Retinal Toxicity of Nitric Oxide Released by Administration of a Nitric Oxide Donor in the Albino Rabbit

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Purpose. Nitric oxide (NO), which has been identified as an endothelium-derived relaxing factor, might be involved in regulation of retinal circulation and intraocular pressure. Recently, it was suggested that NO might also be related to neuronal excitotoxicity mediated by the N-methyl-D-aspartate receptor and to the pathologic changes induced by some kinds of uveitis. However, ocular toxicity of NO released by an NO donor has not been clearly demonstrated. In the current study, NO neurotoxicity in the retina was investigated.

Methods. S-nitroso-N-acetyl-DL-penicillamine (SNAP, 200 nmol) was injected into the vitreous of albino rabbits as an NO donor. The changes of retinal function were evaluated at 1, 2, and 3 hours and 1 and 4 weeks after SNAP injection, using electroretinogram and visual-evoked potentials. Histologic changes of the retina were also examined.

Results. Injection of SNAP reduced the a-wave amplitude. In contrast, the amplitudes of the oscillatory potentials were increased during the 3-hour observation period. Histologic examination showed vacuolar degeneration and loss of the nuclei of the photoreceptors. In the inner retina, some ganglion cells were lost, and cell density in the internal nuclear layer was decreased.

Conclusion. Retinal toxicity of NO was demonstrated functionally and histologically, suggesting that NO may play a pathophysiologic role in retinal ischemia or in degenerative retinal diseases. Invest Ophthalmol Vis Sci. 1997;38:2540-2544.

Nitric oxide (NO) has been identified as an endothelium-derived relaxing factor, and its physiological functions have been investigated in various fields of medicine. Nitric oxide increases cyclic guanosine monophosphate (cGMP) by stimulating guanylyl cyclase. In the blood vessels, NO causes smooth muscle relaxation, leading to vasodilation and increased blood flow. An NO synthase (NOS) inhibitor has been reported to suppress the relaxation of isolated porcine ophthalmic arteries as well as the dilation of dog retinal arteries elicited by bradykinin, acetylcholine, or substance P.\(^1\)\(^2\) Nitric oxide synthase also exists in the ciliary muscle, the trabecular meshwork, and the canal of Schlemm,\(^3\) and its activity might be decreased in the eyes of patients with primary open-angle glaucoma.\(^4\) These findings suggest that NO might be involved in regulation of the retinal circulation and intraocular pressure, by modulating smooth muscle tone.

Besides its significant influence on smooth muscle, NO appears to be involved in neurotransmission in the central nervous system. Excitotoxicity of neurons mediated by the N-methyl-D-aspartate (NMDA) receptor, which is stimulated by the release of glutamate after ischemia, seems to be an important cause of ischemic damage to the central nervous system, because NMDA antagonists can diminish the severity of such damage.\(^5\) Excitation of neurons by the binding of glutamate to the NMDA receptor increases the influx of Ca\(^{2+}\), which stimulates NOS. Because an NOS inhibitor lessened NMDA neurotoxicity, it seems that NO is related to neuronal excitotoxicity mediated by the NMDA receptor.\(^6\) Some neuronal cells of the retina (amacrine and ganglion cells) contain NOS;\(^7\) therefore, NO may have a role in retinal neurotransmission and may participate in the development of ischemic retinal damage.

Other than constitutive NOS (cNOS) expressed in...
vascular endothelial cells and in some of the neurons mentioned earlier, inducible NOS (iNOS), which was originally described in macrophages, also exists in the posterior pole, particularly in the retinal pigment epithelium and in Müller cells. Once iNOS is stimulated in such pathologic conditions as uveitis, a much higher amount of NO is released for a longer period than that derived from cNOS. This is considered to be cytotoxic and to lead to histologic changes in the eye. To investigate whether NO can actually influence retinal function and induce retinal toxicity, we examined the functional and histologic changes of the rabbit retina after intravitreal administration of an NO donor.

MATERIALS AND METHODS

Materials

To serve as an NO donor, SNAP was purchased from Wako (Osaka, Japan). N-acetyl-DL-penicillamine (NAP), a SNAP analogue lacking the nitrosocompound, was purchased from Sigma Chemical Company (St. Louis, MO). As NO trapping agents, carboxy-PTIO (Wako) and rabbit hemoglobin (Sigma Chemical) were used. These reagents were initially dissolved in dimethyl sulfoxide (DMSO; Wako) to make $10^{-4}$ M solutions because of insolubility in water, and the final concentration was adjusted to $10^{-2}$ M by further dilution with BSS.

Animals

Albino rabbits (2.4 to 2.8 kg) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. They were housed in a room with a 12-hour light-dark diurnal cycle. Only the right eye was carefully patched to avoid all stimulation. For recording, we used a photic stimulator (SLS 4100), a biophysical amplifier (AVM-10), and an averager (DAT-1100; Nihon-Kohden, Tokyo, Japan). Bandpass filters were set at 0.5 to 100 Hz for VEP and ERG a and b waves, and at 50 to 300 Hz for ERG oscillatory potentials.

The ERG was performed with a 20-J light stimulus set 20 cm in front of the eye, and recordings were made from the active electrode on the cornea by averaging four responses to the light stimulus at 0.1 Hz. The oscillatory potentials that appeared first and second were referred to as OP1 and OP2, respectively. We measured the amplitude and the latency of the a, b, OP1, and OP2 waves. After the ERG, VEPs were recorded from the active electrode on visual area 1 by summating 32 responses to a 0.6-J light stimulus at 1 Hz. The first negative peak with a latency of 20 msec was defined as N1, and its latency was measured as an indicator of visual function. Analogue data were recorded by a rectilinear pen system and were simultaneously stored and digitized, using a microcomputer (MacLab 2e, AD Instruments, Castle Hill, Australia). Analysis of various parameters was performed by the microcomputer, using the stored data. Measurements were carried out at 1, 2, and 3 hours and 1 and 4 weeks after SNAP injection. Before each recording, dark adaptation was allowed for 60 minutes.

Histologic Examination

Animals were killed with intravenous pentobarbital sodium for histologic examination. The eye was enucleated, cut open at the corneal limbus, and placed in
a- and b-waves oscillatory potentials

before injection
2 hours
4 weeks

FIGURE 1. Representative changes of electroretinogram after S-nitroso-N-acetyl-DL-penicillamine injection. The a-wave amplitude was reduced after injection, whereas the oscillatory potentials were transiently enhanced after injection.

0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde and 0.25 M sucrose. After fixing for 24 hours, the posterior segment of the inferior retina was excised. After rinsing in buffer, the tissue was post-fixed in 1% OsO$_4$ for 60 minutes and dehydrated through a graded ethanol series. The tissue was embedded in Epon, and 1-µm transverse sections were cut parallel to the medullary rays and stained with toluidine blue. Ultrathin sections were stained by uranyl acetate and lead citrate and were observed by a transmission electron microscope (H800, Hitachi, Tokyo, Japan). These sections were cut approximately 2 mm inferior to the optic disc.

Statistical Analysis

Data are expressed as mean values ± SEM. Two-way interactions were analyzed by repeated measures analysis of variance, and statistical comparisons between two groups were done using Student’s t-test. The difference was accepted as significant at P < 0.05.

RESULTS

Representative ERG waveforms obtained before and after SNAP injection are shown in Figure 1. The amplitude of the a wave was reduced 2 hours after SNAP injection and did not recover by 4 weeks after injection. In contrast, the amplitudes of the oscillatory potentials were elevated after SNAP injection (Fig. 2B). The maximum effect was seen at 2 hours after injection, when the level was 168.9 ± 9.8% of baseline. The amplitude returned to the previous level 4 weeks after injection. Two-way interaction was also significant by repeated measures analysis of variance (P = 0.002), and the difference was significant relative to that in control samples.

The amplitude of the a wave was reduced to 81.7 ± 4%, 78.2 ± 4.8%, and 73.5 ± 7.1% of baseline at 1, 2, and 3 hours after SNAP injection, respectively, and a further decline continued throughout the study. Two-way interaction was significant by repeated measures analysis of variance (P = 0.001), and amplitudes differed significantly from that in control samples at every examination point (P < 0.01; t-test; Fig. 2A). In contrast, the amplitudes of the oscillatory potentials (OP$_1$ plus OP$_2$ amplitude) were elevated after SNAP injection (Fig. 2B). The maximum effect was seen at 2 hours after injection, when the level was 168.9 ± 9.8% of baseline. The amplitude returned to the previous level 4 weeks after injection. Two-way interaction was significant by repeated measures analysis of variance (P = 0.002), and the difference was significant relative to that in control samples.
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FIGURE 3. Cross-sections of the retina at different times after S-nitroso-N-acetyl-DL-penicillamine injection (A) in controls, (B) 8 hours and (C) 4 weeks after S-nitroso-N-acetyl-DL-penicillamine injection, respectively. Some ganglion cells and some photoreceptor cell nuclei have been lost, and the cell density in the inner nuclear layer is reduced (C) 4 weeks after injection. Some photoreceptor cell nuclei have degenerated and show pyknosis at (B) 8 hours after injection. GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer. Bar = 30 μm.

samples at 1, 2, and 3 hours after SNAP injection (P = 0.005, 0.001, and 0.019, respectively; Student's t-test; Fig. 2B). The VEP latency was delayed a maximum of 105.1 ± 2.5% of baseline at 4 weeks after treatment, but this was not significant. Other parameters (the a-wave latency, b-wave amplitude, b-wave latency, and OP1 and OP2 latencies) did not show significant changes.

The effects of NAP and NO scavengers on the influence of SNAP on the ERG were also investigated for 3 hours after treatment. NAP had no effect on the ERG parameters (n = 4). When 200 nmol carboxy-PTIO was injected before SNAP, the elevation of oscillatory potentials was almost completely inhibited (n = 4, data not shown). However, carboxy-PTIO itself affected the ERG a wave and significantly delayed its latency. Hemoglobin was also tested for its effect on the a-wave amplitude (n = 4). It was maintained at 93.1 ± 3%, 93.8 ± 0.6%, and 90.1 ± 1.7% of baseline by pretreatment with hemoglobin at 1, 2, and 3 hours after SNAP injection, respectively.

The histologic changes of the retina caused by SNAP are demonstrated in Figures 3 and 4. Four weeks after injection, SNAP caused the distortion of the retinal architecture and a decrease of photoreceptor cell nuclei (Fig. 3C). In the inner retina, some ganglion cells were lost, and the density of cells in the inner nuclear layer was also reduced (Fig. 3C). These findings may have been time dependent, in that the changes were not apparent at 8 hours after injection, although the uniform size and staining intensity of photoreceptor cell nuclei was already disturbed (Fig. 3B). Electron microscopic study of the outer retina at 4 weeks after SNAP injection demonstrated vacuolar degeneration of the photoreceptor layer. There was degeneration of photoreceptor cell nuclei with pyknosis, and glial proliferation was observed between the nuclei (Fig. 4).

DISCUSSION

The effects of NO are controversial; it is both neuroprotective and neurodestructive. Under physiological conditions, NO can be converted between different redox forms, the nitric oxide radical (NO·) and the nitrosonium ion (NO+), and it has distinct properties in these different states. The existence of these different redox species may explain the paradoxical effects of NO. It is accepted that NO· has potential neurotoxicity, because it regulates the killing of bacteria by macrophages and participates in the development of uveitis. In contrast, NO+ may suppress NMDA receptor activity and Ca2+ influx and thus may be neuroprotective against NMDA neurotoxicity.

The most striking findings in the current study were that SNAP caused impairment of the ERG a wave and produced photoreceptor cell degeneration. Transient enhancement of oscillatory potentials was observed simultaneously. These changes appeared to be caused by NO, because NAP had no effect on ERG findings, and NO scavengers reduced the ERG changes caused by SNAP. Although some ganglion cells were lost after SNAP injection, this did not lead to significant prolongation of VEP latency. It has been suggested that cGMP opens the light-sensitive Na+ channels and produces the dark currents in photoreceptor cells. Cyclic GMP hydrolysis triggered by light then closes these channels and generates a hyperpolarized potential, the ERG a wave. Therefore, it seems possible that a tonic increase of conductance by NO-activated cGMP reduced a-wave amplitude. The oscillatory potentials reflect radial current flow within the retina.
the inner retina, the origin of which is unknown, although bipolar and amacrine cells have been implicated in its generation. An elevated cGMP level might influence current flow or increase the light response of these cells to enhance the oscillatory potentials.\(^6\)\(^9\) However, cGMP was found to be unrelated to NMDA neurotoxicity in a study in vitro.\(^6\)

Nitric oxide is a low molecular weight gas and can easily pass through the cell membrane; therefore, NO injected into the vireous should affect the whole retina, and indeed, we found damage in all retinal layers. It should be noted that the photoreceptor cells were vulnerable to NO; their nuclei underwent degeneration 8 hours after SNAP injection, when the inner retina seemed to be relatively preserved. These findings were different from the results of an ischemia–reperfusion study,\(^11\) in which NO generated by intrinsic NOS selectively damaged the inner retina, with reduction of ganglion cells and the density of the inner nuclear layer. The retinal impairment induced in the ischemia–reperfusion study might have been influenced by the distribution of NMDA receptors and NOS, as well as by the ischemic stress itself. Our study was aimed at detecting direct toxicity of NO in the retina, and a different pattern of retinal damage was noted.

Results obtained in a study in vitro have shown that vulnerability to NO depends on the neuronal activities of NOS and superoxide dismutase, in that some cells enriched with NOS are tolerant of NO-mediated neurotoxicity,\(^6\)\(^9\) and superoxide dismutase can reduce this toxicity.\(^9\) Recently, peroxynitrite was reported to be an important factor in NO-related neurotoxicity.\(^6\)\(^9\) NO react rapidly with the superoxide anion (O\(_2^-\)) and generates peroxynitrite,\(^9\) a reaction that can be blocked by superoxide dismutase. Peroxynitrite decomposes into the highly reactive hydroxyl radical (OH\(^-\)), which is considered to be more toxic than NO, because OH\(^-\) produces lipid peroxidation and leads to cell death.\(^6\)\(^9\) Accordingly, vulnerability to NO might be related to these enzymes and to the tissue (O\(_2^-\)) level. The NOS immunoreactivity of the photoreceptor layer has been shown to be lower than that of the inner retina.\(^7\) Also, an immunohistochemical study has shown that superoxide dismutase is mainly present in the inner retina,\(^12\) although the greater part of the oxygen consumption in the retina occurs in the photoreceptor cells. High oxygen consumption and relatively low superoxide dismutase activity may lead to high peroxy- nitrite levels in the photoreceptor cells, which may also help to explain the discrepancy. With the ultimate goal of developing appropriate medical treatment, the retinal toxicity of NO should be investigated further.

**Key Words**

electroretinogram, nitric oxide, N-methyl-D-aspartate neurotoxicity, nitric oxide donor, visual-evoked potentials

**References**