Inhibition of Apoptosis and Reduction of Intracellular pH Decrease in Retinal Neural Cell Cultures by a Blocker of Carbonic Anhydrase

Eva M. Kniep, Cora Roehlecke, Nurdan Özkucur, Alexander Steinberg, Friedemann Reber, Lilla Knels, and Richard H. W. Funk

Purpose. Methylglyoxal and glyoxal are intermediates of advanced glycation end products (AGEs). These substances, as well as hydrogen peroxide, induce retinal neurons to reduce their intracellular pH and augment their production of reactive oxygen species, leading to apoptosis. Because these processes may play a role in diabetic retinopathy, the authors undertook this study to investigate the protective action of dorzolamide, an inhibitor of carbonic anhydrase, on retinal neural cells.

Methods. E1A-NR3 cells were incubated with varying concentrations of glyoxal, methylglyoxal, and H_2O_2 for different periods of time in the presence or absence of dorzolamide. Apoptotic changes were determined by cytofluorometry after the cells were incubated with appropriate dyes and antibodies. The parameters studied were DNA strand breaks (TUNEL assay), subdiploid DNA content (sub-G1 assay), annexin V binding, reactive oxygen species intermediates production, active caspase-3, N_ϵ-(carboxymethyl)lysine (a glycation product), and intracellular pH.

Results. Optimal conditions for detection of the cell-protecting effect of dorzolamide were incubation with 0.6 to 0.8 mM glyoxal or methylglyoxal for 5 hours or with 0.1 mM H_2O_2 for 30 minutes, respectively, followed by 20-hour incubation with fresh medium. All apoptotic changes were reduced in the assays in which dorzolamide was included.

Conclusions. Dorzolamide reduced the damage inflicted on retinal neural cells by agents that induced apoptosis and, therefore, can be considered a neuroprotectant. (*Invest Ophthalmol Vis Sci.* 2006;47:1185–1192) DOI:10.1167/iovs.05-0555

Neuroprotection is a topic of a growing number of studies[1–9] because many diseases of the eye (e.g., certain forms of glaucoma,[10] diabetic retinopathy,[11,12] and age-related macular degeneration [AMD][13]) lead to neurodegeneration, especially of the retinal neurons. It may be possible to rescue neural cells that already have lost their function but maintain their structural integrity. In retinal ganglion cells (which, in this respect, are the most vulnerable cell population[14]), functional injury precedes the onset of structural damage.[15] Recovery may restore their function. Until now, however, few substances have had a defined neuroprotective capacity and could be associated with a causal mechanism. Such substances must be safe and easily applied to the eye.

While testing the vasodilating capacity of carbonic anhydrase (CA) blockers in post vivo whole mounts of the rat retina (the cells were held viable in a special observation chamber[16,17]), we found that the intracellular pH (pH_i) of the neurons in the retinal whole mounts remained at higher levels in the CA blocker–treated cells than in the untreated retina cells.[18] This observation was the starting point of the idea that holding the pH_i at normal levels would have an antiapoptotic effect. Because of these results, CA blockers should counteract apoptosis. CA has been demonstrated in many tissues, including cells of the brain and eye,[19–23] and apoptosis is often associated with decreased cytosolic pH.[24–29] In neurons, ischemia or oxidative stress leads to decreased pH_i, rendering the cells susceptible to further damage.[30,31]

Reduced pH_i promotes apoptosis by favoring caspase activation (optimum pH for caspase-3, 6.6–6.8[32]) and activation of DNase II,[33] but it is controversial whether low pH leads to enhanced production of free radicals (reactive oxygen species [ROS])[34] or vice versa.[28] Some studies indicate that acidification of cytosol was inhibited without alteration of the apoptotic response.[24,29] We conclude from these observations that decreased cytosolic pH favors apoptosis. Until now, it has been unknown whether decreased cytosolic pH plays a role in signaling cell death.

Advanced glycation end-products (AGEs) and hydrogen peroxide lead to acidification of the cytoplasm, elevated ROS production, and apoptosis.[35–40] In the present study, we tested the pH-stabilizing capacity and putative neuroprotective effect of dorzolamide, a blocker of CAII,[41] which is applied as eye drops in patients with glaucoma to lower the intraocular pressure and to improve the microcirculation in the posterior eye. To model cell damage, we administered the reactive AGE intermediates glyoxal and methylglyoxal and ROS-inducing H_2O_2 to the rat retinal cell line E1A-NR3.

Materials and Methods

Cells

The rat retinal cell line E1A-NR3[42] was kept in DMEM containing 10% FCS, 2 mM glutamine, 1% nonessential amino acids, and 1% vitamins (Biochrom, Berlin, Germany).

Reagents

Dorzolamide was a gift from Merck (Rahway, NJ). Glyoxal, methylglyoxal, propidium iodide, and bisbenzimide were purchased from Sigma (Deisenhofen, Germany), and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF AM), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) were obtained from Molecular Probes (Leiden, The Netherlands). Earle balanced salt solution (EBSS) consisted of

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140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 25 mM HEPES, pH 7.3.

Immunocytochemistry

For immunocytochemistry, cells were grown on glass coverslips coated with collagen IV (5 μg collagen IV/mL 0.05M HCl for 60 minutes). Monoclonal mouse antihuman Thy-1 (CD90; Acris, Hiddenhausen, Germany) was added directly to the cell cultures (final dilution, 1:200) and kept for 45 minutes in the incubator. After washing and incubation with secondary antibody Cy3-conjugated goat anti-mouse (1/1000; Jackson ImmunoResearch Labs, West Grove, PA), cells were fixed with 4% paraformaldehyde. For staining of CAII and neuron-specific enolase (NSE), cells were fixed with acetone/methanol (1:1) at −20°C and incubated with goat anti-mouse CAII (1:50; Santa Cruz Biotechnology, Heidelberg, Germany) or monoclonal mouse anti-human NSE (1:50; Alexis, Grünberg, Germany), respectively. Secondary antibodies were FITC-conjugated donkey anti-goat (1:100) and Cy3-conjugated goat anti-mouse (1:1000; both from Jackson ImmunoResearch Labs). Nuclei were stained with 1 μg/mL bisbenzimide. Controls were performed with the primary antibodies omitted. Cells were viewed with a fluorescence microscope (IX81; Olympus Biosystems, Planegg, Germany) equipped with a camera (Coolpix SIS; Olympus).

Apoptosis Induction

Cells were seeded into T25 flasks (1×10^5/mL). After 24 hours, cells were subconfluent, and glyoxal, methylglyoxal, or hydrogen peroxide was added to achieve the concentrations indicated in the figures. Glyoxal and methylglyoxal were added to the culture medium, whereas incubation with hydrogen peroxide was performed in Hanks balanced salt solution (HBSS). When dorzolamide was included in the assays, it was added immediately before the apoptosis-inducing agents. After the indicated incubation periods, the supernatants were removed (detached cells were spun down and returned to the flasks), and incubation was continued for another 20 hours. The supernatant was collected, and detached cells were spun down. Adherent cells were collected after trypsin treatment (trypsin-EDTA; Biochrom). Adherent and detached cells were pooled, washed with PBS containing 1% horse serum, and submitted to the respective apoptosis assays. For each assay, 10^5 cells were applied.

Apoptosis Assays

Fluorescent cells were measured in a FACSCalibur (Becton-Dickinson, Heidelberg, Germany). The assay for subdiploid DNA (sub-G1 peak) was performed as described by Nicoletti et al.[43] Briefly, cells were fixed in cold 70% ethanol for 5 minutes. Fifty microliters PBS with 1% horse serum containing 1 μg propidium iodide and 10 μg RNase were added, and the mixture was incubated for 30 minutes at room temperature. Apoptotic nuclei revealed a subdiploid DNA peak. The percentage of apoptotic cells was calculated from the histograms of propidium iodide–stained DNA. Necrotic cells were determined by incubation of unfixed cells for 5 minutes with propidium iodide (0.01 mg/mL) and cytofluorimetric analysis. TUNEL assay was performed with a fluorescein in situ cell death detection kit (Roche, Mannheim, Germany), according to the directions of the manufacturer. Annexin V binding was studied using FITC-conjugated annexin V (Immuno Tools, Friesoythe, Germany), which was diluted 100-fold with binding buffer containing 10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$. Fifty microliters of this solution was added to cell pellets, and suspensions were incubated for 10 minutes at room temperature. For the determination of ROS, CM-H_2DCFDA was dissolved in dimethyl sulfoxide to achieve 1 mM. This was diluted 700-fold with PBS, and 50 μL was added to each cell pellet. Suspensions were incubated at 37°C for 30 minutes. Green fluorescent cells were measured cytofluorometrically. For the determination of carboxymethyllysine (CML)–modified proteins, cells were permeabilized with PBS containing 0.5% saponin and 0.5% BSA and were incubated with polyclonal rabbit anti–CML antiserum (a kind gift of Erwin D. Schleicher[44]) for 30 minutes at 4°C. Secondary antibody was FITC-conjugated anti–rabbit IgG (Sigma). Activation of caspase-3 was measured using the polyclonal rabbit anti–active caspase-3 (PharMingen, Hamburg, Germany). Binding of both antibodies was analyzed with the FACSCalibur.

Analysis of Mitochondrial Membrane Potential

Cells were grown on chambered coverglass (Nalge Nunc, Naperville, IL) coated with collagen IV, as described. After treatment with methylglyoxal (0.8 mM) or methylglyoxal together with dorzolamide (0.3 mM), respectively, for 2 hours, cells were stained with JC-1 (stock solution; 0.2 mg/mL in dimethyl sulfoxide [DMSO]) diluted to 1 μg/mL with medium) for 30 minutes at 37°C. Pictures were taken using a microscope (IX81; Olympus) equipped with a dual-view imaging system.

Measurement of pH_i

Intracellular pH was determined as described by Franck et al.[45] Briefly, cells were washed with EBSS and incubated with 10 μM BCECF AM in EBSS (stock solution; 1 mM BCECF AM in DMSO) for 25 minutes at 37°C. Fluorescence was measured at 530 nm and 650 nm. Ratios of the fluorescence emissions were plotted, and pH values were read from a standard curve.

Statistical Analysis

Unless stated otherwise in the figure legends, all assays were performed at least in triplicate, the bars represent mean ± SEM of three or more independent assays, and histograms represent examples of series of similar experiments.

Results

Cells

E1A-NR3 is a rapidly proliferating rat retinal precursor cell line that has been used in several previous studies as a model for

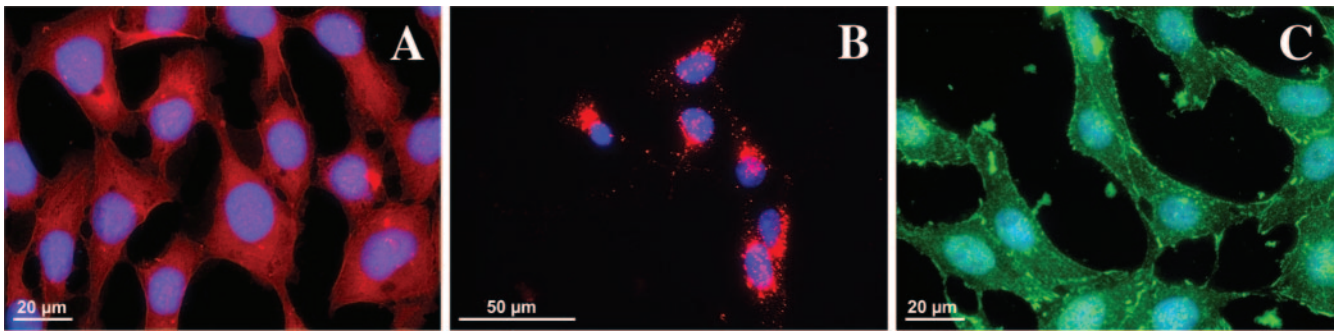

Figure 1. Detection of NSE, Thy-1, and CAII. Cells were stained with the respective antibodies, as described in Materials and Methods. (**A**) Anti-NSE. (**B**) Anti–Thy-1. (**C**) Anti-CAII. All cells on the slides stained positive with all three antibodies. Control staining with secondary antibodies was negative. Each image represents a section of one of three independent stainings.

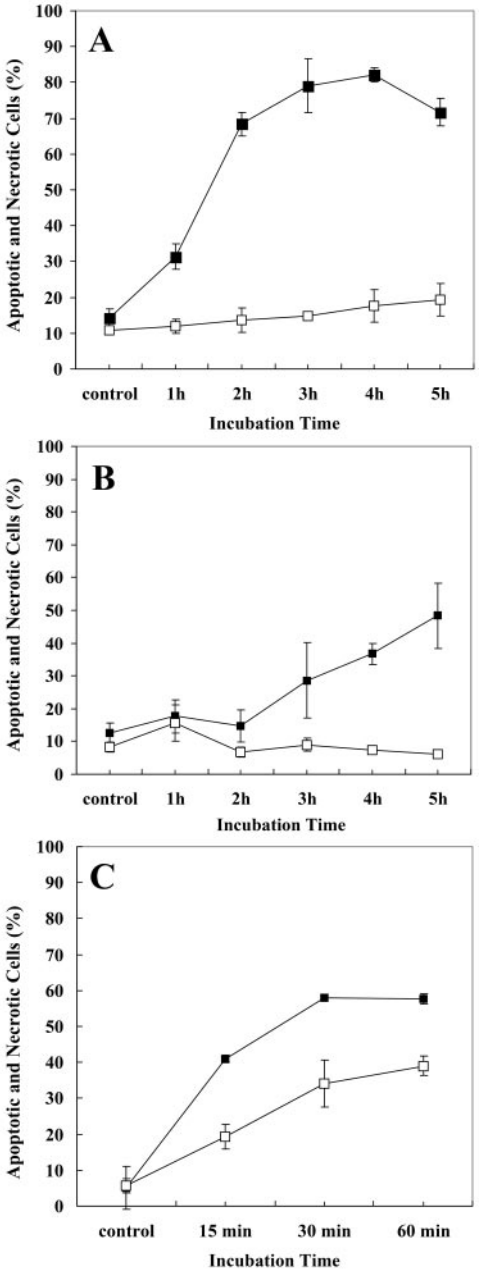

FIGURE 2. Apoptosis and necrosis induced by methylglyoxal, glyoxal, and H_2O_2. Cells were incubated with the respective agents. Glyoxal and methylglyoxal, respectively, were added to the medium; incubation with H_2O_2 was carried out in HBSS. After the indicated periods, supernatants were removed (detached cells were spun down and returned to the flasks), and incubation was continued with fresh medium for 20 hours. Cells were harvested after treatment with trypsin (again, the detached cells were included). Apoptosis (*closed squares*) was calculated from the sub-G1 peaks, and necrosis (*open squares*) was calculated from the fluorescence of propidium iodide taken up by dead cells. (**A**) 0.8 mM methylglyoxal. (**B**) 0.8 mM glyoxal. (**C**) 0.1 mM H_2O_2. Data represent the mean ± SEM of three independent experiments.

retinal neurons.[11,46–48] Subpopulations of the cell line express retinal and neuronal markers and respond to specific neurotransmitters.[49] In our experiments, all cells were positive for NSE (Fig. 1A) and expressed the Thy-1 antigen, a marker for retinal ganglion cells.[50] All cells also contained CAII, an enzyme that exerts influence on intracellular and extracellular pH (Fig. 1C).

Induction of Apoptosis

Apoptosis was induced by treatment with glyoxal, methylglyoxal, or hydrogen peroxide (for time frames and doses, see Figs. 2A–C). A pulse with the respective agent was given, followed by incubation with medium overnight. Apoptosis was quantified by determining the percentage of cells with a subdiploid DNA content. With this method, the necrotic cells were not measured with apoptotic cells because the necrotic cells showed a normal DNA peak. Methylglyoxal was the most effective inducer of apoptosis: after treatment with 0.8 mM for 2 to 5 hours, 70% to 80% of the cells showed nuclei with a subdiploid DNA content (Fig. 2A). With the same concentrations of glyoxal, the increase of apoptotic cells was slower (Fig. 2B). With 0.1 mM hydrogen peroxide, incubation for 15 to 30 minutes was sufficient to drive the cells into apoptosis, but apoptotic cells were always accompanied by a considerable number of necrotic cells (Fig. 2C). Fewer cells became necrotic with brief direct exposure to toxic agents than with exposure for the entire duration (not shown). Hydrogen peroxide concentrations lower than 0.1 mM did not lead to detectable apoptosis, even with prolonged incubation periods (not shown).

Apoptotic Changes of the Cells and Their Inhibition by Dorzolamide

Dorzolamide was added simultaneously with the toxic agents. Incubation of the cells with methylglyoxal and dorzolamide led to a reduction of apoptotic cells (compared with incubation with methylglyoxal alone) in a concentration-dependent manner (Fig. 3). With dorzolamide alone, no effects on the cells were detected (Table 1). Furthermore, the proportion of necrotic cells (which was normally between 10% and 20% of the total cell number) was not changed in the presence of dorzolamide (Table 1).

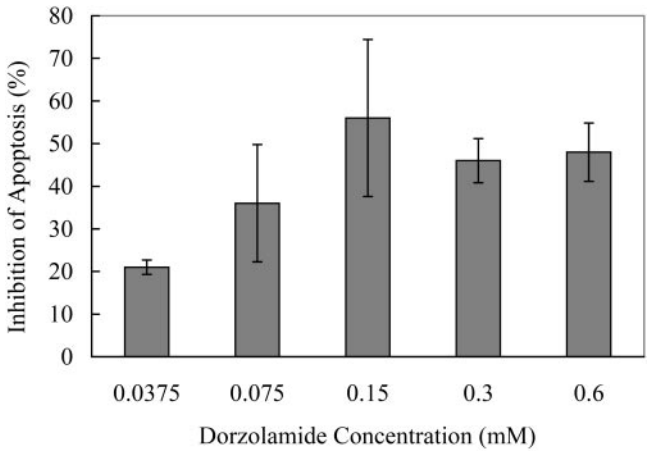

FIGURE 3. Inhibition of methylglyoxal-induced apoptosis by dorzolamide. Dorzolamide was added to cell cultures to achieve the indicated concentrations. Methylglyoxal was added immediately afterward (final concentration, 0.8 mM). After 4 hours, the medium was changed and incubation was continued for 20 hours. Cells were harvested after trypsin treatment and combined with the cells that had detached during the incubation and assayed with the sub-G1 test. Apoptosis and percentage inhibition was calculated from the percentage of cells with subdiploid DNA content; samples without dorzolamide represented 0% inhibition. Data represent the mean ± SEM of three to six independent experiments (*first and last bars*, three experiments; *three middle bars*, six experiments).

TABLE 1. Comparison of Total Cell Numbers, Necrotic Cells, and Apoptotic Cells after Treatment with Glyoxal, Methylglyoxal and Hydrogen Peroxide with and without Dorzolamide

Treatment	Total Cell Number (% of Control)	Proliferation in 25 h (-fold)	Necrotic Cells (%)	Apoptotic Cells (%)
Control	100	4.0	10 ± 2	9 ± 4
0.3 mM Dorzolamide	97 ± 2	4.0	12 ± 3	13 ± 8
0.8 mM Methylglyoxal	42 ± 18	1.7	19 ± 1	71 ± 3
0.8 mM Methylglyoxal + 0.3 mM Dorzolamide	70 ± 13	2.8	15 ± 6	53 ± 8
0.8 mM Glyoxal	16 ± 3	0.6	8 ± 1	43 ± 3
0.8 mM Glyoxal + 0.3 mM dorzolamide	18 ± 5	0.7	7 ± 0	14 ± 3
0.1 mM H_2O_2	73 ± 9	3.0	28 ± 6	42 ± 9
0.1 mM H_2O_2 + 0.3 mM dorzolamide	80 ± 8	3.2	32 ± 2	21 ± 2

Cells were treated for 5 h (line 1–6) or 1 h (line 7 and 8), respectively, followed by 20 h incubation with fresh medium as described in the legend of Figure 2. Total cells were counted with a CASY (Schärfe System, Reutlingen, Germany), necrotic and apoptotic cells were determined as described in Materials and Methods. Data represent the mean ± SEM of 6 independent experiments.

The inhibitory effect of dorzolamide on cell damage was most prominent when incubation periods were just long enough to achieve apoptosis. Prolonged incubation with methylglyoxal led to decreased protection (Fig. 4). Dorzolamide in concentrations higher than 0.3 mM provided no further benefit (Fig. 3). By changing the medium at the end of the incubation period with methylglyoxal and dorzolamide, both agents were removed. Substituting dorzolamide for the rest of the incubation time did not change the results (not shown).

Treatment of cells with glyoxal or hydrogen peroxide reduced the number of apoptotic cells when dorzolamide was present during incubation with the respective agent (Fig. 5; Table 1). To exclude the possibility that dorzolamide simply destroyed the apoptotic cells—thus rendering them undetectable in the cytofluorometer (this would also have reduced the percentage of apoptotic cells)—or else influenced their percentage by stimulating proliferation, we considered the total cell numbers. Control cells and cells treated with dorzolamide alone proliferated fourfold during the 25-hour incubation period. Incubation with methylglyoxal for 5 hours followed by 20-hour incubation with fresh medium reduced proliferation to less than half that of the control. Furthermore, 71% of these cells became apoptotic. Thus, methylglyoxal reduced the proliferation of cells and rendered the major portion of cells apoptotic. When dorzolamide was applied with methylglyoxal, the fraction of apoptotic cells was reduced to 53%. There were fewer total cells than in controls, but their number was considerably greater than after treatment with methylglyoxal alone. These data could be interpreted on the assumption that dorzolamide, applied in the presence of methylglyoxal, had an effect on proliferation and on apoptosis or that it might have affected only proliferation because there was a comparable absolute number of apoptotic cells in the assays with and without dorzolamide. With glyoxal and hydrogen peroxide, on the other hand, total cell numbers were comparable in the assays with and without dorzolamide; accordingly, the absolute number of apoptotic cells was reduced by dorzolamide (Table 1).

To determine during which step of the apoptosis cascade dorzolamide might have interfered, we investigated apoptosis and the protective action of dorzolamide through additional methods.

Classical TUNEL assay revealed DNA nicks in cells after treatment with glyoxal and fewer nicks in the presence of dorzolamide (Fig. 6A). Similarly, the exposure of phosphatidylserine on plasma membranes of cells treated with methyl-

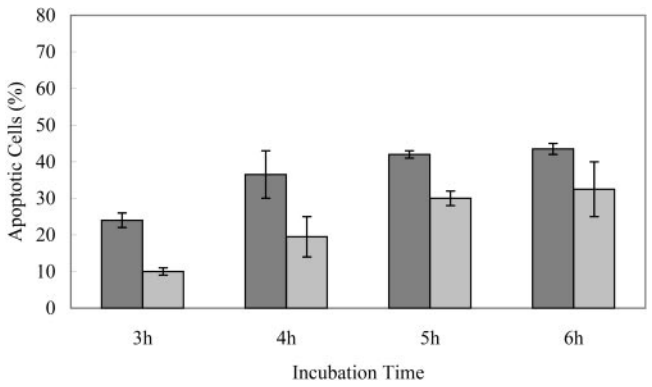

FIGURE 4. Apoptosis induced by methylglyoxal for different time periods in the presence and absence of dorzolamide. Cells were treated with 0.8 mM methylglyoxal without (*dark bars*) or with (*light bars*) 0.3 mM dorzolamide. After the indicated time intervals, the supernatants were removed and incubation was continued with fresh medium. Detached cells were treated as described in the legend to Figure 2. Apoptosis (%) minus the apoptosis measured with control cells without methylglyoxal is shown. Data represent the mean ± SEM of three independent experiments.

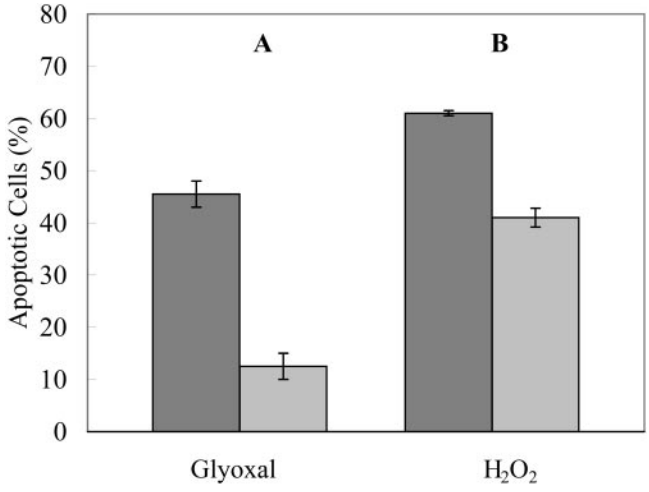

FIGURE 5. Inhibition of glyoxal- and hydrogen peroxide–induced apoptosis by dorzolamide. (**A**) Cells were incubated with 0.3 mM glyoxal (*dark bar*) or with 0.3 mM glyoxal together with 0.3 mM dorzolamide (*light bar*) for 24 hours. (**B**) Incubation with 0.3 mM hydrogen peroxide (in HBSS, as described in the legend to Fig. 2) for 30 minutes without (*dark bar*) or with (*light bar*) 0.3 mM dorzolamide. After 30 minutes, the HBSS containing hydrogen peroxide was exchanged for medium. Data represent the mean ± SEM of four (*dark bars*) or three (*light bars*) independent experiments.

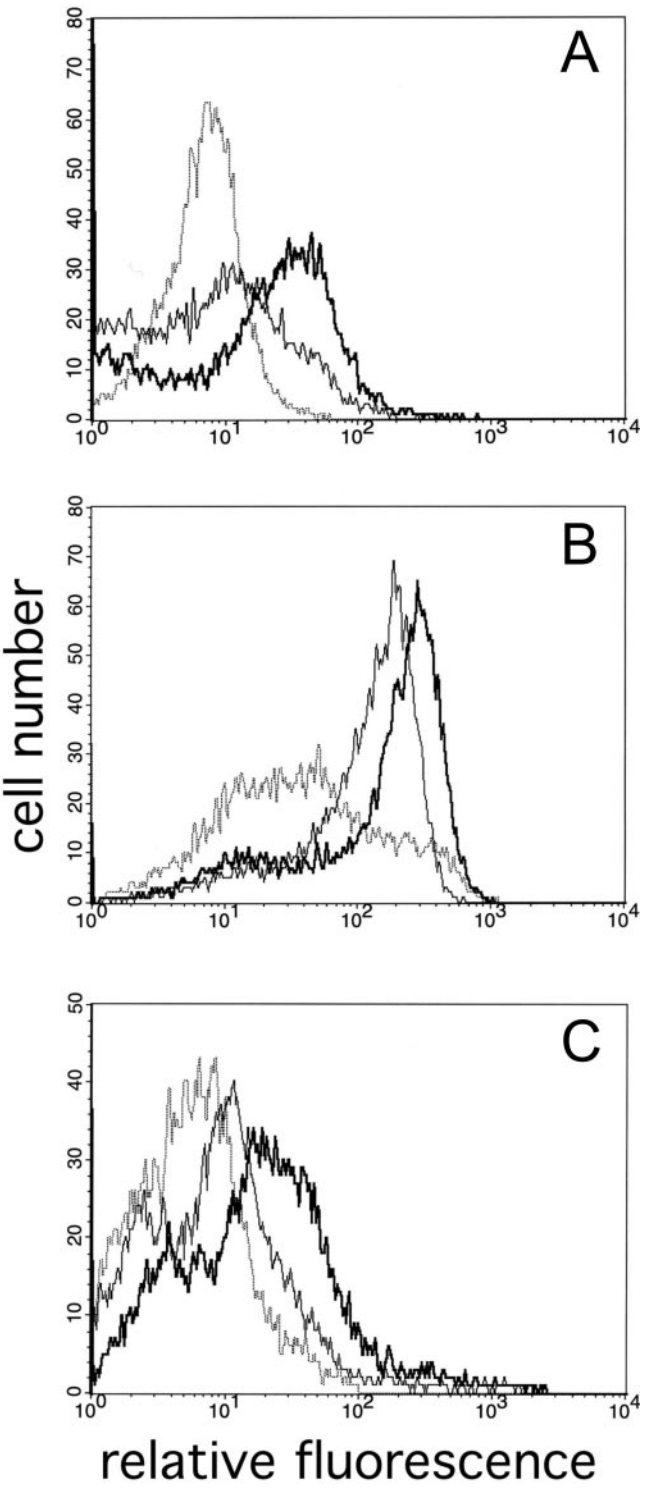

FIGURE 6. Dorzolamide effect revealed by TUNEL assay, binding of annexin V, and production of ROS. (**A**) TUNEL assay. *Dotted line*: control cells; *thick line*: cells treated with 0.1 mM glyoxal for 24 hours; *thin line*: glyoxal + 0.075 mM dorzolamide. (**B**) Binding of FITC-conjugated Annexin V. *Dotted line*: control cells; *thick line*: cells incubated with 0.6 mM methylglyoxal for 5 hours, followed by overnight incubation in fresh medium; *thin line*: methyglyoxal + 0.075 mM dorzolamide. (**C**) Production of ROS; cell treatment as described in panel (**B**). Assays were performed as described in Materials and Methods. Histograms are characteristic examples of several similar experiments.

glyoxal (measured by binding of annexin V) was reduced by dorzolamide (Fig. 6B). The production of ROS was only slightly enhanced by the treatment of cells with methylglyoxal, but the reduction of ROS on cell treatment with dorzolamide was significant (Fig. 6C).

We further investigated changes in the mitochondrial membrane potential. Intact cells in the untreated controls were stained red by JC-1 aggregates. Loss of membrane polarization after exposure to methylglyoxal led to green staining by JC-1 monomers. When the treatment with methylglyoxal was performed in the presence of dorzolamide, the membrane potential was intact in most cells (Figs. 7A–C). Counts of red and green cells revealed approximately 75% green cells (depolarized mitochondrial membranes) in preparations treated with methylglyoxal and 25% green cells in preparations treated with a combination of dorzolamide and methylglyoxal. Controls contained 1% green cells. In contrast to the other manifestations of cell damage we observed, changes in mitochondrial membrane potential were already detectable 1 hour to 2 hours after the toxic agent was added. With glyoxal and hydrogen peroxide, similar results were obtained (not shown).

A crucial step in the apoptosis cascade is the activation of caspase-3. Therefore, we tested the cells for the presence of active caspase-3 after incubation with glyoxal with and without the simultaneous addition of dorzolamide. The amount of activated caspase-3 was reduced to the levels found in control cells when dorzolamide was present during glyoxal treatment (Fig. 8A).

CML-modified proteins belonged to the AGE structures found in cells after pathophysiological changes. We looked for CML modifications after treating the cells with the apoptosis-inducing substances. In contrast to the other manifestations of cell damage, which were similar irrespective of the applied agent, only glyoxal led to significant CML modification. All glyoxal-treated cells bound CML-specific antibody. Under the influence of dorzolamide, binding of the antibody was reduced dramatically (Fig. 8B).

Changes in Intracellular pH

Decreased pH_i is discussed as an early event in apoptosis. We performed assays to detect changes of pH_i after treatment of cells with hydrogen peroxide and methylglyoxal. Hydrogen peroxide provoked a moderate decrease of pH_i, which was not significantly reduced by dorzolamide (not shown). In contrast, administration of methylglyoxal led to an explicit decline of cytosolic pH. In the assays that contained dorzolamide along with methylglyoxal, this acidification was strongly reduced (Fig. 9).

DISCUSSION

In the present study we used glyoxal, methylglyoxal, and hydrogen peroxide to induce AGEs and ROS in a neuronal retinal cell line. The goal was to mimic processes that occur during the degeneration of retinal neurons. In vivo, AGEs are produced during pathophysiological changes relevant for neurodegeneration,[51,52] retinopathy,[53,54] and AMD.[55] ROS, which can be generated by AGEs,[36] is involved in neurodegenerative diseases.[56] AGEs and ROS can provoke acidification of the cytosol. Because of the pH-stabilizing property of dorzolamide,[18] we undertook the present study to explore whether this inhibitor of CAII is capable of protecting neuronal cells from damage through AGEs and ROS. To investigate whether it is possible to rescue cells from apoptotic death, we first tried to determine the conditions that drive retinal neural cells into apoptosis without producing too many necrotic cells. With glyoxal, methylglyoxal, and hydrogen peroxide, this was

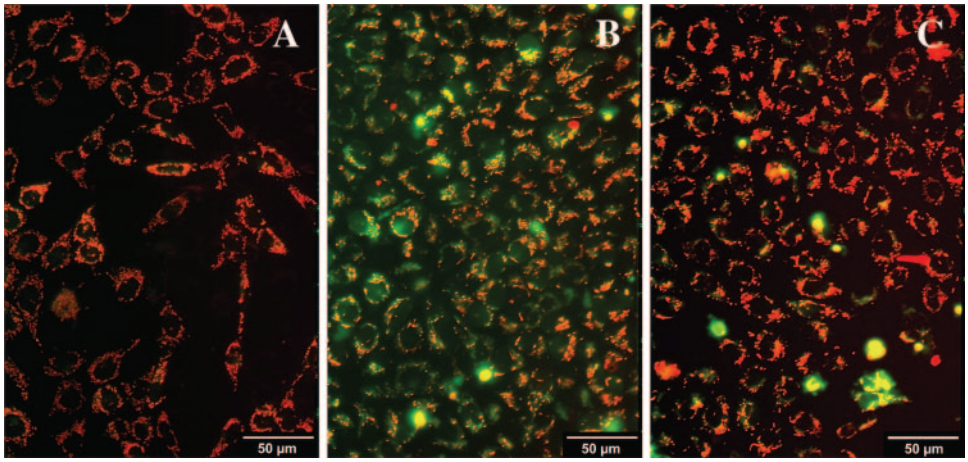

FIGURE 7. Influence of dorzolamide on the loss of mitochondrial membrane potential caused by methylglyoxal. Cells were grown on chambered coverslides coated with collagen IV and stained with JC-1 (2×10^4 cells/mL cultivated for 24 hours). (**A**) Control cells with mostly intact mitochondria stained red from JC-1 aggregates. (**B**) Cells damaged by incubation with 0.8 mM methylglyoxal for 2 hours stained green from JC-1 monomeres. (**C**) After treatment with methylglyoxal in the presence of dorzolamide (0.3 mM), there were fewer green cells in the population; most cells retained intact mitochondria. In each case, a characteristic section of one of five similar experiments is shown.

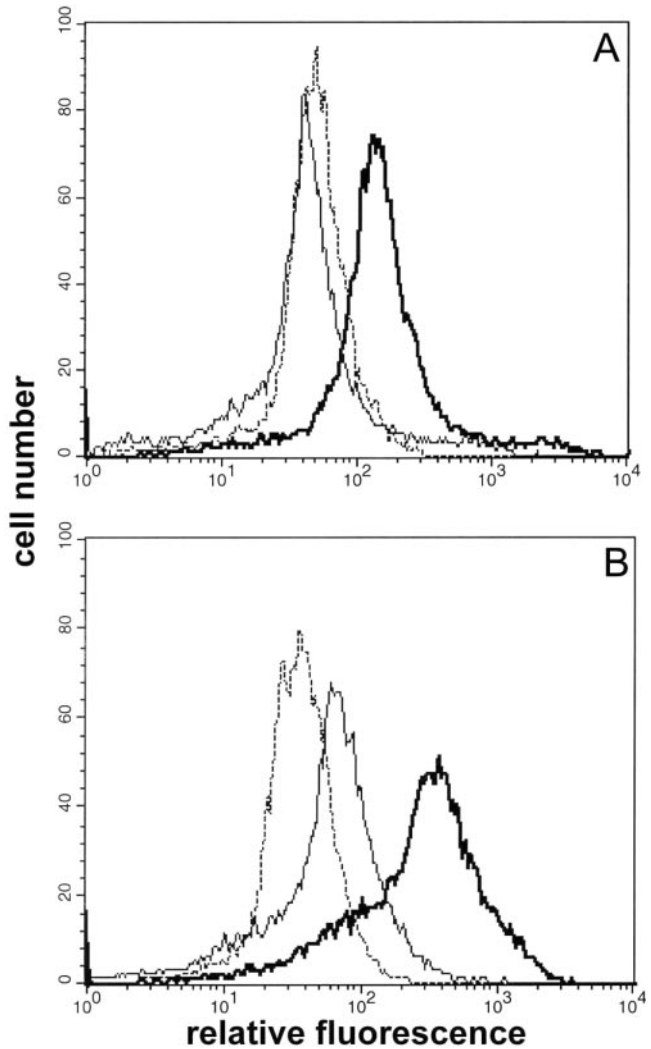

FIGURE 8. Influence of dorzolamide on the augmented occurrence of active caspase-3– and CML modified proteins resulting from glyoxal treatment. (**A**) Active caspase-3. (**B**) CML. After treatment with glyoxal (0.8 mM/5 h followed by 20-hour incubation with fresh medium) in the absence (*thick line*) and in the presence (*thin line*) of 0.3 mM dorzolamide, the cells were harvested as described in the legend to Figure 2 and incubated with anti–active caspase-3 or anti-CML, respectively, for 30 minutes. *Dashed line*: untreated control cells. Histograms are characteristic examples of three similar experiments.

achieved by treating the cells for a duration insufficient for the cells to manifest apoptotic changes, then removing the stimuli and continuing the incubation. During this second incubation, a significant amount of cells became apoptotic. If the stimuli were present for the whole length of incubation time, a higher percentage of necrotic cells was found. The AGE inducers glyoxal and methylglyoxal exerted different effects on the cells: glyoxal concentrations, which led to ratios of apoptosis comparable to those obtained with methylglyoxal, completely inhibited cell proliferation. Conversely, cells continued to grow in the presence of methylglyoxal, though at a reduced rate. In addition, CML modification was only observed after treatment with glyoxal. Consequently, the AGEs induced by glyoxal or methylglyoxal might have been different.

The first incubation period with hydrogen peroxide was shorter than it was with glyoxal or methylglyoxal (Fig. 2), yet apoptosis was always accompanied by necrosis of cells amounting to at least half the number of the apoptotic cells. With all three agents, up to 80% of the cells became apoptotic, as determined by calculating the percentage of cells with subdiploid DNA content. Apoptosis could be decreased by 20%

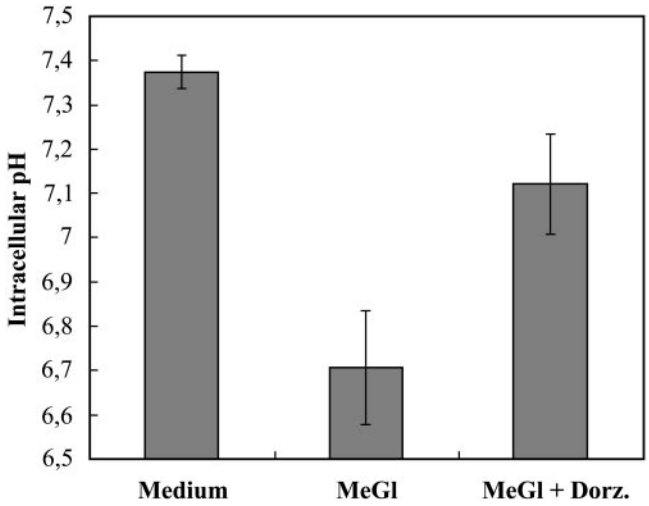

FIGURE 9. Effect of dorzolamide on the decrease of intracellular pH after incubation with methylglyoxal. Cells were left untreated (medium) or were treated with 0.8 mM methylglyoxal for 4 hours followed by 20-hour incubation in fresh medium (MeGl), or 0.3 mM dorzolamide was present together with methylglyoxal (MeGl + Dorz.). Cells were harvested as described in the legend to Figure 2 and were stained with BCECF AM. Data represent the mean ± SEM of three independent experiments.

to 70% (depending on the strength of the apoptosis signal) when the CAII inhibitor dorzolamide was present during incubation. The effects of glyoxal, methylglyoxal, and hydrogen peroxide were similarly diminished. We tested the induction of apoptosis and its inhibition by dorzolamide with a whole series of assays, from ROS production, translocation of phosphatidylserine, and loss of mitochondrial membrane potential—considered early events in the course of apoptosis[34,57,58]—to late events such as the cleavage of DNA. With all the assays we found a protective effect of the CAII blocker, suggesting that it interferes early in the apoptosis cascade.

The possible cause of pH_i reduction after treatment of cells with glyoxal or methylglyoxal has been discussed in a recent paper by our group.[40] Here the alterations in cytosolic pH were found very early after the onset of the cell stress. Thus, monitoring pH_i can show the impairment of the cell function long before apoptosis takes place. The decrease of pH_i may be caused by changes in proton pump activity, the Na^+/H^+ exchanger, or CO_2/H_2CO_3 metabolism,[59] whereas CA could cause acidification. Furthermore, the efficiency of activation of caspase-3 by cytochrome *c* has been found to be pH sensitive, with an optimum pH of 6.6 to 6.8.[32] Thus, one can imagine that stabilizing the pH_i at higher levels may reduce the tendency toward apoptosis. Studies of the membrane and intracellular localization of CAII and of other CA types are necessary for the cell type used in the present study.

Oxidative stress is closely linked to acidification in mitochondria and in the cytoplasm.[60] Inhibition of acidification would reduce the amount of radicals generated and, consequently, would reduce the radical-mediated induction of a caspase-independent pathway of apoptosis.[2] We found reduced ROS production under the influence of dorzolamide.

In our study, dorzolamide, a blocker of the cytoplasmic enzyme CAII,[61] inhibited all manifestations of apoptosis that we observed. Thus, it is evident that dorzolamide exerted a neuroprotectant function in our cell model.

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