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Dual involvement of Caspase-4 in Inflammatory and ER Stress-induced Apoptotic Responses in Human Retinal Pigment Epithelial Cells

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Abstract

PURPOSE—To investigate the functional involvement of caspase-4 in human retinal pigment epithelial (hRPE) cells.

METHODS—Expression and activation of caspase-4 in hRPE cells were measured after stimulation with pro-inflammatory agents IL-1 β (2 ng/ml), TNF- α (20 ng/ml), lipopolysaccharide (1000 ng/ml), γ -interferon (500 U/ml), or monocyte co-culture in the absence or presence of immunomodulating agents cyclosporine (3 or 30 ng/ml), dexamethasone (10 μ M), or IL-10 (100 U/ml), as well as endoplasmic reticulum (ER) stress inducers thapsigargin (25 nM) or tunicamycin (3 or 10 μ M). The onset of ER stress was determined by expression of GRP78. The involvement of caspase-4 in inflammation and apoptosis was further examined by treating the cells with caspase-4 inhibitor ZLEVD-fmk, caspase-1 and -4 inhibitor Z-YVAD-fmk and pan-caspase inhibitor Z-VAD-fmk.

RESULTS—Caspase-4 mRNA expression and protein activation were induced by all the pro-inflammatory agents and ER stress inducers tested in this study. Caspase-4 activation was blocked or reduced by dexamethasone, and IL-10. Elevated ER stress by pro-inflammatory agents and ER stress inducers was shown by increased expression of the ER stress marker GRP78. The induced caspase-4 and caspase-3 activities by tunicamycin, and the stimulated IL-8 protein expression by IL-1 β were markedly reduced by caspase-4 inhibitor Z-LEVD-fmk. While caspase-4 inhibitor Z-LEVD-fmk and caspase-1 and -4 inhibitor Z-YVAD-fmk reduced tunicamycin-induced hRPE apoptotic cell death by 59 and 86%, respectively, pan-caspase inhibitor Z-VAD-fmk completely abolished the induced apoptosis.

CONCLUSION—Caspase-4 is dually involved in hRPE pro-inflammatory and proapoptotic responses. Various pro-inflammatory stimuli and ER stress induce hRPE caspase-4 mRNA synthesis and protein activation. The ER stress-induced hRPE cell death is caspase- and, in part, caspase-4-dependent.

INTRODUCTION

Caspases are a family of cytosolic, aspartate-specific, cysteine proteases involved in apoptosis, inflammation, proliferation, and differentiation.^{1–4} At least 17 members of the caspase family have been identified, of which 13 are found in humans.⁵ Human caspase-4 was cloned independently in three laboratories and designated as ICH2,⁶ ICE_{rel}II,⁷ and TX.⁸ The caspase-4 gene is expressed ubiquitously in various tissues with the exception of brain.^{6, 7} While human caspase-4 has no corresponding mouse orthologue,¹ human caspase-4 and -5 are possibly the orthologues of mouse caspase-11.¹ Caspase-4 cDNA

exhibits 68% sequence homology with human caspase-1.⁷ As with caspase-1, caspase-4 is also composed of a large prodomain (p22) and two small domains (p20 and p10), that are cleaved upon activation.⁷ Transient expression of the cloned caspase-4 gene causes apoptotic cell death in fibroblasts,⁷ Sf9 insect cells,⁶ and COS cells.⁸ Subsequent studies have confirmed the apoptotic role of caspase-4 in endoplasmic reticulum (ER) stress-induced cell death.⁹⁻¹²

The ER is responsible for folding, maturation, and storage of membrane and secreted proteins. ER is also the major organelle that stores second messenger calcium ions which sense and respond to changes in cellular homeostasis. ER stress occurs when the cellular demand for ER function exceeds its capacity. Overloading of unfolded protein aggregates triggers a signaling cascade of events, called unfolded protein response (UPR). Excess UPR leads to irreversible commitment to cell death. There is accumulating evidence to suggest involvement of caspase-4 in ER stress-induced apoptosis. First, caspase-4 is mainly localized to the ER.⁹ Second, caspase-4 is closely associated with many essential proteins in ER stress-induced cell death pathways, including 1) GRP78, a well known marker of ER stress;¹⁰ 2) CARD-only protein (Cop or pseudo-ICE), a regulator of procaspase-1,¹¹ 3) Apf1, a protein involved in death protease-mediated cell death;¹² and 4) TRAF6, a member of the TNF receptor-associated factor.¹³ Third, caspase-4 inhibitor Z-LEVD-fmk selectively and effectively blocks ER stress-induced apoptosis in many cancer cells, such as neuroblastoma cells,¹⁴ lung and esophageal cancer cells,¹⁵ Jurkat cells,¹⁶ and melanoma cells.¹⁷ Fourth, knocking down caspase-4 expression by siRNA in multiple myeloma cells,¹⁸ leukemia cells,¹⁹ glioma cell lines²⁰ and neuroblastoma cells,⁹ introducing antisense oligonucleotides to lymphoblastoid AHH-1 cells,²¹ expressing catalytically inactive caspase-4, and microinjecting anti-caspase-4 antibodies into HeLa cells,²² all abolish ER stress-induced cell death. Conversely, overexpression of caspase-4 in COS-7 cells induces activation of caspase-3 and -9, the two well-known death proteases.²³

Chromosomal mapping reveals that human caspase-4 gene is co-localized within a cluster of functionally related genes, caspase-1, -5, -12 as well as caspase-1 pseudogenes, ICEBERG, COP and INCA in human chromosome 11q22-23.²⁴ The chromosomal co-localization of caspase-4 with inflammatory caspases implies that these caspases are derived from a common ancestor through gene multiplication and share common functions in innate immunity and inflammation. Despite the common acceptance that caspase-4 is a member of the inflammatory caspase family. Most of previous functional studies have focused on the role of caspase-4 in apoptosis. So far only one study has shown that caspase-4 is involved in inflammation, having demonstrated its role in lipopolysaccharide (LPS)-induced inflammatory responses.¹³

In this study we investigated the functional involvement of caspase-4 in hRPE cells. Our data showed that caspase-4 is involved in both inflammation and apoptosis in hRPE cells.

Materials and Methods

Materials

Recombinant human IL-1 β , TNF- α , γ -INF, and IL-10 were purchased from R&D System (Minneapolis, MN). Dexamethasone, cyclosporine, and tunicamycin were purchased from Sigma-Aldrich (St. Louis, MO). The cell-permeable general caspase inhibitor Z-V-A-D (OMe)-fluoromethylketone was from Bachem Americas, Inc. (Torrance, CA). The caspase-1 and -4 inhibitor, Z-Y-V-A-D (OMe)-fmk, and caspase-4 inhibitor, Z-L-E-V-D-FMK (z-LEVD-fmk), were from R&D and BioVision (Mountain View, CA), respectively. The mouse monoclonal antibody (4B9) against caspase-4 was from Abcam (Cambridge, MA). The goat polyclonal antibody against GRP-78 was from Santa Cruz Biotechnology (Santa

Cruz, CA). QIAshredder and RNeasy Mini Kit were purchased from Qiagen (Valencia, CA). Reverse transcription system was obtained from Invitrogen (Carlsbad, CA). RQ1 RNase-free DNase was purchased from Promega (Madison, WI). The Cell Death Detection ELISA and In Situ Cell Death Detection Kits were purchased from Roche Molecular Biochemicals (Indianapolis, IN). The EnVision G/2 System/AP (Permanent Red) was obtained from DAKO Co (Carpinteria, CA). All other reagents were obtained from Sigma-Aldrich.

Cell isolation and culture

The hRPE cells were isolated within 24 hr of death from the donor eyes as previously described.²⁵⁻²⁸ In brief, the sensory retina tissue was separated gently from the hRPE monolayer, and the hRPE cells were removed from Bruch's membrane with papain (5 U/ml). The hRPE cells were cultured in Dulbecco's modified Eagle's /Ham's F12 nutrient mixture medium (DMEM/Ham's F12), containing 15% fetal bovine serum, penicillin G (100 U/ml), streptomycin sulfate (100 µg/ml), and amphotericin B (0.25 µg/ml) in Falcon Primaria culture plates to inhibit fibroblast growth. The hRPE monolayers exhibited uniform immunohistochemical staining for cytokeratin 8/18, fibronectin, laminin, and type IV collagen in the chicken-wire distribution characteristic for these epithelial cells. The identity of hRPE cells in the culture was confirmed by apical immunohistochemical staining of Na⁺-K⁺ ATPase. Cells were subcultured, grown to reach confluency, and exposed to the same medium, but containing reduced serum (5%) for further experiments. The cells were in culture up to four to six passages. A total of three donors have been used in this study. For each experiment at least two donors have been used, of which a typical result is shown.

Monocyte isolation and hRPE-monocyte co-culture

Human monocytes were freshly isolated from the peripheral blood of healthy volunteers as described previously.²⁶ In brief, peripheral blood was drawn into a heparinized syringe and 1:1 diluted in 0.9% saline. Mononuclear cells were separated by density gradient centrifugation. The cells were washed and then layered onto density gradient (Fico-Lite monocytes, 1.068 g/ml) for the enrichment of monocytes. The isolated cells were then washed, cytopun onto a glass slide, stained with Diff-Quick, and differentially counted. The purity of the cell was >97%. For hRPE cells: monocyte co-culture, enriched monocyte populations (3×10⁵) were overlaid onto untreated or pretreated near-confluent hRPE cultures (2×10⁵) for 6 hr. Post co-culture the monocytes were removed as previously described,²⁶ and hRPE cells were subjected to further analyses.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

The total cellular RNA was isolated from hRPE cells by QIAshredder and RNeasy Mini Kit according to manufacturer's protocol. The cDNA synthesis reaction was set up according to the protocol for a reverse transcription system. Briefly, 5 µg of RNA was added to the reaction mixture with RT Superscript III (200 U/µl) and 1 µl Oligo d (T)₂₀ (0.5 µg/µl) in a total volume of 20 µl. Linear range of the β-actin PCR reaction was predetermined by using a series of cycles from 15 to 35 cycles. The mid-linear portion of the response curve was selected as the condition for semi-quantitative PCR. The primer and condition for caspase-4 PCR was as described by Lin et al.²⁹ and confirmed by first examining three cycles (15, 25 and 35) and then cycle 30 and 32. The reaction was initiated by adding 0.15 µl of Taq DNA polymerase (5 U/µl) to a final volume of 20 µl. The resultant cDNAs were amplified through 32 and 20 cycles for caspase-4 and β-actin, respectively. The primer sequences for human caspase-4 genes were as follows: 5'-CAGACTCTATGCAAGAG AAGCAACGTATGGCAGGA-3' (Forward), 5'-CACCTCTGCAGGCCTGGACAATG ATGAC-3' (Reverse). To ensure that an equal amount of templates was used in each amplification reaction, human β-actin sense (5'-GTGGGGCGCCCCAGGCACCA-3') and

antisense (5'-GCTCGGCCGTGGTGGTGAAGC-3') primers were used in parallel. The following conditions were used in RT-PCR reaction for caspase-4 and β -actin: denaturation at 95°C for 45 sec (caspase-4) or 1 min (β -actin), annealing at 65°C for 1 min (caspase-4) or at 62°C (β -actin) for 1 min, and extension at 72°C for 1 min (caspase-4) or 2 min (β -actin) for 32 (caspase-4) or 20 (β -actin) cycles. RT-PCR products were analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Assays for apoptosis

Cell Death Detection ELISA was performed according to the manufacture's protocol. Briefly, the hRPE cells were seeded and grown in 96-well plates until cells were close to confluence. After treating the cells with or without varieties of inducers or inhibitors for 24, 48 or 72 hr, apoptosis was quantified by using a Cell Death Detection ELISA kit. Cultures were lysed with the lysis buffer of the kit and then the cytoplasmic fraction was transferred into the wells coated by streptavidin in the microplate modules for further analysis. Next, the immnoreagent was added into each well, which contained anti-histone-biotin and anti-DNA-peroxidase. The immnoreagents bond to or reacted with the histone and DNA-part of the mono- and oligonucleosomes, that were out of the cytoplasm of cells during apoptosis. After removal of the unbound components with wash, the substrate 2, 2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) was added to determine the amount of peroxidase by reading absorbance difference between A_{405nm} and A_{490nm} in an ELISA reader as a measurement of apoptosis.

TUNEL Staining

The cells were stained with TdT-mediated dUTP nick end labeling (TUNEL) according to the manufacturer's protocol. Briefly, hRPE cells were fixed and incubated with TUNEL mixtures for 1 hr at 37°C. The incorporated fluorescein was detected by sheep anti-fluorescein antibody conjugated with horseradish peroxidase using the substrate diaminobenzidine (DAB). hRPE cells were distinguished by the subsequent labeling with anti-vimentin antibody, alkaline phosphatase labeled polymer and permanent red substrate (EnVision G/2 system, DAKO Co). The stained cells were analyzed by light microscopy. Apoptotic cells in the cultures were quantified by counting the number of TUNEL-positive cells in five random microscope fields.

Caspase-3 Activity

Caspase-3 activity was assayed using a cellular caspase-3 activity assay kit (Biomol; Plymouth Meeting, PA), according to the manufacturer's protocol. Briefly, cell extracts were added to the microtiter wells, and the reaction was initiated by adding 200 μ M Ac-DEVD-pNA substrate. In parallel, the samples were reacted with this substrate in the presence of 0.1 μ M Ac-DEVD-CHO, a specific caspase-3 inhibitor, to measure the nonspecific hydrolysis of the substrate. Absorbance was read at 405 nm in a microtiter plate reader at the indicated time intervals.

Statistic Analysis

Various assay conditions were compared using ANOVA and t-test in Statview, and $p < 0.05$ was considered to be statistically significant. Values represent means $\pm$ SEM.

Results

Expression and activation of Caspase-4 in response to pro-inflammatory stimulation

To determine the involvement of caspase-4 in pro-inflammatory response by hRPE cells, a group of known pro-inflammatory agents were selected for this study, including IL-1 β ,

TNF- α , LPS, γ -interferon (γ -INF) and monocyte co-culture. The concentrations of IL-1 β (2 ng/ml), TNF- α (20 ng/ml), LPS (1000 ng/ml) and γ -INF (500 U/ml), and the conditions for monocyte-hRPE co-culture used in this study have been shown to maximally stimulate pro-inflammatory responses in hRPE cells.^{26-28,30} After treating hRPE cells with above agents for 6 hr, the total cellular mRNA was isolated and subjected to RT-PCR analysis. In order to compare caspase-4 mRNA levels expression of the house keeping gene β -actin was used to monitor gel loading. As shown in Figure 1A, treatment of hRPE cells with IL-1 β , TNF- α , LPS, or monocyte co-culture each increased caspase-4 mRNA synthesis by 0.7, 0.5, 0.5, or 0.6 fold, respectively.

Next, the whole lysates from the hRPE cells treated under the same conditions, but for 24 hr were subjected to Western blotting analysis. Activation of caspase-4 protein was determined by appearance of cleaved caspase-4 products (Figure 1B). Data showed that IL-1 β , TNF- α , LPS, γ -INF, or monocyte co-culture each activated caspase-4 protein.

The activation of caspase-4 by pro-inflammatory agents suggests that caspase-4 may participate in pro-inflammatory responses in hRPE cells. To prove this is the case, IL-1 β -induced IL-8 protein production was examined by Western blots of the whole cell lysates from the hRPE cells treated with 2ng/ml IL-1 β in the presence or absence of caspase-4 inhibitor Z-LEVD-fmk. As we have shown in the past,²⁷ IL-1 β induced IL-8 protein production significantly above the basal levels. As shown in Figure 1C, the presence of caspase-4 inhibitor completely eliminated the induced IL-8 protein production.

Effect of dexamethasone, IL-10 and cyclosporine on IL-1 β - and γ -INF-induced activation of caspase-4

As expression and activation of caspase-4 were induced by IL-1 β and γ -INF, we next investigated if anti-inflammatory agents could neutralize this induced caspase-4 activation by these two pro-inflammatory agents. As expected, treatment with dexamethasone (1 μ M) and IL-10 (100 U/ml) reduced IL-1 β - and γ -INF-induced cleavage of procaspase-4 by 60 and 33%, and 15 and 50%, respectively (Figure 2). On the other hand, cyclosporine (3ng/ml) inhibited the γ -INF induced caspase-4 activation by 47%, but markedly increased the IL-1 β induced caspase-4 activation by more than twofold.

Induction of caspase-4 mRNA synthesis and protein activation by ER stress-inducers tunicamycin or thapsigargin

Tunicamycin and thapsigargin are the two well known ER stress inducers that act respectively by blocking N-glycosylation of newly synthesized proteins and Ca²⁺-ATPase, which maintains Ca²⁺ homeostasis in the ER. We used these two agents to study apoptotic involvement of caspase-4. Similar to the results from using pro-inflammatory agents as described above, tunicamycin (3 μ M) and thapsigargin (25ng/ml) both moderately increased caspase-4 mRNA by 0.3 and 0.4 fold, respectively (Figure 3A). Ten μ M tunicamycin induced caspase-4 cleavage that appeared as early as 24 hr post-stimulation, and the activation remained up to 72 hr (Figure 3B). The induced caspase-4 activation was reduced by co-incubation with caspase-4 inhibitor Z-LEVD-fmk (2 μ M) and caspase-1 and -4 inhibitor Z-YVAD-fmk (2 and 20 μ M) (Figure 3C).

Since executioner caspase-3 is the downstream target of caspase-4,^{10,23} we next assessed caspase-3 activity in the presence or absence of 2 μ M caspase-4 inhibitor ZLEVD-fmk. The caspase-3 activity was determined by caspase-3 specific cleavage of substrate Ac-DEVD-pNA following treating hRPE cells with 3 μ M tunicamycin. Consistent with previous reports,^{10,23,31} caspase-4 inhibitor completely abolished the tunicamycin-induced activation of caspase-3 (Figure 3D).

ER stress in response to apoptotic and inflammatory stimuli

Tunicamycin has been widely used to induce ER stress. To confirm the existence of ER stress when caspase-4 was activated by this agent, expression of GRP78, a specific marker of ER stress, was examined. In untreated hRPE cells, GRP78 protein was barely detectable by Western blots (Figure 4A). Induction of GRP78 production appeared at 24 hr after treating hRPE cells with 10 μ M tunicamycin. The induced GRP78 expression continued to increase up to 48 hr.

Tunicamycin treatment also triggered a significant increase in expression of the anti-apoptotic protein Bcl-2 in as early as 24 hr after stimulation, and the increase sustained up to 2.5 fold at 48 hr after the treatment (Figure 4B). On the other hand, tunicamycin also increased the pro-apoptotic protein Bax in a similar trend, but modestly by only 0.7 fold. As a result, the Bcl-2/Bax ratios were enhanced 1.1 fold by tunicamycin.

As GRP78 has been shown having immunosuppressive activity,^{31,32} we then examined expression of GRP78 after treating hRPE cells with IL-1 β , TNF- α , LPS, or overlaid monocytes. As shown in Figure 5A, all of these treatments increased hRPE GRP78 expression. In monocyte co-culture, for example, the enhanced GRP78 protein levels appeared as early as 2 hr and reached steady state in 8 hr.

The induced GRP78 expression by IL-1 β was modestly inhibited by dexamethasone by 38% (Figure 5B). In contrast, cyclosporine (3ng/ml) and IL-10 markedly enhanced the induced GRP78 protein production by near twofold (Figure 5B). Furthermore, either γ -IFN alone or in combination with dexamethasone, cyclosporine (3ng/ml) or IL-10 did not affect GRP78 expression. Of note, cyclosporine at 30ng/ml appeared to be cytotoxic and caused strong ER stress. The latter was demonstrated by 5 folds increase in GRP78 protein production in IL-1 β treatment and threefold in γ -IFN treatment.

Involvement of caspase-4 in tunicamycin-induced apoptotic cell death

Cell death detection ELISA kits were used to measure apoptotic cell death at the same concentrations used for caspase-4 activation. At 72 hr post-treatment, tunicamycin induced substantial hRPE cell apoptotic death compared to untreated control cells which had undetectable levels of cell death. Arbitrarily taking the ELISA readings under tunicamycin treatment as 100% cell death, the induced hRPE apoptosis was inhibited by caspase-4 inhibitor Z-LEVD (2 μ M) and caspase-1 and -4 inhibitor Z-YVAD-fmk (20 μ M) by 59 and 86%, respectively (Figure 6). The pan-caspase inhibitor Z-VAD-fmk (50 μ M) completely abolished the induced hRPE cell death.

To further validate the tunicamycin-induced apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) staining was performed. After treating hRPE cells with tunicamycin (10 μ M) for 24 and 48 hr, 13% and 24% of the cells exhibited apoptotic cell death as detected by TUNEL assay. In the presence of the caspase-4 inhibitor Z-LEVD-fmk (2 μ M) the tunicamycin-induced apoptotic cell death at 24 and 48 hr was reduced by 62 and 53%, respectively. In contrast to Z-LEVD-fmk, pan-caspase inhibitor Z-VAD-fmk almost completely blocked the induced apoptotic cell death. Furthermore, treatment of hRPE cells with IL-1 β (IL-1, 2ng/ml), TNF- α (TNF, 20ng/ml) and lipopolysaccharide (LPS, 1000ng/ml) for 24 hr (Figure 6B, left), or with cyclosporine (CsA, 3ng/ml), IL-10 (100 U/ml) and dexamethasone (Dex, 1 μ M) for 72 hr (Figure 6B, right) did not result in apparent cell death as compared with tunicamycin control.

DISCUSSION

Human caspase-12 was originally thought to play distinctive roles in inflammation and apoptosis, corresponding to those of caspase-12 in mice. However, as most human populations express only the C-terminal truncated form of caspase-12, the role of this caspase in human apoptosis has been challenged.^{2, 33, 34} The short form of human caspase-12 is not a null product in hRPE cells since we have recently shown that it may play an immunomodulatory role in these cells.³⁵ Phylogenetic analyses suggest that caspase-4 and -5 may be the functional counterpart of caspase-12 in human.⁴ The data from the present study support the notion that human caspase-4 may correspond to murine caspase-12, involving in both inflammation and apoptosis.³⁶

Despite the accumulating evidence supporting an apoptotic role of caspase-4, negative results have been reported in tunicamycin- and thapsigargin-induced apoptosis in human multiple myeloma cell lines,³⁷ homoharringtonine-induced apoptosis in MUTZ-1 cells,³⁸ and Z alpha-1 antitrypsin-induced apoptosis in HEK293 cells.³⁹ While apoptosis usually does not require denovo synthesis of caspases, ER stress may increase mRNA expression of certain caspases including caspase-4.⁴⁰ Our study demonstrated that pro-inflammatory stimuli (IL-1 β , TNF- α , LPS, γ -INF and monocyte co-culture) induced both mRNA synthesis and activation of caspase-4 in hRPE cells. The IL-1 β -induced IL-8 protein production was reduced by the caspase-4 inhibitor Z-LEVD-fmk, suggesting that caspase-4 is involved in the pro-inflammatory responses of hRPE cells. These results are consistent with a previous study in which the LPS-induced IL-8 mRNA synthesis and protein production are reduced by caspase-4 knockdown in human THP1 monocytic cell lines.¹³ On the other hand, the ER-stress inducer tunicamycin induced expression of GRP78, a specific marker of ER stress. While tunicamycin increased expression of proapoptotic Bax, this study also found increased expression of the antiapoptotic Bcl-2 protein, resulting in a increased Bcl-2/ Bax ratio. In most cases the ER stress-induced apoptotic responses is regulated by or dependent on the BCL-2 family of proteins.⁴¹ The Bcl-2/ Bax ratio has been clinically used as indicator of susceptibility of tumor cells to the induction of apoptosis by chemotherapeutic agents.⁴² However, the involvement of Bcl-2 and Bax in tunicamycin-induced apoptosis has not been well characterized. Increased Bcl-2 protein expression by tunicamycin has been reported in one previous report.⁴³ The increased Bcl-2 expression as shown in our study, however, may not affect caspase-4 activation since a previous study has shown that activation of caspase-4 by tunicamycin is only slightly affected by over-expression of Bcl-2.⁹ The increased Bcl-2/ Bax ratio by tunicamycin may suggest an early protective response of hRPE cells to apoptotic stimuli by enhanced expression of the anti-apoptotic protein Bcl-2 to counteract the increase in pro-apoptotic protein Bax.

Furthermore, in response to ER stress, hRPE cells increased production and activation of caspase-4. The latter is consistent with a recent report in ARPE19 cells, an immortalized hRPE cell line.⁴⁰ These authors showed that treatment of ARPE19 cells with tunicamycin and thapsigargin simulated activation of caspase-4, although the effect of tunicamycin on caspase-4 mRNA expression was negligible. In agreement with this recent report, apoptotic hRPE cell death by staurosporine was insensitive to caspase-4 inhibitor, thus caspase-4 is unlikely involved in staurosporine-induced mitochondrial apoptotic pathway (data not shown). We further demonstrated in this study that tunicamycin-induced caspase-4 activation and apoptotic hRPE cell death were highly sensitive to inhibition by caspase-4 inhibitor Z-LEVD-fmk (59%), implying that ER stress-induced apoptosis in hRPE is in part via activation of caspase-4. The difference in potency between caspase-4 inhibitor Z-LEVD-fmk (59% inhibition) and pan-caspase inhibitor Z-VAD (98% inhibition) suggests the existence of parallel caspase-4-independent apoptotic pathways that are also induced by ER stress. Although apoptotic cell death can be caspase-independent,⁴⁴ the hRPE apoptotic cell

death induced by ER stress was totally caspase-dependent, because this induction was completely abolished by a pan-caspase inhibitor. The caspase inhibitor Z-YVAD-fmk has been used as a caspase-4 inhibitor,^{45, 46} but it is more frequently used as caspase-1 inhibitor.⁴⁷ In fact, Z-YVAD-fmk inhibits both caspase-1 and -4.⁴⁸ The stronger inhibition of apoptosis by Z-YVAD-fmk (86%) suggests that caspase-1 is likely to be responsible for the ER stress-induced, caspase-4-independent hRPE cell death. Caspase-1 and -4 have been shown to coordinate in TNF- α -, but not tunicamycin-induced cell death in Cop-transfected HeLa cells.¹¹ Our data also show that caspase-4 inhibitor Z-LEVD-fmk abolishes both tunicamycin-induced activation of caspase-3 and apoptotic hRPE cell death, suggesting that caspase-3, the central effector of apoptosis, acts downstream from caspase-4 in the ER stress-induced RPE apoptotic pathway as reported in other cell types.^{10, 23, 31, 46, 49} Taken together, our hRPE data support the proposal by Momoi et al. (2004)³⁶ that caspase-4 may be a functional surrogate of the truncated human caspase-12.

The pro-inflammatory agents tested in this study, including IL-1 β , TNF- α and LPS, upregulated the ER stress marker, GRP78. However, when treating hRPE cells with these three agents at the concentrations used for other experiments in this study for up to 24 hr, none of these agents caused apoptotic cell death. Upregulation of GRP78 in response to inflammation suggests its immunosuppressive and protective role.^{31, 32} Moreover, drugs dexamethasone and cyclosporine, and anti-inflammatory cytokine IL-10, when added together with pro-inflammatory agent IL-1 β , exerted differential consequences on the inductions of GRP78 expression. While dexamethasone reversed IL-1 β -induced increases in hRPE GRP78, cyclosporine at 3 and 30ng/ml enhanced the already elevated GRP78 production caused by IL-1 β . The different impact by dexamethasone and cyclosporine, as indicated by ER stress marker GRP78 response, suggests potential risks of long term of treatment with some anti-inflammatory drugs such as cyclosporine. Indeed, when we treated hRPE cells with cyclosporine (30ng/ml) for 72 hr, all hRPE cells died. In contrast, neither dexamethasone (10 μ M) nor IL-10 (100 U/ml) induced noticeable apoptosis under the same conditions. The cyclosporine concentration used in this study (3-30ng/ml) was well within the drug concentrations used clinically (5 to 100 ng/ml),⁵⁰ and 30 ng/ml of cyclosporine appeared to be cytotoxic in this study. Indeed, cyclosporine itself has been shown to induce ER stress and GRP78, during nephrotoxicity and apoptotic cell death,⁵¹ whereas dexamethasone has been shown effective in treating acquired glomerular diseases by suppressing ER stress and GRP78.⁵²

The signaling pathway mediating caspase-4 pro-inflammatory responses remains elusive. Caspase-4 does not appear to act via caspase-1 activation. Activation of caspase-4 requires both dimerization and proteolysis, a feature that combines the requirement for activation of initiator caspases such as caspase-1 (dimerization) and effector caspases such as caspase-3 (interdomain cleavage).⁵³ Caspase-4 does not promote maturation of caspase-1 substrate pro-IL-1 β ,⁶ and currently no specific substrates have been identified for caspase-4. These observations suggest that the pro-inflammatory mechanisms involving caspase-4 differ from that by caspase-1.^{6, 8} It has been proposed that caspase-4 may function mainly via NF- κ B signal pathway in inflammatory responses.¹³ Our previous studies have shown that NF- κ B pathway is essential for expression of IL-8 and other cytokines in hRPE cells and inhibition of NF- κ B activation effectively blocks IL-1 β -induced cytokine production in hRPE cells.³⁰ As knockdown of caspase-4 gene by siRNA significantly reduces NF- κ B activation and nuclear translocation,¹³ this mechanism for caspase-4 in the IL-8 pathway is likely. Caspase-4 has been shown associated with activation of signal transducer TRAF6.¹³ In response to stimulation by pro-inflammatory cytokines, TRAF6 activates I κ B kinase in the NF- κ B pathway.⁵⁴ Thus TRAF6 may also be involved with ER stress response-induced hRPE IL-8 expression.

This study shows that caspase-4 appears a key mediator of apoptosis and inflammation in hRPE cells, underscoring its potential value as a novel therapeutic target. The pathophysiologic relevance of this dual role in hRPE responses warrants further investigation. Inflammatory cytokines are essential mediators of the innate immune response. Since hRPE cells play important roles in ocular functions under normal and diseased conditions, ^{27,55} caspase-4-mediated cytokine expression could be relevant to many non-infectious and infectious retinal diseases, such as proliferative vitreoretinopathy, ⁵⁶ age-related macular degeneration, ⁵⁷ uveitis, ⁵⁸ and endophthalmitis.⁵⁹ On the other hand, apoptotic cell death is an established responses in many ocular diseases, such as aged related macular degeneration, diabetic retinopathy, retinitis pigmentosa, retinal ischemia, photoreceptor degenerations, and glaucoma.⁶⁰⁻⁶³ Blockade of caspase-3, the target downstream of caspase-4, may represent a therapeutic strategy in protection of retinal degeneration.^{64,65} Therefore, it is of interest to investigate if caspase-4 is involved under those diseased conditions. Further delineating the signaling pathway, regulation and functional roles of caspase-4 may suggest novel strategies for developing therapies for ocular disease.

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REFERENCES

1. Martinon F, Tschopp J. Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ.* 2007; 14:10–22. [PubMed: 16977329]
2. Scott AM, Saleh M. The inflammatory caspases: guardians against infections and sepsis. *Cell Death Differ.* 2007; 14:23–31. [PubMed: 16977333]
3. Cornelis S, Kersse K, Festjens N, Lamkanfi M, Vandenabeele P. Inflammatory caspases: targets for novel therapies. *Curr Pharm Des.* 2007; 13:367–385. [PubMed: 17311555]
4. Lamkanfi M, Kalai M, Vandenabeele P. Caspase-12: an overview. *Cell Death Differ.* 2004; 11:365–368. [PubMed: 14685161]
5. Eckhart L, Ballaun C, Hermann M, VandeBerg JL, Sipos W, Uthman A, Fischer H, Tschachler E. Identification of novel mammalian caspases reveals an important role of gene loss in shaping the human caspase repertoire. *Mol Biol Evol.* 2008; 25:831–841. [PubMed: 18281271]
6. Kamens J, Paskind M, Hugunin M, Talanian RV, Allen H, Banach D, Bump N, Hackett M, Johnston CG, Li P, Mankovich JA, Terranova M, Ghayur T. Identification and characterization of ICH-2, a novel member of the interleukin-1 beta-converting enzyme family of cysteine proteases. *J Biol Chem.* 1995; 270:15250–15256. [PubMed: 7797510]
7. Munday NA, Vaillancourt JP, Ali A, Casano FJ, Miller DK, Molineaux SM, Yamin TT, Yu VL, Nicholson DW. Molecular cloning and pro-apoptotic activity of ICERelII and ICERelIII, members of the ICE/CED-3 family of cysteine proteases. *J Biol Chem.* 1995; 270:15870–15876. [PubMed: 7797592]
8. Faucheu C, Blanchet AM, Collard-Dutilleul V, Lalanne JL, Diu-Hercend A. Identification of a cysteine protease closely related to interleukin-1 beta-converting enzyme. *Eur J Biochem.* 1996; 236:207–213. [PubMed: 8617266]
9. Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, Manabe T, Yamagishi S, Bando Y, Imaizumi K, Tsujimoto Y, Tohyama M. Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. *J Cell Biol.* 2004; 165:347–356. [PubMed: 15123740]
10. Jiang CC, Chen LH, Gillespie S, Wang YF, Kiejda KA, Zhang XD, Hersey P. Inhibition of MEK sensitizes human melanoma cells to endoplasmic reticulum stress-induced apoptosis. *Cancer Res.* 2007; 67:9750–9761. [PubMed: 17942905]

11. Wang X, Narayanan M, Bruey JM, Rigamonti D, Cattaneo E, Reed JC, Friedlander RM. Protective role of Cop in Rip2/caspase-1/caspase-4-mediated HeLa cell death. *Biochim Biophys Acta*. 2006; 1762:742–754. [PubMed: 16920334]
12. Hu Y, Benedict MA, Wu D, Inohara N, Núñez G. Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc Natl Acad Sci U S A*. 1998; 95:4386–4391. [PubMed: 9539746]
13. Lakshmanan U, Porter AG. Caspase-4 interacts with TNF receptor-associated factor 6 and mediates lipopolysaccharide-induced NF-kappaB-dependent production of IL-8 and CC chemokine ligand 4 (macrophage-inflammatory protein-1). *J Immunol*. 2007; 179:8480–8490. [PubMed: 18056395]
14. Oda T, Kosuge Y, Arakawa M, Ishige K, Ito Y. Distinct mechanism of cell death is responsible for tunicamycin-induced ER stress in SK-N-SH and SH-SY5Y cells. *Neurosci Res*. 2008; 60:29–39. [PubMed: 18029041]
15. Pataer A, Hu W, Xiaolin L, Chada S, Roth JA, Hunt KK, Swisher SG. Adenoviral endoplasmic reticulum-targeted mda-7/interleukin-24 vector enhances human cancer cell killing. *Mol Cancer Ther*. 2008; 7:2528–2535. [PubMed: 18723497]
16. López-Antón N, Rudy A, Barth N, Schmitz ML, Pettit GR, Schulze-Osthoff K, Dirsch VM, Vollmar AM. The marine product cephalostatin 1 activates an endoplasmic reticulum stress-specific and apoptosome-independent apoptotic signaling pathway. *J Biol Chem*. 2006; 281:33078–33086. [PubMed: 16945918]
17. Chen LH, Jiang CC, Watts R, Thorne RF, Kiejda KA, Zhang XD, Hersey P. Inhibition of endoplasmic reticulum stress-induced apoptosis of melanoma cells by the ARC protein. *Cancer Res*. 2008; 68:834–842. [PubMed: 18245485]
18. Nawrocki ST, Carew JS, Maclean KH, Courage JF, Huang P, Houghton JA, Cleveland JL, Giles FJ, McConkey DJ. Myc regulates aggresome formation, the induction of Noxa, and apoptosis in response to the combination of bortezomib and SAHA. *Blood*. 2008; 112:2917–2926. [PubMed: 18641367]
19. Rahmani M, Davis EM, Crabtree TR, Habibi JR, Nguyen TK, Dent P, Grant S. The kinase inhibitor sorafenib induces cell death through a process involving induction of endoplasmic reticulum stress. *Mol Cell Biol*. 2007; 27:5499–5513. [PubMed: 17548474]
20. Pyrko P, Kardosh A, Wang W, Xiong W, Schöenthal AH, Chen TC. HIV-1 protease inhibitors nelfinavir and atazanavir induce malignant glioma death by triggering endoplasmic reticulum stress. *Cancer Res*. 2007; 67:10920–10928. [PubMed: 18006837]
21. Lin R, Sun Y, Li C, Xie C, Wang S. Identification of differentially expressed genes in human lymphoblastoid cells exposed to irradiation and suppression of radiation-induced apoptosis with antisense oligonucleotides against caspase-4. *Oligonucleotides*. 2007; 17:314–326. [PubMed: 17854271]
22. Kamada S, Washida M, Hasegawa J, Kusano H, Funahashi Y, Tsujimoto Y. Involvement of caspase-4 (-like) protease in Fas-mediated apoptotic pathway. *Oncogene*. 1997; 15:285–290. [PubMed: 9233763]
23. Yukioka F, Matsuzaki S, Kawamoto K, Koyama Y, Hitomi J, Katayama T, Tohyama M. Presenilin-1 mutation activates the signaling pathway of caspase-4 in endoplasmic reticulum stress-induced apoptosis. *Neurochem Int*. 2008; 52:683–687. [PubMed: 17942194]
24. Nadiri A, Wolinski MK, Saleh M. The inflammatory caspases: key players in the host response to pathogenic invasion and sepsis. *J Immunol*. 2006; 177:4239–4245. [PubMed: 16982854]
25. Elnier SG, Elnier VM, Pavilack MA, Todd RF 3rd, Mayo-Bond L, Franklin WA, Strieter RM, Kunkel SL, Huber AR. Modulation and function of intercellular adhesion molecule-1 (CD54) on human retinal pigment epithelial cells. *Lab Invest*. 1992; 66:200–211. [PubMed: 1346541]
26. Bian ZM, Elnier SG, Yoshida A, Elnier VM. Human RPE-monocyte co-culture induces chemokine gene expression through activation of MAPK and NIK cascade. *Exp Eye Res*. 2003; 76:573–583. [PubMed: 12697421]
27. Elnier VM, Strieter RM, Elnier SG, Baggiolini M, Lindley I, Kunkel SL. Neutrophil chemotactic factor (IL-8) gene expression by cytokine-treated retinal pigment epithelial cells. *Am J Pathol*. 1990; 136:745–750. [PubMed: 2183623]

28. Elnor VM, Strieter RM, Pavilack MA, Elnor SG, Remick DG, Danforth JM, Kunkel SL. Human corneal interleukin-8. IL-1 and TNF-induced gene expression and secretion. *Am J Pathol.* 1991; 139:977–988. [PubMed: 1951636]
29. Lin XY, Choi MS, Porter AG. Expression analysis of the human caspase-1 subfamily reveals specific regulation of the CASP5 gene by lipopolysaccharide and interferon-gamma. *J Biol Chem.* 2000; 275:39920–39926. [PubMed: 10986288]
30. Bian ZM, Elnor SG, Yoshida A, Kunkel SL, Su J, Elnor VM. Activation of p38, ERK1/2 and NIK pathways is required for IL-1beta and TNF-alpha-induced chemokine expression in human retinal pigment epithelial cells. *Exp Eye Res.* 2001; 73:111–121. [PubMed: 11428868]
31. Wang M, Wang P, Liu YQ, Peng JL, Zhao XP, Wu S, He FR, Wen X, Li Y, Shen GX. The immunosuppressive and protective ability of glucose-regulated protein 78 for improvement of alloimmunity in beta cell transplantation. *Clin Exp Immunol.* 2007; 150:546–552. [PubMed: 17956578]
32. Panayi GS, Corrigall VM. BiP regulates autoimmune inflammation and tissue damage. *Autoimmun Rev.* 2006; 5:140–142. [PubMed: 16431346]
33. Saleh M, Vaillancourt JP, Graham RK, Huyck M, Srinivasula SM, Alnemri ES, Steinberg MH, Nolan V, Baldwin CT, Hotchkiss RS, Buchman TG, Zehnbauser BA, Hayden MR, Farrer LA, Roy S, Nicholson DW. Differential modulation of endotoxin responsiveness by human caspase-12 polymorphisms. *Nature.* 2004; 429:75–79. [PubMed: 15129283]
34. Fischer H, Koenig U, Eckhart L, Tschachler E. Human caspase 12 has acquired deleterious mutations. *Biochem Biophys Res Commun.* 2002; 293:722–726. [PubMed: 12054529]
35. Bian ZM, Elnor SG, Elnor VM. Regulated expression of caspase-12 gene in human retinal pigment epithelial cells suggests its immunomodulating role. *Invest Ophthalmol Vis Sci.* 2008; 49:5593–5601. [PubMed: 18791174]
36. Momoi T. Caspases involved in ER stress-mediated cell death. *J Chem Neuroanat.* 2004; 28:101–105. [PubMed: 15363495]
37. Obeng EA, Boise LH. Caspase-12 and caspase-4 are not required for caspase-dependent endoplasmic reticulum stress-induced apoptosis. *J Biol Chem.* 2005; 280:29578–29587. [PubMed: 15975932]
38. Jie H, Donghua H, Xingkui X, Liang G, Wenjun W, Xiaoyan H, Zhen C. Homoharringtonine-induced apoptosis of MDS cell line MUTZ-1 cells is mediated by the endoplasmic reticulum stress pathway. *Leuk Lymphoma.* 2007; 48:964–977. [PubMed: 17487741]
39. Miller SD, Greene CM, McLean C, Lawless MW, Taggart CC, O'Neill SJ, McElvaney NG. Tauroursodeoxycholic acid inhibits apoptosis induced by Z alpha-1 antitrypsin via inhibition of Bad. *Hepatology.* 2007; 46:496–503. [PubMed: 17559149]
40. Koyama Y, Matsuzaki S, Gomi F, Yamada K, Katayama T, Sato K, Kumada T, Fukuda A, Matsuda S, Tano Y, Tohyama M. Induction of amyloid beta accumulation by ER calcium disruption and resultant upregulation of angiogenic factors in ARPE19 cells. *Invest Ophthalmol Vis Sci.* 2008; 49:2376–2383. [PubMed: 18515580]
41. Hetz CA. ER stress signaling and the BCL-2 family of proteins: from adaptation to irreversible cellular damage. *Antioxid Redox Signal.* 2007; 9:2345–2355. [PubMed: 17854276]
42. Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. *Nat Rev Cancer.* 2002; 2:647–656. [PubMed: 12209154]
43. Zhu L, Xiang R, Dong W, Liu Y, Qi Y. Anti-apoptotic activity of Bcl-2 is enhanced by its interaction with RTN3. *Cell Biol Int.* 2007; 31:825–830. [PubMed: 17379544]
44. Bröker LE, Krut FA, Giaccone G. Cell death independent of caspases: a review. *Clin Cancer Res.* 2005; 11(9):3155–3162. [PubMed: 15867207]
45. Liang B, Song X, Liu G, Li R, Xie J, Xiao L, Du M, Zhang Q, Xu X, Gan X, Huang D. Involvement of TR3/Nur77 translocation to the endoplasmic reticulum in ER stress-induced apoptosis. *Exp Cell Res.* 2007; 313:2833–2844. [PubMed: 17543302]
46. Li J, Xia X, Ke Y, Nie H, Smith MA, Zhu X. Trichosanthin induced apoptosis in HL-60 cells via mitochondrial and endoplasmic reticulum stress signaling pathways. *Biochim Biophys Acta.* 2007; 1770:1169–1180. [PubMed: 17570595]

47. Guo H, Petrin D, Zhang Y, Bergeron C, Goodyer CG, LeBlanc AC. Caspase-1 activation of caspase-6 in human apoptotic neurons. *Cell Death Differ.* 2006; 13:285–292. [PubMed: 16123779]
48. Perche O, Doly M, Ranchon-Cole I. Transient protective effect of caspase inhibitors in RCS rat. *Invest Ophthalmol Vis Sci.* 2008; 86:519–527.
49. Kim SJ, Zhang Z, Hitomi E, Lee YC, Mukherjee AB. Endoplasmic reticulum stress-induced caspase-4 activation mediates apoptosis and neurodegeneration in INCL. *Hum Mol Genet.* 2006; 15:1826–1834. [PubMed: 16644870]
50. Kurtz RM, Elner VM, Bian ZM, Strieter RM, Kunkel SL, Elner SG. Dexamethasone and cyclosporin A modulation of human retinal pigment epithelial cell monocyte chemotactic protein-1 and interleukin-8. *Invest Ophthalmol Vis Sci.* 1997; 38:436–445. [PubMed: 9040477]
51. Pallet N, Bouvier N, Bendjallabah A, Rabant M, Flinois JP, Hertig A, Legendre C, Beaune P, Thervet E, Anglicheau D. Cyclosporine-induced endoplasmic reticulum stress triggers tubular phenotypic changes and death. *Am J Transplant.* 2008; 8:2283–2296. [PubMed: 18785955]
52. Fujii Y, Khoshnoodi J, Takenaka H, Hosoyamada M, Nakajo A, Bessho F, Kudo A, Takahashi S, Arimura Y, Yamada A, Nagasawa T, Ruotsalainen V, Tryggvason K, Lee AS, Yan K. The effect of dexamethasone on defective nephrin transport caused by ER stress: a potential mechanism for the therapeutic action of glucocorticoids in the acquired glomerular diseases. *Kidney Int.* 2006; 69:1350–1359. [PubMed: 16531978]
53. Karki P, Dahal GR, Park IS. Both dimerization and interdomain processing are essential for caspase-4 activation. *Biochem Biophys Res Commun.* 2007; 356:1056–1061. [PubMed: 17400183]
54. Wajant H, Henkler F, Scheurich P. The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. *Cell Signal.* 2001; 13:389–400. [PubMed: 11384837]
55. Hecquet C, Lefevre G, Valtink M, Engelmann K, Mascarelli F. Activation and role of MAP kinase-dependent pathways in retinal pigment epithelium cells: JNK1, P38 kinase, and cell death. *Invest Ophthalmol Vis Sci.* 2003; 44:1320–1329. [PubMed: 12601065]
56. Charteris DG, Hiscott P, Grierson I, Lightman SL. Proliferative vitreoretinopathy. Lymphocytes in epiretinal membranes. *Ophthalmology.* 1992; 99:1364–1367. [PubMed: 1407970]
57. Lopez PF, Grossniklaus HE, Lambert HM, Aaberg TM, Capone A Jr, Sternberg P Jr, L'Hernault N. Pathologic features of surgically excised subretinal neovascular membranes in age-related macular degeneration. *Am J Ophthalmol.* 1991; 112:647–656. [PubMed: 1957899]
58. Koizumi K, Poulaki V, Doehmen S, Welsandt G, Radetzky S, Lappas A, Kociok N, Kirchhof B, Joussen AM. Contribution of TNF-alpha to leukocyte adhesion, vascular leakage, and apoptotic cell death in endotoxin-induced uveitis in vivo. *Ophthalmol Vis Sci.* 2003; 44:2184–2191.
59. Petropoulos IK, Vantzou CV, Lamari FN, Karamanos NK, Anastassiou ED, Pharmakakis NM. Expression of TNF-alpha, IL-1beta, and IFN-gamma in *Staphylococcus epidermidis* slime-positive experimental endophthalmitis is closely related to clinical inflammatory scores. *Graefes Arch Clin Exp Ophthalmol.* 2006; 244:1322–1328. [PubMed: 16544114]
60. Nickells RW, Zack DJ. Apoptosis in ocular disease: a molecular overview. *Ophthalmic Genet.* 1996; 17:145–165. [PubMed: 9010866]
61. Heathcote JG. Apoptosis and oncosis in ocular disease. *Can J Ophthalmol.* 1995; 30:298–300. [PubMed: 8574975]
62. Campochiaro PA. Potential applications for RNAi to probe pathogenesis and develop new treatments for ocular disorders. *Gene Ther.* 2006; 13:559–562. [PubMed: 16195702]
63. Algvare PV, Marshall J, Seregard S. *Acta Ophthalmol Scand.* 2006; 84:4–15. [PubMed: 16445433]
64. Bode C, Wolfrum U. Caspase-3 inhibitor reduces apoptotic photoreceptor cell death during inherited retinal degeneration in tubby mice. *Mol Vis.* 2003; 9:144–150. [PubMed: 12724642]
65. Chen TA, Yang F, Cole GM, Chan SO. Inhibition of caspase-3-like activity reduces glutamate induced cell death in adult rat retina. *Brain Res.* 2001; 904:177–188. [PubMed: 11516428]

Figure 1. Stimulation of human RPE caspase-4 mRNA synthesis (A) and protein activation (B), and blockade of IL-1 β -induced IL-8 production by caspase-4 inhibitor Z-LEVD-fmk (Z-LEVD) (C)

The hRPE cells were cultured either without (untreated; Ctl) or with IL-1 β (IL-1, 2ng/ml), TNF- α (TNF, 20ng/ml), lipopolysaccharide (LPS, 1000ng/ml), γ -interferon (γ -IFN, 500 U/ml) or overlaid monocytes (RM), and incubated for 6 hr (A) or 24 hr (B). The data shown represent results from a typical experiment. (A) Steady-state caspase-4 mRNA determined by RT-PCR. The fold changes were calculated by normalization against β -actin and comparison with untreated control. (B) Western blots of Caspase-4 and actin proteins. The arrow-pointed bands are presumably either nonspecific bands or intermediately cleaved caspase-4.¹⁰ (C) hRPE cells were pretreated with caspase-4 inhibitor Z-LEVD-fmk (2 μ M) for 30 min and then co-incubated with IL-1 β for additional 24 hr. Proteins from whole hRPE cell lysates were subjected to Western blotting analysis by anti-IL-8 antibody.

Figure 2. The effect of dexamethasone (Dex), cyclosporine (CsA) and IL-10 on caspase-4 activation by IL-1 β (A and C) and γ -IFN (B and C) in hRPE cells
The hRPE cells were pretreated with Dex (1 μ M), CsA (3 ng/ml) or IL-10 (100 U/ml) for 30 min and then co-incubated with IL-1 β (2 ng/ml) and γ -IFN (500 U/ml) for additional 24 hr. Proteins from whole hRPE cell lysates were detected by anti-caspase-4 antibody specific for pro-caspase-4 and cleaved caspase-4. The fold changes of the cleaved caspase-4 were calculated by relative density between treated and untreated samples as determined by densitometry after normalization with actin protein.

Figure 3. Stimulation of hRPE caspase-4 mRNA synthesis (A), protein activation (B, C), and caspase-3 activity (D) by tunicamycin (Tu) or thapsigargin (Tha)

The hRPE cells were cultured either without (untreated; Ctl) or with tunicamycin (3 or 10 μ M), or thapsigargin (25 ng/ml) for 6 (A), 24 (B, D), 48 (B, C) or 72 (B) hr. In C and D, hRPE cells were pretreated with or without caspase-4 inhibitor Z-LEVD-fmk (Z-LEVD) and caspase-1 and -4 inhibitor Z-YVAD-fmk (Z-YVAD). A. To determine the steady-state levels of caspase-4 mRNA, total RNA was isolated and subjected to semi-quantitative RT-PCR. The fold changes were expressed as ratios between treated and untreated samples after normalization by β -actin. B and C, Caspase-4 protein production and activation. Western blots of proteins from the whole cell lysates treated or untreated were detected by anti-caspase-4 antibody. D. Caspase-3 activity was determined by cleavage of substrate Ac-DEVD-pNA and the absorbance was read at 405 nm. Protein content of each sample was determined with a BCA assay. Values represent means $\pm$ SEM, n=4, ***, P<0.001.

Figure 4. Time-dependent effects of tunicamycin (Tu) on GRP78 (A), Bcl-2 and Bax (B) protein expression

The hRPE cells were cultured either without or with tunicamycin (10 μM) for 24 and 48 hr. The hRPE whole cell lysates were subjected to Western blotting analysis for GRP-78, Bcl-2 and Bax expression. The data shown represent results from a typical experiment. The fold changes were calculated by normalization of band density with actin and assigned control value as 1.

Figure 5. Expression of GRP78 protein in hRPE cells that were stimulated by pro-inflammatory agents (A) or in combination with anti-inflammatory agents (B)

The hRPE cells were cultured either without (Ctl, control) or with IL-1 β (IL-1, 2 ng/ml), TNF- α (TNF, 20ng/ml), lipopolysaccharide (LPS, 1000ng/ml), γ -interferon (γ -IFN, 500 U/ml) or overlaid monocytes (RM) for 2 to 24 hr (A, middle and bottom panels) or 24 hr (A, top, and B). In B and C, cultures were pre-treated with or without dexamethasone (Dex, 1 μ M), cyclosporine (CsA, 3 or 30ng/ml) or IL-10 (100 U/ml) for 30 min, and then co-incubated with IL-1 β and γ -IFN for additional 24 hr. Whole hRPE cell lysates were subjected to Western blotting analysis for GRP-78 expression. The fold changes of cleaved caspase-4 were determined by the ratios between treated and untreated samples of the band densities which were quantified by densitometry and normalized by actin.

Figure 6. ER stress-induced hRPE apoptotic cell death

A. hRPE cells were cultured either without or with 10 μ M tunicamycin in the presence or absence of caspase-4 inhibitor Z-LEVD-fmk (Z-LEVD), caspase-1 and -4 inhibitor Z-YVAD-fmk (Z-YVAD), or pan-caspase inhibitor Z-VAD-fmk (Z-VAD) for 72 hr. **B.** The hRPE cells were cultured either without (Ctl, control) or with IL-1 β (IL-1, 2ng/ml), TNF- α (TNF, 20ng/ml), lipopolysaccharide (LPS, 1000ng/ml) and tunicamycin (3 μ M) for 24 hr (left), or with cyclosporine (CsA, 3ng/ml), IL-10 (100 U/ml), dexamethasone (Dex, 1 μ M) and tunicamycin (10 μ M) for 72 hr (right). Apoptosis was determined by absorbance difference between A_{405nm} and A_{490nm} using cell death detection ELISA kit. For comparisons, the stress inducer-treated cells were arbitrarily assigned as 100% apoptotic cell death by tunicamycin. ***, <0.001; **, P<0.01; *, P<0.05 as compared to tunicamycin treatment without inhibitors.

Figure 7. Quantification of the effects of ER stress-induced hRPE cell death by TUNEL assays HRPE cells were cultured either without or with 10 μ M tunicamycin in the presence or absence of caspase-4 inhibitor Z-LEVD-fmk (Z-LEVD) or pan-caspase inhibitor Z-VAD-fmk (Z-VAD) for 24 or 48 hr. **A**, TUNEL staining (dark brown), 400X. The hRPE cells were stained by vimentin (red). On the top, unstimulated hRPE cells (cultures and in TUNEL assay), and on the bottom, the hRPE cells treated with tunicamycin showing nuclear condensation and cell shrinkage. **B**, Data are expressed as percentage of TUNEL-positive hRPE cells. Values represent means $\pm$ SEM. ***, <0.001 ; **, $P<0.01$ as compared to tunicamycin treatment without inhibitors.