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European Journal of Pharmacology 452 (2002) 263–268

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Nipradilol inhibits apoptosis by preventing the activation of caspase-3 via *S*-nitrosylation and the cGMP-dependent pathway

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Received 28 June 2002; accepted 23 August 2002

Abstract

To study whether nipradilol, which is used as an ophthalmic solution for the treatment of glaucoma, has a cytoprotective effect, we investigated its effect on the apoptosis induced by serum withdrawal in PC12 cells. Nipradilol has α1- and β-adrenoceptor-blocking and nitric oxide (NO)-donating properties. We also investigated the effects of timolol, prazosin and *S*-nitroso-*N*-acetylpenicillamine (SNAP) on PC12 cell death. Serum withdrawal from PC12 cells resulted in apoptosis, and the survival rate was decreased in a time-dependent manner. The addition of nipradilol to the medium showed a cytoprotective effect on PC12 cell death in a dose-dependent manner, but timolol and prazosin did not. We measured caspase-3 activity to clarify the mechanism of the inhibition of apoptosis in the presence or absence of dithiothreitol (DTT). The caspase-3 activity could be reactivated by DTT. In addition, to investigate the relationship of the cGMP-dependent pathway to the nipradilol-induced cytoprotective effect, we tested the effect of the protein kinase G inhibitor KT5823. KT5823 partially reversed the nipradilol-mediated cytoprotective effect. These results indicate that the cytoprotective effect of nipradilol in PC12 cell death was due to the caspase-3 inhibition mediated by NO-related *S*-nitrosylation and activation of protein kinase G.

Keywords: Nipradilol; PC12 cell; Apoptosis; Nitric oxide (NO); *S*-nitrosylation

1. Introduction

Nipradilol (3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2*H*-1-benzopyran) is used as an ophthalmic solution for the treatment of glaucoma (Kanno et al., 2000, 1998). It is known that nipradilol has non-selective β-adrenoceptor (Kawashima et al., 1984; Uchida et al., 1983) and selective α1-adrenoceptor antagonist effects (Kou et al., 1984; Ohira et al., 1985). This compound also has a vasodilating activity due to nitric oxide (NO) released from its nitroxy moiety (Adachi et al., 1995; Aniya et al., 1996; Hayashi et al., 1997). It is reported that nipradilol has a neuroprotective effect on *N*-methyl-D-aspartate (NMDA)-induced retinal cell damage and that effective concentrations of nipradilol reach the posterior retina after topical application (Mizuno et al., 2001). Mizuno et al. concluded that the protective effect of nipradilol was due to the NO released from the compound because both β- and selective ∂1-adrenoceptor antagonists had no protective effect. However, it is not clear how NO released from nipradilol prevents cell damage.

It is known that NO in some cells acts as an anti-apoptic agent (Beauvais et al., 1995; Genaro et al., 1995; Mannick et al., 1994). NO inhibits trophic factor deprivation-induced apoptosis in primary neurons (Estevez et al., 1998b) and PC12 cells (Anastasiadis et al., 2001) by the inhibition of caspase-3 activity. It is thought that the inhibition of caspase activity by NO is caused by two different mechanisms. One is by indirectly inhibiting caspase-3-like activation via a cGMP-dependent mechanism; the other is by direct inhibition through *S*-nitrosylation of cysteine residues in caspases (Kim et al., 1997). Generally, it is reported that NO can inhibit apoptosis by direct inhibition through protein *S*-nitrosylation (Li et al., 1997) as well as through cGMP-dependent pathways.

Our objective is to clarify the mechanism of the protective effect in nipradilol. We selected PC12 cells because the mechanisms of apoptosis induced by serum withdrawal and of apoptosis inhibited by NO donors are well documented. In this report, we suggest that the protective effect of

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PII: S0014-2999(02)02329-4

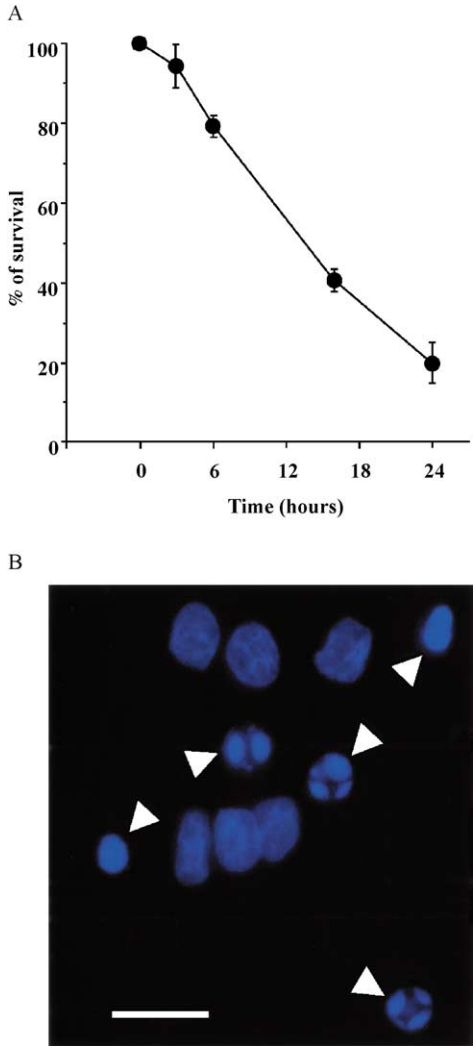

Fig. 1. PC12 cell death was caused by serum deprivation. (A) The time course of survival of the PC12 cells after serum deprivation. Percentage of survival was determined by counting intact nuclei. (B) The nuclear morphology of PC12 cells 24 h after serum deprivation. Arrows indicate nuclear fragmentation. Bar = 20 mm.

nipradilol is mediated through *S*-nitrosylation of caspase and activation of the protein kinase G by the NO nipradilol releases.

2. Materials and methods

2.1. Cell culture

RPMI 1640 and × 100 antibiotics (containing penicillin and streptomycin) were purchased from Life Technologies (Gaithersburg, MD). Undifferentiated PC12 cells were maintained in RPMI 1640 medium containing 10% horse serum and 5% fetal bovine serum with 2 mM glutamine and antibiotics in collagen-coated dishes at 37 °C with 95% air–5% CO_2.

2.2. Cell viability assay

PC12 cells maintained in RPMI medium containing 10% horse serum and 5% fetal bovine serum were washed with serum-free RPMI 1640 medium three times and replated onto collagen-coated 24-well plates at a density of 2×10^5 cells per well. Cells were treated with various concentrations of nipradilol, timolol maleate (a β-adrenoceptor antagonist, Wako, Osaka, Japan), prazosin hydrochloride (an α1-adrenoceptor antagonist, Wako) or *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) (Wako). We used 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazol-1-yloxy-3-oxide potassium (carboxy-PTIO, Wako) and KT5823 (protein kinase G inhibitor, Calbiochem, San Diego, CA) to investigate a mechanism of nipradilol-induced protective effect. To measure cell viability, cells were stained with 10 μM Hoechst 33258 (bisbenzimide; Molecular Probe, Eugene, OR) for 30 min at 37 °C and then fixed with 4% paraformaldehyde/0.1 M phosphate buffer (PBS). The cells were observed under a fluorescence microscope (Leica Q550) with an A4 filter (ex: BP360/40, em: BP470/40). Because Hoechst 33258 stains all nuclei, we could easily distinguish the apoptic cells, which have pyknotic or irregular shaped nuclei, from the viable cells. The nuclei were counted in a hematocytometer. Counts were performed on four wells.

2.3. Preparation of cell lysate

Cells were washed with ice-cold PBS and centrifuged. The lysis buffer (50 mM 2-hydroxyethypiperazine *N*-2-ethansulfonic acid (HEPES), pH 7.4, 5 mM 3-[(3-choramidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)) containing a protease-inhibitor cocktail for mammalian cells (Roche Diagnostics, Tokyo, Japan) was added to the cell

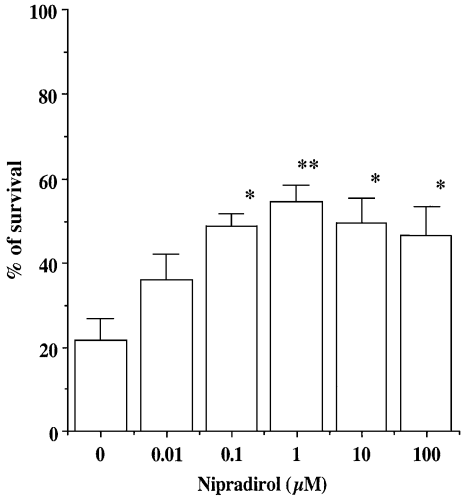

Fig. 2. Effect of nipradilol on PC12 cell death induced by serum withdrawal. Nipradilol has a cytoprotective effect at concentrations higher than 0.1 μM. $*P<0.05$, $**P<0.01$ compared to control group. Percentage of survival was determined by counting intact nuclei.

pellet, and it was kept on ice for 30 min. The lysed cells were centrifuged at 14,000 × *g* for 15 min at 4 °C. The supernatant was used for the measurement of caspase-3 activity and Western blot analysis. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL).

2.4. Caspase-3 activity

Activity of caspase-3 was measured by using a fluorogenic substrate peptide, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC; Sigma, St. Louis, MO). Cell lysate were incubated with 100 μM substrate peptide in 200 μl of incubation buffer (20 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM dithiothreitol (DTT) or not, 2 mM EDTA) at 37 °C for 60 min. The released AMC was measured by Spectra MAX Gemini (ex: 360 nm, em: 460 nm, Molecular Devices, Menlo Park, CA). Caspase-3 activity was expressed as nanomoles of AMC per minute per milligram of protein.

2.5. Western blot analysis

For the Western blot of cleaved caspase-3, cell lysates (30 μg protein) were separated on 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidine difluoride (PVDF) membrane. The membrane was hybridized with a cleaved caspase-3 antibody (1:1000, Cell Signaling Technology, Beverly, MA) and then washed with TBST (20 mM Tris–HCl, 500 mM NaCl, 0.05% Tween-20) three times. Alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1000, Promega, Madison, WI) was used as the second antibody. Protein bands were visualized using a Fluo-S MAX chemiluminescence system (Bio-Rad, Tokyo, Japan).

2.6. Statistical analysis

Statistical analysis was performed using StatView 4.11J software for Macintosh. Bonferroni/Dunn's multiple range test was used for planned comparisons among the various treatment groups. The level of significance was set at $*p<0.05$ and $**p<0.01$. Values are expressed as the mean ± S.D., and the p value is given for differences with the control group.

3. Results

PC12 cell death was caused by serum deprivation (Fig. 1A, B). The number of surviving cells showed a

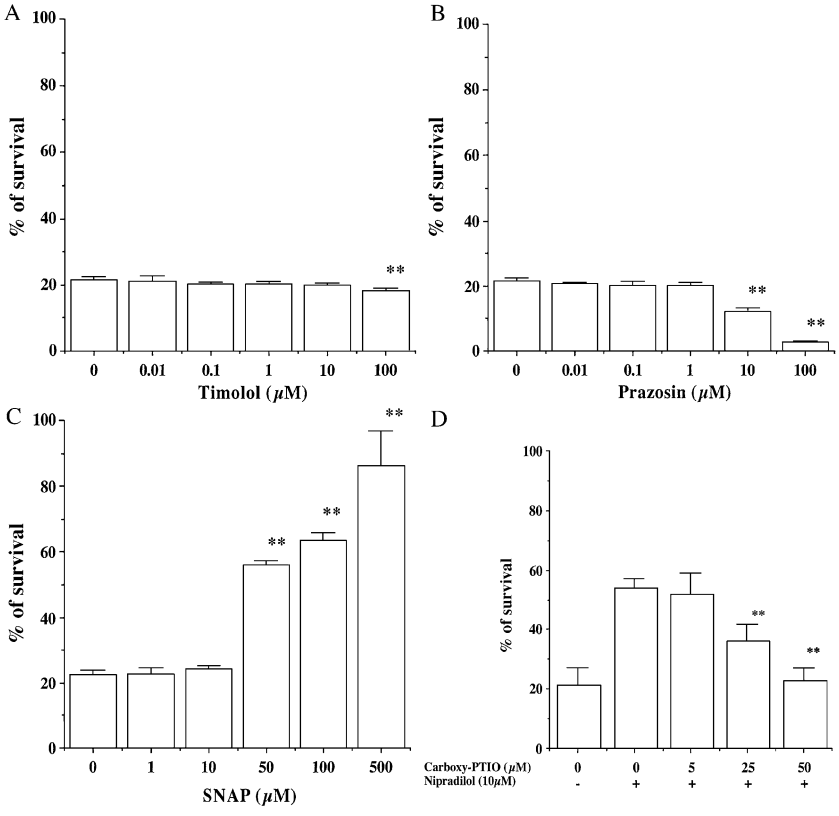

Fig. 3. Effect of timolol (A), prazosin (B), SNAP (C) and carboxy-PTIO (D) on PC12 cell death induced by serum withdrawal. The carboxy-PTIO was added to medium containing 10 μM nipradilol. Timolol and prazosin have a cytotoxic effect in a high-dose range. SNAP has a protective effect on PC12 cell death. The addition of carboxy-PTIO counteracts the protective effect of nipradilol in a dose-dependent manner. $*P<0.05$, $**P<0.01$ compared to the addition of nipradilol. Percentage of survival was determined by counting intact nuclei.

time-dependent decrease (Fig. 1A). The apoptic cells stained with Hoechst 33258 were easily identified by the nuclear morphology (Fig. 1B). Nipradilol prevented PC12 cell death, induced by serum deprivation in a dose-dependent manner (Fig. 2). It was previously known that nipradilol has nonselective β- and selective α1-adrenoceptor-blocking properties. In addition to these effects, nipradilol has a nitric-oxide-donating action. Therefore, we examined a nonselective β-adrenoceptor, timolol, a selective α1 adrenoceptor antagonist, prazosin, and a NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), to clarify the mechanism of the protective effect of nipradilol. Timolol and prazosin did not prevent PC12 cell death (Fig. 3A, B), but the NO donor SNAP showed a protective effect similar to nipradilol on PC12 cells in a dose-dependent manner (Fig. 3C). The addition of carboxy-PTIO to the medium containing 10 μM nipradilol inhibited the protective effect of nipradilol on PC12 cell death induced by serum withdrawal (Fig. 3D). These results suggest that the protective effect of nipradilol results from NO donation.

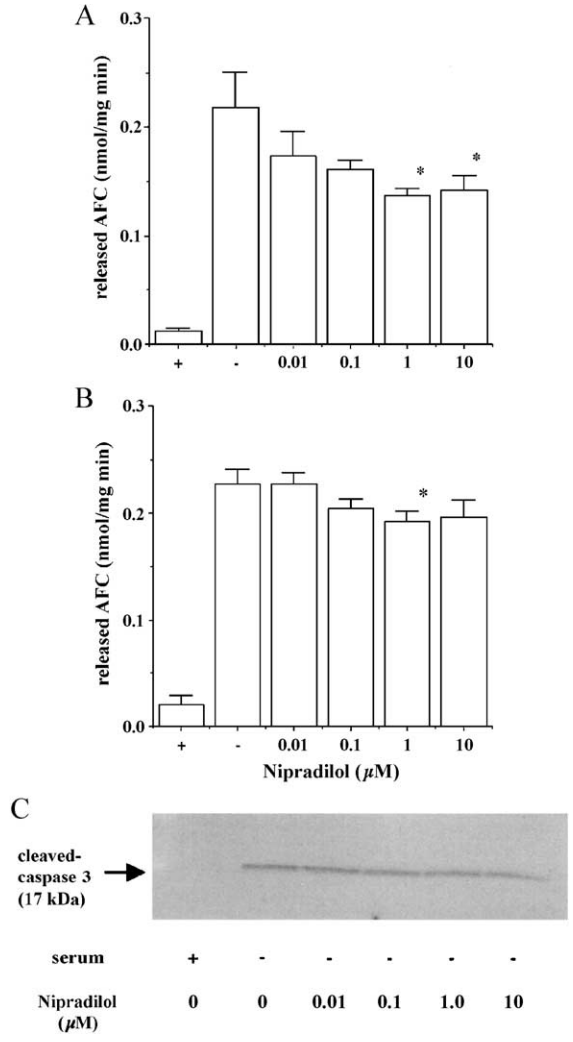

Fig. 4. Cytosolic caspase-3 in PC 12 cells 16 h after serum deprivation was investigated. Activity was measured without (A) or with dithiothreitol (DTT) (B) and Western blot analysis (C). The caspase-3 inhibited by *S*-nitrosylation can be reactivated by the addition of DTT. We measured caspase-3 activity with and without the presence of DTT. Western blot analysis showed that caspase-3 protein did not differ between the control- and nipradilol-treated groups. Arrow indicates cleaved caspase-3 (17 kDa).

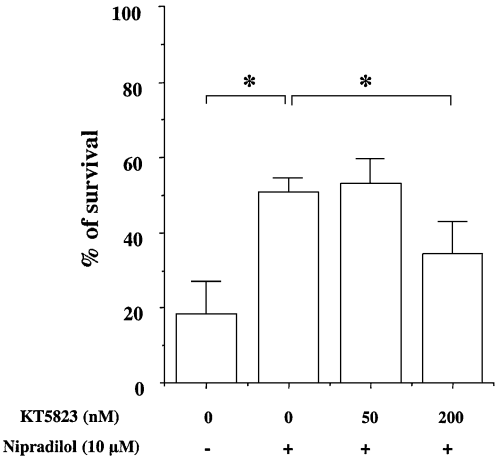

Fig. 5. Effect of KT5823 on nipradilol-induced cytoprotection. Protein kinase G inhibitor partially reversed the nipradilol-induced cytoprotective effect. (* $P<0.05$) Percentage of survival was determined by counting intact nuclei.

Because apoptosis in PC12 cells is induced by serum deprivation, which is related to the caspase cascade, we examined the caspase-3 activity with a fluorometric assay. The caspases can be reactivated by the addition of a reducing agent, such as DTT. To confirm the *S*-nitrosylation of nipradilol-mediated protection, we assayed caspase-3 activity with no DTT present. Caspase-3 activity in serum-deprived PC12 cells was 40 times that of the control group 16 h after serum deprivation. The increase of caspase-3 activity was slightly reduced by the addition of nipradilol with or without DDT (Fig. 4A, B). The addition of nipradilol to PC12 cells also slightly decreased caspase-3 cleavage in the Western blot analysis (Fig. 4C). These results indicate that the protective effect of nipradilol may be partially mediated through the inhibition of caspase-3 activity by NO-mediated *S*-nitrosylation. Finally, we tested the effect of the cGMP-dependent pathway on nipradilol-induced protection. The protein kinase G inhibitor KT5823 partially reversed the nipradilol-mediated cytoprotective effect (Fig. 5).

4. Discussion

This study achieved three goals. The first was to investigate whether nipradilol had a cytoprotective effect. The second was to identify the major mechanism of the protective effect of nipradilol because it has three effects: nonselective β-, and selective α1-adrenoceptor-blocking properties, and NO donation. The third goal was to clarify the mechanism of this effect of nipradilol.

Our results demonstrated that nipradilol had a cytoprotective effect on PC12 cell death induced by serum withdrawal and that this effect is due to nitric oxide it releases. We found that the cytoprotective mechanism of nipradilol has two pathways. One is the release of NO from nipradilol, which directly inhibits caspase-3-like activity via *S*-nitrosylation of cysteine residues in caspases, and the other is the cGMP-dependent mechanism.

The role of NO in cells is complex. Response to NO depends on cell type and cell conditions. NO causes apoptosis in mouse dendritic cells and differentiated PC12 cells (Heneka et al., 1998; Yamamoto et al., 2000). On the other hand, there are many reports that NO protects PC12 cells from apoptosis, and the mechanisms have been previously studied (Reimann-Philipp et al., 2001; Terwel et al., 2000). Specifically, NO protects motor (Estevez et al., 1998a,b) and sympathetic neurons including PC12 cells (Kim et al., 1999) from apoptosis.

Although nipradirol showed a cytoprotective effect on apoptosis induced by serum withdrawal in a dose-dependent manner, the protective effect of nipradilol reduced over a dose of 10 μM. Timolol, a nonselective β-adrenoceptor antagonist, had no effect, and the α1-adrenoceptor antagonist, prazosin, showed cell toxicity over 100 μM. The α1-blocking effect of nipradilol is about 100 times weaker than prazosin (Kou et al., 1984). The protective effect of nipradilol in a 10 or 100 μM may be affected by the α1-blocking properties of nipradilol. To confirm the role of NO in nipradilol-mediated protection, the effects of carboxy-PTIO on PC12 cells were examined in the presence of nipradilol. The addition of carboxy-PTIO inhibited the protective effect of nipradilol in a dose-dependent manner. These results showed the protective effect of nipradilol was due to NO released from the compound.

NO-related inhibition of apoptosis mediated through cGMP-dependent pathways, inhibition of Bcl-2 protein degradation and *S*-nitrosylation of caspases is reported. In PC12 cells, the major role of NO in inhibition of apoptosis is in the cGMP-dependent pathway (Beauvais et al., 1995; De Nadai et al., 2000; Estevez et al., 1998b; Wirtz-Brugger and Giovanni, 2000). Kim et al (1999) reported that a soluble guanylate cyclase inhibitor attenuated NO-mediated inhibition of apoptic cell death and that a cGMP analog, 8-bromo-cGMP, blocked apoptic cell death, caspase-3 activity and activation, and cytochrome *c* release. Therefore, we examined caspase-3 using active caspase-3 antibody in Western blotting. However, the amount of caspase-3 was almost unchanged in the nipradirol-added group and in the control group.

Therefore, we investigated the other mechanism of caspase inhibition, which is not related to protein level. Stamler et al. reported that NO can modify enzyme function by *S*-nitrosylation of protein thiol groups (Stamler, 1994; Stamler et al., 2001). The caspases are a family of cysteine proteases. Caspases are also reversibly inhibited by NO-related *S*-nitrosylation (Li et al., 1997). Our findings, which demonstrate the difference in caspase-3 activity with or without DTT, indicate direct *S*-nitrosylation of caspase catalytic cysteine residues by NO because DTT was able to reverse the NO inhibition. In addition, the protein kinase G inhibitor KT5823 partially inhibited the cytoprotective effect induced by nipradilol. These results suggest that nipradilol acts as a NO donor like *S*-nitroso-*N*-acetylpenicillamine. However, there were some differences in the protective effect between nipradilol and *S*-nitroso-*N*-acetylpenicillamine in our experiment that might be explained by the character of nipradilol. Nipradilol releases NO mediated by the intracellular enzyme, glutathione S transferase (Aniya et al., 1996). Therefore, it is thought that NO release by nipradilol is slower than that from NO donors such as *S*-nitroso-*N*-acetylpenicillamine and sodium nitro prusside. In our present study, apoptosis in PC12 cells was shown at 6 h after serum withdrawal, and about 80% of the cells had normal-featured nuclei. The caspase-3 activity of the cells 6 h after serum withdrawal was about 20 times that of the control (data not shown). One possibility is that NO release from nipradilol is too late to inhibit caspase-3 activation through a cGMP-dependent pathway.

Nipradilol is an ophthalmic solution for the treatment of glaucoma (Kanno et al., 2000, 1998). Axotomy and retinal ischemia model have been used as animal models for the study of neuronal cell death caused by glaucoma. It is reported that NO acts as a neuroprotective agent on ischemia-induced retinal cell death (Adachi et al., 1998a,b; Lam and Tso, 1996; Maynard et al., 1996), and in our previous study (Nakazawa et al., 2002). Our study showed that nipradilol has a cytoprotective effect mediated through NO release. These results indicate that nipradilol is an effective ophthalmic solution for patients with glaucoma not only because it reduces intraocular pressure but also because it protects neurons.

Acknowledgements

This research was supported in part by the Kowa Company, Ltd. (Tokyo, Japan) and by a Grant-in-Aid for Scientific Research (no. 13771009) from the Ministry of Education of the Japanese Government.

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