischemia inducing selective destruction of the inner retina for the protection assays.

Inhibition of NMDA receptors by MK-801 (3–10 mg/kg) significantly ameliorated ischemic damage when the drug was systemically applied prior to ischemic insult. These doses are comparable to those used in the studies of brain ischemia (e.g., Gill and Woodruff, 1990; Nakamura et al., 1993). Doses higher than 10 mg/kg could not be tested because these doses of MK-801 given under pentobarbital anesthesia were lethal. Together with the present results and those of our previous in vitro (Kashii et al., 1996) and in vivo studies (Morizane et al., 1997), it was concluded that NMDA receptors are the predominant route of glutamate neurotoxicity which plays an important role in retinal ischemia. Thus, we examined the effects of L-NAME, a competitive NO synthase inhibitor, to investigate the role of NO in ischemic damage of the retina. L-NAME reduced ischemia-induced damage of the ganglion cell layer, inner plexiform layer and inner nuclear layer, although the recovery of the inner nuclear layer was not statistically significant. The results of this study are consistent with the previous report of Geyer et al. (1995), in which N^{ω}-nitro-L-arginine almost completely abolished ischemic damage of the retina. Moreover, D-NAME, the inactive enantiomer of L-NAME, had no effect on ischemia-induced retinal damage, suggesting that the protective effect of L-NAME is due to its specific effect on NO synthase. Our previous study indicated that intravitreal

injection of L-NAME prevents NMDA-induced retinal damage by its specific action on NO synthase (Morizane et al., 1997). These results indicate that inhibition of retinal NO synthase activity by L-NAME reduces excitotoxic damage to the inner retinal layers. Moreover, glutamate release from the retina is found in rabbits subjected to pressure-induced ischemia (Louzada-Junior et al., 1992). Therefore, it is suggested that NO plays a crucial role in NMDA receptor-mediated glutamate neurotoxicity involved in retinal ischemia.

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