Cholecystokinin octapeptide antagonizes apoptosis in human retinal pigment epithelial cells

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Abstract

Although cholecystokinin octapeptide-8 is important for neurological function, its neuroprotective properties remain unclear. We speculated that cholecystokinin octapeptide-8 can protect human retinal pigment epithelial cells against oxidative injury. In this study, retinal pigment epithelial cells were treated with peroxynitrite to induce oxidative stress. Peroxynitrite triggered apoptosis in these cells, and increased the expression of Fas-associated death domain, Bax, caspase-8 and Bcl-2. These changes were suppressed by treatment with cholecystokinin octapeptide-8. These results suggest that cholecystokinin octapeptide-8 can protect human retinal pigment epithelial cells against apoptosis induced by peroxynitrite.

Key Words: nerve regeneration; retinal pigment epithelial cells; peroxynitrite; cholecystokinin octapeptide; apoptosis; Fas-associated death domain; Bax; Caspase-8; Bcl-2; neural regeneration

Introduction

Retinal pigment epithelial (RPE) cells are a monolayer of cuboidal cells located between the photoreceptors of the neurosensory retina and the choroidal capillary bed. RPE cells are involved in visual signal processing. Age-related macular degeneration is an idiopathic retinal degenerative disease, and is the leading cause of irreversible vision loss among people over the age of 65. RPE cell apoptosis is an important feature of the advanced forms of age-related macular degeneration (Yang et al., 2005; Koyama et al., 2008).

Oxidative stress may cause RPE cell apoptosis (Sinha et al., 2013). RPE cells are exposed to continual oxidative stress throughout life (Rodriguez and Beconi, 2009; Sankaralingam et al., 2010; Aghani et al., 2011a, b; Guven et al., 2011). Previous investigations on oxidative stress injury caused by oxygen free radicals emphasized the contribution of hydrogen peroxide (Wijeratne et al., 2005), nitric oxide (Jang et al., 2010; Ru et al., 2011) and superoxide anion. Nitric oxide and superoxide react to produce peroxynitrite, which, along with its derivatives, are strong oxidants (Drake et al., 2002; Gebicka and Didik, 2010).

Cholecystokinin octapeptide (CCK) is a peptide originally discovered in the gastrointestinal tract, and subsequently found in the mammalian brain. The C-terminal sulfated octapeptide fragment of cholecystokinin (CCK-8) constitutes one of the major neuropeptides in the brain. CCK-8 contributes to numerous physiological functions. For example, CCK is involved in the neurobiology of anxiety, depression, psychosis, cognition, nociception and feeding behavior (Noble, 2007; Oz et al., 2007; Merino et al., 2008; Hamamura et al., 2010). In addition, CCK can protect cholinergic neurons against basal forebrain lesion caused by brain injury (Sugaya et al., 1992). In this study, we treated human RPE cells with the oxidative stress inducer peroxynitrite, and evaluated the neuroprotective effects of CCK-8.

Materials and Methods

Synthesis of peroxynitrite

Peroxynitrite was obtained by reacting ice-cold solutions of sodium nitrite (0.6 mol/L) and H₂O₂ (0.7 mol/L) in acidic medium (0.6 mol/L HCl) and rapidly quenching the reaction in NaOH (1.5 mol/L), as described previously (Koppenol et al., 1996; Thiagarajan et al., 2004). The reaction mixture solution was frozen at −20°C, and the peroxynitrite concentrated in the upper layer was collected. Concentration was measured at 302 nm using a molar extinction coefficient of 1,670/mol/cm (Koppenol et al., 1996; Thiagarajan et al., 2004).

RPE cell culture and intervention

Human eyes from eight donors (26–56 years of age) were obtained following eyeball rupture in our hospital. The eyes had an intact posterior segment and RPE layers. For RNA extraction, tissues were suspended in RNA preservation solution (Ambion; Austin, TX, USA) and stored at 4°C until processing. The experimental procedures complied with the Declaration of Helsinki.

For RPE isolation, the anterior segment, iris, lens and vitreous of each eye were carefully extracted. After removal of the tissue punches, posterior poles were cut into quadrants.
Each quadrant was rinsed with sterile PBS, and the neural retina was gently teased away from the RPE. After removal of the retina, RPE cells free of choroidal contamination were collected. They were cultured in Dulbecco’s modified Eagle medium/F-12 human amniotic membrane nutrient mixture (DMEM/F-12; Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (Sigma-Aldrich) in a humidified incubator at 37°C and 5% CO₂. The medium was changed every 3 days.

RPE cells were obtained from the second or third generation, and then divided into control, peroxynitrite and CCK-8 groups. The control group was treated with saline; the peroxynitrite group was treated with peroxynitrite, 16 mmol/L; the CCK-8 group was treated with CCK-8, 10 nmol/L, after addition of peroxynitrite, 16 nmol/L. All changes were observed at 6, 12 and 24 hours after treatment.

Changes in RPE cell morphology observed by electron microscopy
Cell samples were fixed in 2.5% glutaraldehyde in PBS, postfixed in 2% buffered osmium tetroxide for 2 hours, and then dehydrated in a graded ethanol series. Specimens were embedded in Epon. Thin sections were cut on an ultra microtome and double stained with uranyl acetate and lead citrate. Electron micrographs were taken on a JEM-2000 electron microscope (JEOL, Tokyo, Japan) operating at 80 kV.

Assessment of RPE cell apoptosis by detecting DNA laddering
Agarose gel electrophoresis was used to detect DNA laddering, an indicator of apoptosis, as described previously (Herrmann et al., 1994).

Assessment of RPE cell apoptosis by fluorescence activated cell sorting
RPE cells were collected, washed with PBS, and adjusted to 1 × 10⁶ cells/mL. 5 µL Annexin V-FITC and 10 µL propidium iodide (10 µg/mL, Sigma) were added to a 100-µL aliquot of suspended cells, and then incubated for 15 minutes in a dark room at room temperature. 1 × 10⁶ cells were collected and analyzed using Cell Quest software 3.0 (Becton Dickinson, San Jose, CA, USA).

Expression of Fas-associated death domain (FADD), Bax, caspase-8 and Bcl-2 in RPE cells detected by western blot analysis
RPE cells were washed twice with cold Hanks’ balanced salt solution and lysed with RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 0.5% deoxycholate and 0.1% SDS, pH 8.0) supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Lysates were cleared by centrifugation. Total protein in the supernatants was measured using Bradford assay (Bio-Rad, Hercules, CA, USA), with bovine serum albumin used to generate the standard curve, according to the manufacturer’s instructions. Protein (30 g) was electrophoresed on a 12.5% SDS-polyacrylamide gel overlaid with a 3.6% polyacrylamide stacking gel. The proteins were transferred to a nitrocellulose membrane (Bio-Rad) with a Mini Trans-Blot apparatus (Bio-Rad), according to the manufacturer’s directions. Mouse anti-human β-actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used as positive control. The filters were blocked in 0.1 mol/L PBS containing 5% skim milk and 0.05% Tween-20 for 1 hour at room temperature. They were then incubated overnight at 4°C with mouse anti-human FADD (1:1,000, Abnova, Taipei, Taiwan, China), Bax (1:800, Abnova) or caspase-8 (1:600, Abnova) monoclonal antibody or rabbit anti-Bcl-2 polyclonal antibody (1:200, Santa Cruz Biotechnology). After five washes in 0.1 mol/L PBS containing 0.05% Tween 20, the filters were incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,000; Cell Signaling, Beverly, MA, USA) and goat anti-rabbit IgG (1:1,000, Cell Signaling), washed, visualized in ECL solution (Amersham Biosciences, Arlington Heights, IL, USA) for 10 minutes, and exposed to film (X-Omat, Fuji, Kanagawa, Japan) for 7 to 10 minutes. Finally, the filters were incubated in a stripping buffer (2% SDS, 0.7% 2-mercaptoethanol, 62.5 mmol/L Tris-HCl, pH 6.8) for 30 minutes at 65°C. Protein levels were quantified by absorbance.

Expression of caspase-8 and bcl-2 mRNA in RPE cells evaluated with real-time polymerase chain reaction (RT-PCR)
Total RNA was isolated from cultured RPE cells (RNeasy; Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s protocols. After isolation and DNase treatment (DNA-free; Ambion), RNA quantitation was performed (RiboGreen RNA, Molecular Probes, Eugene, OR, USA). Equal amounts of RNA were then used to synthesize first-strand cDNAs with a cDNA synthesis kit (iScript; Bio-Rad). RT-PCR for caspase-8 and bcl-2 mRNA was performed with a detection system (iCycler IQ; Bio-Rad). Cycling parameters for caspase-8: denaturation at 94°C for 1 minute; amplification for 34 cycles at 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 1 minute. Cycling parameters for Bcl-2: denaturation at 95°C for 2 minutes; amplification for 50 cycles at 95°C for 15 seconds, 60°C for 15 seconds and 72°C for 15 seconds. Primers for RT-PCR are given in Table 1. The presence of a single melting temperature peak per primer pair and 2% agarose gel analysis confirmed the identity of the PCR products. Each RT-PCR experiment was repeated at least three times.

Statistical analysis
Data were expressed as mean ± SD. Statistical significance was determined by one-way analysis of variance, followed by the Fisher post hoc test for multiple comparisons. A P < 0.05 value was considered statistically significant difference.

Results
Effect of peroxynitrite and CCK-8 on the morphology of RPE cells
Under the transmission electron microscope, we observed nuclear fragmentation and chromatin marginalization
induced by peroxynitrite in RPE cells. Compared with the peroxynitrite group, apoptotic features (loss of microvilli and chromatin condensation, fragmentation and marginalization) were less apparent in the CCK-8 group (Figure 1).

Effect of peroxynitrite and CCK-8 on apoptosis in RPE cells

Agarose gel electrophoresis revealed no DNA laddering in the control group, while there was typical DNA laddering in the peroxynitrite group. Compared with the peroxynitrite group, DNA laddering in the CCK-8 group was significantly weaker (Figure 2).

Fluorescence activated cell sorting analysis showed that the number of apoptotic RPE cells in the peroxynitrite group was increased at 6, 12 and 24 hours compared with the control group ($P < 0.05$). RPE cell apoptosis in the CCK-8 group was decreased at 6, 12 and 24 hours compared with the peroxynitrite group ($P < 0.05$; Figure 3).

Effect of peroxynitrite and CCK-8 on protein levels of FADD, Bax, caspase-8 and Bcl-2 in RPE cells

Western blot analysis showed that the expression of the apoptosis-related proteins FADD, Bax, caspase-8 and Bcl-2 was up-regulated in a time-dependent manner in the peroxynitrite group compared with the control group ($P < 0.01$). CCK-8 suppressed the changes induced by peroxynitrite ($P < 0.01$; Figure 4).

Effect of peroxynitrite and CCK-8 on caspase-8 and bcl-2 mRNA expression in RPE cells

RT-PCR showed that peroxynitrite markedly upregulated caspase-8 and bcl-2 mRNA expression in the RPE cells, compared with the control group ($P < 0.01$). CCK-8 suppressed the changes induced by peroxynitrite ($P < 0.01$; Figure 5).

Discussion

Recently, we demonstrated that Fas/FasL interactions are critical for maintaining immune privilege in the eye. Cell
death induced by Fas/FasL is important for the induction of apoptosis in RPE cells (Hao et al., 2010, 2011a, c, d). Because apoptosis in the eye is a rapid, yet tightly modulated process, we examined whether signals in the ocular microenvironment regulate apoptosis in RPE cells. There is very little information on RPE cell signaling through the Fas/FasL-caspase-8 pathway, despite being very important for eye diseases such as age-related macular degeneration and proliferative vitreoretinopathy (Kroll et al., 2007; Stone, 2007; Meleth et al., 2011; Querques et al., 2011). Consequently, it is crucial to determine how apoptosis in the eye is regulated by Fas/FasL-caspase-8 signaling, and whether anti-apoptotic factors can counteract cell death.

To better understand the mechanisms regulating apoptosis in the eye, in the present study, we focused on the effects of FADD and caspase-8 signaling in RPE cells.

It is thought that the relative balance between anti- and pro-apoptotic signaling determines the viability of a cell. RPE cells are critically important for neural retinal function. Thus, RPE cells exposed to oxidative stress are likely to express many anti-apoptotic proteins and genes. Indeed, we found that apoptosis-related proteins and genes were expressed in cultured RPE cells. Expression of FADD, caspase-8 and Bax were upregulated by peroxynitrite. These changes were suppressed by CCK-8. In general, the expression of the apoptosis-related proteins under basal conditions mirrored mRNA expression as determined by RT-PCR. In addition, caspase-8 protein levels paralleled caspase-8 mRNA expression as determined by RT-PCR. In addition, apoptosis-related proteins under basal conditions mirrored mRNA expression as determined by RT-PCR. In addition, caspase-8 protein levels paralleled caspase-8 mRNA expression as determined by RT-PCR.
sion after peroxynitrite stimulation, and Bcl-2 protein levels paralleled bcl-2 mRNA expression after CCK-8 administration. To our knowledge, these data are the first to demonstrate an involvement of FADD and caspase-8 in RPE cell apoptosis. The pattern of FADD and caspase-8 expression under basal conditions and after exposure to peroxynitrite stimulus is very cell type specific.

Normal cell growth requires a precisely controlled balance between cell death and survival. This involves activation of different types of intracellular signaling cascades within the cell. While some types of signaling proteins promote apoptosis, or programmed cell death, other proteins within the cell can promote survival. Bcl-2 can protect RPE cells from apoptosis in response to several different types of stimuli. We infer one way that Bcl-2 may promote cell survival is by phosphorylating and thereby inhibiting the proapoptotic protein Bad. This leads in turn to the inhibition of effector caspases such as caspase-8. Under these conditions, Bcl-2 inhibits apoptosis early in the caspase cascade, antagonizing the activation of the apoptotic initiator, caspase-8. This inhibition of apoptosis may involve suppression of caspase-8 recruitment to the death domain receptors. This role in regulating initiator caspases is an entirely novel role for the Bcl-2 proteins and suggests a new mechanism by which these proteins promote cell survival.

Taken together, our findings show that Bcl-2 overexpression suppresses oxidative stress events. Our data suggest that Bcl-2, rather than Bax, is an important endogenous RPE cell anti-apoptotic factor, which is consistent with other reports (Banga et al., 2007; Ploner et al., 2008; Teijido and Dejean, 2010; Willimott and Wagner, 2010; Vogler et al., 2011). Further studies are needed to provide further support for this contention. These results highlight the importance of cell-type specificity in therapeutic strategies for inducing apoptotic cell death. Members of the Bcl-2 protein family are crucial apoptosis regulators. We evaluated the expression of Bcl-2 mRNA by RT-PCR, which showed that levels were regulated by CCK-8. An increase in Bcl-2 expression prevented RPE cell apoptosis in an oxidative stress model. In addition, we readily detected Bax protein in cultured human RPE cells by western blot analysis. Similarly, other investigators have identified Bax protein in RPE cells (Letai, 2009; Lalier et al., 2011; Robinson et al., 2011).

Peroxynitrite upregulated FADD, Bax and caspase-8, indicating that it modulates RPE cell apoptosis (Jung et al., 2009; Hirschberg et al., 2010; Holthoff et al., 2010; Gaupels et al., 2011; Juhász et al., 2011). Oxidative stress-induced RPE cell apoptosis has been proposed as a major pathophysiological mechanism in age-related macular degeneration and proliferative vitreoretinopathy (Agrawal et al., 2007; Coleman et al., 2008). Apoptosis is the result of a cascade of gene expression. Numerous genes have been found to contribute to the regulation of apoptosis. It is thought that apoptosis is regulated by an interaction between gene expression and signaling cascades initiated at the cell surface (Ferrington et al., 2006; Zhou et al., 2010; Hao et al., 2011b; DiBase et al., 2012). The multifunctional protein FADD and caspase-8 could participate in the mechanisms of RPE cell apoptosis induced by peroxynitrite. The death inducing signaling complex formed by Fas receptor, FADD and caspase-8 is a pivotal trigger of apoptosis. The Fas-FADD death inducing signaling complex represents a receptor platform, which once assembled, initiates apoptosis. A highly oligomeric network of homotypic protein interactions comprised of the death domains of Fas and FADD is at the centre of the death inducing signaling complex. Thus, characterizing the Fas-FADD interaction is crucial for understanding cell death induction. Scott et al. (2009) successfully isolated the human Fas-FADD death domain complex and reported its crystal structure. The complex has a tetrameric arrangement of four FADD death domains bound to four Fas death domains. An opening of the Fas death domain exposes the FADD binding site and simultaneously generates a Fas-Fas bridge. The result is a regulatory Fas-FADD complex bridge governed by weak protein-protein interactions revealing a model where the complex itself functions as a mechanistic switch. This switch prevents accidental death inducing signaling complex assembly, yet allows for assembly and clustering upon a sufficient stimulus.

In addition to revealing a previously unknown mode of death domain interactions, these results further uncover a mechanism for receptor signaling solely by oligomerization and clustering events (Álvaro-Bartolomé et al., 2010; Thorenoor et al., 2010). Accumulating evidence suggests that apoptosis plays an important role in numerous pathophysiological processes (Matsuda et al., 2009; Drakos et al., 2011). A better understanding of the molecular mechanisms involved in the pathogenesis of RPE cell death is required to develop new therapeutic approaches.

FADD and its apoptotic partner, caspase-8, have also been implicated in necrosis. FADD is intriguing in that T-cell receptor-induced proliferation is blocked in FADD-defective T cells. FADD appears to help keep T-cell receptor-induced programmed necrotic signaling in check during early phases of T-cell clone expansion (Osborn et al., 2010; Ikner and Ashkenazi, 2011). Death domain complexes are key protein arrangements in the regulation of various cellular signaling events. One of the most prominent death domain complexes first described in the initiation of apoptosis is formed by the transmembrane receptor Fas, the cytosolic adaptor protein FADD and caspase-8, and is referred to as the Fas/FADD/caspase-8 death inducing signaling complex. The recent structure of the Fas/FADD death domain complex reveals how formation of this signaling platform can be stringently regulated by Fas receptor clustering to form a death domain network (Salvesen and Riedl, 2009; Li et al., 2010; Wang et al., 2010). The formation of the death-inducing signaling complex is a direct indicator of the activation of the Fas-caspase-8 signaling pathway. The production of cytokines such as type I interferon is an essential component of innate immunity. A reduction in FADD and TRIM21 (TRIM21 is a member of a large family of proteins that can impart ubiquitin modification onto its cellular targets) levels leads to higher interferon-γ induction, IRF7 phosphorylation, and lower titers of RNA virus in infected cells (Garcia-Fuster et al., 2009; Matsumura et al., 2009; Young et al., 2011).
Caspases are a family of aspartate-specific cysteine proteases responsible for the biochemical and morphological changes that occur during the execution phase of apoptosis. The hierarchical ordering of caspases has been clearly established using dATP-activated cell lysates to model the intrinsic pathway induced by initial mitochondrial perturbation. In the model, caspase-9, the initiator caspase, directly processes and activates the effector caspases, caspase-3 and caspase-7. Active caspase-3 then processes caspase-2 and caspase-6, and subsequently, the activated caspase-6 processes caspase-8 and caspase-10. The processing of caspase-2 and caspase-6 occurs within the cytoplasm and active caspase-6 is then responsible for both the processing of caspase-8 and the cleavage of caspase-6 substrates (Inoue et al., 2009). Gene-targeted mice show that caspase-8 is essential for hepatocyte killing (O’Reilly et al., 2004; Kaufmann et al., 2009). Furthermore, L. interrogans-induced apoptosis in macrophages is mediated by caspase-3 and caspase-6 activation through a FADD-caspase-8-dependent pathway, independently of mitochondrial cytochrome c-caspase-9 signaling (Jin et al., 2009).

The Bax protein is pivotal for the apoptotic process. Bax, which resides in an inactive form in the cytosol of healthy cells, is activated during the early stages of apoptosis and becomes associated with mitochondria through poorly understood mechanisms. A cysteine is present in the loop between the two transmembrane alpha helices of Bax (Letal, 2009; Lalier et al., 2011; Robinson et al., 2011).

In conclusion, we speculate that CCK-8, along with antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, protect RPE cells from oxidative-stress-induced apoptotic cell death by modulating FADD and caspase-8 signaling.

Author contributions: All authors were responsible for the study design, implementing the experiment, and evaluating the results. Liu Y drafted the manuscript. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

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