Antiapoptotic Properties of α-Crystallin–Derived Peptide Chaperones and Characterization of Their Uptake Transporters in Human RPE Cells

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Purpose. The chaperone proteins, α-crystallins, also possess antiapoptotic properties. The purpose of the present study was to investigate whether 19 to 20-mer α-crystallin–derived mini-chaperone peptides (α-crystallin mini-chaperone) are antiapoptotic, and to identify their putative transporters in human fetal RPE (hfRPE) cells.

Methods. Cell death and caspase-3 activation induced by oxidative stress were quantified in early passage hfRPE cells in the presence of 19 to 20-mer αA- or αB-crystallin–derived or scrambled peptides. Cellular uptake of fluorescein-labeled, α-crystallin–derived mini-peptides and recombinant full-length αB-crystallin was determined in confluent hfRPE. The entry mechanism in hfRPE cells for α-crystallin mini-peptides was investigated. The protective role of polycaprolactone (PCL) nanoparticle encapsulated αB-crystallin mini-chaperone peptides from H_2O_2-induced cell death was studied.

Results. Primary hfRPE cells exposed to oxidative stress and either αA- or αB-crystallin mini-chaperones remained viable and showed marked inhibition of both cell death and activation of caspase-3. Uptake of full-length αB-crystallin was minimal while a time-dependent uptake of αB-crystallin–derived peptide was observed. The mini-peptides entered the hfRPE cells via the sodium-coupled oligopeptide transporters 1 and 2 (SOPT1, SOPT2). PCL nanoparticles containing αB-crystallin mini-chaperone were also taken up and protected hfRPE from H_2O_2-induced cell death at significantly lower concentrations than free αB-crystallin mini-chaperone peptide.

Conclusions. αA- and αB-crystallin mini-chaperones offer protection to hfRPE cells and inhibit caspase-3 activation. The oligopeptide transporters SOPT1 and SOPT2 mediate the uptake of these peptides in RPE cells. Nanodelivery of αB-crystallin–derived mini-chaperone peptide offers an alternative approach for protection of hfRPE cells from oxidant injury.

Keywords: α-crystallin, chaperone peptides, oxidative stress, RPE protection, oligopeptide transporters

The superfamily of small heat shock proteins (sHSPs) has attracted considerable attention in recent years because of its multifunctional cellular properties. The human genome encodes 10 members of the sHSP family, among which αA-crystallin and αB-crystallin are considered important members.[1] Both αA- and αB-crystallins have been studied extensively in the lens for their chaperone and related functions. However, recent studies have identified several novel functions for αA- and αB-crystallins in retina and other tissues in addition to their well-recognized chaperone function.[2] Both α-crystallins are expressed in RPE cells and in the retina; higher expression of αB-crystallin was found in the RPE while αA-crystallin was found mostly in photoreceptors and astroglial and Müller cells.[3,4] Furthermore, αB-crystallin was shown to be upregulated with several stress stimuli and to translocate to nuclei and mitochondrial compartments of RPE cells.[3,5] Microarray and proteomic analysis and histological studies have revealed that α-crystallin accumulates in drusen.[2,6,7] Subsequent studies demonstrated the expression of αB-crystallin in RPE cells overlying drusen.[2,8] and αB-crystallin has been suggested to be a biomarker for AMD.[8] Since overexpression of full length αB-crystallin reduced susceptibility to oxidative stress-induced apoptosis in RPE cells,[9,10] we suggested that αB-crystallin may have therapeutic potential.[5] Since full length αB-crystallin has limited entry into nonstressed cells,[2,5] we considered the possibility that peptides

of αB-crystallin may retain antiapoptotic activity while having enhanced cellular uptake.

The structure, hydrophobicity, and ionic charge are the major factors that determine the chaperone activity of α-crystallins.[11] Potential sequences of α-crystallins that exhibit chaperone activity have been identified.[12,13] We previously identified a 19-mer sequence corresponding to beta3 and beta4 regions of αA-crystallin domain, which has similar chaperone activity in vitro to that of αA-crystallin.[12] Further studies have revealed that the 19-mer peptide sequence of αA-crystallin inhibits fibril formation of Aβ-amyloid peptides and suppresses the toxic action of Aβ-peptide in rat pheochromocytoma (PC12) cells.[11] Similarly, interactive sequences consisting of residues 73 to 92 in αB-crystallin are able to prevent the aggregation of substrate proteins similar to the action of native αB-crystallin.[13]

Transport systems that mediate the uptake of oligopeptides are known in mammalian cells. With respect to RPE, two novel Na^+-coupled transport systems for oligopeptides—namely, sodium-coupled oligopeptide transport system 1 and 2 (SOPT1 and SOPT2)—have been identified.[14,15] SOPT1 is fully Na^+-dependent, while SOPT2 is partially Na^+-dependent. Both transporters have overlapping substrate specificity and can recognize an array of both endogenous as well as synthetic peptides regardless of their amino acid sequence.[14,16–18] Substrate selectivity studies have revealed that SOPT1 and SOPT2 accept oligopeptides consisting of five or more amino acids.[14,16–18]

We wished to study whether α-crystallin mini-chaperones elicit antiapoptotic properties that were shown previously to be associated with parent proteins. Secondly, we also wished to determine the mechanisms of uptake of these α-crystallin mini-chaperones in human RPE cells and to characterize the putative oligopeptide transporter(s). Thirdly, we investigated the uptake of nanoparticle encapsulated αB-crystallin mini-chaperones and determined their protective effect from oxidant-induced cell death in RPE cells.

Materials and Methods

Materials

A 19-mer of αA-crystallin, a 20-mer of αB-crystallin, and a 20-mer of scrambled sequence (Table 1) were custom-synthesized with a purity of > 98% as per the manufacturer (NeoPeptide, Cambridge, MA). Because both the mini-chaperones have significant sequence homology, we have used one scrambled peptide that contained all the amino acids in 20-mer αB-crystallin mini-chaperone peptides. For cellular uptake studies, αA-crystallin and αB-crystallin–derived chaperone peptides were fluorescein-labeled at the Lys residue of the C-terminus (NeoPeptide). The synthetic opioid peptide DADLE (H-Tyr-D-Ala-Gly-Phe–D-Leu-OH) was obtained from Bachem Americas, Inc. (Torrance, CA). Deltorphin II (H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) and Gly-Gly-IIe, polycaprolactone (PCL) and polyvinyl alcohol (PVA) were obtained from Sigma-Aldrich (St. Louis, MO).

RPE Cell Culture

The protocol for the preparation and use of cultured human fetal RPE cells was approved by the University of Southern California Institutional Review Board under protocol #HS-947005, (continuing review approved January 25, 2012) and adhered to the tenets of the Declaration of Helsinki. hfRPE cells were isolated from human fetal eyes (gestational age 16–18 weeks) obtained from Advanced Bioscience Resources, Inc. (ABR, Alameda, CA). Primary cultures of hfRPE cells were established as described previously and used at passages 3 to 4.[19]

Table 1. Peptide Sequences Used in the Study

Nature of Peptide	Amino Acid Sequence
αA-crystallin–derived mini-peptide	DFVIFLDVKHFSPEDLTVK
αB-crystallin–derived mini-peptide	DRFSVNLDVKHFSPEELKVK
Scrambled α-crystallin peptide	DLPLKKNVEDKFHRSFVESV

Apoptosis Assay

Confluent serum-starved hfRPE cells were cotreated with 200 μM tert-Butyl hydroperoxide (tBH) and either 32 μM (75 μg) of αB-crystallin mini-chaperone or αA-crystallin mini-chaperone for 4 hours. Cell death was measured by TdT-mediated dUTP nick-end labeling following the manufacturer's protocol (TUNEL; In Situ Cell Death Detection Kit; Roche Applied Science, Indianapolis, IN). For every experiment, more than 1000 cells were counted from each of nine defined fields from each slide under each condition. Three independent experiments were performed for each experimental condition. TUNEL positive cells were counted and data were expressed as percent of total cells undergoing cell death.[5]

Immunoblot Analysis

Protein was extracted from the cells at the end of indicated experiments. Equal amounts of protein (50 μg) were resolved on 15% Tris-HCl polyacrylamide gels. Membranes were probed with rabbit polyclonal anticleaved caspase-3 (1:1000; Cell Signaling, Danvers, MA). After incubation with the corresponding secondary antibodies, signals were detected using a chemiluminescence system. GAPDH was used as a loading control.

Fluorescein Labeling of Recombinant Human αB-Crystallin and α-Crystallin–Derived Mini-Chaperone Peptides and PCL-Encapsulated αB-Crystallin Mini-Chaperone and Uptake by hfRPE Cells

Recombinant human full-length αB-crystallin was labeled with fluorescein isothiocyanate (FITC) using a commercially available kit (Dojindo Molecular Technologies, Rockville, MD) following the manufacturer's instructions.[5] Custom FITC-labeled α-crystallin–derived peptides were purchased commercially (NeoPeptide). Serum-starved hfRPE cells were incubated with αB-crystallin mini-chaperone (1.7 μM) for 5 to 30 minutes. Similarly, hfRPE cells were incubated with 1.7 μM fluorescein (FITC)-labeled full-length αB-crystallin for 5 to 30 minutes. Uptake of labeled α-crystallin–derived chaperone peptides or full-length αB-crystallin by hfRPE cells was examined under a spinning disc confocal microscope (PerkinElmer, Waltham, MA). A similar protocol was followed for labeling and uptake of PCL-encapsulated αB-crystallin mini-chaperone.

Characteristics of Uptake of α-Crystallin Mini-Chaperone Peptides by Oligopeptide Transporters

hfRPE cells (0.5×10^6 cells/well) were seeded in 24-well cell culture plates for 4 days. Uptake of [^{3}H]-Deltorphin II and [^{3}H]-DADLE in RPE cells was measured as described.[14,15,17,20] After the cells were washed once in uptake buffer, uptake was initiated by adding 0.25 mL of uptake buffer containing 0.1 to 0.25 μCi of [^{3}H]-Deltorphin II or [^{3}H]-DADLE. Based on the experimental condition, the final concentration of these peptides during uptake varied between 10 to 40 nM. Time course studies were carried out initially to determine optimum

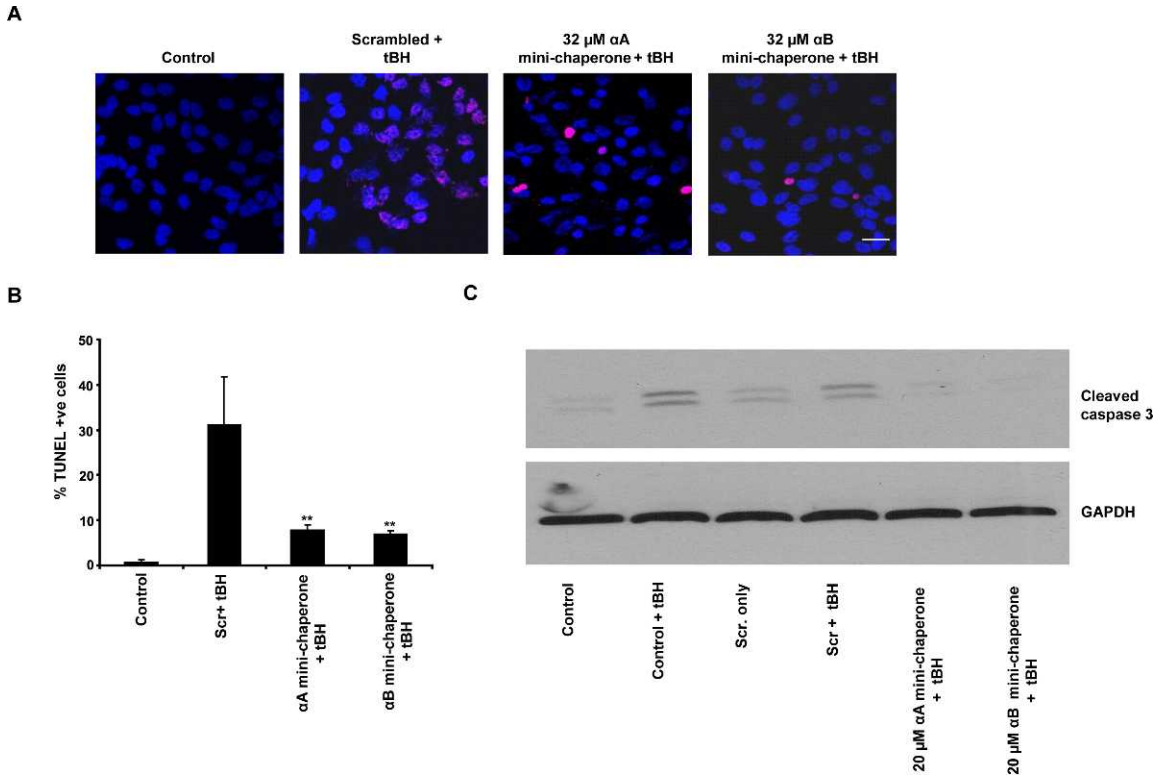

Figure 1. Suppression of oxidant-induced cell death by αA- or αB-crystallin–derived mini-chaperone peptides. (**A**) hfRPE cells were treated with 200 μM tBH or 200 μM tBH plus 32 μM of either αB-crystallin–derived or αA-crystalline–derived mini-chaperone peptide for 4 hours. Apoptosis was assessed by TUNEL staining. Confocal images of TUNEL-positive cells (*red*) and nuclei (*blue*) with and without cotreatment with αA, αB-crystallin–derived peptides are shown. (**B**) Quantification of percent dead cells by TUNEL assay. Apoptosis was significantly higher in cells cotreated with tBH and scrambled peptide when compared with cells cotreated with either αA- or αB-crystallin–derived mini-chaperone peptide and tBH. *Asterisks* indicate $P < 0.01$ versus scrambled crystallin-derived peptides treated with tBH. (**C**) Exogenously added α-crystallin mini-chaperones protect hfRPE cells from tBH-induced oxidative stress by inhibiting activation of caspase-3. hfRPE cells were treated with 200 μM tBH either alone or in the presence of 20 μM αA- or αB-crystallin mini-chaperone in serum-free medium for 4 hours. Caspase-3 activation was prominent in control cells and in cells cotreated with scrambled peptide and 200 μM tBH. Scr represents scrambled α-crystallin mini-peptide, and αA- and αB- represent αA-crystallin–derived mini-chaperone peptide and αB-crystallin–derived mini-chaperone peptide, respectively.

uptake and accordingly, subsequent uptake measurements were performed for 30 minutes representing initial uptake rates.

Uptake of FITC-Labeled αB-Crystallin–Derived Peptide by Human RPE Cells

hfRPE cells were seeded in chamber slides (Nalge Nunc International, Chicago, IL) with an initial density of 5000 cells/chamber and cultured for 24 hours. After 24 hours, cells were washed with PBS and subsequently incubated with 10 nM FITC-labeled αB-crystallin mini-chaperone for 15 minutes in the absence or presence of 10 μM DADLE. Subsequently, cells were washed with PBS and fixed with 4% paraformaldehyde for 5 minutes at room temperature. Samples were mounted with fluorescent dye containing DAPI and visualized under a fluorescence microscope.

Formulation of PCL-Encapsulated Nanoparticles of αB-Crystallin–Derived Mini-Chaperone Peptide

αB-crystallin mini-chaperone–loaded nanoparticles were prepared using w/o/w solvent evaporation method. Briefly, 100 mg of PCL was dissolved in 2 mL of dichloromethane. To this, 20 mg/mL solution of either αB-crystallin mini-chaperone or scrambled α-crystallin mini-peptide in 1 mL of deionized water was added, the container was placed on ice, and the contents sonicated at 9 W for 30 seconds using a probe sonicator (Misonix S3000; Qsonica, LLC, Newtown, CT). This primary emulsion was then further emulsified in 10 mL of 2 % PVA containing 10% sodium chloride (NaCl) by sonication for 2 minutes at 30 W on ice. The w/o/w emulsion thus formed was added dropwise to 50 mL of 2% PVA containing 10% NaCl while stirring at 7000 rpm on a magnetic stirrer. The particles were allowed to harden and the residual solvent was evaporated by stirring for 3 hours at room temperature followed by rotary evaporation (Buchi Rotavapor R200; Buchi Analytical Inc., New Castle, DE) under vacuum for 2 hours at 40°C in a heated water bath. The nanoparticles were then harvested by centrifugation at 34,000*g* for 50 minutes at 4°C, followed by two washes with 50 mL of 0.5% PVA each time to remove unencapsulated drug. The particles were lyophilized for 48 hours and stored at 4°C until further use. To prepare blank particles, αB-crystallin mini-chaperone was excluded from the above procedure.

Characterization of Nanoparticles

To measure the particle size, a dilute suspension of nanoparticle was made in deionized water. The mean hydrodynamic diameter was measured based on the intensity of scattering by the particles at 173° angle using a commercial molecular size analyzer (Zetasizer Nano ZS; Malvern Instruments Ltd., Worcestershire, UK). An average of 11 scans was performed

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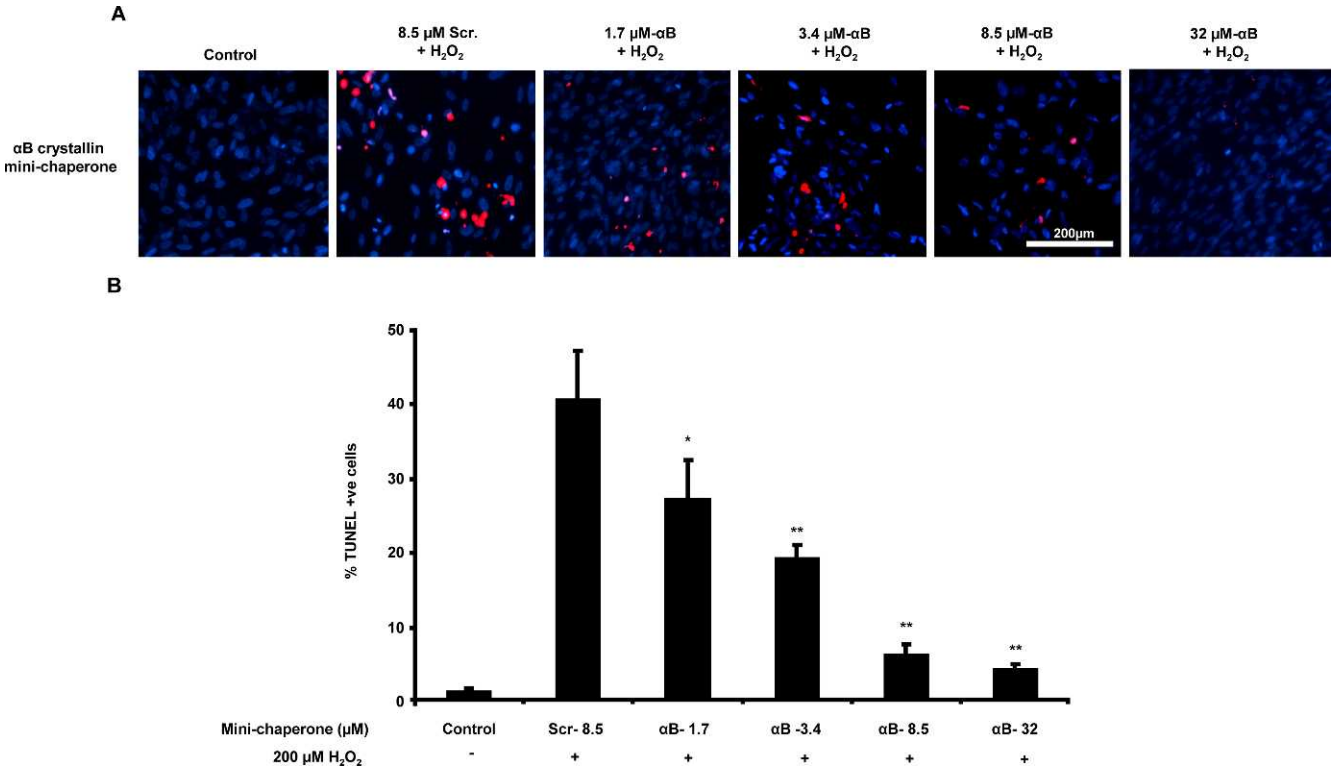

FIGURE 2. Dose-dependent protection by αB-crystallin–derived mini-chaperone from stressed hfRPE cells. hfRPE cells were coincubated with varying doses of αB crystallin mini-chaperone and 200 μM H_2O_2 for 24 hours, and cell death was assessed by TUNEL assay. Confocal images of TUNEL-positive cells (*red*) and nuclei (*blue*) are shown. (**B**) Quantification of TUNEL-positive cells. Data are presented as percent of TUNEL-positive cells. $^*P < 0.05$, $^{**}P < 0.01$.

for each sample. The polydispersity index as well as the zeta potential of the particles was also measured. For surface morphology, nanoparticles were viewed by transmission electron microscopy (Philips, Eindhoven, Netherlands).[21]

Mini-Chaperone Peptide Loading

For drug loading estimation, 5 mg of the nanoparticle was digested in 1 mL of dicholoromethane and vortexed for 1 hour. We determined peptide content with and without dicholoromethane to ensure that this solvent does not cause peptide denaturation (see Supplementary Methods section for detailed procedure). Thereafter, 5 mL of deionized water was added and vortexed for another 2 hours to extract the drug in water. The water layer was separated from the organic layer by centrifugation at 13,000*g* for 5 minutes. The upper water layer was collected and total protein content was measured using a reagent kit (Micro BCA Protein Assay Reagent Kit; Thermo Fisher Scientific, Rockford, IL) as per manufacturer's manual. This method measures specifically the amount of protein contained in each sample. Briefly, the standard curve was prepared from a pure sample of αB-crystallin mini-chaperone peptide or scrambled α-crystallin peptide and thus the total protein (representing the mini-chaperone peptide content) in the water layer was measured using the respective standard curve. The following equations were used to calculate the loading and encapsulation efficiency: Theoretical Loading (%) = Total Amount of Mini-Chaperone Added/(Total Amount of Mini-Chaperone Added + Total Amount of PCL Taken) × 100; Actual Loading (%) = (Amount of Mini-Chaperone Extracted/Amount of Nanoparticles Taken) × 100; Loading Efficiency (%) = (Actual Loading [%]/Theoretical Loading [%]) × 100.

Cell Death Studies

Cell death was studied in hfRPE cells cotreated with varying doses of nanoparticles (0.34, 0.68, or 1.7 μM) and 200 μM H_2O_2 for 24 hours. TUNEL-positive cells were counted and data were expressed as percent of total cells undergoing cell death.[5]

Data Analysis

The kinetic parameters (K_t and V_{max}) were determined by nonlinear regression analysis and the values confirmed by linear regression analysis according to the Eadie-Hofstee transformation of the Michaelis-Menten equation (Sigma Plot, v. 6.0; SPSS, Inc., Chicago, IL). Statistical analysis was performed with one-way ANOVA followed by Tukey's posthoc test. A $P < 0.05$ was taken as statistically significant. All experiments were repeated three times, and measurements were made in duplicate for each experimental condition. Data are presented as the mean ± SEM.

RESULTS

Mini-Chaperone Peptides Derived From α-Crystallin Protect hfRPE Cells From Oxidative Injury

To study whether α-crystallin mini-chaperones offer protection to hfRPE cells from oxidative stress, we coincubated hfRPE cells with 200 μM tBH and 32 μM α-crystallin mini-chaperones for 4 hours. We selected this concentration for α-crystallin mini-chaperone based on our pilot dose-response studies (Kannan R, et al. *IOVS* 2010;51: ARVO E-Abstract 1441). Apoptotic cell death was assessed by TUNEL assay. Our data revealed that both αA and αB-crystallin mini-chaperones

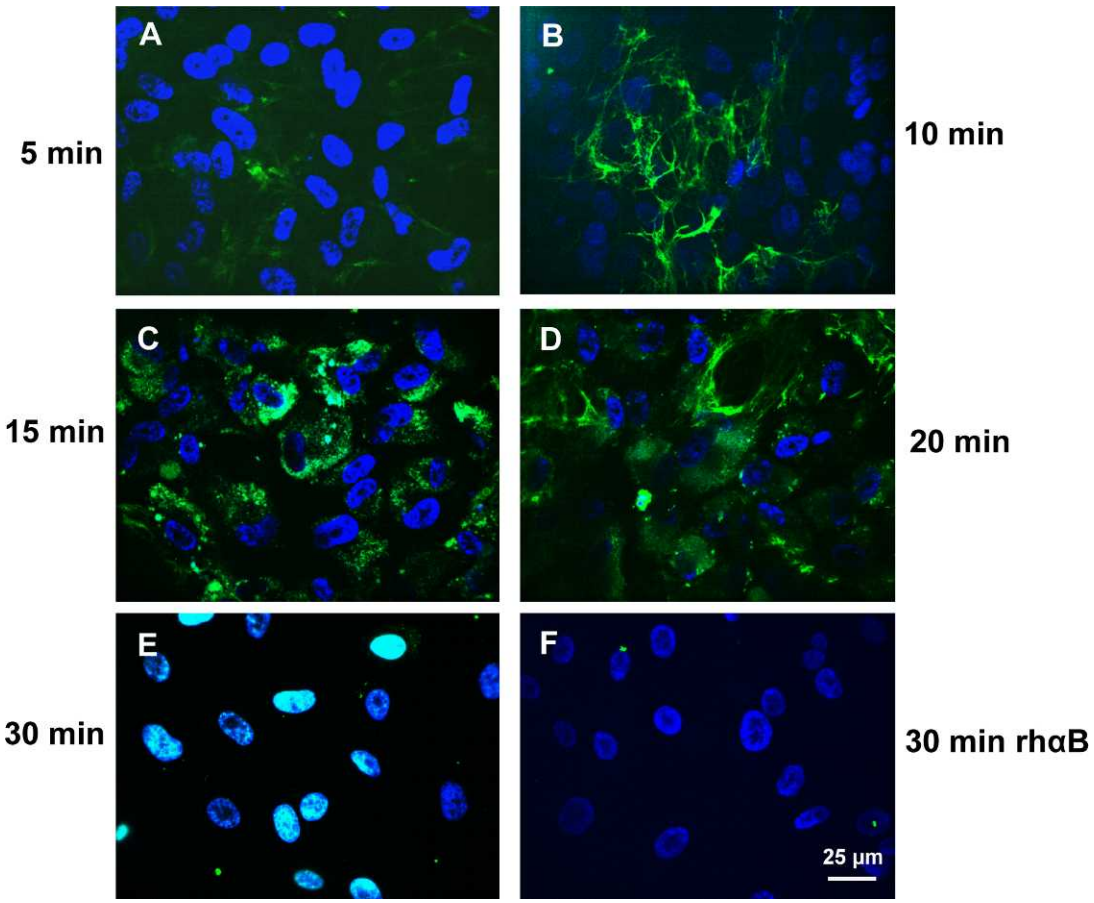

FIGURE 3. Time-dependent uptake and translocation of αB-crystallin–derived mini-chaperone peptides and FITC-labeled full-length αB-crystallin in hfRPE cells. Serum-starved RPE cells were incubated with 1.7 μM of FITC-labeled αB crystallin mini-chaperone for 5 to 30 minutes. Panels A through E show time-dependent uptake of labeled αB-crystallin mini-chaperone by RPE. Fluorescent signal was evident in the cytosol up to 20 minutes, followed by nuclear translocation in 30 minutes (Fig. 3E). Minimal uptake of full-length αB-crystallin was observed (Fig. 3F) in 30 minutes.

protected hfRPE cells from apoptotic cell death (Fig. 1A). This protection was highly significant ($P < 0.01$) when compared with scrambled peptides (Fig. 1B). Immunoblot analysis showed that inhibition of apoptosis by α-crystallin mini-chaperones was accompanied by a significant decrease in caspase-3 activation, which was not observed with a nonspecific scrambled peptide (Fig. 1C). Both αA-crystallin mini-chaperone and αB-crystallin mini-chaperone displayed this protective effect against tBH.

The dose-dependent effect of H_2O_2 treatment by hfRPE cells in the presence of 1.7, 3.4, 8.5, and 32 μM αB-crystallin mini-chaperone is shown in Figure 2. The corresponding TUNEL staining for 8.5 μM scrambled α-crystallin peptide is also shown (Fig. 2A). A progressive decrease in the number of TUNEL-positive cells with increasing concentration of αB-crystallin mini-chaperone was observed. The inhibition of cell death was significant at all mini-chaperone concentrations studied and amounted to 66%, 47.1%, 15.4%, and 11.9%, respectively (Fig. 2B).

Time-Dependent Uptake of Exogenously Added Fluorescein-Labeled Full-Length αB-Crystallin and αB-Crystallin Mini-Chaperone Peptide by RPE Cells

hfRPE cells were incubated with 1.7 μM fluorescein-labeled αB-crystallin mini-chaperones or full-length αB-crystallin for 5 to 30 minutes in serum free medium. As seen in Figure 3, mini-chaperone peptide uptake by hfRPE cells was time-dependent, first beginning at 5 minutes, becoming prominent in the cytosol at 10 to 15 minutes, predominantly perinuclear at 20 minutes, and nuclear at 30 minutes (Figs. 3A–E). Consistent with previous work,[5] we did not observe any cellular uptake of full-length αB-crystallin over this time period (Fig. 3F).

We next examined the nature of uptake of αA and αB-crystallin mini-chaperones by hfRPE cells by comparing their abilities to compete with DADLE,[17] a preferred synthetic substrate for SOPT2, for the uptake process. αA and αB-crystallin mini-chaperones inhibited labeled DADLE uptake in a dose-dependent manner (Fig. 4A). The inhibition of DADLE uptake was also seen with scrambled α-crystallin peptide, suggesting that the oligopeptide transporter mediating DADLE uptake does not distinguish between 19 and 20-mer α-crystallin mini-chaperones and the nonchaperone scrambled peptides (Fig. 4A). The IC_{50} values from these studies were 0.15 ± 0.02 μM for αA-crystallin mini-chaperone, 0.49 ± 0.13 μM for αB-crystallin mini-chaperone, and 0.59 ± 0.13 μM for scrambled peptide. αB-crystallin mini-chaperone also inhibited the uptake of deltorphin II, a known substrate for SOPT1.[14] A unique feature of SOPT1 is that its activity is stimulated by small peptides (dipeptides and tripeptides). Thus, while the two oligopeptide transport systems (SOPT1 and SOPT2) exhibit similar substrate specificity, they can be differentiated by the opposing modulating effects of the dipeptides and tripeptides.[15] Not only did 25 μM of αA and αB-crystallin mini-chaperone inhibited unstimulated deltorphin II uptake in hfRPE cells, they also inhibited the

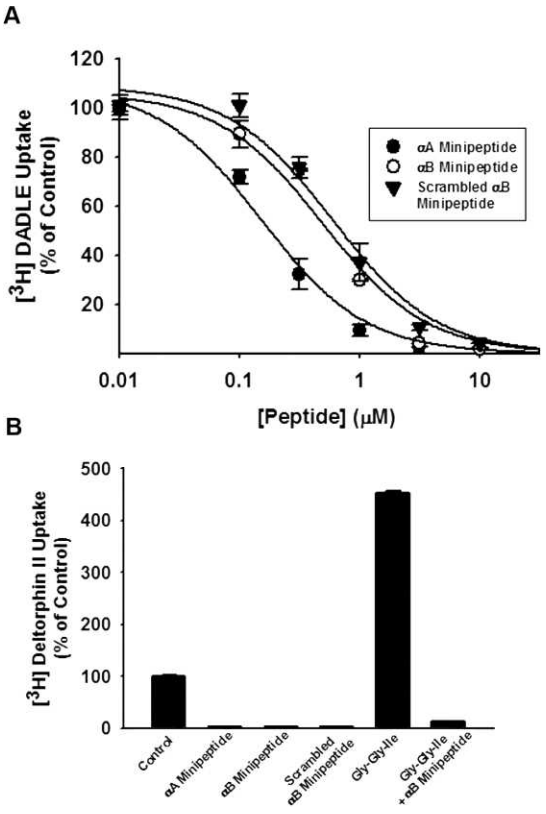

Figure 4. Inhibition of DADLE and deltorphin II uptake in hfRPE cells by αA or αB crystallin-derived mini-chaperone peptides. (**A**) Uptake was performed with [^{3}H]-DADLE (10 nM) for 30 minutes in the absence or presence of increasing concentrations of αA- and αB-crystallin–derived mini-peptides and a scrambled peptide. The IC_{50} values calculated from the dose-response studies were: αA-crystallin–derived mini-peptide, 0.15 ± 0.02 μM; αB-crystallin–derived mini-peptide, 0.49 ± 0.13 μM; scrambled peptide, 0.59 ± 0.13 μM. (**B**) Inhibition of deltorphin II uptake by αA- or αB-crystallin–derived mini-peptides in RPE cells. Uptake measurements were made for 30 minutes with [^{3}H]-deltorphin II (40 nM) in the absence or presence of α-crystallin–derived mini-peptides (25 μM). Uptake of deltorphin II was also determined in the absence or presence of Gly-Gly-Ile (1 mM), a tripeptide that stimulates the uptake of deltorphin II via SOPT1. The α-crystallin mini-peptides potently inhibited deltorphin II uptake in the absence as well as in the presence of Gly-Gly-Ile.

uptake of deltorphin II in the presence of the stimulating tripeptide Gly-Gly-Ile (1 mM; Fig. 4B).

Cellular Uptake of αB-Crystallin–Derived Mini-Chaperone and Effect of DADLE on Uptake

Even though αB-crystallin mini-chaperone inhibited the uptake of deltorphin II and DADLE, preferred substrates for SOPT1, and SOPT2, respectively,[14,17] it is possible that the mini-chaperone blocks their uptake by simply interacting with the substrate-binding site on the transporter without itself being translocated across the membrane. To rule out this possibility, we used FITC-tagged αB mini-chaperone and performed confocal studies to monitor directly the entry of the fluorescence-labeled peptide into the cells. These experiments provide evidence for intracellular localization of αB-crystallin mini-chaperone (Fig. 5). The FITC-labeled mini-chaperone entered into hfRPE cells. This entry process was almost completely blocked in the presence of excess amounts of DADLE, suggesting that DADLE competed with αB-crystallin mini-chaperone for the transport process (Fig. 5). These data provide further evidence that transport of αB-crystallin mini-chaperone by RPE cells is mediated by SOPT1 and SOPT2.

Saturation Kinetics and Kinetic Parameters of Inhibition of DADLE and Deltorphin II Uptake by αB-Crystallin–Derived Mini-Chaperone Peptide

Kinetics of uptake of various concentrations of DADLE (0–25 μM) by hfRPE cells was studied in the presence of 0.5 μM αB-crystallin mini-chaperone (Fig. 6A). Eadie-Hofstee plot of the data showed that there was no change in the maximal velocity (V_{max}) of uptake (1.3 ± 0.1 vs. 1.2 ± 0.1 nmole/mg protein/30 min, $P < 0.05$). However, the Michaelis constant increased from 2.3 ± 0.3 μM to 5.4 ± 0.4 μM, $P < 0.01$) in the presence of αB-crystallin mini-chaperone, suggesting competitive inhibition (Fig. 6B). Saturation kinetics of deltorphin II uptake in the presence or absence of αB-crystallin–derived mini-chaperone is shown in Figure 7A. Uptake of deltorphin II was measured at increasing concentrations (0–250 μM). The uptake was saturable whether the uptake was measured in the presence or absence of αB-crystallin (Fig. 7A). In the presence of αB-crystallin mini-chaperone peptide, the Michaelis constant increased to 60.1 ± 15.6 μM while the control value in the absence of the mini-chaperone was 26.3 ± 4.5 μM. However, there was no significant change in the V_{max} (absence of the mini-chaperone, 1.8 ± 0.1 nmol/mg protein/30 min; presence of the mini-chaperone, 1.9 ± 0.3 nmol/mg protein/30 min; Fig. 7B).

Characterization of αB-Crystallin Mini-Chaperone Encapsulated PCL Particles and Efficiency of Protection of RPE Cells From Oxidant Injury

We wished to study whether delivery of α-crystallin mini-chaperone peptide in the form of nanoparticles could enhance the protective function in hfRPE cells. For this purpose, we generated PCL-encapsulated nanoparticles containing αB-crystallin mini-chaperone and scrambled α-crystallin peptides. The characteristics of PCL nanoparticles, namely the mean hydrodynamic diameter, polydispersity index, zeta potential, theoretical, and actual drug-loading and encapsulation efficiency as well as particle size and morphology by electron microscopy are presented in Table 2 and Figure 8, respectively. To rule out the possibility that dichloromethane interacts with the mini-peptide, the peptide content of αB-crystallin mini-chaperone was measured before and after dichloromethane treatment and was found to be unchanged (194 ± 40 μg/mL and 191 ± 15 μg/mL, respectively). Thus, exposure to dichloromethane did not interfere with the Micro BCA assay (Micro BCA Assay Reagent Kit; Pierce Chemical, Rockford, IL). This was further confirmed by the circular dichroism spectrum that was unaltered before and after exposure of the peptide to dicholoromethane (See Supplementary Table S1 and Supplementary Figure S1 for αB-crystallin mini-peptide secondary structure and CD spectrum). We first determined the uptake of PCL-encapsulated αB-crystallin mini-chaperone by RPE cells. As shown in Figure 9A, there was a time-dependent uptake of PCL nanoparticle containing αB-crystallin mini-chaperone by hfRPE cells; particles resided in the cytosol at 30 minutes, moved to the perinuclear region at 1 hour, and were found within the nucleus at 4 hours. However, the uptake and nuclear translocation of PCL-αB-crystallin took longer time than that of αB-crystallin mini-chaperone alone (see Fig. 3). Further, we determined the dose-dependent effect of PCL-coated particles containing the αB-crystallin mini-chaperone and compared the efficiency in protection from H_2O_2-induced apoptosis with nonencapsulated free αB-crystallin mini-chaperone peptide.

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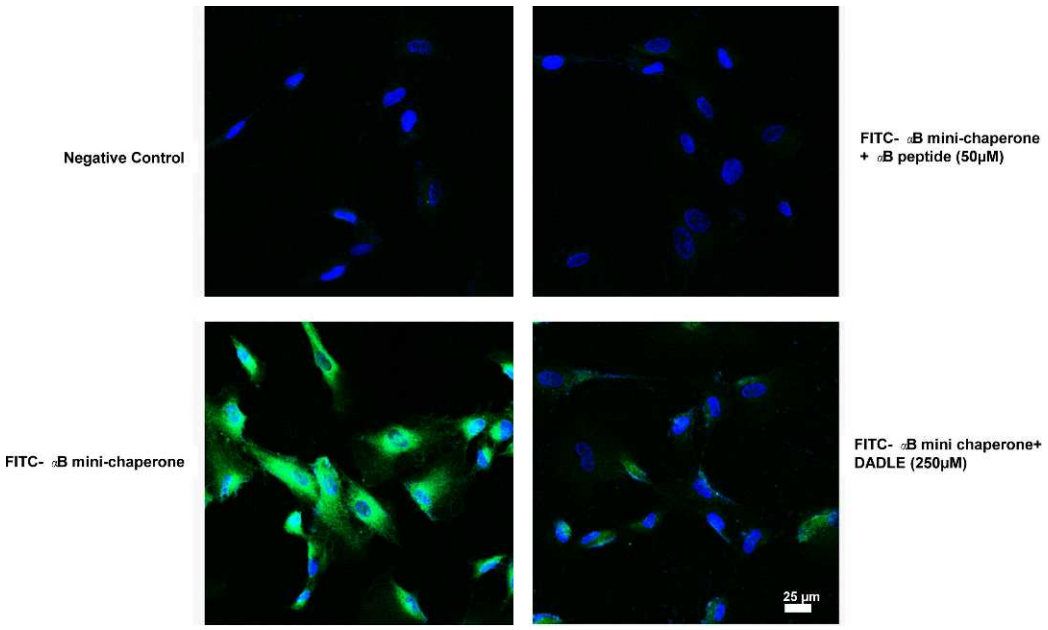

Figure 5. Confocal images showing inhibition of fluorescein-labeled αB-crystallin–derived mini-chaperone peptide by DADLE. Fluorescein-labeled αB-crystallin mini-chaperone (10 nM) was used to show peptide entry into cells. Unlabeled peptide along with nuclear staining by DAPI served as negative control. The images show the entry of fluorescein-labeled αB-crystallin mini-chaperone into human fRPE cells and the inhibition of this entry process in the presence of excess amounts of unlabeled αB-crystallin mini-chaperone or DADLE.

Nanodelivery of αB-crystallin mini-chaperone in the form of PCL particles significantly inhibited H_2O_2-induced cell death assessed by TUNEL staining at all doses studied ($P < 0.01$) as compared with PCL particle alone (Figs. 9B, 9C). The inhibition of apoptosis was ~79% with the lowest concentration studied (0.34 μM). Comparing the inhibitory level of PCL encapsulated αB-crystallin mini-chaperone peptide with that of free αB-crystallin mini-chaperone peptide (Fig. 2), it is evident that nanoparticle delivery of the peptide is more efficient in arresting oxidant-induced apoptosis at lower concentrations than the free peptide (Figs. 9B, 9C). A > 72% inhibition of apoptosis was achieved with the lowest concentration of PCL-αB-crystallin mini-chaperone peptide studied, namely 0.34 μM. These results suggest that αB-crystallin mini-chaperone PCL-encapsulated particles are more efficient than the free peptide in arresting hfRPE apoptosis.

Discussion

In the present study, we have used hfRPE cells for studying transport and protective properties of α-crystallin–derived mini-chaperone peptides. We performed our studies in hfRPE cells as it has been known that these early passage cultures closely represent the phenotype of healthy RPE in vivo.[22,23] It was further shown that primary/early passage cultures of fetal and adult human RPE cells are superior to transformed RPE cell types such as ARPE-19 and D407.[23] As well, ARPE-19 and adult RPE cultures may have differential responses to oxidant challenge.[24] In addition, use of hfRPE cells may have an advantage over adult human RPE cells in that, adult human RPE cells show an age-dependent decline in mitochondrial function and antioxidant potency.[25]

In the present study, we found that mini-chaperone peptides derived from αA and αB-crystallins exhibited anti-apoptotic properties. Both αA and αB-crystallin mini-chaperone peptides protected RPE from H_2O_2-induced cell death and inhibited caspase-3 activation. Nanoparticle encapsulation increased the efficiency of hfRPE protection from cell death induced by oxidant stress. Further, unlike the full-length αB-crystallin, αB-crystallin mini-chaperones and PCL–αB-crystallin mini-chaperones exhibited prominent uptake by hfRPE cells and showed time-dependent nuclear localization. Using preferred oligopeptide substrates, we could demonstrate that the uptake of α-crystallin mini-chaperones was mediated by two related sodium-dependent oligopeptide transporters, SOPT1 and SOPT2. Further, our studies showed that nanoencapsulation of αB-crystallin mini-chaperone peptide inhibited oxidant-induced apoptosis more efficiently than the mini-chaperone peptide alone.

In addition to the well-known chaperone properties of α-crystallins, recent work from our laboratory as well as others have demonstrated antiapoptotic properties of α-crystallin against multiple stress stimuli that include H_2O_2, staurosporine, tumor necrosis factor-alpha, UV radiation, hypoxia, ceramide, and tunicamycin.[3–5,9,10,26–30] Overexpression of αB-

Table 2. Characterization of PCL Nanoparticles

No.	Particles	Mean Hydrodynamic Diameter, nm	Polydispersity Index	Zeta Potential, mV	Theoretical Loading, %	Actual Loading, %	Encapsulation Efficiency, %
1	PCL-blank	341	0.21	−2.4	–	–	–
2	PCL–αB-crystallin mini-chaperone	342	0.17	−1.5	16.67	3.58	21.48
3	PCL-scrambled α-crystallin	321	0.16	−4.1	16.67	3.26	19.56

Investigative Ophthalmology & Visual Science

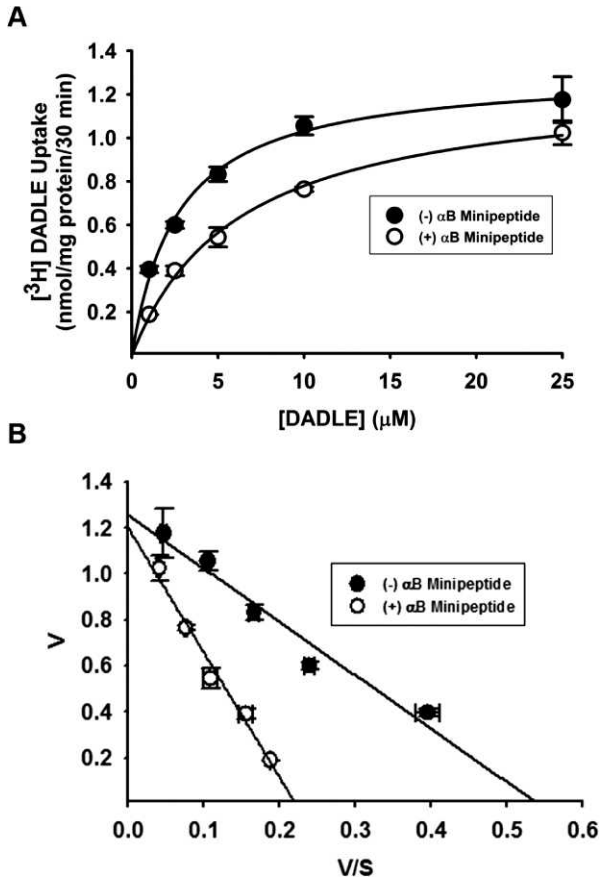

FIGURE 6. Kinetics of inhibition of DADLE uptake by αB-crystallin–derived mini-chaperone peptide. (**A**) Uptake of DADLE was measured for 30 minutes at increasing concentrations in the absence or presence of αB-crystallin–derived mini-peptide (0.5 μM). (**B**) Eadie-Hofstee plot to determine the kinetic parameters for DADLE uptake in the absence and presence of the α-crystallin mini-peptide.

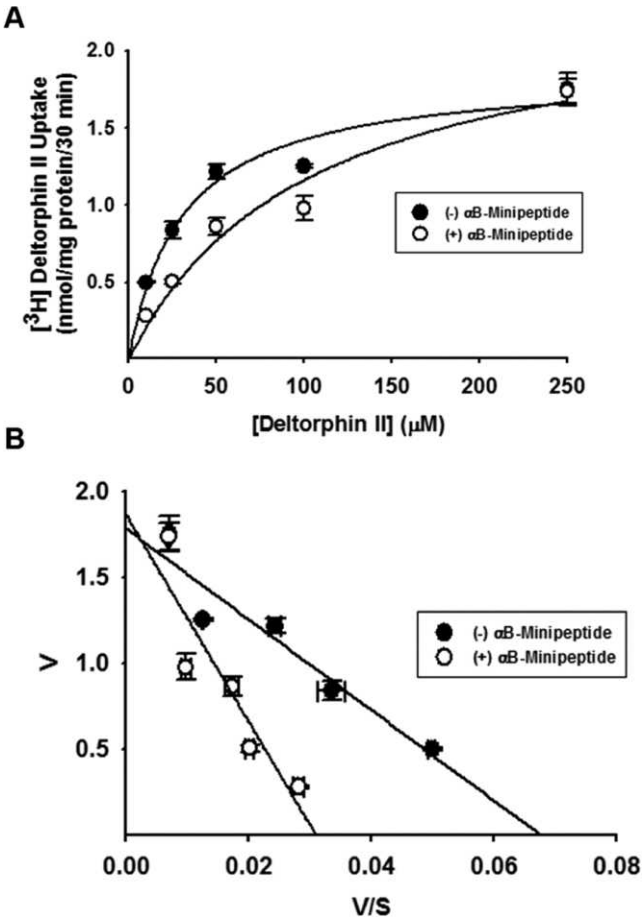

FIGURE 7. Kinetics of inhibition of deltorphin II uptake by αB-crystallin–derived mini-chaperone peptide. (**A**) Uptake of deltorphin II was measured for 30 minutes at increasing concentrations in the absence or presence of αB-crystallin–derived mini-peptide (0.5 μM). (**B**) Eadie-Hofstee plot to determine the kinetic parameters for deltorphin II uptake in the absence and presence of the α-crystallin mini-peptide.

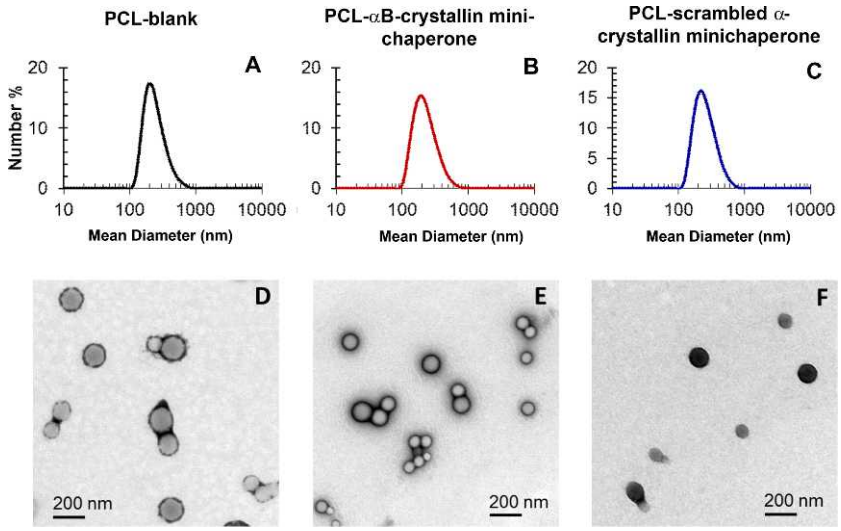

FIGURE 8. Characteristics of PCL nanoparticles particle size, surface morphology, and mean size distribution of the PCL nanoparticles are presented in panels (**A–C**). The surface morphology of the particles by transmission electron microscopy is presented in panels (**D–F**).

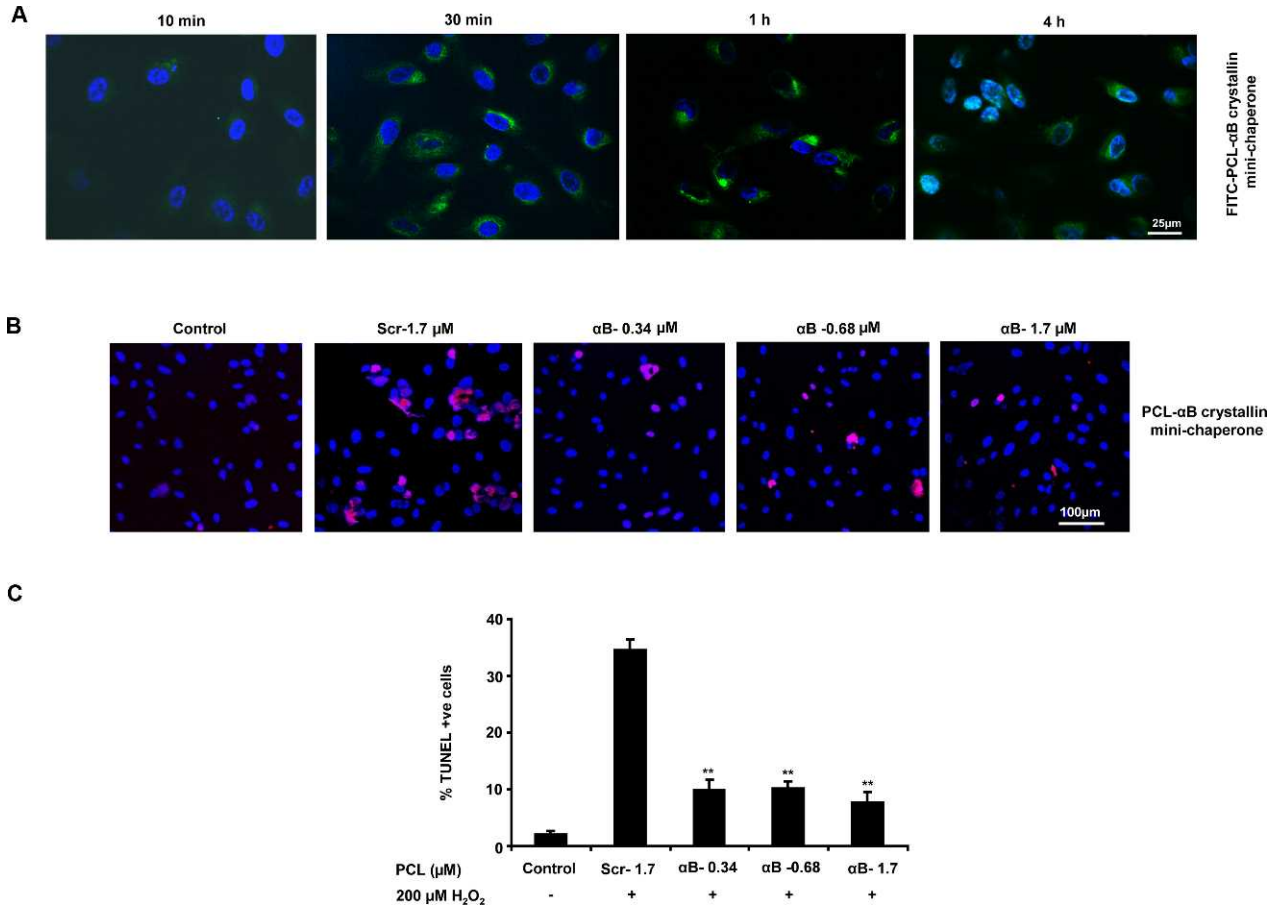

Figure 9. Cellular uptake of PCL-encapsulated αB-crystallin mini-chaperone peptide nanoparticles and protection from cell death of stressed RPE cells. (**A**) Serum-starved hfRPE cells were incubated with 1.7 μM of FITC-labeled PCL–αB-crystallin mini-chaperone for 10 minutes to 4 hours. Cytosolic localization was prominent at 30 minutes to 1 hour, while nuclear localization was found at 4 hours. (**B**) hfRPE cells were cotreated with varying doses of PCL–αB-crystallin mini-chaperone and 200 μM H_2O_2 for 24 hours, and cell death was assessed by TUNEL assay. Confocal images of TUNEL-positive cells (*red*) and nuclei (*blue*) are shown. (**C**) Quantification of TUNEL-positive cells. Data are presented as percent of TUNEL-positive cells. $^{**}P < 0.01$.

crystallin was found to offer protection while silencing rendered cells susceptible to apoptosis from oxidative injury. Signaling mechanisms of cellular protection varied depending on the imposed stress stimuli.[31] Further, recent work by Pasupuleti et al.[32] found that the antiapoptotic function of αA-crystallin in Chinese hamster ovary cells and HeLa cells is linked to its chaperone function. It is of interest that Kurnellas et al.,[33] while studying structure-activity relationship between chaperone activity and therapeutic (protective) function of 19- to 20-mer peptides, found that this correlation was also evident for several sHSPs in addition to αB-crystallin mini-chaperone.

It is well known that specific regions exist in αB-crystallin structure that are sites of interaction with other proteins and thus exhibit chaperone functions. For example, Ghosh et al.[34] found that two interactive sites containing amino acids residues 73 to 85 and 131 to 141 belonging to the αB-crystallin core domain exhibited chaperone activity similar to that of parent αB-crystallin. We identified 19- to 20-mer sequences with chaperone function from αA-crystallin and αB-crystallin.[12,13] The individual amino acid, the location, and the chain length of the amino acid sequence are important determinants of the function of a specific peptide. For example, while a 19-mer peptide consisting of a 70 to 88 sequence of αA-crystallin is antiapoptotic, sequences 66 to 80 of a 15-mer peptide cause aggregation and toxicity.[11,35] Thus, it was of interest to determine whether selected α-crystallin–derived peptides with chaperoning activities exhibit protective antiapoptotic function against oxidative stress in RPE cells. We also characterized the uptake of αA- and αB-crystallin mini-chaperones in RPE cells and identified the transporters that are responsible for the entry of these peptides into RPE cells.

Our studies show contrasting findings between the full-length αB-crystallin and the αB-crystallin–derived mini-chaperone with respect to uptake by hfRPE cells. While full-length αB-crystallin showed minimal or no cellular uptake, prominent uptake of the αB-crystallin mini-chaperone was observed. Thus, it is likely that extracellular action of αB-crystallin may contribute to its recently reported anti-inflammatory effects in in vivo model systems.[36,37] On the other hand, the αB-crystallin mini-chaperone is taken up by hfRPE by specific transporters so that the mini-chaperone may act both intracellularly and extracellularly. Nanoparticles containing αB-crystallin mini-chaperone showed a prominent and uniform uptake by hfRPE cells. This is consistent with our previous findings that ARPE-19 cells can internalize nano- as well as microparticles through nonsaturable processes involving phagocytosis or pinocytosis, depending on particle size.[38,39] Both of these mechanisms were also shown for RPE cells in vivo.[40] Furthermore, encapsulation of a 19-mer oligonucleotide by poly (D-L-lactide-co-glycolic acid) nanoparticle enhanced uptake of the oligopeptide.[39] Further studies will be needed to fully elucidate the mechanism of entry of PCL particles into hfRPE cells.

We found that both αA and αB-crystallin mini-chaperones protected RPE cells from H_2O_2-induced cell death, and inhibited caspase-3 activation as was previously shown for the parent protein αB-crystallin.[41–43] It is of interest that αB-crystallin was found to interact with caspase 8,[44] with Bax and Bcl,[31,43,45] or with mitochondrial voltage-dependent anion channels (VDAC), translocase of outer mitochondrial membranes 20 kDa.[42] It is plausible that these or similar mechanisms also operate with αB-crystallin mini-chaperone peptide, but needs to be confirmed by further work.

Nanoparticle delivery increased the protective efficiency as evidenced by the protection offered with very low doses of PCL-coated αB-crystallin, which was about 4-fold greater than the nonencapsulated αB-crystallin mini-chaperone peptide. The possibility that nanoparticles simply enhance delivery of the chaperone resulting in increased intracellular levels cannot be excluded at this time. The increased efficiency of the nanoparticle delivery of chaperone could also be attributed to the longer retention of the PCL-αB-crystallin in the cytosol where it could interact with procaspase-3 or its cleavage intermediates. In support, it has been reported in different cells that αB-crystallin can directly interact with the precursors of caspase-3 to suppress its activation.[43–45] One may also speculate that the mini-chaperones, once they enter the cell, may also upregulate antioxidant genes. In this context, it is of interest that our previous work showed that αB-crystallin modulated glutathione (GSH) levels in mitochondria and mediated transport of GSH via MRP1, the recently identified transporter of GSH in RPE cells.[19] Thus, whether α-crystallin mini-chaperones participate in the regulation of redox proteins could be worthy of investigation.

The two novel sodium-dependent oligopeptide transporters SOPT1 and SOPT2 have been functionally characterized in fRPE cells.[14,15] These transporters transport a wide array of endogenous and synthetic opioid peptides and also other peptides such as the iron-regulatory peptide hormone hepcidin.[15,20] Using the synthetic substrates deltorphin II and DADLE as the preferred substrates for SOPTI and SOPT2, respectively, we could establish the expression of SOPT1 and SOPT2 in hfRPE cells at the functional level. These transporters are distinct from the H^+-coupled peptide transporters PEPT1 and PEPT2.[46] While there is a considerable overlap between SOPT1 and SOPT2 with respect to substrate specificity, they are distinguishable from each other because of the opposing modulatory effects of di- and tripeptides.[15,47] Here we report for the first time that α-crystallin–derived mini-chaperone peptides of 19 to 20-amino acid chain length are high-affinity substrates for these two oligopeptide transport systems in hfRPE cells. αA- and αB-crystallin mini-chaperones significantly inhibited the uptake of DADLE and deltorphin II, showing that these peptides compete with these substrates for SOPT1 and SOPT2. The finding that αA- and αB-crystallin mini-chaperones are transported into hfRPE cells via the oligopeptide transporters is likely to have significant application in the exploitation of these transporters for therapeutic intervention of retinal diseases such as AMD. Peptides and peptidomimetic drugs, being hydrophilic, cannot cross the plasma membrane by diffusion; as such, these transporters hold great potential of delivering the α-crystallin mini-chaperone peptides and related substrates to the retina for treatment of retinal diseases. Detailed characterization of these RPE transporters will require that they be cloned and that specific antibodies be developed.

It may be envisaged that the mini-chaperones derived from α-crystallin have multifunctional properties in addition to being antiapoptotic as is the case with the full-length α-crystallin. Furthermore, as mentioned earlier, they may also function extracellularly apart from being efficient intracellular oxyradical scavengers. In this context, the extracellular anti-inflammatory properties of αB-crystallin have been successfully applied therapeutically in the experimental autoimmune encephalomyelitis model.[36] These authors have further shown that the chaperone region of the crystallin domain of the sHSPs is mainly responsible for the anti-inflammatory effect in vivo. Neutralization of the extracellular misfolded oligomers and suppression of oligomer toxicity by sHSPs, including αB-crystallin, were reported in a recent study, thus providing a valuable strategy for therapeutic interventions against diseases stemming from extracellular protein aggregation.[48] We have shown recently that the α-crystallin chaperone counteracts the cataractogenic effect of α-crystallin mutants.[49] Future work is likely to offer valuable insight into the beneficial, therapeutic use of α-crystallin minichaperones in the prevention of retinal diseases involving oxidative stress such as AMD.

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