Ethambutol Is Toxic to Retinal Ganglion Cells via an Excitotoxic Pathway

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Purpose. Ethambutol is an essential medication in the management of tuberculosis. However, it can cause an optic neuropathy of uncertain etiology. Ethambutol toxicity was therefore studied in rodent retinal cells, and agents that might block its toxicity were considered.

Methods. The toxicity of ethambutol and related agents was evaluated in rodent retinal dissociated cell preparations and whole eyes. Calcium fluxes and mitochondrial function were evaluated by fluorescent and staining techniques. For in vivo assays, adult rats were administered oral ethambutol over a 3-month period. Cell survival was assessed by stereology.

Results. Ethambutol is specifically toxic to retinal ganglion cells in vitro and in vivo. Endogenous glutamate is necessary for the full expression of ethambutol toxicity, and glutamate antagonists prevent ethambutol-mediated cell loss. Ethambutol causes a decrease in cytosolic calcium, an increase in mitochondrial calcium, and an increase in the mitochondrial membrane potential.

Conclusions. The visual loss associated with ethambutol may be mediated through an excitotoxic pathway, inasmuch as ganglion cells are rendered sensitive to normally tolerated levels of extracellular glutamate. Ethambutol perturbs mitochondrial function. Its toxicity may depend on decreased ATPase activity and mitochondrial energy homeostasis. Glutamate antagonists may be useful in limiting the side effects seen with ethambutol. (Invest Ophthalmol Vis Sci. 1999;40: 190-196)

Ethambutol is in widespread therapeutic use as an antituberculous agent. Its most common side effect is acquired red-green dyschromatopsia, a phenomenon usually associated with optic neuropathy. Attempts to experimentally model ethambutol toxicity have been difficult, because the drug has a broad spectrum of effects that are species- and dosage-dependent. All the animal and human data suggest, however, that ethambutol is toxic to retinal neurons or retinal ganglion cell axons (which make up the optic nerve). In piscine retina, ethambutol is toxic to horizontal or cone cells. Ethambutol can also cause discoloration of the tape-mum lucidum in dogs and retinal detachment in cats. In monkeys and humans, ethambutol is toxic to the optic nerve, a finding consistent with the observation that ethambutol causes acquired dyschromatopsia.

The pattern of cell loss seen with ethambutol toxicity, specifically the loss of retinal ganglion cells, is strikingly similar to that seen with glutamate-mediated damage. Studies in the central nervous system over the past four decades have found that traumatic and ischemic neuronal injury are mediated by excessive levels of excitatory amino acids, especially glutamate. Work in many laboratories has established that glutamate-induced retinal toxicity in neonatal mice and coinced the term “excitotoxicity” for this type of neuronal damage. Sisk and Kuwabara, and subsequently Siliprandi and coworkers, injected glutamate or related agonists intravitreally in adult rats and observed marked loss of the ganglion cell layer. Glutamate causes loss of ganglion cell bodies and their axons, the optic nerve. Work in many laboratories has established that the predominant form of excitotoxicity of retinal ganglion cells is mediated by overstimulation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor, which in turn leads to excessive levels of intracellular calcium.

One well-characterized NMDA antagonist is dizocilpine (MK-801), an open-channel blocker of the NMDA receptor, that can protect neurons from ischemic necrosis in gerbils and rats. However, MK-801 also impairs many normal neuronal functions, can cause injury or reversible neuronal swelling at therapeutic concentrations, and is unlikely to ever have clinical utility.
Memantine (1-amino-3,5-dimethyladamantane hydrochloride) is known to have anti-Parkinsonian and antiepileptic properties and is an analogue of amantadine (1-adamantane-mine hydrochloride). 27–28 Amantadine is a well-known antiviral agent that has been used clinically for >20 years in the United States. 29–31 Memantine has been used to treat Parkinson's disease in Europe for the past decade with few side effects. 2/i2H

We consequently explored the toxicity of ethambutol to retinal cells both in vitro and in vivo and the role of glutamate and its antagonists in this toxicity.

METHODS
All experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinal preparations and viability assays were from postnatal rats as previously described. 34,50 Retinal ganglion cells were identified by DiI fluorescence (retrogradely transported from a prior injection site in the superior colliculus). Retinal ganglion cells were scored for viability in masked fashion. The ability of cells to take up and cleave fluorescein diacetate was used as an index of their viability.

In vivo assays, five adult rats were administered ethambutol for 90 days at a daily dose of 25 mg/kg. Five control animals were administered vehicle only. Five rats were administered oral ethambutol and intraperitoneal memantine (1 mg/kg per day); one group of five rats received memantine alone. After 90 days the animals were killed, and retinal ganglion cells were back-labeled with horseradish peroxidase; retinas were then whole mounted and cell survival was assessed by stereological techniques. 55 Ganglion cells with demonstrable horseradish peroxidase-labeling were scored as viable. A second group of eyes was sectioned and stained with hematoxylin and eosin for masked counts of cells in each retinal layer.

Fluorescent assays were carried out as previously described, using an Image 1 system (Universal Imaging, West Chester, PA). 38 For these assays, ganglion cells were not back-labeled but instead were identified by morphology.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay for mitochondrial activity 40 was performed on ganglion cell dissociations 24 hours after plating and treatment with 10 μM ethambutol or 1 μM 2,2’(ethyleneimino)diethylurea (EDBA) or without any added drug. At 24 hours, the dissociations were washed, and media containing MTT at a concentration of 500 ng/ml was added to each dish. Each dish was then returned to a 5% CO2 incubator and incubated at 37°C for 3 hours. One hundred retinal ganglion cells for each group were then examined on an inverted microscope for MTT deposition (blue dye) and photographed. Images were digitized and the MTT intensity was quantified as previously described. 41

Values for all in vitro experiments were normalized to control (1) and represent the mean ± SD; the number of high-power fields needed to visualize 150 retinal ganglion cells was used as a baseline for all experimental determinations. Two-way analysis of variance (ANOVA) with post hoc Scheffé tests for multiple comparisons was used to assess effects between drugs and dose levels. 42 LD50 was estimated by probit analysis. 43 A two-tailed P < 0.05 was considered statistically significant. The GLM procedure in SAS software was used for statistical analysis (version 6.12; SAS Institute, Cary, NC). For analysis of the MTT data, one-way ANOVA and multiple unpaired t-tests with a Bonferroni correction for multiple comparisons were used to evaluate differences in staining between control, ethambutol-, and EDBA-treated groups.

RESULTS
To identify which cells in the eye were damaged by ethambutol, cultured dissociated rat retinas were incubated for 24 hours with increasing concentrations of ethambutol or its metabolite EDBA. 44 Ethambutol and EDBA both were toxic to retinal ganglion cells in a dose-dependent fashion (Fig. 1A). The LD50 for ethambutol toxicity was 10 μM, similar to the concentration found in the cerebrospinal fluid of humans taking ethambutol. 39

Ethambutol was selectively toxic to retinal ganglion cells in vivo. Rats were administered ethambutol orally at a daily dose of 25 mg/kg (human dosage is 15–25 mg/kg per day). Animals were killed after 90 days, and the retinas were evaluated. Ethambutol caused a marked loss of cells from the retinal ganglion cell layer but not other layers (Fig. 2A). Quantification by stereological analysis of retinal whole mounts revealed a loss of 35% of the cells in the retinal ganglion cell layer (Fig. 2B). Cell counts of retinal cross sections (performed in masked fashion on all layers) revealed pathology only in the ganglion cell layer (data not shown). Intraocular injections of EDBA demonstrated identical histopathology (not shown).

The selective loss of retinal ganglion cells seen with ethambutol and EDBA is strikingly reminiscent of that seen with glutamate analogues, suggesting a related mechanism. We hypothesized that if ethambutol injures retinal ganglion cells through an excitotoxic pathway, then glutamate antagonists should protect the retina from ethambutol-mediated damage. Accordingly, retinal cultures were incubated overnight with ethambutol and one of several antagonists to glutamate receptors. The NMDA channel antagonists MK-801, 2-amino-5-phosphonovalerate (APV), and memantine protected ganglion cells from ethambutol toxicity (Fig. 1B). In contrast, the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) did not protect ganglion cells from ethambutol. Similarly, incubation with memantine and MK-801, but not CNQX, blocked the toxicity of EDBA (not shown).

Similar results were observed in vivo. Rats were administered ethambutol (25 mg/kg per day) and memantine (1 mg/kg intraperitoneally per day), and ganglion cell survival was assessed at 90 days. Memantine protected retinal ganglion cells from ethambutol toxicity in vivo (Fig. 2B).

One explanation for these results would be if ethambutol was an NMDA-receptor agonist. NMDA and related compounds cause a rapid and pronounced rise in intracellular calcium. However, there was no change in intracellular calcium when concentrations of 10 μM to 1 mM ethambutol were applied to ganglion cells loaded with either fura-2 or fluo 3 (60 cells examined, data not shown), suggesting that ethambutol is not a direct NMDA-receptor agonist. Alternatively, endogenous glu-
**FIGURE 1.** (A) Toxicity of ethambutol and 2,2'(ethylenediamino)-dibutyric acid (EDBA). Dose–response curve showing the fraction of surviving retinal ganglion cells exposed to increasing concentrations of ethambutol (left) and EDBA (right) in an in vitro retinal dissociation. Retinal ganglion cells were killed in a dose-dependent fashion by ethambutol and EDBA; the half-maximal effects were as follows: ethambutol, 10 μM; EDBA, 1 μM.

* Statistical difference, P < 0.01. (B) N-methyl-D-aspartate (NMDA) antagonists attenuate ethambutol toxicity, and endogenous glutamate is necessary for the expression of ethambutol toxicity. Left: dissociated retinal cells were incubated with either no added drug (control), 10 μM ethambutol alone, or ethambutol added with the following drugs: 12 μM dizocilpine (the open-channel NMDA antagonist, MK-801), 100 μM APV (the specific NMDA-receptor antagonist 2-amino-5-phosphonovalerate), 12 μM memantine, or 100 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). * Statistical difference, P < 0.01. These drugs had no effect on retinal ganglion cell viability in the absence of ethambutol (not shown).

The NMDA antagonists MK-801, APV, and memantine can protect the retinal ganglion cell against ethambutol toxicity; non-NMDA antagonists are ineffective. Right: as noted, approximately half of the retinal ganglion cells incubated with 10 μM ethambutol died within 24 hours compared with sibling control cultures. In the presence of 10 μM ethambutol, treatment with glutamate-pyruvate transaminase (GPT) with the cosubstrate sodium pyruvate prevented cell loss. Treatment with either GPT compound alone or with heat-inactivated GPT (HT) plus pyruvate was ineffective at protecting ganglion cells from ethambutol-mediated cell death. GPT or pyruvate alone or in combination, or heat-treated GPT with pyruvate had no effect on retinal ganglion cell survival (data not shown). Endogenous glutamate is necessary for the full expression of ethambutol toxicity. Eth, ethambutol; Mem, memantine; Pyr, pyruvate.

tamate could mediate NMDA-receptor activation. We therefore pretreated cultures with glutamate-pyruvate transaminase (GPT) to degrade endogenous glutamate. This pretreatment protected retinal ganglion cells from ethambutol toxicity (Fig. 1B), indicating that endogenous glutamate was necessary for ethambutol toxicity. Parallel experiments with EDBA showed similar results (not shown). This suggested that ethambutol or EDBA rendered retinal ganglion cells sensitive to endogenous levels of glutamate; levels to which they were ordinarily resistant.
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Mitochondria play a critical role in buffering the calcium influx that results from stimulation of the NMDA receptor. To see whether ethambutol perturbed mitochondrial calcium levels, mitochondrial calcium levels were measured with the fluorescent dye rhod-2. The addition of ethambutol to labeled retinal ganglion cells led to an increase in rhod-2 fluorescence (Fig. 3A), consistent with an increase in mitochondrial calcium.

To establish the source of this mitochondrial calcium flux, cells were incubated with calcium green 1-AM to detect changes in cytosolic calcium (because calcium green is sensitive to small changes in cytosolic calcium). There was a net decrease in fluorescence when retinal ganglion cells were exposed to ethambutol, indicating a decrease in cytosolic calcium (Fig. 3B). Ethambutol had no effect on either calcium green or rhod-2 fluorescence in the presence of the respiratory chain complex I inhibitor rotenone or uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone. The increase in mitochondrial calcium and the decrease in cytosolic calcium seen after exposure to ethambutol suggest that ethambutol mediates a flow of calcium from cytosolic pools into mitochondria and that this flux is dependent on mitochondrial respiration.

Mitochondrial function was assayed by MTT staining, a relative measure of mitochondrial dehydrogenase activity and an indirect indicator of cell viability. Retinal ganglion cells were treated with either ethambutol or EDBA (in the presence of MK-801 to ensure cell viability), and MTT staining was quantified. Highly significant differences were detected among the three groups by ANOVA, F(2,279) = 207.48, P < 0.0001. When normalized to an intensity of 1 for control retinal ganglion cell staining, control cells had 1.00 ± 0.13 MTT staining (mean ± SD), compared with 0.70 ± 0.12 MTT staining for ethambutol-treated cells and 0.65 ± 0.12 for EDBA-treated cells. Treatment with ethambutol and EDBA significantly diminished MTT staining (P < 0.0001 for both), consistent with moderately decreased mitochondrial function.

Discussion

These results indicate that ethambutol and EDBA are specifically toxic to retinal ganglion cells both in vitro and in vivo. Stimulation by endogenous levels of glutamate through the NMDA channel is required for toxicity, indicating that ethambutol-induced ganglion cell death is secondary to glutamate excitotoxicity. Ethambutol and EDBA had no effect on the NMDA channel itself. They neither directly stimulated nor altered the kinetics of the channel. Ethambutol and EDBA, however, reduced mitochondrial activity. Consequently, ganglion cells were rendered more sensitive to normally tolerated...
Figure 3. Ethambutol mediates a flow of $\text{Ca}^{2+}$ from the cytosol into the mitochondria, an increase in mitochondrial membrane potential, and a decrease in mitochondrial function. Retinal ganglion cells were labeled with either rhod-2 (A), calcium green (B), or JC-1 (C). Addition of ethambutol is indicated by the arrow. Ethambutol caused an increase in rhod-2 fluorescence, indicating an increase in mitochondrial calcium. Addition of ethambutol also caused a decrease in calcium green intensity, suggesting a corresponding decrease in cytosolic calcium. Both changes could be blocked by incubation with either of the mitochondrial uncouplers/inhibitors (10 $\mu$M rotenone, not shown, or 10 $\mu$M carbonylcyanide $p$-trifluoromethoxyphenylhydrazone). Identical results were seen with 2,2'(ethylenediamino)-dibutyric acid (EDBA). These data suggest that ethambutol causes a shift in intracellular calcium from cytosol to mitochondria. For JC-1, approximately 20 cells were analyzed in each of five separate experiments; 53% of cells responded with an increase in intensity.
external levels of glutamate by a mechanism involving mitochondrial energy homeostasis.

Ethambutol and EDBA both chelate zinc (log Ka 17.6 and 16.4, respectively), and ethambutol toxicity is more pronounced in humans with depressed serum zinc levels.\(^1\) One possibility for the underlying mechanism of ethambutol toxicity is therefore chelation of intracellular zinc. Several targets for zinc chelation might explain these findings. One or more zinc-dependent inhibitory factors are known to modulate ATP synthesis and the preservation of mitochondrial homeostasis.\(^5\) Certain endonucleases are modulated by zinc, and their dysregulation can lead to DNA cleavage and cell death. Cu-Zn-superoxide dismutase (SOD-1) is critical in protecting mitochondria from superoxide anion, but it is unlikely that the relatively weak zinc chelators used in these experiments could dissociate zinc from SOD-1. A more likely target is the ATPase inhibitory protein IF1, which is itself inhibited by zinc. Zinc chelation would allow IF1 to inhibit ATPase activity, decreasing ATP synthesis and simultaneously increasing mitochondrial membrane potential. This would parallel the effect of oligomycin on the ATPase.\(^6\) The ensuing rise in mitochondrial calcium would exacerbate excitotoxicity by compromising mitochondrial buffering of glutamate-induced cytoplasmic calcium fluxes. Diminished ATP levels would inhibit mitochondrial dehydrogenase activity.

In summary, ethambutol and EDBA are toxic to retinal ganglion cells in vitro and in vivo, and stimulation by endogenous levels of glutamate through the NMDA receptor is required for toxicity. Ethambutol results in a decrease in cytosolic calcium, an increase in mitochondrial calcium, and an increase in the mitochondrial membrane potential. Ganglion cells are rendered sensitive to normally tolerated levels of extracellular glutamate, possibly by a mechanism involving decreased ATPase activity and mitochondrial energy homeostasis. Glutamate antagonists may therefore be useful in limiting side effects seen with ethambutol administration. Alternatively, ethambutol derivatives, which are unable to cross the blood-brain barrier, might maintain antimycobacterial activity without inducing optic neuropathy.

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**References**


