Histological Protection by Nilvadipine against Neurotoxicity Induced by NOC12, a Nitric Oxide Donor, in the Rat Retina

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In the present study, we histologically examined the effects of nilvadipine on neuronal injury induced by intravitreal (i.v.) N-methyl-D-aspartate (NMDA) (200 nmol/eye) and intravitreal NOC12 (400 nmol/eye), a nitric oxide donor, in the rat retina. Morphometric evaluation at 7 d after injection of NMDA or NOC12 showed that treatment with nilvadipine (1 mg/kg, i.v.) 15 min prior to injection of NMDA or NOC12 dramatically reduced the retinal damage. These results suggest that nilvadipine protects neurons against excitotoxic injury in the rat retina in vivo at least in part via an antioxidative effect.

Key words nilvadipine; N-methyl-D-aspartic acid (NMDA); voltage-dependent calcium channel; L-type; antioxidant

Excitotoxic injury is considered important in retinal diseases, such as glaucoma, including open angle glaucoma, closed angle glaucoma and normal-tension glaucoma, and central retinal vessel occlusions. Endogenous substances such as glutamate, oxygen free radicals, nitric oxide and calcium are among the pathological causes of the injury. Glutamate is the principal excitatory neurotransmitter in the central nervous system, including retina. However, stimulation of some of the glutamate receptor by excess amount of glutamate under pathologic conditions such as hypoxia and ischemia-reperfusion is toxic to neuronal cells. The activation of the N-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptor, followed by excess Ca\(^{2+}\) influx via NMDA receptor-operated channels is thought to be involved in the predominant mechanism of neuronal excitotoxicity. In fact, excitotoxicity caused by the elevation of glutamate concentration in the retinal extracellular space near the glutamate receptor channels is thought to be one of the mechanisms of neuronal cell death induced by glaucoma. MK-801, an NMDA receptor channel blocker, has been reported to prevent retinal damage induced by retinal ischemia-reperfusion. Activation of NMDA receptors depolarizes neurons and subsequently activates voltage-dependent Ca\(^{2+}\) channels. We previously reported that cilnidipine, a dual L/N-type Ca\(^{2+}\) channel blocker, has a beneficial effect on retinal ischemia-reperfusion injury. Therefore, activation of VDCCs after stimulation of NMDA receptor is likely involved in the mechanism of retinal excitotoxic injury.

Nilvadipine, a dihydropyridine L-type VDCC blocker, has selective and long-lasting effects on cerebral arteries compared with other calcium channel blockers such as nicardipine, nifedipine and diltiazem. Nilvadipine was effective in several experimental models of cerebral ischemia. In addition, nilvadipine has been reported to inhibit glutamate-induced apoptotic cell death by blocking Ca\(^{2+}\) influx via L-type VDCC in the purified retinal ganglion cells, and to have beneficial effects on the retinal ischemia-reperfusion injury. Nilvadipine increases blood velocity and blood flow in the optic nerve head, choroid and retina of rabbits, and had little effect on systemic blood pressure in subjects without hypertension.

In contrast to cilnidipine and nilvadipine, we previously reported that amlodipine, another dihydropyridine L-type VDCC blocker, did not inhibit retinal injury induced by ischemia-reperfusion, suggesting that other mechanism than blocking Ca\(^{2+}\) influx via L-type VDCC may be important for retinal neuroprotection against excitotoxicity by L-type VDCC blockers. In fact, cilnidipine blocks not only L-type but also N-type VDCCs, and nilvadipine has also been reported to block not only L-type but also T-type VDCCs and to exert an antioxidant effect that is greater than the effects of nimodipine, nicardipine, and amlodipine.

In the present study, to clarify whether an antioxidant effect of nilvadipine is involved in its neuroprotective effect, we assessed the effect of nilvadipine on retinal injury induced by intravitreal injection of NOC12, a nitric oxide (NO) donor compound, in rats.

METHODS

Animals All experimental procedures used in this study conformed to Procedures for animal experiments were approved by the Committee for Animal Experiments at Kitasato University and the Guiding Principles for the Care and Use of Laboratory Animals, which has been approved by the Japanese Pharmacological Society. Male Sprague-Dawley rats weighing 230–300 g (Charles River Japan, Kanagawa, Japan) were housed 4–6 per cage in a room under controlled temperature (22±5°C), humidity (55±5%), and a 12-h light/dark cycle, and were allowed free access to regular rat chow and tap water.

Intravitreal Injection Intravitreal injection was performed as previously described with some modifications. Briefly, rats were anesthetized with ketamine (90 mg/kg intraperitoneally) and xylazine (10 mg/kg, i.p.; Tokyo Kasei, Tokyo, Japan). Injection was performed with a 33-gauge needle connected to a 25-µL microsyringe (MS-N25, Ito Seisakusho, Fuji, Japan). The tip of the needle was inserted approximately 1 mm behind the corneal limbus. Five microliter of the drug solution described below was administered into one eye and the vehicle was ad-
ministered into another eye as the control.

**Preparation of Drugs** Nilvadipine (1 mg/kg; gift from Astellas Pharma Inc., Tokyo, Japan) were intravenously administered 15 min before intravitreal injection of NMDA or NOC12. We prepared 100 mg/mL stock solutions of nilvadipine in a solution of polyethylene glycol 400:99.5% ethanol=1:1, and diluted them to working concentrations with saline. This dose of nilvadipine was reported to reduce retinal ischemia-reperfusion injury in the rats. NOC12 (Nacalai Tesque, Kyoto, Japan) was dissolved in saline. NOC 12 (Dojindo Laboratories, Kumamoto, Japan) was dissolved in 5 µL of sodium phosphate buffer, containing 0.03 M NaOH.

**Histological Evaluation** The method for histological evaluation was described previously with some modifications. Animals were euthanized by overdose of pentobarbital sodium 7 d after 60 min of ischemia and both eyes were enucleated. Enucleated eyes were fixed with Davidson solution, comprised of 37.5% ethanol, 9.3% paraformaldehyde, 12.5% acetic acid and 3% glutaraldehyde for 1–12 h at room temperature. The fixed eye was bisected through the optic nerve head in the vertical meridian with a microtome blade (Histo Cutter Super #35 Type, Micro Glass, Tokyo, Japan) and embedded in paraffin after removing a lens. Five micrometer horizontal sections through the optic nerve head of the eye were cut along the vertical meridian of the eye so as to contain the entire retina from the ora serrata in the superior hemisphere to the ora serrata in the inferior hemisphere using a microtome (HM325, Microm International, Walldorf, Germany) and a microtome blade (Histo Cutter Super #35 Type, Micro Glass). The sections were stained with hematoxylin and eosin, and subject to morphometry. The sections showed oblique regions were excluded to avoid artifacts. The total number of the cells in the retinal ganglion cell layer (GCL) was counted for a length of 250 µm on either side of the optic nerve head beginning approximately 1 mm from the center of the optic nerve head in four independent sections using a light microscope (Optiphot-2, Nicon, Tokyo, Japan). No attempt was made to distinguish the cell types in the GCL, and displaced amacrine cells were not excluded from the counts. Digital photographs (digital camera [DP11, Olympus, Tokyo, Japan] connected to a light microscope) were taken so that ca. 0.25 mm of retina appeared in each photograph, with sections ca. 1 mm from the center of the optic nerve head chosen.

Thickness measurements of the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), and the outer nuclear layer (ONL) were performed. These parameters of each eye subjected to ischemia were normalized with those of the corresponding intact opposite eyes and are presented as percentages. We did all of the morphometrical analysis in a blind fashion.

**Statistical Analysis** The data represent the means±S.E.M. of three rats per group. Student’s t-test was used for the comparison between the two groups. Differences were considered to be statistically significant when the p values were less than 0.05.

**RESULTS**

At first, the effects of nilvadipine on retinal injury induced by intravitreal injection of NMDA (200 nmol) in rats were tested. Typical photomicrographs of the retina taken seven days after NMDA injection are shown in Figs. 1A–D. In the vehicle-treated group, degenerative changes were observed in GCL and IPL of the NMDA-treated eye (Fig. 1B), but such changes were not seen in the contralateral vehicle-treated retina (Fig. 1A). Intravenous treatment with nilvadipine at 1 mg/kg 15 min before injection of NMDA markedly reduced the retinal degeneration (Fig. 1D).

Next, the effects of nilvadipine on retinal injury induced by intravitreal injection of NOC12 (700 nmol) in rats were tested. Typical photomicrographs of the retina taken seven days after NOC12 injection are shown in Figs. 2A–D. In the vehicle-treated group, degenerative changes were observed in GCL and IPL of the NOC12-treated eye (Fig. 2B), but such changes were not seen in the contralateral vehicle-treated retina (Fig. 2A). Intravenous treatment with nilvadipine at 1 mg/kg 15 min before injection of NOC12 markedly reduced the retinal degeneration (Fig. 2D).

**DISCUSSION**

The present study clearly demonstrated that intravenous treatment with nilvadipine ameliorated NMDA-induced retinal degeneration. We also showed intravenous treatment...
with nilvadipine ameliorated NOC12-induced retinal degeneration. Both apoptosis and necrosis are involved in NMDA and NOC12-induced injury in the rat retina.25) It has been reported that retinal ischemia-reperfusion injury is almost completely reduced by an antagonist of NMDA receptor such as MK-801, and by an NO synthase inhibitor, such as N(G)-monomethyl-L-arginine monoacetate salt (L-NMMA).26) It is widely thought that glutamate and NO are absolutely crucial factors in the mechanism of retinal ischemia-reperfusion injury. Therefore, NMDA and NO-donor will cause the retinal injury similar to retinal ischemia-reperfusion injury. In fact, nilvadipine reduced the number of terminal deoxy-nucleotidyl transferase mediated deoxyuridine triphosphate nick-end labeling (TUNEL)-positive cells in the ischemic-reperfused rat retina.19) In the present study, nilvadipine almost completely reduced the histological damage induced by NMDA and NOC12, suggesting that nilvadipine should reduce both apoptosis and necrosis in our model.

We used 1 mg/kg nilvadipine, because this dose of the drug was reported to reduce retinal ischemia-reperfusion injury in the rats,19) and Takahara et al. reported that 100 µg/kg nilvadipine did not reduce the infarct size in a cerebral ischemia-reperfusion model.27) Because 100 µg/kg nilvadipine showed a hypotensive effect and an increase of the blood flow of the central blood vessels,26) we cannot deny the possibility that these effects may be involved in the protective effect of nilvadipine. However, another report demonstrated that nilvadipine (10–100 nm) protected against the glutamate-induced neurotoxicity in the purified retinal ganglion cells.28) This report suggests that the effect of the drug on hemodynamics is not necessary for its retinal protective effect.

Activation of NMDA glutamate receptor is reported to lead to the activation of NO synthase.27) Activation of NO synthase has been shown to be involved in the mechanism of ischemia-reperfusion injury in the rat retina.11) Nilvadipine exerts an antioxidant effect that is greater than the effects of nimodipine, nicardipine, and amlodipine.22) To clarify whether the antioxidant effect of nilvadipine is involved in its neuroprotective effect, we examined the effect of nilvadipine on the retinal injury induced by exogenous NOC12, an NO donor compound. In the present study, nilvadipine significantly reduced the injury induced by NOC12. This result is consistent with the previous report showing that nilvadipine prevented oxidation of low-density lipoprotein cholesterol.28) These data suggest that the antioxidant effect may be important for the protective effects of nilvadipine on excitotoxicity in the retina. In contrast, it has been reported that the maximum plasma concentration of nilvadipine (1 mg/kg, intramuscular (i.m.)) was about 44.4 ng/mL, i.e., 0.12 µM.19) This value is 250 times lower than the reported IC50 for anti-oxidant potency in vitro (25.1 µM).22) Although we cannot clarify this discrepancy, we speculate that the effective concentration for anti-oxidant potency may be different between in vitro and in vivo. To prove our hypothesis, analysis using anti-nitrotyrosine antibody are needed. Unfortunately, we have not been able to find the optimal experimental condition for the experiment yet. We understand that this issue is one of our remaining issues.

It has been reported that 100 µM cilnidipine had a radical scavenging activity.29) But this concentration of cilnidipine showed only almost 25% reduction of dihydroxybenzoic acid formation from salicylate by H2O2. Therefore, IC50 for anti-oxidant potency in vitro of cinidipine may be over µM range. In our previous study, 100 µg/kg cilnidipine was treated, so the estimated maximum plasma concentration of cilnidipine should be at most around tens of nano molar, 10000 times lower than the concentration used in the in vitro study. We speculate that anti-oxidant potency of nilvadipine is greater than that of cilnidipine, and that this is because cilnidipine did not reduce the NOC12-induced retinal injury in our previous report.19) We think that the possibility that radical scavenging activity is involved in the protective effect of cilnidipine on the NMDA-induced retinal injury is low.

Another possibility for the mechanism of the protective effect of nilvadipine is the blocking effect on VDCCs. It has been demonstrated that nilvadipine ameliorates glutamate-induced cell death of purified RGCs, although other calcium channel blockers, nifedipine and diltiazem, do not.30) In addition, increase of the glutamate-evoked intracellular Ca2+ concentration was significantly reduced by nilvadipine, but not nifedipine and diltiazem, in the purified RGCs.30) We reported that amlodipine did not inhibit retinal injury induced by ischemia-reperfusion.14) These data suggest that the blocking effect on Ca2+ influx via L-type VDCC may not be sufficient for retinal neuroprotection against excitotoxicity by L-type VDCC blockers. It has been shown that dual T- and L-type VDCC blockers, such as flunarizine30) and lomerizine,11) pre-

Fig. 2. Representative Photomicrographs Showing Histological Appearance of the Vehicle-Injected Control (A), the NOC12-Injected (B), the Nilvadipine-Treated (C), and the NOC12 and Nilvadipine-Treated (D) Retinae Seven Days after Intravitreal Injection

Nilvadipine was treated intravenously 15 min before intravitreal NOC12 or saline (vehicle) injection. Retinal damage is shown in the NOC12-injected (B) retina. In the 1 mg/kg nilvadipine-treated group, retinal structure is preserved (D). Scale bar=50 µm. Original magnification is ×200. Morphometric results at seven days after intravitreal injection of three independent experiments are shown in (E). The data represent the means±S.E.M. of three rats per group. *p<0.05 vs. the control group.
vented death of retinal neurons in a transient retinal ischemia model and glutamate-induced neurotoxicity in vitro.31) Recently, nilvadipine was reported to block both T- and L-type VDCCs in isolated rat hippocampal CA1 pyramidal neurons.32) Therefore, blockade of T-type VDCC may be important for the protective effect of nilvadipine shown in the present study. However, further additional biochemical, molecular biological and physiological experiments are clearly needed to clarify whether T-type VDCC is involved in the mechanism of the protective effect of nilvadipine.

Permeability into the retina is another important issue for the protective effect of the drugs on the retinal tissue. It has been reported that nilvadipine has a greater permeability into the retina and a longer half life in the retina than nifedipine,33) suggesting that nilvadipine effectively exerts its actions in the retina by easily passing through the blood-retinal barrier. This property may account for the protective effects of nilvadipine on retinal degeneration induced by NMDA and NOC12. In fact, nilvadipine has been reported to delayed photoreceptor degeneration in RCS rats,34) and to have beneficial effects on the retinal ischemia-reperfusion injury.19)

In conclusion, we demonstrated that the NMDA-induced retinal injury is blocked by nilvadipine. Nilvadipine also reduced retinal degeneration induced by NOC12, an NO donor. These findings suggest that antioxidative effect of nilvadipine is at least in part involved in its protective effects on excitotoxicity in the retina. Nilvadipine may be useful for the treatment of the retinal diseases related to excitotoxicity, such as glaucoma and central retinal artery occlusion.

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