

# Coenzyme Q10 Instilled as Eye Drops on the Cornea Reaches the Retina and Protects Retinal Layers from Apoptosis in a Mouse Model of Kainate-Induced Retinal Damage

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**PURPOSE.** To evaluate if coenzyme Q10 (CoQ10) can protect retinal ganglion cells (RGCs) from apoptosis and, when instilled as eye drops on the cornea, if it can reach the retina and exert its antiapoptotic activity in this area in a mouse model of kainate (KA)-induced retinal damage.

**METHODS.** Rat primary or cultured RGCs were subjected to glutamate (50  $\mu$ M) or chemical hypoxia (Antimycin A, 200  $\mu$ M) or serum withdrawal (FBS, 0.5%) in the presence or absence of CoQ10 (10  $\mu$ M). Cell viability was evaluated by light microscopy and fluorescence-activated cell sorting analyses. Apoptosis was evaluated by caspase 3/7 activity and mitochondrion depolarization tetramethylrhodamine ethyl ester analysis. CoQ10 transfer to the retina following its instillation as eye drops on the cornea was quantified by HPLC. Retinal protection by CoQ10 (10  $\mu$ M) eye drops instilled on the cornea was then evaluated in a mouse model of KA-induced excitotoxic retinal cell apoptosis by cleaved caspase 3 immunohistofluorescence, caspase 3/7 activity assays, and quantification of inhibition of RGC loss.

**RESULTS.** CoQ10 significantly increased viable cells by preventing RGC apoptosis. Furthermore, when topically applied as eye drops to the cornea, it reached the retina, thus substantially increasing local CoQ10 concentration and protecting retinal layers from apoptosis.

**CONCLUSIONS.** The ability of CoQ10 eye drops to protect retinal cells from apoptosis in the mouse model of KA-induced retinal damage suggests that topical CoQ10 may be evaluated in designing therapies for treating apoptosis-driven retinopathies. (*Invest Ophthalmol Vis Sci.* 2012;53:8295–8302) DOI: 10.1167/iovs.12-10374

Apoptosis of retinal ganglion cells (RGCs), associated or not with elevated intraocular pressure (IOP), is recognized as the main cause of the progressive loss of vision in patients with glaucoma, the first cause of irreversible blindness in developed countries.<sup>1–3</sup>

The discovery that the key to glaucoma is in the connections within the retina to the brain has led to exciting advances in research, giving way to new potential treatments. Whether due to mechanical trauma, decreased blood flow, or other causes, optic nerve axon injury causes changes in RGCs, eventually causing cell death. These specific areas of injured optic nerve axons and RGC loss match the peripheral vision damage from glaucoma. Because the RGC axon stretches from the retina through the optic nerve to the brain, its surrounding cells also become damaged by glaucoma. Within the retina, other cells, such as amacrine cells, degenerate and rewire their connections after RGCs are lost. Thus, in addition to treatments directed at lowering eye pressure, still the mainstay of glaucoma therapy, there may be opportunities to develop neuroprotective treatments directed at the retina and the brain.

Although glaucoma is generally associated with high IOP, increased IOP is not detected in a significant subset of patients who have normal tension glaucoma (NTG). In all cases, hyperstimulation of RGCs consequent to a local increase of glutamate, the master excitatory neurotransmitter, is recognized to be a pivotal mechanism producing glaucoma.<sup>4–6</sup> Furthermore, neurochemical evidence implicates elevated glutamate in the mechanisms of high IOP-induced RGC death in rats.<sup>6</sup> Glutamate acts predominantly through the *N*-methyl-D-aspartate (NMDA) receptors, which trigger RGC apoptosis by inducing calcium influx into the cell.<sup>7</sup> Moreover, via hyperstimulation of the non-NMDA receptors, glutamate upregulates tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), which catalyze plasminogen activation to plasmin, leading to degradation of the extracellular matrix and RGC apoptosis.<sup>8</sup>

Although the precise role of excitotoxicity in human glaucoma is still a matter of considerable debate, mice that represent various models of NTG continue to serve as a basis for investigating neuroprotective strategies aimed at excitotoxic insults to the CNS.<sup>9</sup> Intraocular injection of excitotoxic agents is commonly used to induce a relatively specific insult to RGCs in the absence of increased IOP in rodents.<sup>9</sup> Although the pathogenesis of cell damage in this system does not directly follow that of human glaucoma, insight into RGC neurodegeneration has been gained using this model.

The main cellular trigger of apoptosis is the opening of two mitochondrial transmembrane channels. The most relevant is the mitochondrial permeability transition pore (mPTP), whose opening is accompanied by loss of mitochondrial membrane

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potential ( $m\Delta\Psi$ ) and extrusion to cytoplasm of a variety of apoptotic molecules.<sup>10</sup> The other is the mitochondrial apoptosis-induced channel (MAC), whose opening releases apoptotic molecules without loss of  $m\Delta\Psi$  and mitochondrial integrity.<sup>11</sup>

We have previously shown that coenzyme Q10 (CoQ10) inhibits corneal keratocyte apoptosis both *in vitro*<sup>12</sup> and *in vivo*,<sup>13</sup> with an efficacy that is higher than that of other antioxidants. This result suggests the involvement of mechanism(s) independent from the free radical scavenging function of CoQ10, leading to the disclosure that CoQ10 prevents apoptosis also by inhibiting mitochondrial depolarization.<sup>14</sup> This is in keeping with the evidence obtained by Walter et al.,<sup>15</sup> indicating that some synthetic ubiquinones prevent mitochondrial depolarization, depending on their molecular structure rather than antioxidant properties. More recently, Devun et al.<sup>16</sup> have also reported that the same synthetic ubiquinones could either induce or prevent *in vitro* mPTP opening and thereby apoptosis, depending on the cell type from which the mitochondria are derived.

The recent observation that corneal administration of CoQ10 in patients undergoing vitrectomy markedly increased CoQ10 concentration in the vitreous,<sup>17</sup> prompted us to explore whether CoQ10 instilled as eye drops on the cornea could reach the retina and exert its antiapoptotic activity also in the retinal tissue. To this aim, in cultured RGCs we first observed the antiapoptotic effect of CoQ10. Next, using the rabbit as an experimental model, we evaluated if CoQ10, topically applied to the cornea, effectively reached the choroid/retina. Finally, the mouse model of kainate-induced retinal damage, reminiscent of glaucoma, was used to investigate the possible neuroprotective and antiapoptotic activity of CoQ10.

## MATERIALS AND METHODS

### RGC-5 Cell Line

The rat RGC-5 cell line (obtained from Neeraj Agarwal, University of North Texas Health Science Center, Fort Worth, TX) was cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin G, and 100  $\mu$ g/mL streptomycin, in a humidified incubator in 5% CO<sub>2</sub> at 37°C.

### Primary RGCs

A suspension of primary rat RGCs was obtained from excised retinal membranes of 20 neonatal (5 days old) Wistar rats according to Feher et al.<sup>18</sup> Briefly, the retinal membranes were dissociated by enzymatic treatment with papain (15 U/mL) and collagenase (70 U/mL) in Hank's buffered salt solution (HBSS) containing 1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 0.2 mg/mL bovine serum albumin (BSA), and 0.2 mg/mL DL-cysteine for 30 minutes at 37°C. The preparation was microfragmented with a Pasteur pipette in a solution consisting of 2 mg/mL ovomucoid, 1 mg/mL BSA, and 0.004% DNase, centrifuged at 100g for 5 minutes, and resuspended in phosphate-buffered saline (PBS). The cellular suspension was transferred to a 25-cm<sup>2</sup> tissue culture plastic flask and maintained at room temperature for 30 minutes in the presence of antimacrophage antibody CD172a. Nonadherent cells were transferred to plastic tubes and exposed to RGC-specific antibody Thy-1 (CD90) for 30 minutes. All adherent cells were washed once in PBS, harvested by centrifugation, and resuspended in commercial media (Neurobasal; Invitrogen, Carlsbad, CA) supplement medium containing B27, glutamine (1 mM), gentamycin (10  $\mu$ g/mL), brain-derived neurotrophic factor (40 ng/mL), ciliary neurotrophic factor (50 ng/mL), and forskolin (5  $\mu$ M).

### Treatments

The RGC-5 cell line was treated for 24 hours with the respiratory chain blocker Antimycin A (200  $\mu$ M) or for 72 hours with FBS withdrawal

(0.5%), used as hypoxia- or growth factor starvation-mimetic damaging agents, respectively. The schedule was optimized to commit cells to apoptosis rather than to necrosis as described previously.<sup>14</sup> The primary rat RGCs were plated at a density of 2000 cells onto 6-cm plates and treated with glutamate (50  $\mu$ M) for 24 hours.<sup>6</sup> Two hours before the application of glutamate, cells were pretreated with 10  $\mu$ M CoQ10, dissolved in a commercial vehicle to ensure cellular uptake (0.04% Lutrol F127, now known as Kolliphor P407; Sigma-Aldrich, St. Louis, MO). Vehicle alone-treated cells were used as controls.

### Quantification of RGC Viability

The viability of RGC-5 cells or primary RGCs was evaluated microscopically and confirmed with a commercial reagent (Guava ViaCount Assay with CytoSoft software, cat. #4000-0040; Guava Technologies, Hayward, CA). This assay distinguishes between viable and nonviable cells, based on their differential permeability to two fluorescent nucleic acid stains (Annexin V and 7-AAD) in the reagent.

### Evaluation of Apoptosis with Caspase 3/7 Activity Assay

Caspase 3/7 activity was measured using a commercial assay kit (Caspase-GLO Assay Kit; Promega, Madison, WI). Briefly, cells or tissue were collected and solubilized in a lysis buffer containing 25 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), pH 7.5, 5 mM MgCl<sub>2</sub>, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), and 2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 5000g for 10 minutes, 10  $\mu$ g of protein extracts in 50  $\mu$ L total volume were mixed with 50  $\mu$ L of equilibrated commercial reagent (Caspase-GLO 3/7) and incubated for 1 hour at room temperature. Afterward, luminescence was measured using a luminometer (GloMax 20/20n luminometer; Promega).

### TMRE Staining of Mitochondrial Membrane Potential

The change in  $m\Delta\Psi$  occurring during apoptosis was detected by a fluorescence-based assay. RGC-5 cells, cultured on coverslips, were subjected to apoptotic stimuli as indicated, followed by incubation with 50 nM tetramethylrhodamine ethyl ester perchlorate (TMRE; Sigma-Aldrich) in culture medium for 30 minutes at 37°C, allowing mitochondria to load with the dye in proportion to mitochondrial membrane potential. After washing with PBS, coverslips were mounted on a glass slide with a drop of HEPES-Tris buffer (pH 7.4, prewarmed at 37°C). The glass slide was immediately mounted on a confocal microscope (Nikon TE2000; Nikon Corp., Tokyo, Japan) equipped with a 60 $\times$ A/1.40 oil-immersion objective and live-cell images were acquisitioned using commercial confocal microscopy software (EZ-C1 Software; Nikon Corp.). Further image processing and analysis were performed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsbweb.nih.gov/ij/index.html>) as described before in detail.<sup>19</sup>

### Experiments in Animals

All experiments in animal models were performed in compliance with the Italian law on animal care (No. 116/1992) and in accordance with the guidelines established by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Quantification of CoQ10 in the Rabbit Retina following Corneal Instillation

Three male New Zealand albino rabbits weighing 2.5 to 3.0 kg were anesthetized by intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg), and treated with instillation on corneas of 5 or 15 drops (1 drop = 100  $\mu$ L)/min of a

solution containing 2 mM CoQ10 dissolved in a vehicle containing a commercial reagent (Lutrol F127 [100 g/L], a commercial emulsifier and solubilizer (Kolliphor EL, formerly known as Cremophor EL) 50 g/L, NaCl 0.45%, and benzalconium chloride 0.001% in H<sub>2</sub>O, as previously reported.<sup>12</sup> Rabbits instilled with 5 or 15 drops of the vehicle alone were used as controls. At 2, 7, and 15 minutes after CoQ10 instillations on corneas, venous blood samples (1 mL) were withdrawn from the marginal vein of rabbits ear into heparinized tubes and centrifuged at 1500g for 15 minutes at room temperature to obtain plasma. Fifteen minutes after the beginning of treatments, rabbits were euthanized and each eye was explanted and washed with a physiologic solution before separating each choroid/retina for quantification of CoQ10. For this purpose, each choroid/retina was homogenized in 1 mL of bidistilled water and 2 mL of 95% ethanol/5% isopropanol plus 5 mL hexane to extract CoQ10. After stirring for 2 minutes, to completely oxidize the CoQ10, the mixture was centrifuged at 5000g for 10 minutes at room temperature to separate a supernatant, which was collected, dried under nitrogen stream, lyophilized, and suspended in ethanol. The CoQ10 quantification in plasma and retinal extracts was carried out by high-performance liquid chromatography (HPLC) analysis according to Takada et al.,<sup>20</sup> using a commercial HPLC (Waters 501; Waters, Milford, MA) supplied with a detector at 275 nm. Analyses were carried out on a commercial column (Nucleogen ID C18 column; Macherey-Nagel, Düren, Germany) with a solution of methanol, hexanes, acetic acid, isopropanol (55:9:1:1, vol/vol/vol/vol), and 0.42% sodium acetate as mobile phase at a flow rate of 1.5 mL/min at room temperature.

### The Mouse Model of KA-Induced Retinal Damage

C57BL/6 mice (6–8 weeks old; Harlan Laboratories, Udine, Italy) were anesthetized by intraperitoneal injection of 1.2% tribromoethanol and 2.4% amylene hydrate in distilled water (0.02 mL/g body weight; Avertin; Pierce, Rockford, IL) and subjected to intravitreal injection of 1 µL of 10 mM kainate (KA), the specific agonist for ionotropic glutamate receptors, or control physiological solution (NanoFil syringe; World Precision Instruments, Sarasota, FL) equipped with a 36-gauge beveled needle. One drop of a CoQ10 solution (2.3 mM, Coqun eye drops; Visufarma, Rome, Italy) or saline (–CoQ10 control), was instilled on corneas at 60, 30, 15, and 5 minutes before injection of KA and 15, 30, and 60 minutes and 2, 3, 4, and 23 hours after injection of KA.<sup>21</sup> Mice instilled with CoQ10 eye drops omitting KA intravitreal injection were used as controls. At 24 hours after KA injection, mice were euthanized and the eyes were rapidly removed for caspase 3/7 activity quantification, cleaved caspase 3 immunohistofluorescence analysis, and quantification of RGCs in flat-mounted retinas.

### Immunohistofluorescence Analysis of Cleaved Caspase 3

Mouse eyes were kept immersed overnight at room temperature in a fixative solution containing 4% paraformaldehyde, following immersion in 25% sucrose/PBS for 2 days, and then embedded in paraffin. Retinal sections of 4 µm were stained with cleaved caspase 3 (Asp175) primary antibody (Cell Signaling Technology, Danvers, MA), followed by fluorescent anti rabbit Cy-2-conjugated secondary antibody (Chemicon International, Inc., Temecula, CA) and counterstained with Hoechst 33258 (Sigma-Aldrich). Fluorescence was imaged (Nikon TE2000 Confocal Microscope and EZ-C1 Software; Nikon Corp.) and digitally captured.

### Flat-Mounted Retinas

Eyes were fixed in 4% paraformaldehyde for 30 minutes at room temperature. The corneas and lenses were removed and the remaining eye cups were incubated in 4% paraformaldehyde for another 30 minutes. Retinas were peeled off carefully and washed three times with PBS, and ganglion cells remaining in the retinas were identified as

described in Nadal-Nicolas et al.<sup>22</sup> Briefly, whole retinas were permeabilized in 0.5% Triton-X100 in PBS for 15 minutes at room temperature. The retinas were washed three times with PBS and incubated with 1:100 diluted Brn3a mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer (2% BSA, 2% Triton-X100 in PBS) overnight at 4°C. Next day, the retinas were washed three times with PBS and incubated for 2 hours with Cy3-conjugated secondary antibody (Chemicon International). Subsequently, the retinas were washed three times with PBS and mounted on slides, vitreous side facing upward. Brn3a-positive RGCs in retinas were assessed microscopically using a confocal microscope (Nikon TE2000 using EZ-C1 Software; Nikon Corp.), equipped with a 60×A/1.40 oil-immersion objective and digitally captured. For quantitative analysis, the positive cells were counted in four to six microscope fields of identical size located at approximately the same distance from the optic disc.

### Statistical Analysis

Data are presented as means ± SEM of at least three to four independent experiments. Statistical comparisons were made using unpaired Student's *t*-test or ANOVA followed by Newman–Keuls multiple comparison post test, as appropriate. A value of *P* < 0.05 was considered statistically significant.

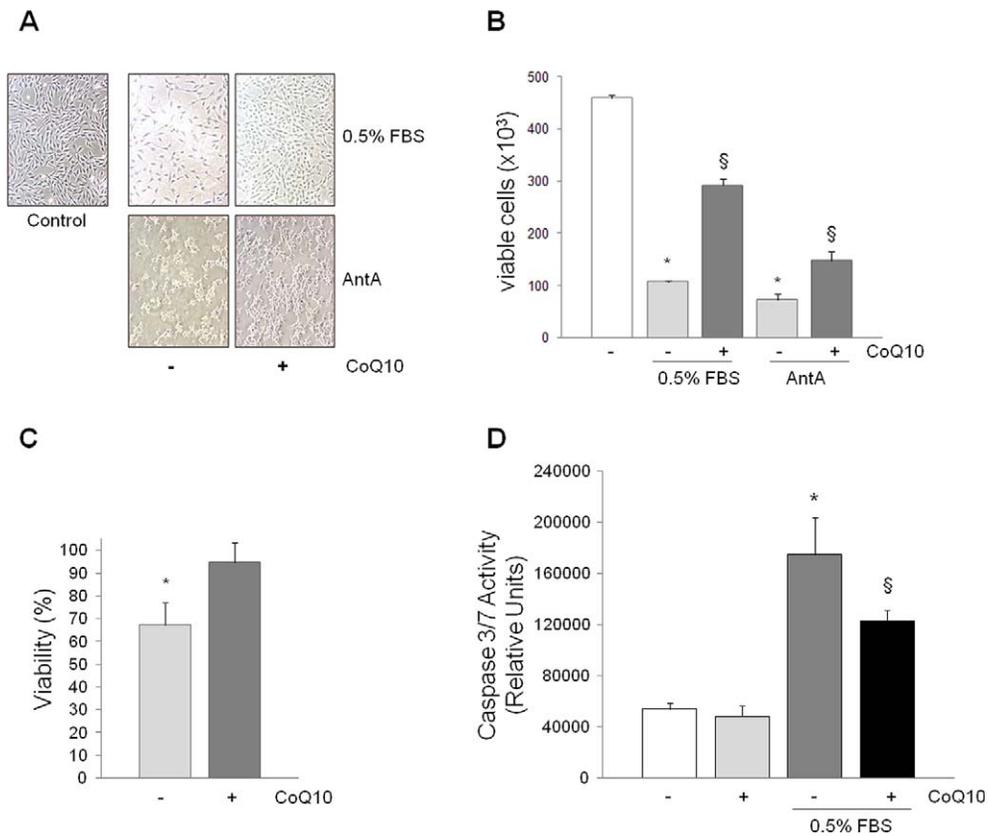
## RESULTS

### CoQ10 Increases RGC Viability and Inhibits Apoptosis

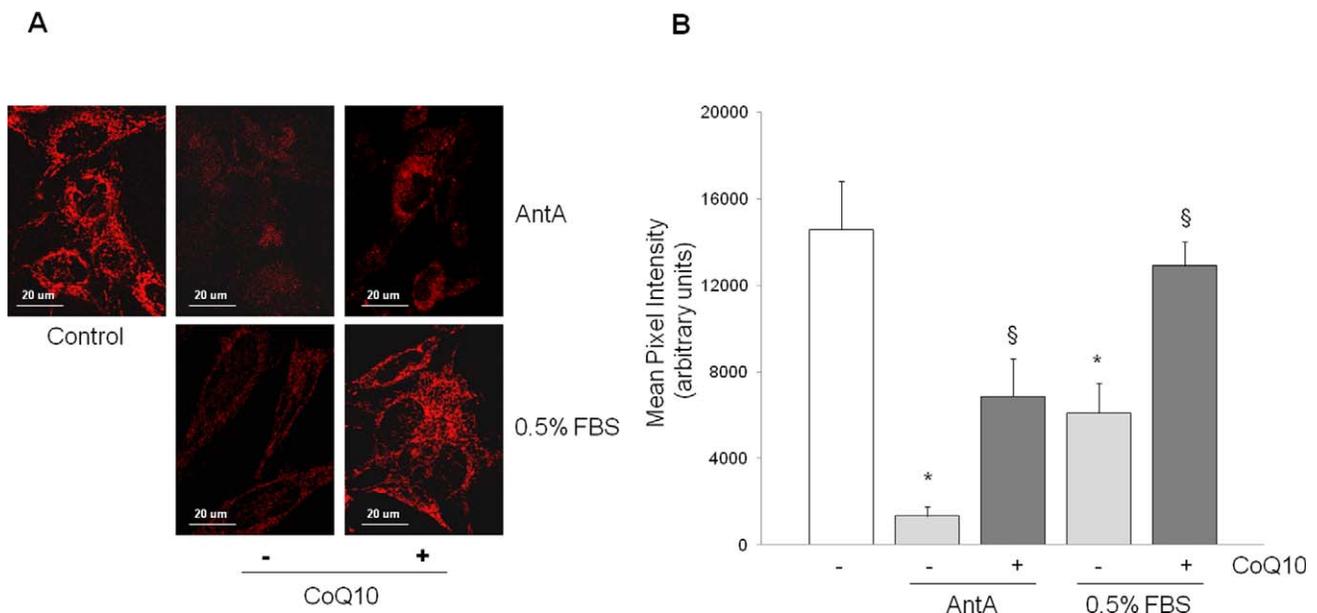
The neuroprotective property of CoQ10 was first evaluated in the cultured rat RGC-5 cell line on the basis of cell viability in response to Antimycin A (200 µM) or serum starvation (0.5% FBS). As we demonstrated previously,<sup>14</sup> both Antimycin A and serum starvation do not induce apoptosis through the generation of free radicals in our experimental conditions. Two hours before application of the apoptotic stimuli, cells were pretreated with CoQ10 (10 µM), dissolved in a commercial reagent (Lutrol [+CoQ10] or with control Lutrol alone [–CoQ10]). Light microscopy examination of RGC-5 cells (Fig. 1A) revealed that CoQ10 prevented the marked drop of living cells caused by both apoptotic stimuli. Scoring of viable cells by fluorescence-activated cell sorting (FACS) analysis (with the ViaCount reagent on Guava PCA) is shown in Figure 1B. Serum starvation for 72 hours reduced RGC-5 living cells to 23% ( $107 \pm 2 \times 10^3$ ) compared with controls ( $465 \pm 6 \times 10^3$ ), but to 63% ( $292 \pm 12 \times 10^3$ ) if cells were pretreated with CoQ10, resulting in a >2.7-fold increment of living cells. The treatment with Antimycin A for 24 hours reduced RGC-5 cell viability to 16% ( $73 \pm 10 \times 10^3$ ) compared with the untreated controls, but to 32% ( $148 \pm 17 \times 10^3$ ) if cells were pretreated with CoQ10, resulting in a 2-fold increase in living cells.

Next, cell viability of primary rat RGCs, following a 24-hour treatment with the excitotoxic amino acid glutamate (50 µM) preceded or not by a 2-hour pretreatment with CoQ10 (10 µM) was evaluated by FACS analysis (Guava PC). Figure 1C shows that glutamate reduced cell viability to approximately two thirds ( $67 \pm 9\%$ ) compared with untreated controls, but did not elicit any significant effect if cells were pretreated with CoQ10 ( $95 \pm 8\%$ ).

To verify if the increased RGC viability was due to apoptosis inhibition by CoQ10, we assessed the ability of CoQ10 to prevent caspase 3/7 activation on RGC-5 cells in response to a 72-hour treatment with serum starvation. As shown in Figure 1D, serum starvation increased caspase 3/7 activity by more than 3-fold, whereas this increase was significantly reduced by approximately 30% when cells underwent a 2-hour pretreatment with CoQ10.



**FIGURE 1.** Evaluation of the number of viable RGC-5 cells, pretreated (+CoQ10) or not (-CoQ10) with 10  $\mu$ M CoQ10 for 2 hours and maintained in conditions of serum starvation (0.5% FBS) for 72 hours or treated with 200  $\mu$ M Antimycin A (AntA) for 24 hours. Untreated RGC-5 cells were used as controls. **(A)** Light microscopy examination of RGC-5 cells. **(B)** FACS analysis of viable and nonviable nucleated cells with commercial reagent (ViaCount on Guava PCA). Data are means  $\pm$  SEM of at least four to five experiments. \* $P < 0.001$  vs. control; § $P < 0.01$  vs. 0.5% FBS and Antimycin A stimulated. **(C)** FACS analysis (Guava PCA) of viability of primary rat RGCs treated for 24 hours with glutamate (50  $\mu$ M) combined (+CoQ10) or not (-CoQ10) with 10  $\mu$ M CoQ10. Data are means  $\pm$  SEM of at least four to five experiments. \* $P < 0.001$  vs. control. **(D)** Analysis of caspase 3/7 activity in RGC-5 cells maintained in conditions of serum starvation (0.5% FBS) for 72 hours and pretreated (+CoQ10) or not (-CoQ10) with 10  $\mu$ M CoQ10 for 2 hours. The caspase 3/7 activity was measured using a luciferase-based chemical assay system. Relative luciferase units are proportional to functional caspase 3/7 activity. Data are means  $\pm$  SEM of three experiments. \* $P < 0.005$  vs. control; § $P < 0.05$  vs. 0.5% FBS.



**FIGURE 2.** TMRE staining of mitochondrial membrane potential. **(A)** The change in  $m\Delta\Psi$  detected with 50 nM TMRE at the 24th hour following treatment with 200  $\mu$ M Antimycin A or at the 72nd hour following serum starvation, preceded or not by pretreatment with 10  $\mu$ M CoQ10. Treated

cells show reduced fluorescence as compared with nontreated uniformly stained controls, indicating loss of mitochondrial membrane potential. The loss was significantly prevented by CoQ10. Each image is representative of at least three independent experiments with similar results. (B) Quantification of TMRE intensity in arbitrary units. \* $P < 0.005$  vs. control; § $P < 0.005$  vs. AntA or 0.5% FBS.

### CoQ10 Inhibits Apoptosis by Preventing the Mitochondrial Depolarization

The ability of CoQ10 to prevent mitochondrial depolarization independently from its antioxidant properties was evaluated by the uptake of TMRE in RGC-5 cells treated with Antimycin A or serum starvation (Fig. 2A). Two hours before application of the apoptotic stimuli, cells were pretreated with 10  $\mu$ M CoQ10 or with vehicle alone. Quantification of TMRE intensity (Fig. 2B) revealed that treatment with Antimycin A for 24 hours ( $1347 \pm 441$  arbitrary units [a.u.]) or serum starvation for 72 hours ( $6090 \pm 1368$  a.u.) induced a marked loss of TMRE staining compared with the untreated controls ( $14,587 \pm 2250$  a.u.). Pretreatment with CoQ10 successfully prevented the mitochondrial depolarization as indicated by the almost 5-fold ( $6860 \pm 1760$  a.u.) or 2-fold ( $12,913 \pm 1135$  a.u.) increase of TMRE staining, respectively.

### CoQ10 after Topical Application on the Rabbit Cornea Rapidly Reaches the Choroid/Retina in a Dose- and Time-Dependent Manner

Next, we tested if CoQ10 applied to the cornea could reach the choroid/retina. For this purpose, New Zealand albino rabbits were sedated and treated with 5 or 15 eye drops at 1 drop/min of either CoQ10 (2 mM) solution or vehicle alone. During treatments (at 2, 7, and 15 minutes) venous blood samples (1 mL from marginal ear vein) were withdrawn for HPLC analysis and the rabbits were euthanized, eyes were explanted, and choroids/retinas were isolated and used for HPLC quantification of CoQ10 (Fig. 3). Instillation of 15 eye drops of CoQ10 solution (Fig. 3A) increased the CoQ10 choroid/retina concentration at the 15th minute by 80% compared with controls ( $1443 \pm 132$  pmol/g vs.  $800 \pm 224$  pmol/g). Moreover, the levels of CoQ10 taken up by choroid/retina were dose-dependent (Fig. 3B) and time-dependent (Fig. 3C) during the first 15 minutes. There were no significant changes in CoQ10 plasma content between CoQ10-treated and vehicle alone-

treated rabbit eyes (data not shown), which excluded transfer of CoQ10 to the retina through hematic circulation.

### Topical Application of CoQ10 in a Mouse Model of KA-Induced Retinal Damage Protects Retinal Cells from Apoptosis

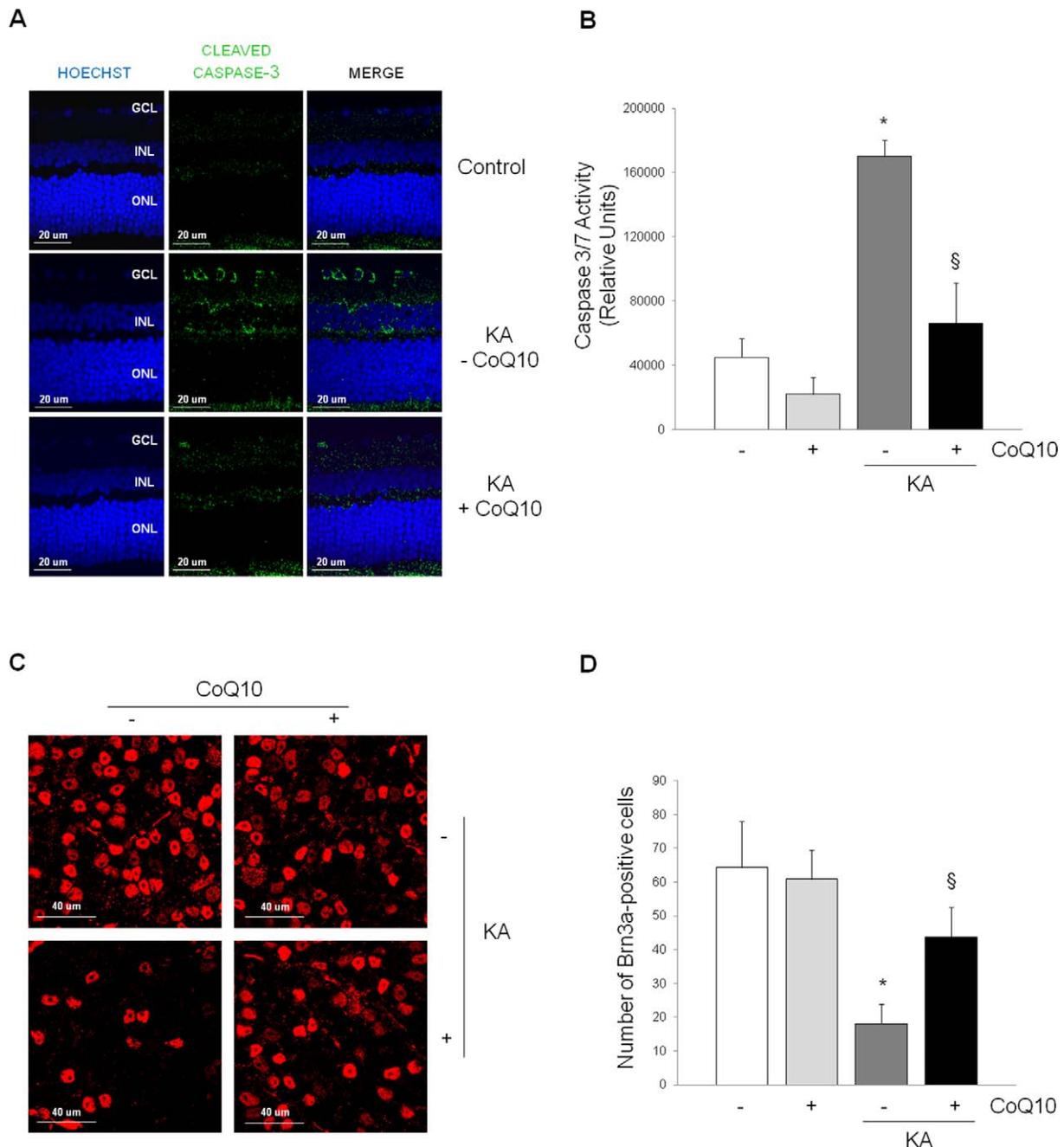
Finally, we investigated whether topical application of CoQ10 eye drops on the cornea of C57BL/6 mice could counteract the retinal cell death induced by intravitreal injections of the glutamate agonist KA (10  $\mu$ M). As shown in Figure 4A, at the 24th hour following KA intravitreal injection a substantial number of cells in the ganglion cell layer (GCL) and the inner nuclear layer (INL) stained positive for the cleaved caspase 3, showing green fluorescent cytoplasm of apoptotic cells. Significantly, the KA-induced apoptotic cell death underwent a dramatic drop in the retina of mice treated with topical application of CoQ10.

To confirm data obtained by cleaved caspase 3 staining analysis in vivo, we quantified the caspase 3/7 activity on retinal extracts. As shown in Figure 4B, intravitreal injection of KA induced a substantial increase (by almost 4-fold compared with untreated controls) in caspase 3/7 activity. Treatment with CoQ10 eye drops abolished the increase in caspase 3/7 activity.

Next, we performed the quantification of RGCs in the GCL in flat-mounted retinas, to determine the extent of retinal cell loss and the level of protection exerted by topical application of CoQ10 in KA-treated mice. At 24 hours after intravitreal injection of KA, the eyes were enucleated and fixed in 4% paraformaldehyde, and the whole retinas were immune-stained with Brn3a antibody to detect the remaining RGCs. Results presented in Figure 4C indicate that at 24 hours after intravitreal injection of KA, the density of the Brn3a-positive RGCs was reduced in treated mice compared with nontreated controls. Instead, topical application of CoQ10 prevented the loss of RGCs and increased number of Brn3a-positive cells in the GCL layer. Quantitative analysis of RGCs presented in



**FIGURE 3.** Quantification of CoQ10 in choroid/retina of New Zealand albino rabbit eye following corneal application. The oxidized CoQ10 (exogenous) in extracted eye tissues was quantified by HPLC. (A) The CoQ10 concentration in the choroid/retina of rabbits treated with 15 eye drops of 2 mM CoQ10 solution was compared with controls treated with vehicle alone. Data are means  $\pm$  SEM of three experiments. \* $P < 0.02$  vs. control. (B) The dose-dependent exogenous CoQ10 concentration in the choroid/retina as a function of the number of eye drops (5 or 15) of 2 mM CoQ10 solution applied to right eyes was compared with control left eyes treated with the vehicle alone. Data are means  $\pm$  SEM of three experiments. \* $P < 0.001$  vs. control. (C) The time course-dependent increase in concentration of exogenous CoQ10 was analyzed at 2, 7, and 15 minutes in the choroid/retina of right eyes, compared with control left eyes. Data are means  $\pm$  SEM of three experiments. \* $P < 0.001$  vs. control; § $P < 0.001$  vs. 7 minutes.



**FIGURE 4.** Application of CoQ10 to the mouse cornea prevents retinal cell apoptosis in response to intravitreal injection of KA. (A) Evaluation of KA-induced apoptosis in retinal sections by immunohistochemistry. Representative photomicrographs of immune-stained retinas from control mice or KA-treated mice either receiving vehicle (–CoQ10) or CoQ10 (+CoQ10) show the Hoechst nuclear staining (left), the cleaved caspase 3 staining (center), and merge (right). GCL, INL, and ONL are indicated. (B) The caspase 3/7 activity in corresponding retinal extracts was measured using a luciferase-based assay system. Relative luciferase units are proportional to functional caspase 3/7 activity. Three animals in each group were used. \* $P < 0.001$  vs. control (–CoQ10 and –KA); § $P < 0.005$  vs. KA-treated (–CoQ10). (C) CoQ10 attenuated KA-induced loss of RGCs; 24 hours following KA intravitreal injection the loss of RGCs in flat-mounted retinas was determined by immunofluorescence staining with antibodies against Brn3a. (D) Quantification of cells indicates that, whereas Brn3a-positive RGCs remained similar in untreated or CoQ10-treated control mice, Brn3a-positive RGCs were reduced significantly in mice treated with KA. In contrast, KA-induced cell loss was reversed when the mice were treated with CoQ10. Three mice in each group were used. \* $P < 0.005$  vs. control (–CoQ10 and –KA); § $P < 0.01$  vs. KA-treated (–CoQ10).

Figure 4D indicated that in the KA-treated mice the number of RGCs was reduced by 72% as compared with untreated controls ( $18.1 \pm 5.8$  and  $64.3 \pm 13.7$ , respectively). Treatment with CoQ10 eye drops increased the number of the Brn3a-positive cells ( $43.8 \pm 8.8$ ) observed in the GCL layer, reducing the loss significantly from 72% to 32%.

## DISCUSSION

Neurodegenerative diseases, including glaucoma,<sup>3,21</sup> involve cell loss by apoptosis as the main pathogenetic event.<sup>23</sup> For this reason antiapoptotic drugs have been viewed as one of the most rational pharmacologic tools for such pathologies.

Notwithstanding a substantial amount of research that is focused on this area, effective treatments for most neurodegenerative diseases do not yet exist.<sup>24</sup>

Previously, we have shown that CoQ10 prevented apoptosis of corneal keratocytes both *in vitro*<sup>12</sup> and *in vivo*<sup>13</sup> in response to therapeutic excimer laser irradiation with markedly higher efficacy compared with that of other antioxidants (vitamins A, C, and E). We then demonstrated that keratocyte apoptosis prevention by CoQ10 also occurred in response to apoptotic stimuli that do not induce the formation of free radicals such as ceramide, hypoxia, and growth factor withdrawal and that this was consequent to the ability of CoQ10 to inhibit mitochondrial depolarization.<sup>14</sup>

The effects of CoQ10 on the vision and, in particular, on the health of the retina have not been widely investigated. Only a few studies have indicated that oral or intravitreal administration of CoQ10 significantly improves eyesight<sup>18,25,26</sup> and protects RGCs against oxidative stress<sup>27</sup> or high IOP-induced ischemia.<sup>6</sup> The capacity of intraocularly injected CoQ10 to reduce RGC apoptosis in a rat model of ischemia/reperfusion damage has also been reported,<sup>25,28</sup> but the molecular mechanisms have not been revealed.

Although the involvement of RGC excitotoxicity as a pathogenetic mechanism of glaucoma is debated, it has been proven that in most cases it could play a key role. The excitotoxicity of KA/glutamate on RGCs is mediated by various pathways. In particular, Sucher et al.<sup>7</sup> reported that glutamate acts predominantly by binding to NMDA receptors, which triggers apoptosis by promoting Ca<sup>2+</sup> influx into the cell and consequent activation of the caspase cascade. Mali et al.<sup>8</sup> demonstrated that KA-induced retinal damage promotes the upregulation of tissue tPA and the induction of uPA, which resulted in plasminogen activation to plasmin, extracellular matrix degradation, and apoptosis in the RGC layer as well as in the inner and outer nuclear layers. In a parallel study, Kumada et al.<sup>29</sup> identified tPA as directly responsible for KA-induced retinal damage independently from cleavage of plasminogen to plasmin. A very recent study reports that KA induces a reactive gliosis that leads to RGC apoptosis by activating retinal matrix metalloproteinase-9, tPA, and uPA.<sup>30</sup> Indeed, the excitotoxicity has been implicated as a contributing factor in human glaucoma.<sup>9</sup> Moreover, the NMDA receptor antagonist MK-801 has been shown to have neuroprotective properties in rat ocular hypertension and optic nerve damage.<sup>31</sup> For this reason, we have chosen the kainate mouse model to serve as a basis for investigating retinal neuroprotective strategy using CoQ10 against damage to RGCs.

Here, we demonstrate that CoQ10 protects RGCs from apoptosis independently from its antioxidant properties, in keeping with the previous results we obtained with corneal keratocytes.<sup>14</sup> Furthermore, if instilled as eye drops on the cornea, CoQ10 can reach the retina without entering into the blood vessels and exert its antiapoptotic activity in this area in a mouse model of KA-induced retinal damage, reminiscent of glaucoma. Our results also indicate that CoQ10 increases RGC viability and inhibits apoptosis in response to different apoptotic stimuli such as glutamate, chemical hypoxia (Antimycin A), and serum withdrawal (FBS 0.5%), by preventing mitochondrial depolarization.

On the basis of these observations, the effectiveness of corneal application of CoQ10 eye drops in protecting RGCs from KA-induced apoptosis in our study indicates that CoQ10 may act by mechanisms that are downstream of and overcome all the above pathways.

Finally, the ability of CoQ10 eye drops to deliver CoQ10 to the retina and to protect the retinal layers from apoptosis in the mouse model of KA-induced retinal damage reminiscent of glaucoma suggests that CoQ10 eye drops have a promising

therapeutic applicability. The ability of CoQ10 to reach the retina following corneal application is supported by an observation of Fato et al.<sup>17</sup> that corneal instillation of a collirium based on CoQ10 in patients undergoing vitrectomy resulted in a marked increase of CoQ10 in the vitreous. Our results are in keeping with a report of Qu et al.,<sup>32</sup> demonstrating that the choroid/retina levels of endogenous CoQ10 decrease with aging concomitantly with progression of apoptosis-related macular degeneration. These authors speculate that enhancing CoQ10 levels in the retina of elderly patients could have therapeutic value.

In conclusion, we propose that CoQ10 eye drops may be evaluated as a novel, simple, and noninvasive therapy for topical treatment of retinopathies with apoptosis as the main pathogenetic mechanism.

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