α2-Adrenoreceptor Agonists Are Neuroprotective in a Rat Model of Optic Nerve Degeneration

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PURPOSE. The neurodegenerative progression of glaucoma is considered to be related not only to primary risk factors such as the elevation of intraocular pressure, but also to mediators of secondary neuronal degeneration. In the present study, the neuroprotective activity of the α2-adrenoreceptor agonists brimonidine, AGN 191103, and clonidine were examined in an animal model that simulates secondary neuronal degeneration of the optic nerve in a way thought to be independent of elevation of intraocular pressure. The β-blocker timolol, currently used clinically to decrease intraocular pressure, was also examined for neuroprotective activity at dosages corresponding to the effective antihypertensive dosage.

METHODS. A single dose of each of the tested compounds was administered intraperitoneally immediately after partial crush injury of the rat optic nerve. Secondary degeneration was measured by determining injury-induced deficits with and without the drug. This was achieved electrophysiologically by measurement of compound action potential amplitude, and morphometrically by counting the retrogradely labeled retinal ganglion cells, representing viable optic nerve axons, in wholemounted retinas.

RESULTS. All three α2-adrenoreceptor agonists, but not timolol, exhibited neuroprotective effects. Treatment immediately after injury with each of these agonists resulted in a dose-dependent attenuation of the injury-induced decrease in compound action potential amplitude. Moreover, after treatment with 100 μg/kg brimonidine administered intraperitoneally, the loss of retinal ganglion cells 2 weeks after injury was three times lower than in saline-treated animals.

CONCLUSIONS. In addition to their known effect of lowering intraocular pressure, α2-adrenoreceptor agonists, unlike timolol, exert a neuroprotective effect. Use of the rat optic nerve model of partial crush injury can serve as a method of screening compounds that are potentially capable of alleviating the progression of secondary neuronal degeneration. (Invest Ophthalmol Vis Sci. 1999; 40:65–73)

Chronic progressive loss of retinal ganglion cells (RGCs) is a predominant pathophysiological mechanism in primary open-angle glaucoma (POAG). Recent research suggests that the neurodegenerative process in POAG may be similar to the process of secondary degeneration described in the context of traumatic injuries to the central nervous system (CNS). After CNS nerve injury, there is elevation in toxic substances in the nerve’s extracellular milieu that can cause the eventual degeneration of neurons that escaped the primary trauma or were only marginally affected by it. Drugs that are capable of neutralizing the toxicity of such substances, competing with their activities, or increasing the resistance of any remaining viable neurons to the stressful conditions can be considered to have neuroprotective potential.

In the present study, on the assumption that the processes of secondary degeneration of neurons after injury of the optic nerve are likely to involve pathways and mediators similar to those in brain trauma, we examined in the rat optic nerve the neuroprotective potential of the α2-adrenoreceptor agonists. Such agonists were chosen as potential neuroprotections in the optic nerve because they have been shown to reduce excitability in the damaged brain, and their receptors are known to be widely distributed in the retina and anterior segment of the eye. Moreover, because they are effective intraocular pressure-lowering agents in animals and humans they seemed to be even more appealing in the context of optic nerve damage than in the damaged brain.

The α2-adrenoreceptor agonists clonidine and dexmedetomidine exhibit anti-ischemic and neuroprotective effects in focal and global cerebral ischemia. α2-Adrenoreceptor activation has been shown to couple with K+ activation, leading to neuronal hyperpolarization, reduced excitability, and release of neurotransmitters during ischemia. More recently, clonidine and xylazine were shown to protect photoreceptor cells from light damage.

To facilitate the screening of compounds for their potential neuroprotective ability in the optic nerve, we have developed a model of acute partial-crush injury of the rat optic nerve. In this model, neuronal damage resulting from the acute injury triggers over 2 to 4 weeks (in the absence of any further external insult) the subsequent (secondary) degeneration of axons that escaped the acute injury or were only marginally damaged by it. The model has been used to study...
Animals were handled according to the ARVO Statement on the pathophysiology of optic nerve and RGCs. Thus for example, mediators of toxicity such as glutamate and nitric oxide, both found in the brain after injury, were also found to be elevated in the eyes of rats after partial lesion of their optic nerves. It was therefore not surprising to find that compounds shown to be efficacious after brain trauma, such as MK-801, a blocker of the N-methyl-D-aspartate receptor pathway, could reduce the progression of neuronal loss when tested in this model.17

The compounds tested here were selected for their high affinity and selectivity for the α2-adrenoceptors, rather than α1-adrenoceptors. Brimonidine and AGN 191103 are agonists that are selective for α2-adrenoceptors but not α1-adrenoceptors.18,19 Clonidine has a high affinity for the α2 receptor, but differs from brimonidine in that it has a lower degree of selectivity for α2 over α1, and is a partial agonist with a different pharmacodynamic profile.20,21

We showed in the present study that the α2-adrenoceptor agonists brimonidine, AGN 191103, and clonidine can attenuate neuronal damage after optic nerve injury. Specifically, we found that brimonidine activates an α2 pathway that results in the protection of axons from degeneration and that this protective effect is blocked by the α2-adrenoceptor antagonist rauwolscine.

METHODS

Animals

Animals were handled according to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Male Sprague-Dawley rats weighing 300 to 400 g from the Weizmann Institute of Science animal house were anesthetized with intraperitoneal (IP) injection of 50 mg/kg ketamine and 0.5 mg/kg xylazine. Before tissue excision the rats were killed by an overdose (170 mg/kg) of IP sodium pentobarbitone. Clonidine was purchased from RBI (Natick, MA), and timolol was obtained from Sigma (St. Louis, MO). Brimonidine and AGN-191.103 were obtained from Allergan (Irvine, CA).

Crush Injury

With the aid of a binocular-operating microscope, lateral canthotomy was performed in the right eyes of rats. The conjunctiva was incised laterally to the cornea, the retractor bulbi muscle was separated, and the optic nerve exposed. Using a calibrated cross-action forceps, a moderate injury was inflicted on the nerve 2 mm from the globe, avoiding the retinal blood supply. The retina was observed after crush to ensure that there was no retinal ischemia.

Electrophysiological Measurements

Electrophysiological recordings from the rat optic nerves were obtained 14 days after the injury. The experimental setup was as described by Assia et al.22 Rats were deeply anesthetized with 170 mg/kg pentobarbitone, the skin was removed from the skull, and the optic nerve was detached from the eyeball. Subtotal decapitation was performed, and the skull was opened with a rongeur. The cerebrum was displaced laterally, exposing the intracranial portion of the optic nerve. Dissection at the level of the optic chiasm allowed removal of the entire nerve, which was transferred to a vial containing fresh saline solution consisting of 126 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 2 mM MgSO4, 2 mM CaCl2, and 10 mM D-glucose, aerated with 95% O2 and 5% CO2 at room temperature. The nerves were kept in this solution, in which their electrical activity remained stable for at least 3 to 4 hours. After 30 minutes of recovery at room temperature, electrophysiological recordings were performed. The nerve ends were connected to two suction Ag-AgCl electrodes immersed in the bathing solution at 37°C. A stimulating pulse was applied through the electrode at the proximal end, and the action potential was recorded by the distal electrode. A stimulator (SD9; Grass, Quincy, MA) was used for supramaximal electrical stimulation at a rate of 0.5 pulses per second. The solution, stimulator, and amplifier had a common ground. The compound action potential (CAP) was amplified 100 times by a microelectrode alternating current amplifier (model 1800; AM Systems, Everett, WA) and digitized (12 bits; 5000 samples/sec), using a multifunction data acquisition I/O board for Nusus (model MO169; National Instruments, Austin, TX) and a data acquisition and management system (LabView 2.2.1.; National Instruments).

Retinal Ganglion Cell Labeling Procedure

Degeneration was assessed morphometrically by retrograde labeling of cell bodies, by applying a dye distally to the site of the lesion. Provided the axons are intact, dye taken up at the site of application could be transported along the axon to the cell body; thus, only those cell bodies with intact axons could be reached and labeled by the dye. The primary neuronal loss (i.e., the immediate loss resulting from the acute injury) was determined in one group of animals by retrograde labeling immediately after injury. Secondary degeneration, with or without drug treatment, was determined by applying the dye at the corresponding site 2 weeks after the injury. Application of the dye involved re-exposure of the nerve without damaging the retinal blood supply. Solid crystals (0.2–0.4 mm in diameter) of the neurotracer dye 4-Di-IO-Asp were deposited 1 mm from the distal border of the injury site. Noninjured optic nerves were similarly labeled. One week later, 170 mg/kg of pentobarbitone was administered to the animals. The retinas were detached from the eye and prepared as flattened whole mounts in 4% paraformaldehyde solution, and the labeled ganglion cells were counted using fluorescence microscopy. Five randomly selected fields, located at approximately the same distance from the disc, were counted from each retina. In some experiments, topographical mapping was monitored by selecting five fields from each zone and obtaining their average cell count.

RESULTS

Electrophysiological Analysis of the Effects of Brimonidine, AGN 191,103, and Clonidine

The measured CAP amplitude was directly proportional to the number of excitable fibers in the nerve: the greater the number of excitable fibers, the greater the amplitude. The effects of different drug dosages on the CAPs of the excised nerves were examined in the rat optic nerve model 2 weeks after partial crush injury and treatment with a single IP injection of 3 μg/kg to 100 μg/kg brimonidine, 1 μg/kg to 30 μg/kg AGN 191103, or 0.1 μg/kg to 30 μg/kg clonidine. The effects of different doses of the α2-adrenoceptor agonists on CAP amplitudes are...
Figure 1. Dose-dependent effect of brimonidine on the compound action potential (CAP) of injured optic nerves. Brimonidine at different dosages was injected intraperitoneally immediately after injury. Results are expressed as mean values of CAP amplitude ± SEM for each dosage, measured from the excised nerve 2 weeks after injury and treatment. * Significant difference (P < 0.01) compared with the vehicle-treated group.

Figure 2. Dose-dependent effect of clonidine on the compound action potential (CAP) of partially crush-injured optic nerves. Clonidine at different dosages was injected intraperitoneally immediately after injury. Results are expressed as mean values of CAP amplitude ± SEM for each dosage, measured from the excised nerve 2 weeks after injury and treatment. * Significant difference (P < 0.05) compared with the vehicle-, 10-μg/kg-, and 30-μg/kg-treated groups.
Dose of AGN-191103 (µg/kg)

FIGURE 3. Dose-dependent effect of AGN 191103 on the compound action potential (CAP) of partially crush-injured optic nerves. AGN 191103 at different dosages was injected intraperitoneally immediately after injury. Results are expressed as mean values of CAP amplitude ± SEM for each dosage, measured from the excised nerve 2 weeks after injury and treatment. * Significant difference (P < 0.05) compared with the vehicle-treated group.

described by bell-shaped curves (Figs. 1, 2, 3). The effect of all three agonists was significant according to analysis of variance (brimonidine, F = 2.725, P = 0.01; clonidine, F = 6.874, P = 0.0004; AGN 191103, F = 9.061, P = 0.0001). The optimal concentration of brimonidine was 100 µg/kg (Fig. 1); at the other concentrations tested, this drug did not significantly affect CAP amplitudes. Significant effects on CAP amplitude were exerted by AGN 191103 at concentrations of 10 µg/kg to 30 µg/kg and by clonidine at 0.1 µg/kg to 1 µg/kg; however, clonidine, even at its maximally effective dose, was less efficacious than brimonidine or AGN 191103. The maximum CAP amplitude with clonidine treatment was 482 ± 35 µV, compared with 863 ± 197 µV with brimonidine and 1406 ± 399 µV with AGN 191103.

Morphometric Measurement of the Effect of Brimonidine on Secondary Degeneration

The primary and secondary effects of the crush injury and the effects of treatment with the different drugs were also measured morphometrically. For this purpose, the neurotracer dye 4-Di-10-Asp was applied distally to the site of the lesion immediately after the injury (to determine primary damage) or 2 weeks after injury and treatment (to determine secondary degeneration). Because, as indicated in the Methods section, fibers damaged either directly or indirectly by the crush injury cannot transport the dye all the way back to their cell bodies, the number of labeled RGCs is a reflection of the number of intact axons. Immediately after partial crush injury, the animals were divided into four groups and IP injections were administered containing brimonidine, saline, rauwolscine HCl (an α2-adrenoceptor antagonist) or brimonidine together with rauwolscine HCl. The results of this experiment are shown in Figure 4. Analysis of variance indicated a significant effect of treatment on the number of surviving neurons at 2 weeks, measured by the number of retrogradely labeled RGCs (F = 4.1, P = 0.01). The number of surviving neurons in the group treated with brimonidine alone was significantly higher (P < 0.05) than those of the other groups. These findings show not only that brimonidine acted as a neuroprotective agent, but also that its protective effect could be blocked by the use of a specific α2-adrenoceptor antagonist, which did not itself affect neuronal survival. It should be noted that immediately after the crush injury, approximately 42% of the axons were found to have survived. Two weeks later, there was only approximately 8% survival in the vehicle-treated group, whereas survival in the brimonidine-treated group was approximately two to three times higher. No differences were observed in the intraocular pressure of eyes of injured nerves treated with vehicle only or brimonidine, or between eyes of noninjured nerves treated with brimonidine or untreated.

Relation between Brimonidine Protection and RGC Distribution in the Retina

After optic nerve injury, the rate of degeneration is reportedly higher in neurons whose cell bodies are located at the retinal periphery than in those with cell bodies at the center.29 pre-
Effects of brimonidine and its antagonist rauwolscine on neuronal survival after partial crush injury of the optic nerve. Optic nerves were moderately crushed, and the animals were immediately treated systemically with the indicated compounds. Two weeks later, spared axons were labeled by application of a dye distally to the primary crush injury. One week later, retinas were excised, and the labeled cells were counted. Results are expressed as means ± SEM of stained retinal ganglion cells (RGCs) per square millimeter of retina (n = 3–5).

Summarily because of differences in cell body vulnerability, mapping of RGC distribution in the retinas of animals 4 weeks after optic nerve crush injury and treatment with brimonidine or vehicle yielded the distribution patterns shown in Figures 5 and 6. Ganglion cell loss was more pronounced at the periphery than close to the optic disc. Brimonidine treatment not only contributed to the increased survival of axons but also affected the distribution pattern of surviving ganglion cells in the retina (Fig. 6A). Calculation of the index of protection, which was defined as the ratio between the numbers of RGCs in retinas of brimonidine-treated and of vehicle-treated injured nerves (Fig. 6B), indicated that the protective effect of brimonidine treatment was more pronounced in neurons whose cell bodies were located at the retinal periphery.

Comparison of the Effects of Brimonidine and Timolol on Neuronal Survival

Morphometric analysis was performed 2 weeks after crush injury and drug treatment to compare the ability of brimonidine to protect the nerve from secondary injury with that of another intraocular pressure-lowering drug, timolol (Fig. 7A). In calculating secondary degeneration, the axons that survived the primary crush (i.e., the number of labeled RGCs counted immediately after the crush) were considered to represent 100% survival, or zero secondary degeneration (Fig. 7B). By 2 weeks after injury and drug treatment, nerves in rats treated with 100 μg/kg brimonidine showed significantly less secondary degeneration than did those in saline-treated control animals.
subjects (P < 0.001). Timolol was tested at doses 10 times higher than the tested dosages of brimonidine, corresponding to their effective dosages as antihypertensive drugs. Timolol had no neuroprotective effect at any of the 3 dosages tested (Fig. 7B). The effect of brimonidine differed significantly (P < 0.01-0.001) from that of timolol at all three dosages. Timolol was tested at doses corresponding to those causing systemic hypotensive effects.

Figure 6. Ganglion cell distribution in retinas with injured optic nerves, 4 weeks after injury and immediate treatment with vehicle or brimonidine. Retinas were divided into five circular zones on the basis of their distance from the center (optic disc). A. 0 to 800 μm; B. 800 to 1600 μm; C. 1600 to 2400 μm; D. 2400 to 3200 μm; E. 3200 to 4000 μm. (A) Average number of retinal ganglion cells (RGCs) per zone, calculated by counting them in four fields in each zone. The graph depicts the means ± SEM of three animals treated with brimonidine and four animals treated with vehicle. (B) Index of protection from secondary degeneration in each zone. The most pronounced effect is seen in the most peripheral zone. The index is the ratio between the RGCs in brimonidine-treated and untreated nerves in each zone.
DISCUSSION

The results of this study show that the α2-adrenoreceptor agonists brimonidine, clonidine, and AGN 191103 reduce (or at least delay) the secondary loss of neurons after partial crush injury of the optic nerve. Partial injury of the optic nerve results in an immediate (primary) degeneration of neurons whose axons were directly damaged, and the delayed (secondary) degeneration of neurons whose axons either escaped the injury or were only marginally damaged. The secondary degeneration may be an outcome of the hostile environment created by the severely damaged neurons undergoing primary degeneration. Although degeneration of the directly injured neurons is inevitable, we have shown in this model, by using compounds shown to be neuroprotective after brain injury, that the undamaged and the marginally damaged neurons are amenable to treatment that reduces or delays their degeneration. Among the factors identified as mediators of secondary neuronal degeneration in the CNS are excitatory amino acids such as glutamate and aspartate, free radicals, nitric oxide, and high levels of potassium and calcium ions. Elevated levels of glutamate have been detected in the aqueous humor of the rat 3 to 7 days after partial crush lesion of the optic nerve, before returning to normal 14 days later. Elevated glutamate levels have been observed in the vitreous of glaucoma patients, and increased amounts of nitric oxide synthase have been detected in the optic nerve head of patients with open-angle glaucoma. High levels of extracellular glutamate and nitric oxide are thought to be important causes of secondary death of cells that have escaped initial injury in CNS trauma. In the visual system, chronic low-level glutamate elevation has been found to kill 42% of RGCs after 3 months. Loss of RGCs in optic neuropathies such as POAG is the pathologic basis for loss of retinal function and vision.

In view of our previous finding of mediators of toxicity in eyes with crushed optic nerves, and because the delayed degeneration demonstrated in the model has been shown to be amenable to neuroprotective treatment, we examined a new family of potentially neuroprotective compounds, for which abundant receptors are known to exist in the retina. The results of the present study show that 0.1 mg/kg brimonidine, injected IP immediately after optic nerve injury, protected spared optic neurons from secondary degeneration (Figs. 1, 3). The neuroprotective effect was evident from the enhanced survival of RGCs and nerve fibers observed 2 weeks after injury in brimonidine-treated rats compared with those in untreated control subjects. Preliminary pharmacokinetic data showed vitreal concentration of 100 nM to 200 nM after IP injections of brimonidine (unpublished data). Clonidine was less effective than brimonidine. Both compounds have approximately the same affinity for the α2-adrenoreceptor. It should also be noted that all three of the tested α2-adrenoreceptor compounds showed decreased activity at higher doses. This type of dose-response curve has also been observed in other classes of neuroprotective agents, such as HU-211, in the rat optic nerve partial-crush injury model and in other models of neuronal survival.

The neuroprotective activity of brimonidine was blocked by the α2-adrenoreceptor antagonist rauwolscine. This finding suggests that the retina contains an α2-adrenoreceptor pathway through which the survival of injured neurons can be
enhanced. A number of such pathways are possible. α2-Adrenoceptors could couple to and activate a PI3K pathway, that has been suggested to be an important signal inhibiting apoptosis. α₂-Adrenoceptor agonists have also been shown to be capable of inducing phosphorylation of mitogen-activated protein kinase in the retina. α₂-Adrenoceptor agonists can also induce hyperpolarization. Under ischemia and trauma conditions cellular energy is decreased, ion gradients are not maintained, and the nerve membrane potential becomes more depolarized and can release glutamate pathologically. Under such conditions, α₂-adrenoceptor agonists would presumably cause a decrease in the release of glutamate from neurons. Clonidine and brimonidine can upregulate the neuronal cell survival agent, basic fibroblast growth factor. It is not yet known which, if any, of these pathways may be operative in reducing secondary neuronal loss. The use of α₂ agonists should be tested in other models of neuronal injury (e.g., retinal ischemia) to determine the scope of the neuronal survival pathways activated by α₂-adrenoceptors. In view of the already known activity of the α₂ agonists as antihypertensive drugs, we tested in the present study another antihypertensive compound not from the same family to find whether the two activities are related. We found that the second antihypertensive drug timolol had no neuroprotective effect, suggesting that the two activities were not necessarily linked. Human testing and confirmation of the neuroprotective activity of α₂ agonists such as brimonidine, may lead to the development of a new class of drugs for the treatment of glaucoma that not only lower intraocular pressure, but are also neuroprotective.

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References


