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PERK and XBP1 differentially regulate CXCL10 and CCL2 production

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Abstract

Inflammation plays a key role in the pathogenesis of many retinal degenerative diseases related with photoreceptor dysfunction/degeneration. However the involvement of photoreceptor cells in inflammatory reactions is largely unknown as they are not considered as inflammatory cells. In this study, we assessed whether photoreceptor cells can produce CCL2 and CXCL10, two important players in inflammation during endoplasmic reticulum (ER) stress. After photoreceptor 661W cells were treated with ER stress inducer thapsigargin (TG), induction of ER stress increased CXCL10 and CCL2 expression at both mRNA and protein levels, which was significantly blocked by an ER stress blocker 4-phenylbutyrate. ER stress contains three pathways: PERK, ATF6 and IRE1 α . Knockdown of PERK attenuated TG-induced CXCL10 and CCL2 mRNA expression, associated with significant decreases in phosphorylation of NF- κ B RelA and STAT3. In contrast to PERK, knockdown of XBP1, which is activated by IRE1 α -mediated splicing, robustly enhanced TG-induced CXCL10 and CCL2 expression and phosphorylation of NF- κ B RelA and STAT3. Blockade of NF- κ B or STAT3 markedly diminished TG-induced CXCL10 and CCL2 expression. The specific roles of PERK and XBP1 in CXCL10 and CCL2 expression were further investigated by treating photoreceptor cells with advanced glycation end products (AGE) and high glucose (HG), two of the major contributors to diabetic complications. Similarly, AGE and HG induced CXCL10 and CCL2 expression in which PERK was a positive

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regulator while XBP1 was a negative regulator. These studies suggest that photoreceptors may be involved in retinal inflammation by expressing chemokines CXCL10 and CCL2. PERK and IRE1 α /XBP1 in the unfolded protein response differentially regulate the expression of CXCL10 and CCL2 likely through modulation of ER stress-induced NF- κ B RelA and STAT3 activation.

Keywords

Chemokine; ER stress; photoreceptor; NF- κ B; STAT3

1. Introduction

Vision is the most important sense for human beings and almost 30% of the sensory input to the brain is generated from the retina (Jayakody et al., 2015). Photoreceptors are specialized neurons in the retina. Their biological function is mainly recognized to convert light into neural signals during visual perception. Loss of photoreceptors during retinal degenerative diseases such as retinal detachment, retinitis pigmentosa, age-related macular degeneration and others is a leading cause of blindness in developed countries (Jayakody et al., 2015; Murakami et al., 2013). Although inflammation is well appreciated to play a key role in the pathogenesis of these diseases, the involvement of photoreceptors in inflammatory reactions is largely unknown. Two recent papers showing that photoreceptors are the major source of superoxide in diabetic retinopathy and elimination of photoreceptors prevents retinal inflammation and capillary degeneration indeed suggest photoreceptors can communicate with non-adjacent cells in the retina through undefined mechanisms (Du et al., 2015; Du et al., 2013).

The endoplasmic reticulum (ER) is an intracellular organelle for protein synthesis, folding and trafficking. When ER function is perturbed by various cellular stressors, protein assembly is disturbed, resulting in accumulation of unfolded and misfolded proteins in the ER, which triggers the unfolded protein response (UPR) (Kim et al., 2008; Malhotra and Kaufman, 2007; Zhang et al., 2015). The UPR consists of three pathways mediated by PKR-like ER kinase (PERK), inositol-requiring enzyme α (IRE1 α) and activating transcription factor 6 (ATF6). These proteins normally exist in an inactive state by binding to ER chaperone GRP78 (78-kDa glucose-regulated/binding immunoglobulin protein). During ER stress, GRP78 binds to misfolded proteins and PERK, IRE1 and ATF6 are released. PERK undergoes autophosphorylation and activation. Activated PERK phosphorylates and inactivates the eukaryotic initiation factor 2 α (eIF2 α), leading to the attenuation of protein translation and subsequent reduction of protein load in the ER. On the other hand, phosphorylated eIF2 α induces the translation of certain mRNAs, such as the mRNA encoding the activating transcription factor 4 (ATF4), which mediates the transcription of genes involved in ER homeostasis, anti-oxidative stress and amino-acid metabolism (Kim et al., 2008; Lu et al., 2004). PERK is the only UPR branch that modulates protein synthesis as an adaptive response. However, prolonged PERK activity is correlated with the progression of chronic diseases such as neurodegenerative diseases and diabetes, and blockade of PERK has been shown to be beneficial in a variety of disease contexts (Bell et al., 2016). In contrast to PERK, spliced X-box binding protein-1 (XBP1s), which is generated by splicing

an intron from XBP1 by activated IRE1 α , inhibits inflammation and oxidative stress, and protects neuronal cells from injuries (Casas-Tinto et al., 2011; Hollien and Weissman, 2006; Huang et al., 2015; Kim et al., 2008; Li et al., 2011; Valdes et al., 2014).

In retinal diseases, ER stress is implicated in diabetic retinopathy and glaucoma given that ER stress markers are upregulated in these diseases and modulation of ER stress pathways substantially reduces vascular inflammation, leakage and retinal ganglion cell degeneration (Chen et al., 2012; Doh et al.; Hu et al., 2012; Ito et al.; Li et al., 2009; Makino et al., 2013). The role of ER stress in photoreceptor degenerative diseases is also appreciated. Mutations within the rhodopsin gene cause rhodopsin misfolding, ER stress and UPR, which promotes photoreceptor cell death in autosomal dominant retinitis pigmentosa, whereas the alleviation of ER stress with GRP78 overexpression prevents photoreceptor cell death and preserves its function (Gorbatyuk et al., 2010; Kang et al., 2012; Shinde et al., 2012). In spite of the above progresses, the mechanisms of ER stress-induced photoreceptor degeneration are mainly linked to its activation of apoptotic pathways. Although inflammation is a key player in retinal degenerative diseases and ER stress is a well-known inducer of inflammation, it is unclear whether ER stress regulates inflammatory reactions in photoreceptors.

Chemokines are a group of peptides (8–15kD) that have an essential role in inflammation by mediating leukocyte recruitment and activation (Chen et al., 2004; Zlotnik and Yoshie, 2012). Inflammatory chemokine CCL2, formerly named monocyte chemoattractant protein-1, functions mainly as chemoattractant to recruit and activate monocytes, macrophages and microglia from the blood to the site of inflammation (Charo and Taubman, 2004), which is critical for photoreceptor degeneration in retinal detachment, age-related macular degeneration, retinitis pigmentosa and diabetic retinopathy (Ambati et al., 2003; Guo et al., 2012; Nakazawa et al., 2007; Rangasamy et al., 2014). CXCL10 is another key chemokine to attract monocytes, macrophages, microglia and T cells. It is involved in inflammation and neuronal injury in retinal ischemia reperfusion, Alzheimer's disease, multiple sclerosis and spinal cord injury (Balashov et al., 1999; Glaser et al., 2006; Ha et al., 2015; Krauthausen et al., 2015). CXCL10 level is also increased in multiple models of photoreceptor degenerative diseases (Detrick et al., 2008; Ricker et al., 2010; Rutar et al., 2015). In the present study, we determined whether ER stress induces CCL2 and CXCL10 expression in photoreceptor cells and investigated the potential mechanisms of ER stress-induced CCL2 and CXCL10 expression.

2. Materials and methods

2.1 Cell culture

Mouse photoreceptor cell line 661W (Krishnamoorthy et al., 1999; Tan et al., 2004) was a generous gift from Dr. Muayyad AI-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK). Cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Corning, Corning, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah), 1% Antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA), 40 μ g/L Hydrocortisone 21-hemisuccinate (Sigma-Aldrich, St. Louis, MO), 40 μ g/L progesterone (Sigma-Aldrich), 32 mg/L putrescine (Sigma-Aldrich), and 40 μ L/L β -mercaptoethanol (Thermo Fisher

Scientific). Cells were grown with humidified 5% CO₂ at 37°C. When appropriate, cells were starved with serum free DMEM with 0.1% BSA (Cellgro, Herndon, VA) overnight. All inhibitors were performed 30 minutes before thapsigargin (TG) (Tocris Bioscience, Minneapolis, MN) treatment. In diabetes-related studies, 661W cells were starved in DMEM supplemented with 0.2% FBS overnight and then treated with 200 µg/ml control BSA or AGE (Biovision, Milpitas, CA) for 1–24 hours; or cells were starved in DMEM containing 2% FBS overnight and then treated with 5.5 mM glucose (normal glucose, NG) or 30 mM glucose (high glucose, HG) for 48 hours.

2.2 Retroviral transduction and stable cell line generation

Plasmids including pSuper-puro/luciferase-shRNA control, pSuper-puro-U6-XBP1-shRNA (target sequence 5'-GGA TTC ATG AAT GGC CCT TA-3'), and pSuper-puro-PERK-shRNA (target sequence 5'-AGT GGA AAG CTG AGG TAT A-3') were constructed and 661W cells stably expressing the above plasmids were generated by retroviral transduction as described previously (Sha et al., 2009). Briefly, phoenix cells were transfected with plasmid of interest and VSVG at 2:1 ratio overnight and then replaced with fresh culture media. Media containing retroviruses were harvested 48 hours after transfection and used to transduce 661W cells in the presence of 6 µg/ml polybrene (Sigma-Aldrich) for 24 hours. Stable cell lines were selected in the presence of 1 µg/ml puromycin (Sigma-Aldrich).

2.3 Enzyme-linked immunosorbent assay (ELISA)

Supernatants from cells were collected 24 hours after treatment with vehicle, TG or AGE, or 48 hours after high glucose treatment. The concentrations of CXCL10 and CCL2 in cell-free supernatants were measured with Mouse CXCL10/IP-10/CRG-2 or CCL2 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions and normalized to total protein of the cell lysates. Protein concentration of the cell lysates was determined by a BCA assay (Pierce Biotechnology, Rockford, IL). The optical density (OD) of samples was detected with Synergy™ H1 microplate reader (BioTek, Winooski, VT).

2.4 Western Blotting

For XBP1 blotting, nuclei were extracted using CellLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich). Protein from cells or nuclei were dissolved in 1x SDS loading buffer and separated on SDS-PAGE gels as described previously (Ameri et al., 2014). Subsequently, proteins were electroblotted onto nitrocellulose or PVDF membranes (Bio-Rad Laboratories, Hercules, CA), probed with primary and secondary antibodies, and detected using the enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL). The primary antibodies included the following: Phospho-PERK (Thr980), PERK, Phospho-NF-κB RelA (Ser 468), NF-κB, IκBα, JAK1 and Phospho-STAT3 (Tyr705) from Cell Signaling Technology (Beverly, MA); phospho-JAK1 (Thy1022/Tyr1023) from EMD Millipore (Billerica, MA); STAT3 from BD biosciences (San Jose, CA); XBP1 from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-α-Tubulin (Sigma-Aldrich) and rabbit monoclonal anti-Lamin B1 (Cell Signaling Technology) were used as loading controls. Densitometry analysis was conducted using ImageJ software.

2.5 Quantitative PCR

Total RNA was extracted using TRIzol (Life Technologies, Rockville, MD) according to the manufacturers' recommendations. First-strand cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative PCR was performed using a StepOne PCR system (Life Technologies) and the SYBR Green PCR Master Mix kit (Life Technologies). Primer sequences for mouse transcripts were as follows: HPRT For-5'-GAA AGA CTT GCT CGA GAT GTC ATG-3'; HPRT Rev-5'-CAC ACA GAG GGC CAC AAT GT-3'; CXCL10 For-5'-CAT CCC TGC GAG CCT ATC C-3'; CXCL10 Rev-5'-CAT CTC TGC TCA TCA TTC TTT TTC A-3'; CCL2 For-5'-GGC TCA GCC AGA TGC AGT TAA-3'; CCL2 Rev-5'-CCT ACT CAT TGG GAT CAT CTT GCT-3'; GRP78 For-5'-ACT TGG GGA CCA CCT ATT CCT-3'; GRP78 Rev-5'-ATC GCC AAT CAG ACG CTC C-3'; XBP1s For-5'-TGC TGA GTC CGC AGC AGG TG-3'; XBP1s Rev-5'-GCT GGC AGG CTC TGG GGA AG-3'; ATF4 For-5'-TCC TGA ACA GCG AAG TGT TG-3'; ATF4 Rev-5'-ACC CAT GAG GTT TCA AGT GC-3'; CHOP For-5'-CTG GAA GCC TGG TAT GAG GAT-3'; CHOP Rev-5'-CAG GGT CAA GAG TAG TGA AGG T-3'; ATF6 For-5'-TGC CTT GGG AGT CAG ACC TAT-3'; ATF6 Rev-5'-GCT GAG TTG AAG AAC ACG AGT C-3'. Data obtained from RT-qPCR reaction was analyzed by the $\Delta\Delta$ CT method with HPRT used as the reference gene for normalization.

2.6 MTT Cell survival assay

Cells were seeded at a density of 1×10^4 cells/well in 96-well plates. The following day, cells were starved in DMEM containing 0.1% BSA overnight and then treated with vehicle (DMSO) (Sigma-Aldrich) or 0.01 μ M TG for 24 hours. After treatment, 20 μ l of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was added to each well and plates were incubated at 37 °C cell culture incubator for 1 hour. After media were sucked off, 100 μ l of DMSO was added to each well and plates were shaken at room temperature for 10 minutes to dissolve intracellular MTT formazan crystals, followed by measurement of absorbance at 540 nm. Value of the blank wells with media only was deducted from the measured value.

2.7 Statistical analysis

All experiments were repeated at least three times. Data are expressed as mean $\pm$ standard error of mean (SEM) of all determinations. Group differences were evaluated with one-way ANOVA followed by post-hoc Student's t test. Results were considered significant at $P < 0.05$.

3. Results

3.1 Endoplasmic reticulum (ER) stress induces CXCL10 and CCL2 production in photoreceptor cells

Because of the difficulty of isolation and culture of primary photoreceptor cells, a photoreceptor cell line 661W has been widely used to study mechanisms of photoreceptor dysfunction and degeneration, and develop strategies to protect photoreceptors in a variety of conditions such as light damage, retinitis pigmentosa, diabetic retinopathy, retinal

dystrophy, and retinal detachment (Aslanidis et al., 2015; Besirli et al., 2012; Byrne et al., 2016; Du et al., 2015; Izawa et al., 2016; Jackson et al., 2016; Krishnamoorthy et al., 1999; Lee et al., 2016; Rapp et al., 2014; Scholz et al., 2015; Shi et al., 2015; Tan et al., 2004; Zhang et al., 2014). To determine whether photoreceptor cells can produce chemokines CXCL10 and CCL2 under stress conditions, we treated 661W cells with thapsigargin (TG), which potently induces ER stress at 0.01–0.1 μ M (Bartolome et al., 2012; Hamamura and Yokota, 2007) and is widely used to study ER stress-induced cellular responses (Bartolome et al., 2012; Hamamura and Yokota, 2007; Kim et al., 2008). We treated cells with TG for different time periods and assessed the expression of ER stress-related genes (GRP78, ATF4, CHOP, XBP1s and ATF6) as well as CXCL10 and CCL2. As shown in Figure 1A–E, TG treatment rapidly induced potent upregulation of GRP78, ATF4, CHOP, XBP1s and ATF6. Since the expression of ER stress-related genes is used as a marker of ER stress (Dey et al., 2010; Kennedy et al., 2015; Namba et al., 2007), these results indicate a successful induction of ER stress in our experimental condition. Associated with the increase of ER stress, mRNAs of CXCL10 and CCL2 were robustly increased in a time-dependent manner and reached peak expression at 3 to 6 hours after TG treatment (Figure 1F and 1G). Since TG induced maximal increases in CXCL10 and CCL2 at 6 hours after TG treatment, this time point was selected for further experiments when measuring mRNA expression of CXCL10 and CCL2. Moreover, the protein levels of CXCL10 and CCL2 in conditioned medium were examined by ELISA at 24 hours after treatment. Our data clearly showed that TG treatment significantly increased CXCL10 and CCL2 protein production by 2.2-fold and 1.7-fold, respectively (Fig. 1H and 1I). Therefore, photoreceptor cells are able to produce CXCL10 and CCL2 in response to ER stress.

To validate the role of ER stress in TG-induced CXCL10 and CCL2 expression, 661W cells were treated with 4-phenylbutyrate (4-PBA) which is a chemical chaperone and has been commonly used to attenuate ER stress (Ishimura et al., 2014; Kawasaki et al., 2012; Ozcan et al., 2006). 4-PBA treatment reduced TG-induced CXCL10 and CCL2 mRNA expression by 49% and 58%, respectively (Fig. 2A and 2B), which is consistent with the partial decreases of ER stress as indicated by reduction of TG-induced expression of GRP78, ATF4, CHOP, XBP1s and ATF6 (Fig. 2C–2G). These results indicate that ER stress mediates CXCL10 and CCL2 expression at the level of gene transcription. Correspondingly, the protein levels of CXCL10 and CCL2 were reduced after 4-PBA treatment (Supplemental Fig. 1A and 1B).

3.2 PERK and XBP1 differentially regulate CXCL10 and CCL2 expression during ER stress

PERK is the only unfolded protein response (UPR) branch that modulates protein synthesis as an adaptive response and chronic PERK activation is linked to neurodegenerative diseases (Bell et al., 2016). CXCL10 and CCL2 also play an important role in neurodegeneration, so we presumed that PERK may be activated at earlier time point (e.g. 0–3 hours after TG treatment) than maximal CXCL10 and CCL2 expression was induced, and be potentially involved in ER stress-induced CXCL10 and CCL2 expression. Phosphorylation of PERK, causing PERK to migrate slower on SDS-PAGE gels, can be used as a marker for its activation status (Qi et al., 2011). Treatment of 661W cells with TG resulted in PERK mobility shift as shown in Western blot (Fig. 3A, upper panel). The faster-migrated protein

indicated the unphosphorylated form of PERK, whereas the slower-migrated forms corresponded to phosphorylated PERK. The phosphorylation status of the slower-migrated PERK proteins was further confirmed by blotting with a specific antibody which only recognizes phosphorylated PERK (Thr980) (Fig. 3A, middle panel). These data indicate that TG treatment robustly induces PERK activation in 661W cells.

To determine the role of PERK in CXCL10 and CCL2 expression, we knocked down PERK expression with shRNA. As shown in Figure 3B, there was a 48% reduction of PERK protein in cells transfected with PERK shRNA. Associated with a decrease in PERK expression, TG-induced CXCL10 and CCL2 expression was reduced by 30% and 60% at mRNA level, respectively (Fig. 3C and 3D). Consistently, PERK deletion moderately attenuated TG-induced secretion of CXCL10 and CCL2 (Supplemental Fig. 1C and 1D). To determine whether the effect of PERK knockdown was mediated by a consequent reduction of ER stress, we analyzed the expression of ER stress-related genes. We did not observe any difference in GRP78 and ATF6 expression between cells transfected with control shRNA and PERK shRNA (Fig. 3E and 3F). However, PERK knockdown affected its downstream ATF4/CHOP pathway. It reduced CHOP expression at basal level (Fig. 3G) and reduced ATF4 expression in the absence or presence of TG (Fig. 3H). These results indicate that PERK plays a key role in ER stress-induced CXCL10 and CCL2 expression, and its effect is not due to reduction of ER stress since GRP78 and ATF6 expression was not altered by PERK shRNA.

In contrast to PERK activation, transcription factor XBP1, activated by IRE1 α -mediated splicing, is a major protective molecule in the UPR when cells are under ER stress (Kim et al., 2008). TG treatment increased XBP1s expression (Fig. 4A), indicating this pathway is operative. To investigate the role of XBP1 in ER stress-induced CXCL10 and CCL2 expression, we knocked down XBP1 with shRNA construct, which markedly decreased XBP1s protein level after either vehicle or TG treatment by 35% and 58%, respectively (Fig. 4A). Knockdown of XBP1 resulted in increases of CXCL10 and CCL2 expression at basal level or after TG treatment (Fig. 4B, 4C, Supplemental Fig. 1C and 1D). Expression of GRP78, ATF4 and ATF6 was not altered by XBP1 shRNA (Fig. 4D, 4E and 4F). However, TG-induced CHOP expression was moderately boosted by XBP1 knockdown (Fig. 4G).

Since XBP1s is generated by splicing an intron from XBP1 by activated IRE1 α , we further tested whether inhibition of IRE1 α will duplicate the effects of XBP1 knockdown. We treated 661W cells with IRE1 α specific inhibitor 4 μ 8C (Ma et al., 2016), and determined CXCL10 and CCL2 expression. Similar to XBP1 knockdown, 4 μ 8C increased CXCL10 and CCL2 expression in vehicle-treated cells (Supplemental Fig. 2A and 2B). However, in spite of dramatically reducing TG-induced XBP1s expression (Supplemental Fig. 2C), 4 μ 8C did not enhance CXCL10 expression and only moderately boosted CCL2 level after TG treatment (Supplemental Fig. 2A and 2B). Moreover, 4 μ 8C treatment reduced TG-induced GRP78 and CHOP expression (Supplemental Fig. 2D and 2E). These results are different from the effects of XBP1 knockdown, suggesting that XBP1s is not the only molecule that mediates the effect of IRE1 α in photoreceptor cells during ER stress.

Overall, these studies indicate that PERK and XBP1 are involved in ER stress-induced CXCL10 and CCL2 expression. PERK positively regulates CXCL10 and CCL2 expression whereas XBP1 negatively regulates their expression.

3.3 NF- κ B is a downstream target for PERK and XBP1 in ER stress-induced CXCL10 and CCL2 expression

The NF- κ B family of transcription factors, regulates numerous genes related to inflammation and immune responses (Sanz et al., 2010). It is composed of five members: p50, p52, RelA (p65), c-Rel and RelB, and exists as homo- or hetero-dimers, among which the RelA/p50 dimer is most abundant (Sanz et al., 2010). NF- κ B dimers are bound to an inhibitory protein I κ B (e.g. I κ B α). Upon stimulation, I κ B is phosphorylated and degraded, freeing NF- κ B dimers to translocate to the nucleus and regulate the expression of many inflammatory genes. The transcriptional activity of NF- κ B is further enhanced when RelA is phosphorylated (Viatour et al., 2005). The promoters of CXCL10 and CCL2 contain NF- κ B binding sites and their expressions are regulated by NF- κ B during pathogen infection or inflammatory cytokine stimulation (Brownell et al., 2014; Spurrell et al., 2005; Xing and Remick, 2007), so we investigated whether NF- κ B signaling mediates PERK and XBP1-modulated CXCL10 and CCL2 expression. We analyzed RelA phosphorylation and I κ B α level in TG-treated 661W cells. In cells transfected with control shRNA, there was significant increase in RelA phosphorylation and decrease in I κ B α expression after TG treatment. On the other hand, in cells transfected with PERK shRNA, TG treatment only slightly increased RelA phosphorylation and decreased I κ B α expression (Fig. 5A), indicating TG-induced NF- κ B activation is attenuated by PERK deletion. In contrast, XBP1 knockdown further enhanced TG-induced RelA phosphorylation with decreased I κ B α , reflecting XBP1 deletion promoted NF- κ B activation (Fig. 5B). To examine the role of NF- κ B in TG-induced CXCL10 and CCL2 expression, we treated cells with PDTC, a NF- κ B inhibitor. PDTC treatment partially blocked TG-induced CXCL10 expression (Fig. 5C and Supplemental Fig. 1A) but dramatically attenuated TG-induced CCL2 expression (Fig. 5D and Supplemental Fig. 1B). GRP78 expression was not affected by NF- κ B inhibition (Fig. 5E). Overall, these data suggest that ER stress induces CXCL10 and CCL2 expression by activating the NF- κ B pathway, which is positively regulated by PERK and negatively regulated by XBP1.

3.4 STAT3 is involved in ER stress-induced CXCL10 and CCL2 expression

Since NF- κ B inhibitor only partially blocked TG-induced CXCL10 expression, we reasoned other transcriptional factors might also be involved in this process. The Signal Transducer and Activator of Transcription (STAT) proteins are transcription factors that regulate expression of genes related with development and immune system. Both CXCL10 and CCL2 genes contain STAT binding sites and their expression is regulated by STAT1 and STAT3 (Kok et al., 2009; Lee et al., 2011). Thus we investigated whether STAT1 and STAT3 were involved in PERK and XBP1-regulated CXCL10 and CCL2 expression. We did not detect STAT1 phosphorylation when cells were treated with TG (data not shown). On the other hand, after TG treatment, STAT3 phosphorylation was prominently increased in cells transfected with control shRNA but not in cells with PERK shRNA, indicating that TG-induced STAT3 phosphorylation is dependent on PERK during UPR (Fig. 6A). The

increased STAT3 phosphorylation may be caused by activation of its upstream kinases JAK1 and JAK2. Therefore we analyzed JAK1 and JAK2 phosphorylation. Our data showed that phosphorylated JAK1 (Fig. 6A) but not JAK2 (data not shown) was significantly increased after TG treatment, exhibiting similar profile as phosphorylated STAT3, whereas PERK deletion blunted this response. In contrast, in cells with XBP1 knockdown, the phosphorylation of STAT3 and JAK1 was enhanced at the basal level and TG treatment (Fig. 6B). To further assess the involvement of STAT3 in ER stress-induced CXCL10 and CCL2 expression, we blocked STAT3 activation with a STAT1/3 inhibitor (Stattic) and measured CXCL10 and CCL2 gene expression and secretion. TG-induced CXCL10 and CCL2 expression was significantly reduced by Stattic (Fig. 6C and 6D, and Supplemental Fig. 1A and 1B), without altering GRP78 expression (Fig. 6E). These data suggest that TG induces CXCL10 and CCL2 expression via the activation of STAT3. PERK is a positive regulator of STAT3 activation while XBP1 is a negative regulator of this pathway. ER stress also regulates cell survival. However blocking PERK or STAT3 did not affect TG-induced decrease in cell viability though XBP1 knockdown slightly accelerated TG-induced cell loss (Supplemental Fig. 3), suggesting that different mechanisms are involved in ER stress-induced cytokine expression and cell death.

3.5 PERK and XBP1 regulates CXCL10 and CCL2 expression in photoreceptor cells under diabetes-related conditions

After demonstrating that PERK and XBP1 differentially regulate ER stress activator-induced CXCL10 and CCL2 production in 661W cells, we further investigated whether such mechanism is operative when cells were treated with diabetes-related conditions. AGE is formed by protein glycation during hyperglycemia and plays an important role in diabetic retinopathy and other complications (Singh et al., 2014). Our results revealed that AGE induced CXCL10 and CCL2 expression at transcription level and mRNAs of CXCL10 and CCL2 reached peak expression at 12 hours after AGE treatment (Fig. 7A and 7B). AGE treatment also induced ER stress as indicated by upregulation of PERK phosphorylation, and increased expression of XBP1s, GRP78, ATF4, CHOP and ATF6 (Fig. 7C, 7D and Supplemental Fig. 4) though its effects were much weaker than those induced by TG. Similarly, AGE-induced CXCL10 and CCL2 gene expression and protein secretion were blocked by knockdown of PERK while enhanced by knockdown of XBP1 (Fig. 7E–7H). As hyperglycemia is a major cause of diabetic retinopathy, we further determined CXCL10 and CCL2 in conditioned medium from high glucose-treated photoreceptors and found that PERK and XBP1 regulated CXCL10 and CCL2 levels similar to their actions in TG- or AGE-treated cells (Fig. 8). These studies indicate PERK and XBP1 also play an important role in CXCL10 and CCL2 expression in photoreceptor cells under diabetes-related conditions.

4. Discussion

Photoreceptor dysfunction/death is a cause of many retinal degenerative diseases (Jayakody et al., 2015; Murakami et al., 2013). Studies on photoreceptor injury are mainly focused on how stress conditions initiate intracellular signals that drive photoreceptor apoptosis, necrosis, and/or autophagic cell death (Murakami et al., 2013). Inflammation is recognized

as an important player during photoreceptor death (Ambati et al., 2003; Detrick et al., 2008; Guo et al., 2012; Murakami et al., 2013; Nakazawa et al., 2007; Ricker et al., 2010; Rutar et al., 2015). However, it is largely unknown how photoreceptors communicate with immune cells, as photoreceptors are not considered as inflammatory cells. Extracellular exposure of phosphatidylserine, release of ATP and secretion of high-mobility group box 1 protein from dying photoreceptors have been proposed to serve as “find-me” signal to recruit immune cells (Murakami et al., 2013). In this study we provide the first evidence that ER stress induces CXCL10 and CCL2 gene transcription and protein production in photoreceptor cells in a STAT3 and NF- κ B-dependent manner and PERK positively regulates these pathways whereas XBP1 acts oppositely. As CXCL10 and CCL2 have dominant roles in recruiting mononuclear cells during inflammation, our study suggest that photoreceptors may participate in retinal inflammation by producing chemokines during stress. This study, together with a report that photoreceptors express toll-like receptors (TLR) and produce cytokines when stimulated with TLR ligands (Singh and Kumar, 2015), indicates that photoreceptors can function as inflammatory cells in addition to their basic role as photosensor during visual cycle.

ER homeostasis is tightly monitored by the unfolded protein response (UPR) which controls the ER-to-nucleus signaling cascades (Ron and Walter, 2007). Mild ER stress occurs at physiological conditions and is important for cells to re-establish ER homeostasis and maintain normal cell function (Yang et al., 2010). Nevertheless, excessive ER stress causes cell death, tissue damage and leads to diseases. In the retina, ER stress has been linked to retinal neuronal and vascular degeneration in diabetic retinopathy, glaucoma and photoreceptor degeneration (Chen et al., 2012; Doh et al.; Gorbatyuk et al., 2010; Ha et al., 2015; Hu et al., 2012; Ito et al.; Kang et al., 2012; Li et al., 2009; Liu et al., 2010; Makino et al., 2013; Shinde et al., 2012; Zhu et al., 2013). The contradictory effects of ER stress in cell fates (survival vs. death) are dictated by the balance among UPR branches and their downstream adaptive and cytotoxic pathways (Bell et al., 2016). When PERK is activated, it phosphorylates eIF2 α , which reduces protein synthesis to prevent further influx of nascent polypeptides into the ER while enhancing the translation of certain mRNAs, such as the mRNA encoding ATF4 (Teske et al., 2011). ATF4, as a transcription factor, mediates transcription of genes involved in cell recovery and adaptation (Bell et al., 2016). However, when PERK is chronically activated, it causes neuronal cell dysfunction and apoptosis because of chronic attenuation of protein translation (Bell et al., 2016). In the meanwhile, sustained ATF4 expression activates apoptotic pathways (Bell et al., 2016). Unlike the dual functionality of PERK, upregulation of spliced XBP1 in UPR is neuroprotective as it alleviates ER stress by inducing expression of ER chaperones and ER-associated degradation components, and facilitates lipid synthesis and ER biogenesis pathways (Bell et al., 2016; Casas-Tinto et al., 2011). Spliced XBP1 also enhances cell survival by inducing the expression of molecules for anti-oxidant defense. Overall, the effects of PERK and XBP1 in cell survival and death are usually linked to their modulation of intracellular apoptotic pathways. In this study, we found that PERK and XBP1 differentially regulate the production of CXCL10 and CCL2 in photoreceptors. PERK is a positive regulator of ER stress-induced expression of these two cytokines while XBP1 negatively regulates their expression. Given that CXCL10 and CCL2 play important roles in inflammation which is

critically involved in neuronal injury, our study suggests that the differential regulation of CXCL10 and CCL2 expression by PERK and XBP1 may also participate in their contradictory effects in neuronal cell survival.

ER stress-induced inflammation has been linked to many diseases (Garg et al., 2012). Activated PERK preferably induces translation of selective mRNAs including ATF4, leading to ATF4-dependent upregulation of CHOP. As a transcription factor, ATF4 regulates expression of stress-response genes directly by binding to the promotor region of these genes or interacting with other transcription factors. In monocytes/macrophages, ATF4 exerts its proinflammatory effects through regulating NF- κ B activation, activating the promoter of inflammatory gene (Iwasaki et al., 2014) or binding to transcription factors such as c-Jun (Zhang et al., 2013). In Muller cells, hyperglycemia- and hypoxia-induced ATF4 activation leads to upregulation of inflammatory genes via its crosstalk with hypoxia-inducible factor (HIF)-1 α and c-Jun NH2-terminal kinase (JNK) (Zhong et al., 2012). In retinal endothelial cells, ATF4 mediates hyperglycemia-induced endothelial inflammation and retinal vascular leakage through activation of STAT3 (Chen et al., 2012). Overall, the role of ATF4 in inducing inflammation is generally appreciated though specific pathways by which ATF4 regulates inflammatory molecule expression may differ from cell types and stimuli applied. CHOP, downstream ATF4, also activates NF- κ B or inflammasome pathways by inducing IRAK2 and caspase 11 expression (Endo et al., 2006; Willy et al., 2015). Our study represents the first one to investigate ER stress-induced inflammation in photoreceptor cells. We demonstrated that PERK induces CXCL10 and CCL2 expression by activation of NF- κ B and STAT3. Considering the critical role of ATF4 in inflammation, it is likely that PERK may induce NF- κ B and STAT3 activation through ATF4 or ATF4/CHOP. However, other mechanisms could be operative as well. PERK, as a Ser/Thr protein kinase activated after dimerization and autophosphorylation (Bell et al., 2016), may phosphorylate and activate positive regulators of the NF- κ B and STAT3 pathways. As a result, knockdown of PERK resulted in decreased phosphorylation of NF- κ B RelA and JAK1/STAT3. The PERK/eIF2 α pathway may also attenuate the expression of proteins that inhibit activation or transcriptional activity of NF- κ B and STAT3. This notion has been preliminarily supported by our data that the total level of I κ B α , a protein that binds to NF- κ B and inhibits its activation, is markedly increased after PERK knockdown.

Other than the PERK/eIF2 α /ATF4/CHOP pathway, the IRE1 α /XBP1s branch is also involved in inflammatory reactions. As a transcription factor, XBP1 binds the promoters of genes encoding inflammatory mediators and is required for optimal and sustained production of proinflammatory cytokines in lipopolysaccharide-treated macrophages (Martinon et al., 2010). Our studies, however, identified that XBP1 negatively regulates CXCL10 and CCL2 expression by inhibiting NF- κ B and STAT3 activation. Therefore, XBP1 may exert different effects on inflammation dependent on cellular and environmental contexts. XBP1s is a negative regulator of IRE1 activation. Loss of XBP1 results in IRE1 hyperactivation (Kaser et al., 2008) which may interact with and activate TRAF2 (Urano et al., 2000) and lead to TRAF2-dependent activation of NF- κ B (Kaneko et al., 2003) and JNK/STAT3 signaling (Mao et al., 2016; Prause et al., 2016; Urano et al., 2000). Alternatively, as a transcription factor, XBP1 may negatively regulate NF- κ B and JAK1/STAT3 activation by inducing the expression of molecules (e.g. phosphatases) that can

inactivate NF- κ B and JAK1/STAT3. It has been shown that XBP1s inhibits PI3K/Akt pathway through XBP1-dependent expression of SKIP, a PIP3 phosphatase (Ijuin et al., 2016). Of interest, although XBP1 is a major downstream substrate for IRE1 α , IRE1 α inhibition did not duplicate the effects of XBP1 knockdown. In addition to splicing XBP1 mRNA, IRE1 α cleaves other ER-located mRNAs through regulated IRE1-dependent decay (RIDD) (Ma et al., 2016; Maurel et al., 2014) and regulates protein expression by cleavage of miRNA (Heindryckx et al., 2016; Ma et al., 2016). The difference between IRE1 α inhibition and XBP1 knockdown suggests that other downstream targets of IRE1 α are also involved in TG-induced CXCL10 and CCL2 expression.

While precise mechanisms by which PERK and XBP1 regulate NF- κ B and STAT3 activation in photoreceptor cells during ER stress remain to be elucidated, our findings that ER stress induces CXCL10 and CCL2 expression by activating NF- κ B and STAT3 suggest that blocking the activation of these two transcription factors may be useful to alleviate ER stress-induced inflammation and protect photoreceptor damage. Interestingly, although CXCL10 and CCL2 expression is regulated by both NF- κ B and STAT3, STAT3 plays a predominant role in CXCL10 expression while NF- κ B plays a larger role in CCL2 expression.

5. Conclusions

Our data reveal a novel mechanism that ER stress induces CXCL10 and CCL2 via the activation of NF- κ B and STAT3, and PERK is a positive regulator of this process whereas XBP1 is a negative regulator (Fig. 9). As ER stress occurs in other retinal cells such as endothelial cells and Muller cells in a variety of conditions (Li et al., 2009; Zhang et al., 2015), future studies to examine whether similar mechanisms are also operative in these cells would be important to understand ER stress-induced CXCL10 and CCL2 expression in different cellular contexts and therefore help to better control ER stress-induced retinal inflammation in retinal degenerative diseases as well as chronic diseases including diabetic retinopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Whether photoreceptor cells can produce CCL2 and CXCL10, two important players in inflammation during stress was studied.

Induction of Endoplasmic reticulum (ER) stress in photoreceptor cells induced CXCL10 and CCL2 expression.

PERK was a positive regulator while XBP1 was a negative regulator for CXCL10 and CCL2 expression.

PERK and XBP1 regulates the expression of CXCL10 and CCL2 through regulating NF- κ B RelA and STAT3 activation.

Fig. 1. ER stress induces the production of CXCL10 and CCL2 in photoreceptor cells

Photoreceptor cell line 661W was treated with 0.1% DMSO (veh) or 0.01 μ M thapsigargin (TG). (A–G) Cells were collected for mRNA extraction at indicated time points. The mRNA levels of ER stress-related genes (GRP78, ATF4, CHOP, XBP1s and ATF6), CXCL10 and CCL2 were analyzed by qPCR. * p <0.05 vs 0h; n =3. (H, I) Conditioned medium was collected at 24 hours after treatment and subjected to ELISA to measure CXCL10 and CCL2 production which was normalized to total protein of cell lysates. Cells treated with vehicle were used as reference. * p <0.05 compared with vehicle; n =4.

Fig. 2. Blockade of ER stress attenuates TG-induced CXCL10 and CCL2 expression

Photoreceptor cells were treated with 0.01 μ M TG in the presence or absence of ER stress blocker 4-phenylbutyrate (4-PBA, 4mM) for 6 hours. RNA was extracted and qPCR was performed to assess the expression of CXCL10, CCL2 and ER stress markers (GRP78, ATF4, CHOP, XBP1s and ATF6). Cells treated with 0.1% DMSO (vehicle) were used as reference * $p < 0.05$ vs vehicle control; # $p < 0.05$ vs TG treatment; $n = 4$.

Fig. 3. PERK mediates the expression of CXCL10 and CCL2 during ER stress

(A) Photoreceptor cells were treated with TG for 1 hour and 3 hours, and total and phosphorylated PERK were assessed by Western blot. Equal loading of protein was confirmed with antibody against α-Tubulin. (B) Photoreceptor cells were stably expressed with control shRNA (sh-Con) or PERK shRNA (sh-PERK) and the knockdown efficiency of PERK was evaluated by Western blot and quantified by densitometry. Cells transfected with control shRNA were used as reference. * $p < 0.05$ vs control shRNA. (C–H) Photoreceptor cells stably expressing control or PERK shRNA were treated with 0.1% DMSO (vehicle) or TG for 6 hours. The levels of CXCL10, CCL2, GRP78, ATF6, CHOP and ATF4 mRNA were analyzed by qPCR. Cells transfected with control shRNA and treated with vehicle were used as reference. * $p < 0.05$ vs vehicle-treated cells with control shRNA; # $p < 0.05$ vs TG-treated cells with control shRNA; $n = 3$.

Fig. 4. XBP1 regulates the transcription of CXCL10 and CCL2 expression during ER stress
 Photoreceptor cells stably expressing control shRNA (sh-Con) or XBP1 shRNA (sh-XBP1) were treated with vehicle (veh) or 0.01 μ M TG. (A) 3 hours after TG treatment, the level of XBP1 protein in nucleus was evaluated by Western blot and quantified by densitometry. Lamin B1 served as loading control. Cells transfected with control shRNA and treated with vehicle were used as reference. (B–G) 6 hours after TG treatment, CXCL10, CCL2, GRP78, ATF4, ATF6 and CHOP mRNA levels were analyzed by qPCR. Cells transfected with sh-Con and treated with vehicle were used as reference. * $p < 0.05$ vs vehicle-treated cells with control shRNA; # $p < 0.05$ vs TG-treated cells with control sh-RNA; $n = 4$.

Fig. 5. PERK mediated-CXCL10 and CCL2 expression is dependent on NF- κ B RelA
 (A, B) Photoreceptor cells were stably expressed with control shRNA (sh-Con), PERK shRNA (sh-PERK) or XBP1 shRNA (sh-XBP1) and treated with TG for 3 hours. Phosphorylated and total RelA and I κ B α were evaluated by Western blot and quantified by densitometry. Equal loading of protein was confirmed with antibody against total RelA or α -Tubulin. Cells transfected with control shRNA and treated with vehicle were used as reference. * p <0.05 vs vehicle-treated cells with sh-Con. # p <0.05 vs TG-treated cells with sh-Con; n =3. (C, D, E) Photoreceptors were pretreated with NF- κ B inhibitor PDTC (10 μ M) for 30 minutes and followed with TG treatment for 6 hours. The mRNA levels of CXCL10, CCL2 and GRP78 were analyzed by qPCR. Cells treated with vehicle were used as reference. * p <0.05 vs vehicle treatment; # p <0.05 vs TG treatment; n =3.

Fig. 6. PERK mediated-CXCL10 and CCL2 expression is dependent on STAT3

(A–B) Photoreceptor cells were stably expressed with control shRNA (sh-Con), PERK shRNA (sh-PERK) or XBP1 shRNA (sh-XBP1) and treated with TG for 30 minutes. Phosphorylated and total STAT3 and JAK1 were evaluated by Western blot and quantified by densitometry. Cells transfected with control shRNA and treated with 0.1% DMSO (vehicle) were used as reference. * $p < 0.05$ vs vehicle-treated cells with sh-Con. # $p < 0.05$ vs TG-treated cells with sh-Con; $n = 3$. (C, D, E) Photoreceptors were pretreated with STAT3 inhibitor Stattic (3 μ M) for 30 minutes and followed with TG treatment for 6 hours. The mRNA levels of CXCL10, CCL2 and GRP78 were analyzed by qPCR. Cells treated with vehicle were used as reference. * $p < 0.05$ vs vehicle treatment; # $p < 0.05$ vs TG treatment; $n = 3$.

Fig. 7. PERK and XBP1 oppositely mediate the expression of CXCL10 and CCL2 after AGE treatment in photoreceptor cells

(A-D) Photoreceptor cells were treated with 200 μ g/ml AGE for the indicated time periods. CXCL10 and CCL2 mRNA levels were analyzed by qPCR (A, B). Phosphorylated and total PERK were evaluated by Western blot and quantified by densitometry (C). XBP1s gene expression was analyzed by qPCR (D). Cells treated with vehicle (BSA) were used as reference. * $p < 0.05$ vs 0h; $n = 3$. (E-H) Photoreceptor cells stably expressing control or PERK or XBP1 shRNA were treated with vehicle (BSA) or AGE. The levels of CXCL10 and CCL2 mRNA were analyzed by qPCR at 12 hours after treatment ($n = 4$) (E, F). CXCL10 and CCL2 protein levels in conditioned medium were measured by ELISA at 24 hours after

treatment, and normalized to total protein of cell lysates (n=3) (G–H). Cells transfected with control shRNA and treated with vehicle were used as reference. *p<0.05 vs vehicle-treated cells with control sh-RNA; #p<0.05 vs AGE-treated cells with control shRNA.

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Fig. 8. PERK and XBP1 differentially modulate CXCL10 and CCL2 secretion after high glucose treatment

Photoreceptors stably expressing sh-Con, sh-PERK or sh-XBP1 were cultured in normal glucose (NG, 5.5 mM) or high glucose (HG, 30 mM) for 48 hours and conditioned medium was harvested. CXCL10 and CCL2 protein levels were measured by ELISA and normalized to total protein of cell lysates. * $p < 0.05$ vs NG-treated cells with control shRNA; # $p < 0.05$ vs HG-treated cells with control shRNA; $n = 3$.

Fig. 9.
Proposed signaling pathways in ER stress-regulated expression of CXCL10 and CCL2 in photoreceptor cells.