The Effects of FK506 on Retinal Ganglion Cells after Optic Nerve Crush

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PURPOSE. The purpose of this study was twofold: to determine whether immunophilins were present in the rat retina and to determine the physiologic consequence of their presence.

METHODS. Reverse transcription–polymerase chain reaction (RT-PCR) and Western blot analysis were performed on rat retinal tissue, and the immunophilin FKBP12 was found to be present in retina. Immunohistochemical studies showed the presence of FKBP12 in retinal ganglion cells (RGCs). In rats, optic nerve crush was performed on one side and a sham operation on the other side. By gavage, animals were given 5 mg/kg per day of the FKBP12 ligand FK506 in sterile phosphate-buffered saline (PBS) or in PBS alone. Eight days after nerve crush, the total number of back-labeled RGCs was estimated from retinal wholemounts.

RESULTS. In control eyes, the number of labeled ganglion cells was 74,104 ± 4,166 (mean ± SEM) in rats receiving vehicle and 74,993 ± 3,098 in animals receiving FK506 daily. Eight days after optic nerve crush, 27,775 ± 3,332 labeled ganglion cells were counted in retinas of animals receiving vehicle (n = 11), whereas 33% more ganglion cells (37,118 ± 2,475) were counted in animals receiving FK506 daily (n = 11). This difference was statistically significant (P < 0.05).

CONCLUSIONS. The data presented demonstrate that the immunophilin FKBP12 is present in retina and specifically in RGCs. In addition, the FKBP12 ligand FK506 confers neuroprotection on RGCs after optic nerve crush. This neuroprotection may occur as a result of FK506’s ability to interfere with apoptotic mechanisms after optic nerve crush. (Invest Ophthalmol Vis Sci. 2000;41:1111–1115)

Optic nerve crush is a well established model of delayed retinal ganglion cell (RGC) death. Both glutamate-mediated excitotoxic and apoptotic mechanisms have been proposed. Accordingly, neuroprotective effects have been observed in the optic nerve crush model with pharmacologic agents that block excitotoxicity (e.g., MK-801) with growth factors, and with genetic manipulations such as overexpression of the antiapoptotic factor Bcl-2. In the present study, we explored whether the immunophilins, a novel class of immune modulators also expressed in the central nervous system, might also play a role in modulating RGC death after optic nerve crush.

Immunophilins, including the prototypical molecule FK506-binding protein (FKBP12), are a large group of small cytoplasmic proteins, characterized by their ability to bind and mediate the actions of FK506 and related immunosuppressant agents. They were first described in immune-competent cells such as T cells. FK506 is in widespread clinical use after organ transplantation. The FK506–FKBP12 complex inhibits the phosphatase activity of calcineurin, a Ca2+-calmodulin–dependent phosphatase. This leads to the decreased dephosphorylation of nuclear factor of activated T-cell (NF-AT) preventing its entry into the nucleus and the production of interleukin-2.

Steiner et al. reported in 1992 that immunophilins are also very abundant in the nervous system, although their role in the brain and peripheral nervous system remains uncertain. Recent data suggest that FK506 has properties that modulate neural damage in a variety of paradigms. FK506 reduces ischemic brain damage after middle cerebral artery occlusion when administered up to 60 minutes after occlusion. Also, FK506 has been shown to increase the levels of axotomy-induced growth-associated protein (GAP43) mRNA in dorsal root ganglion, decrease the activation of microglia–macrophages in the white matter of the optic nerve after chronic cerebral ischemia, and promote protection against white matter lesions. Recent data have also shown that FK506 protects hippocampal neurons in culture against glutamate-induced excitotoxicity and apoptosis.

Although immunophilins have been demonstrated in the brain and peripheral nervous system, the presence of FKBP12 in retina has not yet been examined. We present 4 lines of evidence that FKBP12 is present in retina and is physiologically active: detection of mRNA by reverse transcription–polymerase chain reaction; detection of a 12-kDa band on Western blot analysis; immunostaining of RGCs with FKBP12 monospecific antisera; and pharmacologic protection by FK506 against RGC death after experimental optic nerve crush.

MATERIALS AND METHODS

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
RNA Isolation and Reverse Transcription–Polymerase Chain Reaction

Rats were killed with an overdose of chloral hydrate and transcardially perfused with phosphate-buffered saline (PBS). The retinas and brain were removed and immediately stored at −80°C. Total cell RNA was isolated from retina and brain and reverse transcribed into cDNA (Reverse Transcription System; Promega, Madison, WI), according to the manufacturer’s instructions, using 1 μg isolated mRNA. Polymerase chain reaction (PCR) was then performed using oligonucleotide primers. Forward primer 5'-GAG AAA AGC CAG CAT AAA GC-3' and reverse primer 5'-TCT AGA ACT TCG GTG GAA AG-3' were used to amplify 321 bp of the FKBP12 cDNA gene. The PCR product was then subjected to electrophoresis on a 3% agarose gel, and fragments were visualized by ethidium bromide staining.

Protein Isolation and Western Blot Analysis

Animals received overdoses of chloral hydrate and were transcardially perfused with PBS. Eyes were enucleated, retinas removed, and retinas and brain immediately stored at −80°C. Rat cortex and rat retina were homogenized in 50 mM Tris (pH 7.6), 150 mM NaCl, 12 mM CHAPS, 2 mM EDTA, 1% Nonidet P-40, 200 μM phenylmethylsulfonyl fluoride. Human cortex protein (the generous gift of Bradley Hyman, Boston, MA) was prepared as previously described. Total protein was measured by the bicinchoninic acid assay (Sigma, St. Louis, MO). Protein isolated from approximately one half of one retina was analyzed in immunoblots. Human brain extract (50 μg), rat brain extract (100 μg), and rat retinal extract (150 μg) were separated by 10% to 20% polyacrylamide gel electrophoroses (Tris-Tricene gel; Novex, San Diego, CA) and transferred to a membrane (Immobilon-P; Millipore, Bedford, MA) The membrane was probed with a rabbit polyclonal anti-FKBP12 antibody (1:2000, Affinity Bioreagents, Golden, CO) and developed by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunohistochemistry

Animals were killed as described and perfused with PBS followed by 4% paraformaldehyde (PFA). Eyes were enucleated and placed in 4% PFA/3% sucrose solution overnight at 4°C. Eyes were then incubated in 5%, 10%, 15%, and 20% sucrose for 60 to 90 minutes each, embedded in optimal cutting temperature compound, and maintained at −80°C. Cryostat sections (16-μm thick) were cut and sections mounted onto slides (SuperFrost; VWR, West Chester, PA). Mounted sections were soaked in 0.5% Triton X-100 in Tris-buffered saline (TBS) for 20 minutes and rinsed three times in TBS. They were then incubated for 1 hour, with shaking, in 3% nonfat dry milk in TBS. After one rinse in TBS, slides were incubated in 1.5% normal goat serum in TBS with a polyclonal, rabbit anti-FKBP12 antibody (1:500; Affinity Bioreagents) at 4°C overnight in a humidified chamber. The slides were rinsed three times in TBS for 5 minutes each and incubated in goat anti-rabbit Cy3 antibody (1:250; Jackson ImmunoResearch, West Grove, PA) in a humidified chamber for 1 hour at room temperature. Slides were then rinsed again three times in TBS for 5 minutes each, allowed to dry, and coverslipped with antifade medium (Slowfade; Molecular Probes, Eugene, OR), according to the manufacturer’s directions. In negative control experiments, primary antibody was omitted. Sections were viewed with a confocal microscope (model 1024; Bio-Rad, Hercules, CA).

Optic Nerve Crush

Adult male Wistar rats (250–300 g) were anesthetized with a cocktail of ketamine-xylazine-acepromazine and their intraorbital optic nerves exposed after a lateral canthotomy and limbal peritomy. Three millimeters from the globe, on one side only, the nerve was crushed in three 10-second intervals with no. 5 jeweler’s forceps. The tissues were repositioned and antibiotic ointment applied to the eye. The retinal vasculature was then checked by direct ophthalmoscopy, and those animals with interrupted blood supply were excluded. A sham operation was performed in the fellow eye exposing the optic nerve but not crushing it. Two days before death, the RGC were back-labeled using a modification of the technique reported by Koeberle and Ball for axotomy. Each optic nerve was re-exposed and the dural sheath incised longitudinally 1 mm from the globe in both control eyes and eyes that had received an optic nerve crush. Crystals of fluorescein dextran (3000 MW, Molecular Probes) were applied to the exposed optic nerve, and a slurry of crystals dissolved in 10% dimethyl sulfoxide in water was injected into the optic nerve. A piece of gelfoam soaked in this mixture was then applied to each optic nerve, the tissues repositioned, antibiotic ointment applied, and the animal allowed to recover.

Eight days after optic nerve crush, the animals received an overdose of chloral hydrate intraperitoneally and were transcardially perfused with PBS and then 4% PFA in 0.1 M PBS. The eyes were enucleated and soaked in 4% PFA for 30 minutes. The retinas were then removed, flattened on slides, and coverslipped with antifade medium. Retinal wholemounts were counted in a masked fashion.

Sterologic Assessment of RGC Number

Estimates of total RGC number were performed using statistically unbiased stereologic protocols chosen to be advantageous for use in circumstances in which there is heterogeneity among different subregions of the structure under study. In the retina, for example, there are substantial differences in the density of RGCs between the central retina and the periphery, without clear cutoffs identifying individual subregions. Three stereologic tools were used. Sampling was performed using a systematic random sampling protocol that ensured that each part of the retina had an equal, but random, chance of being sampled. A grid was placed over each retina, and 1 of 66 grids was assessed for estimating the total number of back-labeled RGC. The location of the first grid to be counted was determined by choosing the first location between 1 and 66 using a random number table; thereafter, every 66th grid was assessed throughout the entire retina. A stereologic counting chamber with extended exclusion lines was used to perform the counts. This ensured that cells were not double counted, by excluding cells that intersected with two (e.g., top and right) lines of the counting chamber but counting cells that intersected with the other two lines (e.g., bottom and left). The exclusion lines are extended to avoid double counting of curved features. The fractionator approach was taken to calculating total RGC number. By this method, the average number of RGCs counted per counting chamber is multiplied by the total number of possible counting chambers in the structure.
The fractionater provides estimates that are unaffected by shrinkage before, during, and after processing of the tissue. These counting rules do not presuppose any assumptions about the size, shape, or distribution of RGCs in the retina and therefore avoid potential biases in counting. The sampling intensity was targeted to yield a precision of the individual estimates of approximately 5%, in accord with the stereologic literature. Using our protocol, the counting precision, or the coefficient of error, is 4%. This low coefficient of error suggests that the sampling strategy was sufficient to estimate the total number accurately.

Drug
FK506 (Fujisawa, Deerfield, IL) was administered daily by gavage at a dose of 5 mg/kg, beginning the day before the crush procedure. This dose of FK506 was chosen because it was found to be the optimal dose in peripheral nerve regeneration experiments. The contents of FK506 capsules were dissolved in sterile PBS and given to the rats. The control rats were given an equal volume of PBS. There were 11 rats in each group.

Statistics
Groups were compared using unpaired Student’s t-test. Significance was set at 0.05. Power analysis using the variability in RGC number observed in preliminary studies showed that 10 to 12 rats per group would be necessary to demonstrate statistical significance.

RESULTS
Initial experiments were conducted to determine whether FKBP12 mRNA was expressed in retina. RT-PCR was performed on rat brain and retinal tissue and yielded a single band of the appropriate size, 312 bp (Fig. 1). We next sought evidence of FKBP12 at the protein level in retina. Western blot analysis of rat whole retinal lysates revealed a band migrating at 12 kDa, the expected size for the FKBP12 protein (Fig. 2). Whole-brain lysate (rat and human) was used as a positive control and also showed a band at 12 kDa, corroborating previous reports of high levels of FKBP12 expression in the brain.

We next performed immunohistochemical analysis to determine cellular localization of FKBP12 within the retina. Figure 3A shows FKBP12-positive cells in the retina. There is strong labeling in the ganglion cell layer, the inner nuclear layer and the inner portion of the outer segments. Figure 3B demonstrates the low level of nonspecific staining when the primary antibody is omitted. To determine unambiguously whether FKBP12 was in RGCs, RGCs were back labeled with fluorescein dextran and then immunostained for FKBP12. Back-labeled RGCs throughout the ganglion cell layer also labeled with FKBP12 antibody (Figs. 3C, 3D) demonstrating the presence of FKBP12 in RGCs.

Because FKBP12 is present in RGCs, we next examined whether FK506 could confer neuroprotection on those cells (Fig. 4). Animals were treated by gavage with either FK506 (5 mg/kg · d) or an equal volume of PBS for 1 day preceding and...
each day after unilateral optic nerve crush. Eight days after optic nerve crush, an average of 27,775 ± 3,332 (mean ± SEM) labeled RGCs were counted in retinas of animals receiving vehicle control \((n = 11)\), whereas an average of 37,118 ± 2,475 RGCs were seen in the retinas of animals receiving FK506 daily \((n = 11)\). This difference was statistically significant \((P < 0.05)\) by unpaired \(t\)-test, indicating that FK506 conferred a degree of neuroprotection on RGCs after optic nerve crush. In control eyes receiving only sham surgery, the number of labeled RGCs was 74,104 ± 4,166 \((n = 11)\) in rats receiving vehicle control and 74,993 ± 3,098 \((n = 11)\) in animals receiving daily FK506. These values were not statistically different, indicating that FK506 had no effect on baseline RGC number. Overall, in animals receiving vehicle, 39% of RGCs survived 8 days after optic nerve crush compared with 51% of RGCs in animals receiving FK506.

**DISCUSSION**

The purpose of this study was to determine whether immunophilins were present in the rat retina and the physiologic consequence of their presence. The data presented demonstrate that the immunophilin FKBP12 was present in retina and, specifically, in RGCs. In addition, the immunophilin ligand FK506 conferred neuroprotection on RGCs after optic nerve crush.

Although FKBP12 was originally described in its role in immunosuppression, it has subsequently been shown to be widely distributed in the brain. In our initial studies, we extended these observations by showing, by multiple techniques, the robust presence of FKBP12 in retina. Moreover, by back labeling RGCs, we were able to localize the FKBP12 to RGCs unambiguously. This report represents the first descrip-
tion of FKBP12 in the retina and RGCs and prompts consideration that FKBP12 is the immunophilin involved in the recently described in vitro evidence of FK506-mediated protection against glutamate toxicity in retinal cell cultures\textsuperscript{18} and hippocampal neurons.\textsuperscript{10}

Evidence supports both an excitotoxic and an apoptotic component to the molecular mechanisms underlying RGC death. Glutamate, an excitatory neurotransmitter, has been shown to be elevated after optic nerve crush in rats,\textsuperscript{19} suggesting that glutamate may contribute to the death of RGCs. FK506 has been shown to have a protective effect on N-methyl-D-aspartate–treated cortical neurons in culture by inhibiting the production of nitric oxide through a calcineurin-mediated process.\textsuperscript{20} However, numerous markers of apoptosis, including TdT-dUTP terminal nick-end labeling (TUNEL) staining, appear in RGCs after optic nerve injury.\textsuperscript{21} The pharmacologic effects of FK506 and of the FK506-immunophilin complex appear to be directly related to programmed cell death pathways. A recent report suggests that one target of the FK506-immunophilin complex, calcineurin, mediates a critical step in the apoptotic pathway by dephosphorylating BAD, a proapoptotic member of the Bcl-2 family which translocates to the mitochondria after dephosphorylation and forms heterodimers with Bcl-x\textsubscript{L}.\textsuperscript{10} Thus, by inhibiting the calcineurin-mediated dephosphorylation of BAD, the FK506-immunophilin complex promotes cell survival. In hippocampal neurons in culture, for example, FK506 inhibited calcineurin and suppressed mitochondrial targeting of BAD after l-glutamate treatment, suppressing apoptosis.\textsuperscript{10}

Our current data are consistent with the hypothesis that RGCs die through an apoptotic pathway after optic nerve crush, and that this is modulated by a novel therapeutic approach using immunophilin ligands. These data raise the possibility that immunophilin binding agents may have a role in disease conditions characterized by RGC degeneration, including glaucoma.

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References