

Retinal concentration and protective effect against retinal ischemia of nilvadipine in rats

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PURPOSE. Several calcium entry blockers have neuroprotective effects on cellular damage in the brain induced by ischemia. The purpose of this study was to determine whether nilvadipine (NID) crosses the blood-retinal barrier, and if so, whether it can then protect the photoreceptors against retinal ischemia-reperfusion injury.

METHODS. Rats received an intramuscular injection of 1 mg/kg of NID and nifedipine (NIF), and the retinal and serum concentrations were measured. Ischemia was induced by raising the intraocular pressure for 45 minutes. Twenty-four hours after the reperfusion, the number of TUNEL positive cells and retinal ganglion cells (RGCs) were counted, and the thickness of the retina was measured.

RESULTS. After 60 minutes, the concentration of NID, but not NIF, was higher in retina than in the serum. The number of TUNEL-positive cells was fewer and the reduction in the number of RGCs and the thickness of retina was less in the eyes that had received NID than controls.

CONCLUSIONS. The findings show that NID has high permeability to retina compared with NIF, which has less fat solubility than NID, and neuroprotective effect to retinal cells. NID might be useful for the treatment of glaucoma or other retinal diseases that have some relation to apoptosis. (*Eur J Ophthalmol* 2008; 18: 87-93)

KEY WORDS. Apoptosis, Calcium channel blocker, Ischemia-reperfusion, Nilvadipine, Rat, Retina

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INTRODUCTION

Several calcium entry blockers have neuroprotective effects on cellular damage in the brain induced by ischemia (1-4). Two mechanisms are probably involved: relaxation of cerebral vascular smooth muscle and the subsequent increase of cerebral blood flow, and the blockage of excessive calcium influx into brain cells. For therapeutic ophthalmologic use, calcium channel blocking agents can be considered for patients with papillary vasospasm that is provoked by stimuli whose mechanism of vasoconstriction is via calcium channel influx and for patients whose vision is threatened by vasoconstriction (5). Thus, topically applied flunarizine, a calcium channel blocker, reduces the intraocular pressure (IOP) and attenuates injury to the entire retina including the ganglion cells (6).

Nilvadipine (NID, 5-isopropyl-3-methyl-2-cyano-1, 4-dihydro-6-methyl-4-(m-nitrophenyl)-3, 5-pyridinedicarboxylate), is a dihydropyridine type of calcium channel blocker with good fat solubility. It is used as a therapeutic drug for hypertension and ischemic cerebrovascular disease, and has a more selective and long-lasting effect on cerebral arteries than other calcium entry blockers which have less fat solubility such as nifedipine and diltiazem than NID (7). In addition, NID had better access to brain tissue than nifedipine, another dihydropyridine type of calcium entry blocker that is not enough fat soluble, for equivalent doses (100 µg/kg, IV) (8). Furthermore, NID attenuated the liberation of free fatty acids (FFAs) in the ischemic brain of rats in a dose dependent manner. At same doses NID and nifedipine (100 µg/kg, IV), NID attenuated liberation of FFAs more than nifedipine (8). This indicated the benefi-

cial effect of NID would be both mechanism-based and related to the good access to the retina (8).

A recent study reported that NID delayed the inherited degeneration of photoreceptors in RCS rats, and the authors suggested that NID might be used to treat some patients (9). However, there are no reports on the concentration of NID in the retina after systemic injections.

The purpose of this study was to determine whether NID crosses the blood–retinal barrier, and if so, whether it can then protect the photoreceptors against retinal ischemia-reperfusion injury.

MATERIALS AND METHODS

Male Wistar rats, aged 8 weeks and weighting approximately 200 g, were used. The animals were kept on a 12:12 light:dark schedule. All of the experimental procedures conformed to Recommendations from the Declaration of Helsinki and The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23).

Measurement of NID and nifedipine (NIF) concentration in serum and retina

NID and nifedipine (NIF), which is a dihydropyridine-based calcium channel antagonist and has less fat solubility than NID, were dissolved in polyethylene glycol 400. One mg/kg body weight of NID was injected intramuscularly into 25 rats and same amount of NIF to 25 rats. For control, polyethylene glycol 400 alone was injected intramuscularly to 25 rats. At 30, 60, 120, 240, and 480 minutes after the injection, 5 animals in each group were deeply anesthetized with intraperitoneal pentobarbital, blood was collected by cardiac puncture, and saline was injected through heart to wash out the blood from the retina. The retinas were removed from the eyecups and stored at -80°C for later analysis.

The concentrations of unmetabolized NID and NIF in rat serum and retina were measured individually by liquid chromatography/mass spectrometry with high performance liquid chromatography (Alliance 2690: Waters, Milford) and Quattro Ultima (Micromass, Manchester, UK) according to the methods of Carnerup et al (10) and Yasuda et al (11). The lower limit of detection by our method was 0.11 ng/mL for plasma and 6.00 ng/g for retinal tissue for NID, and 1.00 ng/mL for plasma and 6.25 ng/g for retinal tissue for NIF.

The Mann-Whitney test was used to compare NID and NIF concentrations in serum and retina, and $p < 0.05$ was considered statistically significant.

Retinal ischemia-reperfusion

For the experimental animals, 1 mg/kg of NID (0.5 mg/mL in polyethylene glycol 400) was injected intramuscularly. Control rats received the same amount of polyethylene glycol 400. Ten minutes after the injection, rats were anesthetized by an intraperitoneal injection of 75 mg/kg pentobarbital sodium. Five minutes later, the left anterior chamber was cannulated by a 30-G needle that was connected by silicone elastomer tubing to a reservoir of balanced oxyglutathione solution (BSS PLUS[®], Santen, Osaka, Japan). The IOP was raised to 120 mmHg by raising the height of the reservoir. Ophthalmoscopic examination confirmed that the retinal vessels were collapsed and the retina appeared gray demonstrating that the retina was ischemic. After 45 minutes of retinal ischemia, the IOP was returned to normal, and ophthalmoscopy showed a return of the normal circulation and color to the fundus. The body temperature of rats was kept at 37.0°C with a heating pad (KN-474 Homoethermic Blanket Control, Natume, Tokyo, Japan) during ischemia and until the animal recovered from the anesthesia.

TUNEL staining

To demonstrate that NID prevented apoptosis of retinal ganglion cells (RGCs), the presence of apoptotic cells was determined by TdT-mediated dUTP nick-end labeling (TUNEL) staining. Three animals injected with 1 mg/kg of NID and three control animals injected with polyethylene glycol 400 intramuscularly underwent ischemia-reperfusion. They were then perfused through the heart with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) under deep ether anesthesia 24 hours after the beginning of reperfusion, because the peak of apoptosis occurs 24 hours after reperfusion (12). The eyecups were embedded in paraffin, and 6 μm sections were cut.

TUNEL staining was performed with an in situ apoptosis detection kit (Trevigen, Gaithersburg, MD). Labeled nuclei were detected by streptavidin-FITC, and the sections were counterstained with 4, 6-diamidino-2-phenyl indol dihydrochloride (DAPI). The sections were examined under a fluorescence microscope (Carl Zeiss, Jena, Germany), and the sections passing through the optic disc were selected.

Histopathologic studies

Six rats were injected intramuscularly with 1 mg/kg NID (0.5 mg/mL in polyethylene glycol 400), and five control rats received the same amount of polyethylene glycol 400. Seven days after retinal ischemia and reperfusion, rats were perfused through the heart with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M PBS under deep ether anesthesia. The enucleated eyes were kept in the same solution for 30 minutes, and then the anterior segments were cut off and discarded. The eyecups were embedded in paraffin, and 6 μ m sections were cut and stained with hematoxylin and eosin for light microscopy.

The number of the neuronal cells in the ganglion cell layer (GCL) was counted, and the thicknesses of the inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL) were measured in sections passing through the optic disc. All RGCs were counted from ora serrata to ora serrata. Vascular endothelial and glial cell nuclei were morphologically identified and excluded from the cell counts (13, 14). The area around the optic disk was photographed, and the thickness of each layer was measured at 1 mm from the optic disk.

The Mann-Whitney test was used to compare the number of cells in the GCL and the thickness of each layer (IPL, INL, OPL, and ONL) in the two groups. A p value < 0.05 was considered statistically significant.

RESULTS

Retinal concentration of NID and NIF

In the control animals injected polyethylene glycol 400 alone no NID and NIF were detected at any time after injection.

The concentrations of unmetabolized NID in the retina and serum after the intramuscular injection are shown in Figure 1. Thirty minutes after the injection, the mean concentration of NID in the retina and serum was 53.8 ng/g and 44.4 ng/mL, respectively. One hour after the injection, the concentration of unmetabolized NID in the retina had increased to 71.1 ng/g, but decreased in the serum to 37.2 ng/mL. The concentration of NID in retina was significantly higher than that in serum ($p = 0.03$).

At 480 minutes after the injection, the mean concentration

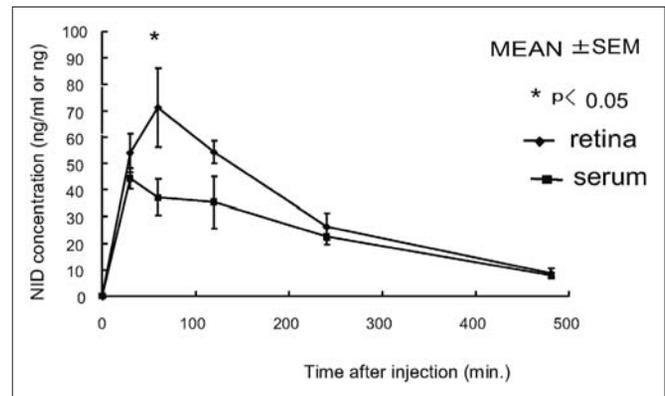


Fig. 1 - Concentrations of non-metabolized nilvadipine (NID) in the retina and plasma after an intramuscular injection of 1 mg/kg of NID.

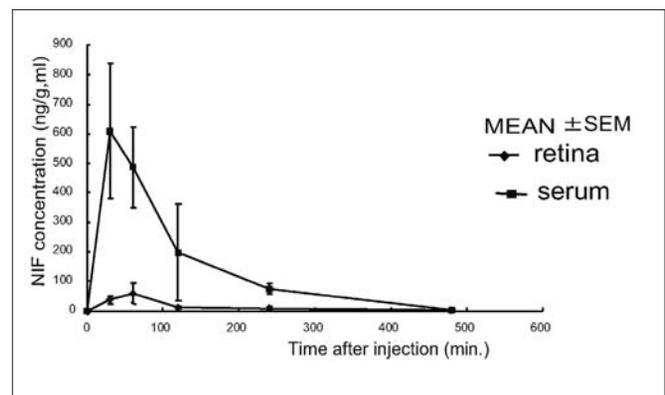


Fig. 2 - Concentrations of non-metabolized nifedipine (NIF) in the retina and plasma after an intramuscular injection of 1 mg/kg of NIF.

of NID in retina and serum was 8.5 ng/g and 8.1 ng/mL, respectively. The terminal half-lives of NID in retina and serum were 1.1 and 0.9 hours, respectively. The ratio of concentration in the retina to plasma varied from 1.0 to 1.9. The concentrations of NIF in the retina and serum are shown in Figure 2. Thirty minutes after the injection, the concentrations of NIF in retina and serum were 37.0 ng/g and 608.6 ng/mL, respectively. Sixty minutes after the injection, the concentration of unmetabolized NIF in retina had increased to 58.0 ng/g, but was decreased in the serum to 486.5 ng/mL. However, the concentration in serum was significantly higher than that in retina after 1 hour and 2 hours. The terminal half-lives of NIF in retina and plasma were 0.9 and 0.6 hours, respectively. The ratio of concentration in retina to plasma varied from 0.06 to 1.0.

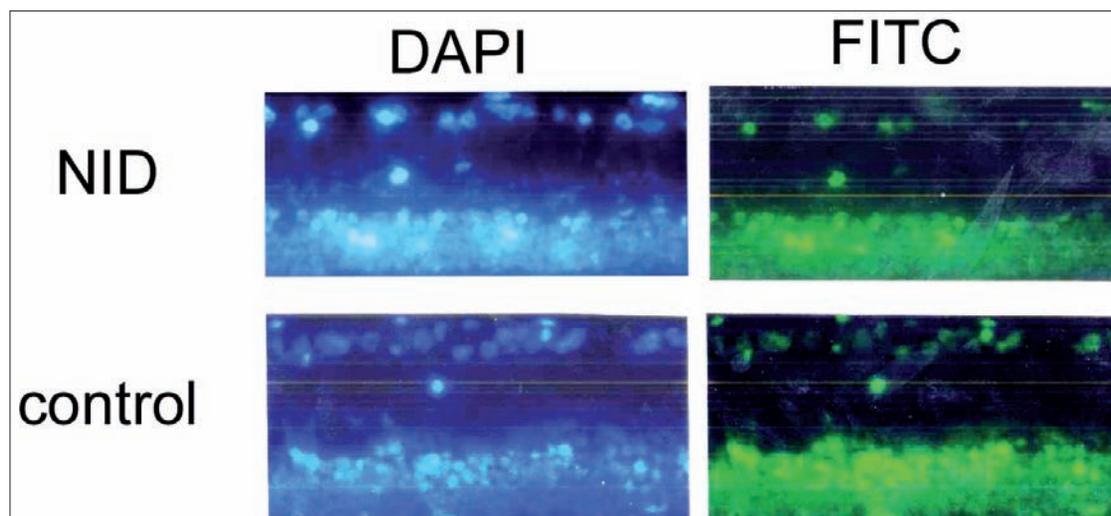


Fig. 3 - Photomicrographs of TdT-mediated dUTP nick-end labeling (TUNEL) stained retinas after an intramuscular injection of nilvadipine or polyethylene glycol 400 (controls). Apoptotic cells were stained by FITC (green) and all nuclei were stained by DAPI (blue).

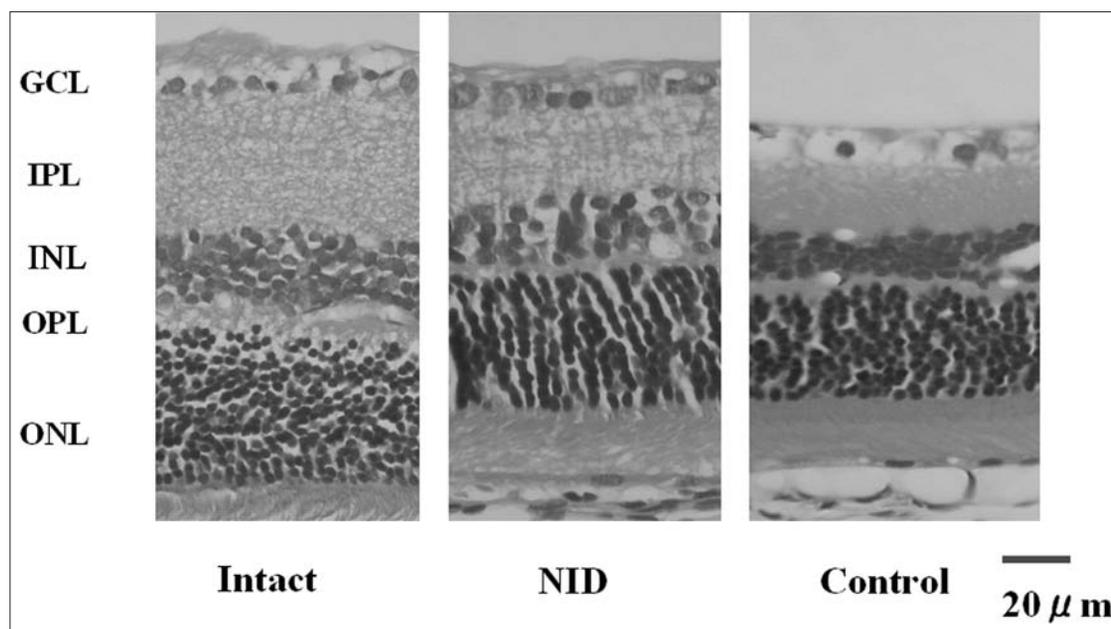


Fig. 4 - Photomicrographs of the retinas of an intact rat, a rat receiving nilvadipine, and a control rat 7 days after reperfusion. Bar = 20 μm.

TUNEL staining and histopathologic studies

Photomicrographs of TUNEL stained rat retinas at 3 mm from optic disc 24 hours after the ischemia with injection of NID and a control animal are shown in Figure 3. The number of TUNEL positive cells looked lower in the group receiving NID than in the control group.

Photomicrographs of the retinas of the intact rats, rats receiving NID with ischemia, and the control rats with ischemia alone are shown in Figure 4. The density of neuronal cells in GCL was lower, and the thicknesses of IPL, INL, and OPL were thinner in the control rats than in eyes

with the NID injection.

The number of cells in GCL after ischemia-reperfusion is shown in Figure 5. The mean \pm standard deviation (SD) of neuronal cells in RGC layer was 657 ± 37 /retinal section in the rats receiving NID (n=6), and 401 ± 23 /retinal section in the control rats without NID (n=5). The number of neuronal cells in the rats receiving NID was significantly higher than control rats ($p=0.04$).

The IPL, INL, and OPL were significantly thicker in the NID-injected rats ($p=0.008$, $p=0.03$, $p=0.0007$, respectively; Fig. 6); however, the differences in the ONL thickness were not significant.

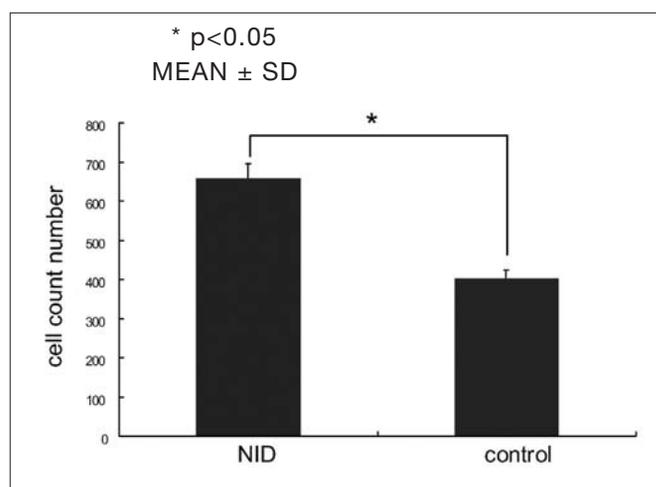


Fig. 5 - Number of retinal ganglion cells 7 days after ischemia-reperfusion injury.

DISCUSSION

The dihydropyridine-based calcium channel antagonists, such as NID and NIF, act preferentially on the vascular network causing vasodilatation at doses which have no effect on heart rate or cardiac contractility (5). These calcium channel antagonists have neuroprotective effects on the sensory retina especially RGCs in *in vivo* or *in vitro* studies.

Besides calcium channel antagonists, the beta-1-adenoreceptor antagonist, betaxolol, can reduce the effects of ischemia on the rat retina when applied topically just before the ischemic insult. They also stimulate the expression of brain-derived neurotrophic factor mRNA (15). Long-term unilateral topical betaxolol leads to increases in tissue blood velocity in the ipsilateral iris and optic nerve head of albino rabbits (16). Intravitreal injection of nipradilol, an $\alpha\beta$ -blocker, reduces the ischemia-reperfusion-induced retinal damage as assessed by electroretinographic and histopathologic observations (14).

Among the different calcium channel antagonists, NID has unique characteristics including increasing the blood velocity and blood flow in the optic nerve head, choroid, and retina of rabbits (17). It also increases the blood velocity in the optic nerve head of patients with normal tension glaucoma (16, 17). NID and lomerizine also increase the tissue blood flow in the optic nerve head of eyes injected with endothelin-1 inducing optic nerve head hypoperfusion (18). Among the calcium antagonists, dilti-

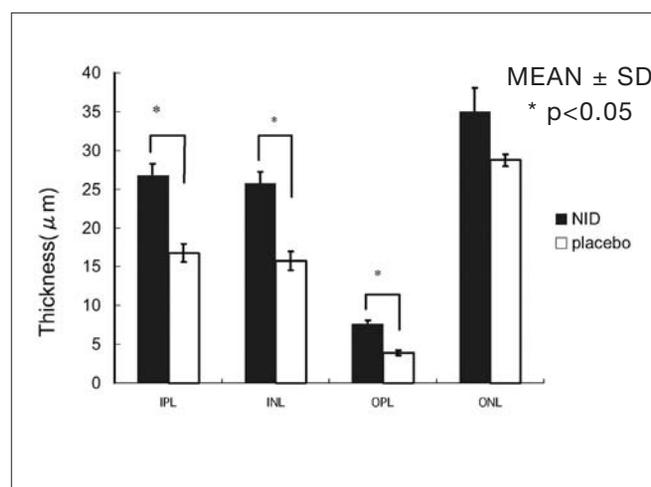


Fig. 6 - Mean thickness of the inner plexiform layer, inner nuclear layer, outer plexiform layer, and outer nuclear layer 7 days after ischemia-reperfusion.

azem, nicardipine, NID, and NIF, only intraperitoneal NID preserved the retinal morphology and electroretinographic responses of RCS rats during the initial stage of retinal degeneration (9).

Calcium-imaging experiments have shown that the glutamate-evoked calcium influx was blocked by NID in approximately 50% of the RGCs, and NID ameliorates glutamate neurotoxicity but NIF or diltiazem, another L type calcium channel blocker, does not prevent glutamate neurotoxicity (19).

NID is a highly lipophilic calcium channel antagonist and is widely distributed in different tissues including the brain in a pharmacokinetic study using [14 C] NID (20). The brain concentration of NID was higher than that of nicardipine after equivalent doses in rats (8). These findings suggest that NID can pass through the blood-brain barrier more easily than the hydrophilic forms.

The higher concentration ratio of NID in the retina to serum after systemic injection of NID and NIF indicated that NID had a greater permeability into the retina than NIF. In addition, the half-life of NID was longer. Thus, NID has better access to the retina, and this may account for the reduced number of apoptotic RGCs and the loss of nuclei in the IPL, INL, and OPL. The results also suggest that the highly lipophilic NID has a higher affinity for calcium channels.

The exact mechanism of the protective activity of NID in the retina has not been determined. However, previous studies have demonstrated that the neurotoxic effects of

low doses of glutamate in purified RGCs are partly blocked by NID but not NIF and diltiazem (19). They speculated this difference may be caused by a high affinity for calcium channel of the high lipophilic NID and NID showed antioxidant effects. Oxidized low-density lipoproteins have numerous effects on the cells of the arterial wall. NID protected low-density lipoprotein cholesterol from *in vivo* oxidation (21).

In addition to the neuroprotective effect of NID, one of the reasons NID has selective and long-lasting effects on the retina compared with other calcium entry blockers may be its high permeability from plasma to retina. This high permeability may cause the high retinal concentration.

In summary, our findings show that NID has high permeability to retina compared with NIF, which has less fat solubility than NID, and neuroprotective effect to retinal cells. NID might be useful for the treatment of glaucoma or other retinal diseases which have some relation to apoptosis.

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