PERK mediates oxidative stress and adipogenesis in Graves’ orbitopathy pathogenesis

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Abstract

We examined endoplasmic reticulum (ER) stress-related gene expression in orbital tissues from patients with Graves’ orbitopathy (GO) and the effects of silencing protein kinase RNA-like endoplasmic reticulum kinase (PERK) in primary orbital fibroblast cultures to demonstrate the therapeutic potential of PERK-modulating agents in GO management. The expression of ER stress-related genes in orbital tissue harvested from individuals with or without GO was studied using real-time PCR. The role of PERK in GO pathogenesis was examined through small-interfering RNA (siRNA)-mediated silencing in cultured primary orbital fibroblasts. Intracellular reactive oxygen species (ROS) levels induced in response to cigarette smoke extract (CSE) or hydrogen peroxide were measured using 5-(and 6)-carboxy-20,70-dichlorodihydrofluorescein diacetate staining and flow cytometry. Cells were stained with Oil Red O, and adipogenesis-related transcription factor expression was evaluated through Western blotting after adipogenic differentiation. PERK, activating transcription factor 4 (ATF4), and CCAAT-enhancer-binding protein (C/EBP)-homologous protein (CHOP) mRNA levels were significantly higher in GO orbital tissues than in non-GO orbital tissues. PERK silencing inhibited CSE- or hydrogen peroxide-induced ROS generation. After adipogenic differentiation, GO orbital fibroblasts revealed decreased lipid droplets and downregulation of C/EBPα, C/EBPβ, and peroxisome proliferator-activator gamma (PPARγ) in PERK siRNA-transfected cells. The orbital tissues of patients with GO were exposed to chronic ER stress and subsequently exhibited enhanced unfolded protein response (especially through the PERK pathway). PERK silencing reduced oxidative stress and adipogenesis in GO orbital fibroblasts in vitro. Our results imply that PERK-modulating agents can potentially be used to manage GO.

Introduction

Graves’ orbitopathy (GO) – also known as thyroid eye disease – is a pathological manifestation of Graves’ disease that involves the infiltration of T cells, B cells, and macrophages into the orbits (Garrity & Bahn 2006, Bahn 2010). Clinical manifestations of GO are largely variable and include orbital pain, ocular discomfort, eyelid retraction, proptosis, and eyelid swelling (Brent 2008). Orbital fibroblasts play a key role in disease
progression, and cellular immunity plays a crucial role in orbital inflammation during the pathogenesis of GO (Khong et al. 2016, Dottore et al. 2021). However, the exact pathological mechanism in the development and progression of GO remains to be established.

The endoplasmic reticulum (ER) is a cellular component that is involved in multiple crucial cellular functions and affects homeostasis (Flamment et al. 2012). ER molecular chaperones and folding enzymes allow new proteins entering the ER lumen to achieve their original conformation and mediate their post-translational modification (Ron & Walter 2007, Halperin et al. 2014). Disruption of this function (such as excessive protein synthesis or accumulation of unfolded or misfolded proteins in the ER lumen) is termed as ‘ER stress’, which can lead to the activation of a cellular response called unfolded protein response (UPR) (Kim et al. 2008, Flamment et al. 2012).

UPR signaling is initiated upon the activation of three ER membrane-bound transducers, namely inositol-requiring enzyme 1 (IRE1), activating transcription factor (ATF) 6, and protein kinase RNA-like ER kinase (PERK) (Ron & Walter 2007, Flamment et al. 2012). Through transcriptional and translational modifications, UPR helps stressed cells adapt to ER stress conditions for survival (Dandekar et al. 2015), and these elaborate adaptive responses exhibit crosstalk with inflammatory and stress signaling pathways (Ron & Walter 2007, Hotamisligil 2010, Dandekar et al. 2015).

Mounting evidence has pointed out that ER stress is involved in the development of autoimmune and inflammatory diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and idiopathic inflammatory myopathies (Todd et al. 2008, Park et al. 2014, Lee et al. 2015, Lightfoot et al. 2015). Moreover, proteomic analysis has revealed aberrant expression of binding immunoglobulin protein (BiP, an ER-stress chaperone that functions as an ER-stress sensor; also known as HSP5A or GRP-78) in thyroid tissues of patients with Graves’ disease (Meng et al. 2017). Recently, we demonstrated that BiP was upregulated in orbital tissues from individuals with GO and that silencing BiP inhibited reactive oxygen species (ROS) generation and adipogenesis in GO orbital fibroblasts, indicating that ER stress is related to GO development (Ko et al. 2021). Although the role of ER stress in GO pathogenesis has been proposed, the downstream signaling pathway(s) have not been previously investigated. Here, we evaluated three major ER stress-signaling pathways (IRE1, PERK, and ATF6) in orbital tissues, demonstrated their roles by modulating PERK expression in primary cultured GO orbital fibroblasts and assessed the potential of PERK modulating agents as therapeutic targets.

Methods

Reagents and chemicals

Fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM), penicillin, and gentamicin were purchased from HyClone Laboratories, Inc. (Logan, UT, USA). PERK small-interfering RNA (siPERK; #4390824) and negative-control siRNA (siCon; #4390843) were purchased from Ambion/Applied Biosystems. Antibodies against p-eukaryotic translation initiation factor (eIF) 2α (RRID:AB_2096481), t-eIF2α (RRID:AB_10692650), ATF4 (RRID:AB_2616025), C/EBP-homologous protein (CHOP; RRID:AB_2089254), p-phosphoinositide 3-kinase (PI3K; RRID:AB_659940), PI3K (RRID:AB_329869), p-AKT (RRID:AB_329825), AKT (RRID:AB_329827), p-extracellular signal-regulated kinase (ERK; RRID:AB_331646), ERK (RRID:AB_330744), p-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB; RRID:AB_330559), NF-κB (RRID:AB_823578), and p-p38 (RRID:AB_331641), p38 (RRID:AB_330713) were obtained from Cell Signaling Technology. Antibodies against PERK (RRID:AB_2762850), peroxisome proliferator-activator gamma (PPARγ; RRID:AB_628115), CCAAT-enhancer-binding protein (C/EBPα (RRID:AB_631233), C/EBPβ (RRID:AB_2078068), and β-actin (RRID:AB_1119529) were obtained from Santa Cruz Biotechnology. Recombinant human interleukin (IL)-1β was purchased from R&D Systems. Oil Red O and hydrogen peroxide solution (H₂O₂; 30%) were purchased from Sigma–Aldrich Corp. Cigarette smoke extract (CSE) was freshly prepared 1 h prior to each experiment from commercially available filtered cigarettes (Marlboro 20 class A cigarettes (8.0 mg tar; 0.7 mg nicotine); Philip Morris Korea, Inc., Seoul, Korea), as described previously (Yoon et al. 2013).

Cell culture and differentiation protocols

Orbital adipose/connective tissues were acquired as surgical waste in orbital decompression surgery from eight individuals with GO (six women, two men; age, 22–68 years). Healthy control tissues were harvested from the post-septal area of six individuals without history or clinical findings of thyroid disease or GO (five women, one man; age, 29–62 years) during upper lid blepharoplasty. All patients with GO had inactive disease (clinical activity scores were less

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than 3) and had stable euthyroidism at the time of surgery. Individuals with GO did not receive steroids, other types of immunosuppressive treatments, or radiotherapy for at least 3 months prior to surgery. The study adhered to the tenets of Declaration of Helsinki, and the study protocol was approved by the Institutional Review Board of Severance Hospital (approval number 4-2019-1025). All patients provided written informed consent.

Primary orbital fibroblast cultures were established as described previously (Kim et al. 2016). Briefly, minced tissue was placed directly in a medium containing equal volumes of DMEM and F12 medium, supplemented with 20% FBS and antibiotics. Primary cultured orbital fibroblast strains were stored in liquid nitrogen until further analysis; cells were used between the second and fifth passages. Adipocyte differentiation was conducted with our previously reported adipocyte differentiation protocol for GO orbital fibroblasts to evaluate the adipogenic potential of orbital fibroblasts (Kim et al. 2016, Ko et al. 2018, 2020). Briefly, after cells reached confluence, the culture media was changed to serum-free DMEM supplemented with biotin, pantothenic acid, transferrin, T3, insulin, and carboxymethylxanthine, with or without IL-1β (10 ng/mL). For the first 4 days, dexamethasone and isobutylmethylxanthine were included in the media. The differentiation was continued for 10 days, during which the media was replaced every 2 to 3 days.

Real-time polymerase chain reaction (PCR)

The mRNA expression of UPR-related genes (PERK, IRE1, ATF6, ATF4, and CHOP) in orbital tissues from individuals with and without GO was evaluated using real-time PCR. RNA isolation from orbital tissue explants and real-time PCR were performed with primer sets (Table 1) as described previously (Kim et al. 2016). All PCR experiments were performed in triplicate, and the mRNA expression in all samples was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative expression was determined using the 2^{ΔΔCt} method as the fold-change of the threshold cycle (Ct) value between healthy subjects and GO patients relative to the expression of the GAPDH control.

**siRNA transfections**

Orbital fibroblasts from individuals with GO were cultured at ~80% confluence in 100 mm plates and transfected with PERK siRNA (siPERK) or siCon with the TransIT-siQUEST reagent (Mirus, PanVera, Madison, WI, USA), as per the manufacturer’s instructions. After transfection, IL-1β (10 ng/mL) was administered to induce inflammation or oxidative stress was induced either with CSE (2%) or H₂O₂ (200 μmol/L). The transfected cells were differentiated to adipocytes for 10 days as described above, to evaluate the potential of adipocyte differentiation.

Western blotting

Western blotting was performed as described previously (Byun et al. 2016, Lee et al. 2019). To assess the reduction of pathogenic GO mechanisms under the condition of silenced PERK expression, orbital fibroblasts were transfected with siPERK or siCon and cultured for 24 h. To evaluate the inhibitory effect of siPERK transfection on PERK expression and its downstream pathways in GO orbital fibroblasts, we analyzed eIF2α phosphorylation and ATF4 and CHOP expression using Western blotting after IL-1β (10 ng/mL) treatment for 24 h or 48 h. The expression of the transcription factors related to adipogenesis (C/EBPα, C/EBPβ, and PPARγ) was analyzed after 10 days of adipogenic differentiation in siRNA-transfected GO orbital fibroblasts. The induction of intracellular signaling proteins was assayed via Western blotting to analyze the expression of PI3K, AKT, ERK, NF-κB, and p38. Relative amounts of protein in each immunoreactive band were quantified through densitometry after normalization with the β-actin levels in the same sample.

**Intracellular ROS measurements**

ROS release was evaluated using 5-(and 6)-carboxy-20,70-dichlorodihydrofluorescein diacetate (H₂DCFDA; Invitrogen/Thermo Fisher Scientific), as described previously (Yoon et al. 2013). Briefly, cultured orbital fibroblasts were transfected with siPERK or siCon and incubated for 24 h. Then, the culture medium was removed, and the cells were washed with PBS, incubated

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**Table 1** Real-time PCR primer sequences.

<table>
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<th>Genes</th>
<th>Primer sequence</th>
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| **PERK** | F: 5′-GAACACGACGATGAGACAGAC-3′  
R: 5′-GTATGACACCAAGGAACC-3′ |
| **IRE1** | F: 5′-GGCAACAGAATAACCATACATAC-3′  
R: 5′-ACCACCCATCCATCCATTG-3′ |
| **ATF6** | F: 5′-CCCTGCTCTACAAGTACATGAG-3′  
R: 5′-CTTCTAACTCCTCCCTTACCAC-3′ |
| **ATF4** | F: 5′-GAACACGAGAAAGCAGGAAACGA-3′  
R: 5′-TCCTCACATGCGGCTCTT-3′ |
| **CHOP** | F: 5′-GCAAGGTATCCATGACAAAC-3′  
R: 5′-GTCCACACCTGTTGCTGA-3′ |
| **GAPDH** | F: 5′-GCCAAGGTCATCCATGACAAAC-3′  
R: 5′-GTCCACACCTGTTGCTGA-3′ |
with 10 mM DCFDA at 37°C for 30 min, and stimulated with CSE (2%) or H$_2$O$_2$ (200 μmol/L) for 30 min (Yoon et al. 2013, Kim et al. 2015, Ko et al. 2018, 2020). Fluorescently stained cells were examined using microscopy at 40× and quantified via flow cytometry.

**Oil Red O staining**

After allowing siRNA-transfected orbital fibroblasts to differentiate for 10 days, the cells were stained with Oil Red O, as described previously (Kim et al. 2016, Ko et al. 2018). Stained cells were visualized and photographed at 40× magnification and the optical density of Oil Red O-stained cell lysates at 490 nm was measured for quantification, according to the manufacturer’s instructions. Optical density assessments were performed in duplicate using cells from different donors; the absorbances were normalized against the absorbance of adipocyte differentiated control cells transfected with siCon.

**Cell viability and apoptosis assays**

An annexin V-FITC Kit (R&D systems) was used to evaluate the effect of PERK silencing on apoptosis in GO and non-GO orbital fibroblasts. Cells were transfected with siPERK or siCon and cultured for 24 h, which was followed by IL-1β treatment (10 ng/mL) for 24 h or 48 h. They were then washed and incubated with annexin V labeled with FITC and propidium iodide (PI) for 15 min. Apoptotic cells were analyzed using FACSCalibur (Becton Dickinson, Cockeysville, MD, USA). Ten thousand cells were excited at 488 nm and emission was measured at 515–545 nm and 600 nm to assess FITC and PI, respectively.

**Statistical analysis**

All experiments were performed using cells from at least three cell strains from three different individuals, which were assayed in duplicate. Differences in continuous data between the experimental and control groups were assessed using Student’s t-test or Wilcoxon rank-sum test in R version 3.1.2 (R Foundation, Vienna, Austria). P values <0.05 were considered to reflect significant differences.

**Results**

**The PERK signaling pathway was upregulated in orbital tissues from individuals with GO**

To determine which of the three ER membrane-associated proteins (PERK, IRE1, and ATF6) are involved in GO pathogenesis, we quantified the expression of PERK, IRE1, and ATF6 mRNA using real-time PCR in orbital tissues of individuals with and without GO (n=6, each). The PERK mRNA expression levels were higher in GO orbital tissues than in non-GO tissues (P=0.002, Fig. 1), whereas the expression levels of IRE1 and ATF6 mRNA did not differ between orbital tissues from individuals with and without GO (P=0.173, and P=0.242, respectively, Fig. 1). The mRNA expression levels of downstream signaling genes in the PERK signaling pathway (ATF4 and CHOP) were also significantly elevated in GO orbital tissues compared to control (P < 0.001, Fig. 1).

**Effect of PERK silencing on its downstream signaling pathway**

To evaluate the effect of PERK silencing on its downstream signaling pathways, orbital fibroblasts from individuals with GO were transfected with siPERK and incubated with IL-1β (10 ng/mL) for 24 h (Fig. 2A) or 48 h (Fig. 2B). Western blotting revealed that transfection with siPERK downregulated PERK expression and consequently reduced eIF2α phosphorylation and ATF4 and CHOP expression (signaling molecules downstream of PERK).

**Silencing PERK suppressed H$_2$O$_2$- or CSE-induced ROS levels**

To verify the anti-oxidative effect of PERK silencing in GO orbital fibroblasts, 200 μmol/L H$_2$O$_2$ or 2% CSE was administered to induce oxidative stress in siPERK or siCon transfectants. Silencing PERK expression attenuated H$_2$O$_2$- or CSE-induced ROS levels (Fig. 3).

**Silencing PERK attenuated adipogenesis in GO orbital fibroblasts**

To investigate the effect of PERK silencing on adipogenesis in GO, orbital fibroblasts from individuals with GO were transfected with siPERK, and then differentiated to adipocytes for 10 days, with or without IL-1β treatment. Silencing PERK decreased lipid droplet accumulation and reduced the number of adipocytes. The optical density at 490 nm of Oil Red O-stained cell lysates was used to quantify the decreased lipid accumulation of siPERK transfectants (Fig. 4A). Western blotting revealed that silencing PERK inhibited the expression of adipogenic transcription factors (C/EBPα, C/EBPβ, and PPARγ) induced by IL-1β during adipogenesis (Fig. 4B).
Effect of silencing PERK on the viability and apoptosis of orbital fibroblasts

To evaluate the effect of PERK silencing on cell viability and apoptosis, orbital fibroblasts from individuals with GO and healthy controls were transfected with siPERK, and treated with IL-1β (10 ng/mL) for 24 h or 48 h. Annexin V-based apoptosis assays revealed that PERK silencing did not induce apoptosis or necrosis in orbital fibroblasts from individuals with or without GO (Fig. 5).

Intracellular signaling pathways related to the PERK pathway

To evaluate the signaling pathways through which PERK mediates its anti-adipogenic and anti-oxidative effects in GO and non-GO orbital fibroblasts, the expression of transcription factors was analyzed after siPERK transfection. PERK silencing reduced IL-1β-induced phosphorylation of PI3K, AKT, and ERK (Fig. 6).

Discussion

Here, we found that PERK, one of the three ER membrane-bound transducers, along with its downstream signaling pathways (ATF4 and CHOP) was upregulated in orbital tissues from individuals with GO compared to the corresponding levels in control. These findings indicated that PERK-mediated UPR was increased in the orbital tissues of individuals with GO. In addition, in vitro studies with GO orbital fibroblasts showed that PERK silencing may have therapeutic potential for treating GO pathogenesis by inhibiting ROS generation and adipogenesis.

In mammals, the proximal signaling events that occur followed by UPR activation involve the release of ER-resident signaling molecules ATF6, IRE1, and PERK from binding to BiP, the ER-resident chaperone, which results in their subsequent activation (Cullinan & Diehl 2006, Hotamisligil 2010). ER stress induction is communicated between stressed cells and other cells in the tissue or organism, such as immune cells. Communication controlled by ER stress triggers inflammation to control tissue damage and aids tissue repair. Under certain conditions, however, ER stress-induced inflammation is related to the exacerbation of disease conditions, which are demonstrated in diabetes, obesity, atherosclerosis, and cancer (Garg et al. 2012).

Immune responses are adversely affected by UPR abnormalities, indicating that UPR dysregulation contributes to the occurrence of autoimmune diseases (Todd et al. 2008, Garg et al. 2012). Proteomic analysis with
thyroid tissue from patients with Graves' disease revealed that BiP was overexpressed, indicating that ER stress may be involved in Graves' disease pathogenesis. Moreover, recent research demonstrated that BiP was expressed at higher levels in orbital tissues from individuals with GO (versus non-GO control subjects) and that silencing BiP curtailed GO-related pathologic responses, namely, inflammatory cytokine production, ROS generation,
and adipogenesis (Ko et al. 2021). Additionally, suppressing protein tyrosine phosphatase 1B, a negative regulator of insulin signaling located on the ER membrane, decreased inflammatory cytokine production, ROS generation, and fibrosis, likely through the reduction of ER stress in GO orbital fibroblasts, indicating that ER stress and UPR are related to the development of GO (Byeon et al. 2020).

The PERK pathway is one of the three major components in UPR, and it is the only pathway that modulates protein synthesis as an adaptive reaction (Bell et al. 2016). Under ER-stress conditions, PERK phosphorylates eIF2α, which reduces protein translation in a generalized manner, reducing the burden of unfolded proteins of the ER (Harding et al. 1999, Zhang 2010). Under chronic or severe ER stress, however, PERK-mediated phosphorylation of eIF2α induces a pathologic response. In mammals, phosphorylated eIF2α can mediate ATF4 translation, which induces the expression of the proapoptotic factor CHOP, thereby subsequently resulting in ER stress-induced apoptosis (Dandekar et al. 2015). Here, we showed that PERK, ATF4, and CHOP were expressed at higher levels in orbital tissues from individuals with GO than in healthy control subjects, indicating that orbital tissues in individuals with GO are under conditions of chronic or severe ER stress.

Protein maturation and disulfide bond formation of proteins in the ER are oxygen-dependent; therefore, the lumen of the ER is highly oxidative where low molecular-weight oxidants are produced and accumulated (Margittai & Banhegyi 2010). ER stress results in ROS accumulation, which promotes oxidative stress. PERK signaling, via activation of the ATF4 transcription factor, coordinates the cross-talk of ER stress with oxidative stress signaling (Cullinan & Diehl 2006). ATF4, which was induced during chronic or severe ER stress via the PERK pathway, is the major transcriptional regulator of the

Figure 4
Effect of PERK silencing on adipogenesis in GO orbital fibroblasts. (A) Adipocyte differentiation was induced for 10 days in siPERK-transfected orbital fibroblasts from individuals with GO. Orbital fibroblasts transfected with siPERK showed attenuated adipocyte differentiation compared with siCon transfectants, as demonstrated through microscopic examination (40×) after Oil Red O staining. The optical density at 490 nm of stained cell lysates also provided quantitative evidence that PERK silencing inhibited lipid droplet accumulation. (B) Western blotting was performed to analyze the expression of C/EBPα, C/EBPβ, and PPARγ after adipocyte differentiation. The experiments were performed in duplicate with cells from three patients. The expression of C/EBPα, C/EBPβ, and PPARγ was demonstrated through densitometry and normalized against the expression of β-actin in the same samples. The results are presented as the mean relative density ratio (%) ± s.d. of three independent experiments (*P < 0.05 and **P < 0.01 vs siCon transfectants). A full color version of this figure is available at https://doi.org/10.1530/JME-21-0057.
Figure 5
Effect of PERK silencing on cell viability and apoptosis. Orbital fibroblasts from individuals with or without GO were transfected with siCon or siPERK for 24 h. After the transfection, cells were incubated for 24 h and 48 h with or without IL-1β (10 ng/mL). Then an Annexin V/FITC Kit was used to determine the degree of apoptosis by detecting phosphatidylserine. The ratio of cells stained with annexin V was analyzed using flow cytometry. Assays were performed using cells from three individuals each, with or without GO; data from a representative experiment are shown. A full color version of this figure is available at https://doi.org/10.1530/JME-21-0057.
hypoxia-related stress response to UPR (Rzymski et al. 2009). In neurons, ATF4 serves as a redox-regulated, death-leading transcriptional activator that propagates the death responses to oxidative stress \textit{in vitro} and those to stroke \textit{in vivo} (Lange et al. 2008). Systemic hyperthyroidism and T cell infiltration into orbital tissues induce oxidative stress conditions in orbit, and oxidative stress from cigarette smoking is a well-known aggravator of GO (Venditti & Di Meo 2006, Marcocci et al. 2012, Lanzolla et al. 2020b). Moreover, based on the role of oxidative stress in the pathogenesis of GO, a therapy with antioxidant agents has been proposed (Lanzolla et al. 2020a). Here, we showed that PERK and ATF4 expression levels were increased in GO orbital tissues and that PERK silencing decreased ATF4 expression levels and curtailed CSE- and H$_2$O$_2$-induced ROS generation in GO orbital fibroblasts.

Chronic activation of ER stress is intimately related to lipid metabolism dysregulation in several metabolically significant cells, including adipocytes (Zha & Zhou 2012). The ER chaperone protein BiP has been detected in lipid droplets isolated from adipocytes, supporting its ER origin (Prattes et al. 2000). Multiple lines of evidence suggest that ATF4 positively regulates adipocyte differentiation. ATF4 overexpression in 3T3-L1 cells enhanced adipogenesis, whereas ATF4 siRNA blocked the differentiation of preadipocytes to adipocytes, and ATF4 depletion decreased adipocyte differentiation in human mesenchymal stem cells (Yu et al. 2014, Cohen et al. 2015). Here, we demonstrated that PERK and ATF4 were expressed at higher levels in orbital tissues from individuals with GO than in control and that PERK knockdown curtailed ATF4 expression and adipogenesis in GO orbital fibroblasts.

Although the main role of UPR in ER stress is to help stressed cells adapt to – and survive under – ER-stress conditions, apoptosis signaling pathways are activated if severe ER stress conditions persist, mainly via CHOP induction and subsequent activation of JNK kinase and caspase-12 (Oyadomari & Mori 2004). Although CHOP expression was exaggerated in orbital tissues from individuals with GO and PERK silencing-dependent CHOP downregulation was noted in this study, the relationship between the PERK pathway and apoptosis was not demonstrated using our \textit{in vitro} model of GO, owing to the resistance of orbital fibroblasts to cell death.

ER stress has been indicated as a crucial contributor to inflammation, and downregulation of NF-κB and proinflammatory cytokines by BiP silencing was observed previously (Ko et al. 2021). However, the PERK pathway can activate and inhibit NF-κB in different cellular contexts. PERK phosphorylates eIF2α, which suppresses...
the translation of IkB, which subsequently frees NF-κB for nuclear translocation. However, PERK also exerts immunomodulatory functions via CHOP, which suppresses NF-κB activation (Garg et al. 2012). In the present study, NF-κB was not affected by PERK silencing; this could be attributed to the dual role of PERK related to inflammation. The association between ER stress and inflammation in GO pathophysiology observed in previous studies is thought to involve pathways other than PERK.

In summary, this study shows that PERK silencing decreased oxidative stress and adipogenesis in an in vitro model of GO, indicating that PERK-mediated UPR pathway activation is associated with the pathogenesis of GO. Although further studies are warranted to demonstrate the in vivo effects of PERK inhibitors in GO before they can be applied clinically, this study offers in vitro background evidence, suggesting that the PERK pathway is a potential therapeutic target for GO management.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data availability
The datasets generated during and/or analyzed during this study are not publicly available due to the IRB recommendation not to send patient data into the public domain but are available from the corresponding author upon reasonable request.

Author contribution statement
JaeSang Ko was involved in conceptualization, funding acquisition, project administration, resources, validation, visualization, writing – original draft, writing – review and editing and supervision; Jin-Young Kim and Min Kyoung Chae were involved in data curation, formal analysis, investigation, methodology and software; Eun Jig Lee was involved in conceptualization and supervision; Jin Sook Yoon was involved in conceptualization, data curation, resources, methodology, supervision and writing – review and editing.

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