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The CXCR3/PLC/IP3-IP3R axis is responsible for the ignition of UPR in intestinal epithelial cells exposed to gliadin peptide, during the onset of celiac disease

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Abstract

Background Coeliac disease is an autoimmune disease that is primarily associated with chronic inflammation of the gut, but can also affect organs outside the gut, from the liver to the skin and CNS. The disease is triggered in predisposed individuals by a peptide mixture (PT) derived from the digestion of gliadin, a component of wheat, which is ingested with food. Although the induction of endoplasmic reticulum stress in intestinal epithelial cells (IECs) upon exposure to PT is known, the underlying molecular mechanisms remain unclear. Identifying the key players in this signaling pathway could therefore help to develop a new effective therapeutic strategy for the treatment of CD patients.

Methods Two CD models were used to identify the molecular mechanism linking extracellular PT and endoplasmic reticulum (ER) stress in the IECs of predisposed individuals exposed to gliadin. These models were an in vitro model based on CaCo-2 cells and an ex vivo model based on our previously described gut ex vivo system (GEVS), both exposed to PT.

Results Our results clearly show that the interaction of gliadin peptides with the transmembrane CXCR3 receptor on IECs leads to a rapid induction of PLC activity that generates IP3 molecules. This second messenger binds to the IP3R located in ER membranes, resulting in calcium efflux from the organelle.

Conclusion The PT-dependent ER stress observed in the IECs of CD patients results from the excessive release of calcium from the ER. Importantly, inhibition of this signaling pathway abrogates ER stress, which in turn attenuates downstream signs of CD, such as TG2 expression and gut permeability dysregulation, as well as inhibits inflammation.

Keywords CD, UPR, CXCR3, PKC, IP3R, IEC

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Introduction

Gliadin peptides (PT), a component of gluten, are known to cause inflammatory responses in the intestinal epithelium, particularly in individuals with celiac disease (CD) [1]. Given the autoimmune nature of the condition, the involvement of the immune system in the pathogenesis of CD has been extensively established [2]. However, the role of intestinal epithelial cells (IECs), which are the body's primary immunological barrier and serve as the primary interface between the body's tissues and the contents of the intestinal lumen—including the microbiota, metabolites, vitamins, and digested or partially digested foods such as gliadin peptides—remains unclear.

Gliadin peptides can bind to the chemokine receptor CXCR3 on intestinal epithelial cells (IECs), triggering a series of intracellular events that result in the disassembly of tight junctions in the intestinal epithelium [3]. This disruption increases intestinal permeability, allowing gliadin and other antigens to pass through the barrier, interact with the immune system, and provoke an inflammatory response. Notably, zonulin levels are significantly elevated in patients with CD during the acute phase and decrease when following a gluten-free diet [3]. However, this phenomenon alone cannot fully account for the complex ignition of CD pathogenesis.

Recent studies have shown that PT exposure of IECs leads to the release of calcium ions from the endoplasmic reticulum (ER), although the exact molecular mechanism remains unclear [4]. Calcium release from the ER is a crucial signaling event that regulates various cellular processes, including the unfolded protein response (UPR) and ER stress. Under ER stress conditions, the accumulation of unfolded proteins in the ER lumen can lead to a passive, progressive release of calcium, which, in turn, exacerbates the stress state [5]. However, persistent, or excessive active calcium release from the ER can also initiate the ER stress signaling pathways setting in motion a vicious cycle of inflammation and cellular dysfunction [6]. Although the molecular basis responsible for passive release, due to impaired ER homeostasis, remains elusive and has been termed '*ER* Ca^{2+}*leak*' [6–8], active release is better characterized and is mainly triggered by ryanodine sensitive receptors (RyRs) or inositol-1,4,5-trisphosphate (IP_3)-sensitive ligand-gated channels known as InsP3 receptors (InsP3Rs or IP_3Rs), which require a specific upstream signaling [4, 6, 9].

Therefore, understanding how extracellular gliadin peptides can trigger the ER stress response in IECs of CD patients and the role of calcium in this process may provide the opportunity to unravel another step in the complex scenario of CD pathogenesis.

It is worth noting that, CD not only affects the gut but, but is also a systemic disease that can damage any organ. It is a complex genetic disorder, with HLA status being the most important genetic factor determining the risk of celiac autoimmunity [1]. Currently, there is no effective therapy available to treat celiac disease, and the only measure is a lifelong gluten-free diet. However, even when gluten is completely removed, full remission is not always achieved [10].

In this study, we investigated the molecular mechanism by which PT triggers the ER stress response in IECs and the role of CXCR3.

Results

Gliadin peptides (PT) actively stimulate Ca^{2+} release from ER, through the PLC/IP3/IP3R axis

It has previously been reported that that IECs exposed to PT release calcium from the ER compartment although the mechanism is still unclear. Importantly, although a progressive passive release of calcium from the ER may be a consequence of the accumulation of unfolded proteins in the lumen of the organelle under ER stress conditions [4], sustained calcium release from the ER compartment also causes ER stress [5, 6, 11]. The latter condition may in fact be the result of activation of Phospholipase C (PLC), which produces IP3, which in turn binds and opens the inositol 1,4,5-trisphosphate (IP3)-sensitive ligand-gated channels (Ca^{2+} channels) known as InsP3 receptors (InsP3R or IP3R) [4, 6, 9]. To test whether the PT-stimulated increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is the result of IP3-mediated active release from the ER, we inhibited the activity of PLC with U73122. To this end, $[Ca^{2+}]_{cyt}$ was measured in CaCo-2 cells in response to acute exposure to PT (1 mg/ml) alone or in combination with U73122 (10 μM; 1 h pretreatment), while the expression of ER stress markers was determined by qPCR 9 h after PT stimulation [12]. Exposure to PT alone induced rapid and sustained increase in $[Ca^{2+}]_{cyt}$ (Fig. 1A) followed by upregulation of ER stress markers ATF4, ATF6 and XBP1s (Fig. 1B) together with downstream TG2 (Fig. 1C). Interestingly, inhibition of PLC completely abrogated PT-stimulated increase in $[Ca^{2+}]_{cyt}$ (Fig. 1A), upregulation of ER stress markers (Fig. 1B) and TG2 mRNA (Fig. 1C). To verify the involvement of this signaling pathway also in vivo, we performed the same experiment with the small intestine of GF mice cultured in GEVS and stimulated for 16 h with PT (2.5 mg/mL) alone or in combination with U73122 (20 μM; 2 h pretreatment). The data presented in Fig. 1D-E show that inhibition of the PLC/IP3/IP3R signaling pathway completely prevented the induction of ER stress (Fig. 1D) and subsequent TG2 upregulation (Fig. 1E).

These data therefore, suggest that PT-stimulated active calcium release from the ER via the PLC/IP3/IP3R signaling pathway is responsible for the induction of ER stress and not a consequence of ER stress induction.

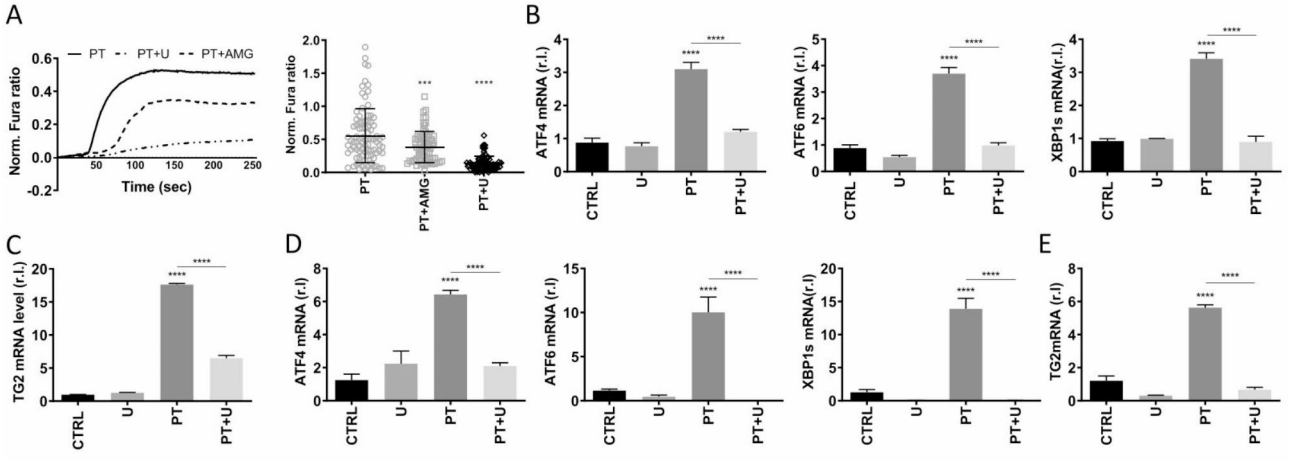

Fig. 1 The PLC/IP3/IP3R axis stimulated by PT induces ER stress in IEC. CaCo-2 cells were loaded with Fura-2/AM (2.5 μM) and exposed to 1 mg/ml PT alone (PT) or in combination with AMG 487 (1 μM; PT + AMG) or U73122 (10 μM; PT + U) and Fura-2 fluorescence was recorded and expressed as Fura-2 ratio (**A**). Data are expressed as mean of peak ± SD. Cells were unexposed or exposed to PT (1 mg/mL; 9 h) alone or in combination with U73122 (U, 10 μM; 1 h pretreatment), and the expression of ATF4, ATF6, XBP1s (**B**), and TG2 (**C**) was evaluated by qPCR. S.i. from GF mice were cultivated in the GEVS and untreated or treated with PT (5 mg/mL) alone or in combination with U73122 (U, 20 μM; 2 h pretreatment) and the expression of ATF4, ATF6, XBP1s (**D**), and TG2 (**E**) was evaluated by qPCR

PT activates PLC through transmembrane CXCR3

Next, we asked how extracellular gliadin peptides activate intracellular PLC. Data published in the literature suggest that the plasma membrane protein CXCR3 could be a potential gliadin peptide receptor [13]. Interestingly, CXCR3 is a G protein-coupled receptor (GPCR) that can stimulate the activation of PLC via a G protein [14]. To test this hypothesis, we first detected the expression of the receptor in CaCo-2 cells as well as in mouse intestinal tissue by Western blotting analysis (Fig. 2A-B). We then exposed CaCo-2 cells to PT (1 mg/mL) in the presence or absence of the CXCR3 antagonist AMG 487 (AMG, 1 μM; 8 h pretreatment) and examined the appearance of Ca^{2+} signaling after acute stimulation, and the subsequent expression of ER stress markers 9 h after the treatment. The data presented in Figs. 1A and 2 show that inhibition of CXCR3 significantly suppressed the PT-dependent increase in cytosolic calcium concentration (Fig. 1A) and completely prevented ER stress (Fig. 2C), along with downstream upregulation of TG2 and downregulation of CFTR (Suppl.S1). These results were also confirmed in vivo, by performing the same experiment in the small intestine of GF mice cultured in GEVS and stimulated for 8 h with PT alone or in combination with AMG 487 (AMG 1 μM, 16 h pretreatment). Analysis of the expression of ATF4, ATF6 and XBP1s by qPCR showed that inhibition of CXCR3 completely prevented the induction of ER stress by PT exposure (Fig. 2D). Moreover, as in the cell model, AMG also prevented PT-stimulated upregulation of TG2 and downregulation of CFTR (Suppl.S2). Finally, complete abrogation of PT-stimulated production/release of the pro-inflammatory cytokines IFNγ, IL17A and IL15 (Fig. 2E) and dysregulation of tissue permeability (Fig. 2F) were also observed under the same experimental conditions.

CXCR3 acts as gliadin peptides receptor onto IECs

To further validate the key role of CXCR3 as a PT receptor and to unleash the UPR, we used the Human Embryonic Kidney 293 (HEK) cells as a model, which are characterized by a negligible expression of CXCR3 at both mRNA and protein levels (Fig. 3A). We then exposed the cells to PT and assessed the expression of CXCR3 using the ER stress inducer thapsigargin (TG, 5 μg/ml) as a control. The data presented in Fig. 3B clearly show that the expression of CXCR3 is not increased in PT-exposed cells compared to TG-exposed cells. These results are consistent with those showing no ER stress induction PT-exposed HEK cells, whereas TG actively increases the expression of ER stress markers (Fig. 3C). Importantly, the inability of PT to induce an ER stress response in HEK cells also abrogated the downstream dysregulation of tight junction proteins such as CDL-2 (Suppl.S3).

Next, we stably overexpressed CXCR3 in HEK cells (Fig. 3D-E) and examined the induction of ER stress in cells exposed to PT. As shown in Fig. 3F, there was a PT-dependent increased expression of the indicated ER stress markers in HEK cells expressing high levels of CXCR3 compared to parental cells (Fig. 3F). Of note, the expression of (endogenous) CXCR3 was also increased in HEK^{CXCR3} cells in the presence of PT (compared to parental HEK, in the same experimental conditions), which is attributable to the induction of ER stress (Fig. 3G).

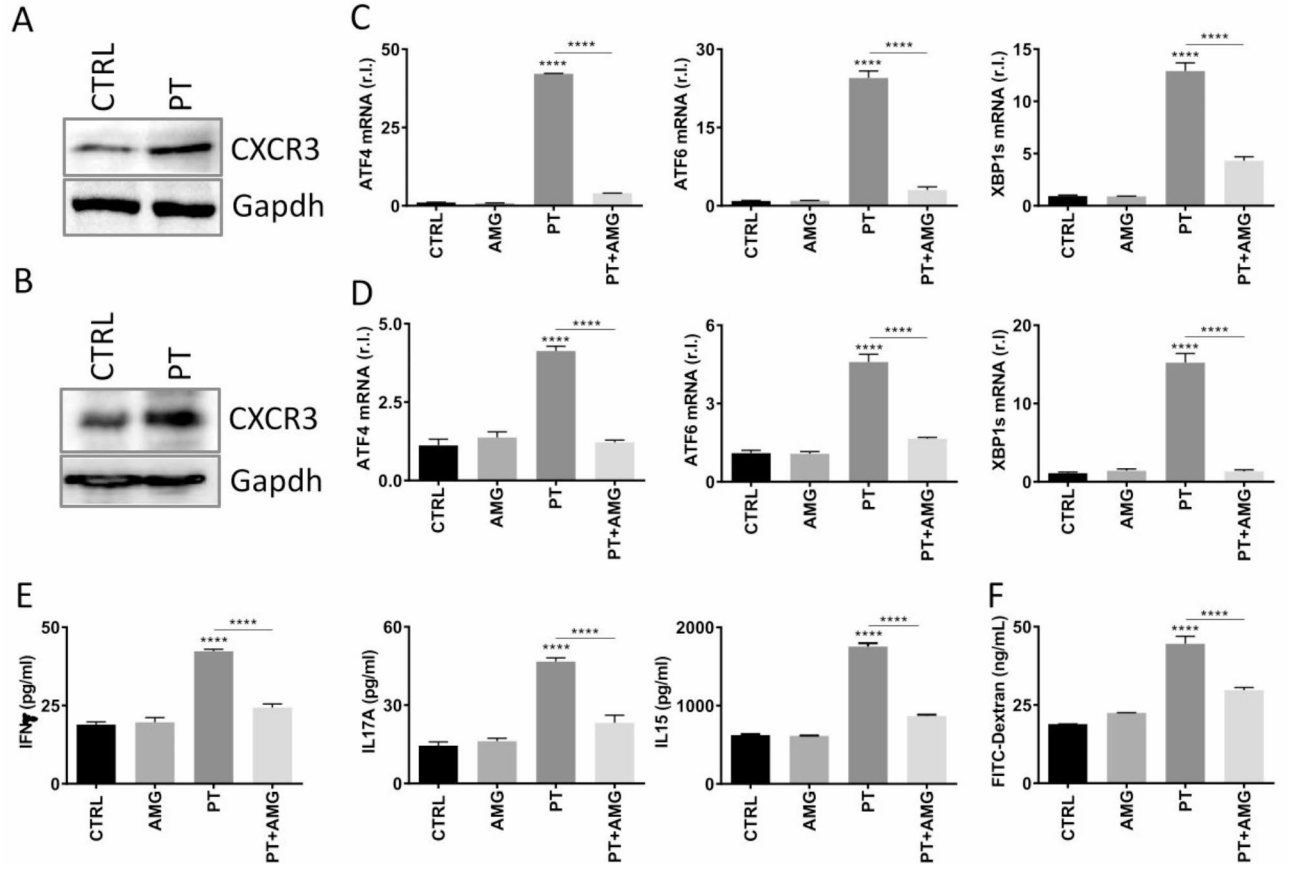

Fig. 2 CXCR3 is stimulated by PT and induces ER stress in IEC. The expression of CXCR3 was evaluated in both CaCo-2 cells (**A**) or s.i. (**B**) unexposed or exposed to PT (1 mg/mL, 9 h, in CaCo-2; 5 mg/mL, 16 h, in GEVS) by western blotting analysis. Cells were unexposed or exposed to PT (1 mg/mL; 9 h) alone or in combination with AMG 487 (AMG, 1 μM; 8 h pretreatment), and the expression of indicated ER stress markers was evaluated by qPCR (**C**). S.i. from GF mice were cultivated in the GEVS and untreated or treated with PT (5 mg/mL) alone or in combination with AMG 487 (AMG, 1 μM; 16 h pretreatment) and the expression of ATF4, ATF6, XBP1s was evaluated by qPCR (**D**). The production of pro-inflammatory cytokines was evaluated by ELISA (**E**), while tissue permeability was evaluated by using the FITC-Dextran protocol (**F**), in the same experimental conditions

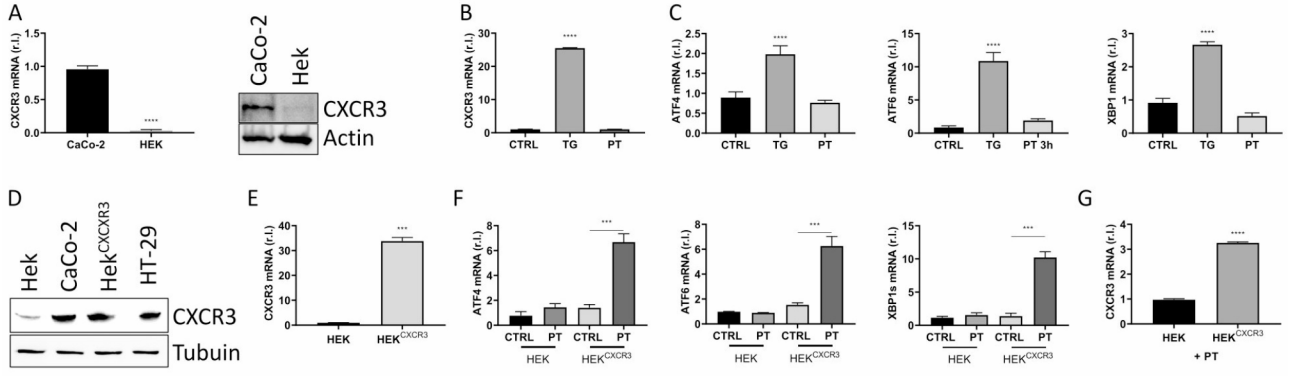

Fig. 3 CXCR3 is strictly required to induce a PT-dependent ER Stress. Basal expression of CXCR3 was evaluated in both CaCo-2 and HEK 293 cells by qPCR (**A**, left panel) or western blotting (**A**, right panel) analysis. HEK cells were unexposed or exposed to TG (10 μg/ml, 3 h) or PT (1 mg/ml, 3–6 h), and the expression of CXCR3 (**B**), or indicated ER stress markers (**C**) was evaluated by qPCR. CXCR3 was stably overexpressed in HEK cells and both protein (**D**) and mRNA (**E**) levels were evaluated by western blotting or qPCR, respectively. Parental (HEK) and CXCR3 overexpressing (HEK^{CXCR3}) HEK cells were unexposed or exposed to PT (1 mg/ml, 3 h), and the expression of indicated ER stress markers was than evaluated by qPCR (**F**). Endogenous CXCR3 was evaluated in both parental and HEK^{CXCR3} exposed to PT (1 mg/ml, 3 h), by qPCR (**G**)

Collectively, these data suggest that the CXCR3/PLC/IP3/IP3R signaling pathway is stimulated by gliadin peptides to induce ER stress and down-stream deregulation of tissue permeability, TG2 and CFTR expression, and pro-inflammatory cytokine production.

Gadd153 enhances the expression of CXCR3

As shown in Fig. 2, increased levels of CXCR3 protein were observed in both Caco-2 cells and small intestine exposed to gliadin peptides (Fig. 2A-B), suggesting a possible positive amplification loop. Interestingly, a possible role of the UPR in regulating CXCR3 expression has been previously suggested [15], although the exact molecular mechanism is still unclear. In addition, when we analyzed the promoter region of the *cxcr3* gene, we found four potential responsive elements (RE) for the C/EBPα TF, which is known to be involved in the regulation of CXCR3 gene expression [16]. Interestingly, we also found a potential RE for other members of the C/EBP family of TFs, which includes Gadd153/CHOP, a downstream TF that is activated under ER stress conditions and is part of the UPR program [17]. To test whether PT-stimulated ER stress is also responsible for the enhanced expression of CXCR3, we exposed both CaCo-2 cells and small intestine of GF mice to PT alone or in combination with the chemical chaperone 4PBA [18–21] and examined the expression of CXCR3 by qPCR. As shown in Fig. 4, inhibition of the UPR completely prevented the PT-dependent increased expression of the receptor (Fig. 4A-B). Next, we examined the expression of Gadd153 in both the in vitro and in vivo models exposed to PT to evaluate the possible involvement of this TF. Our data clearly

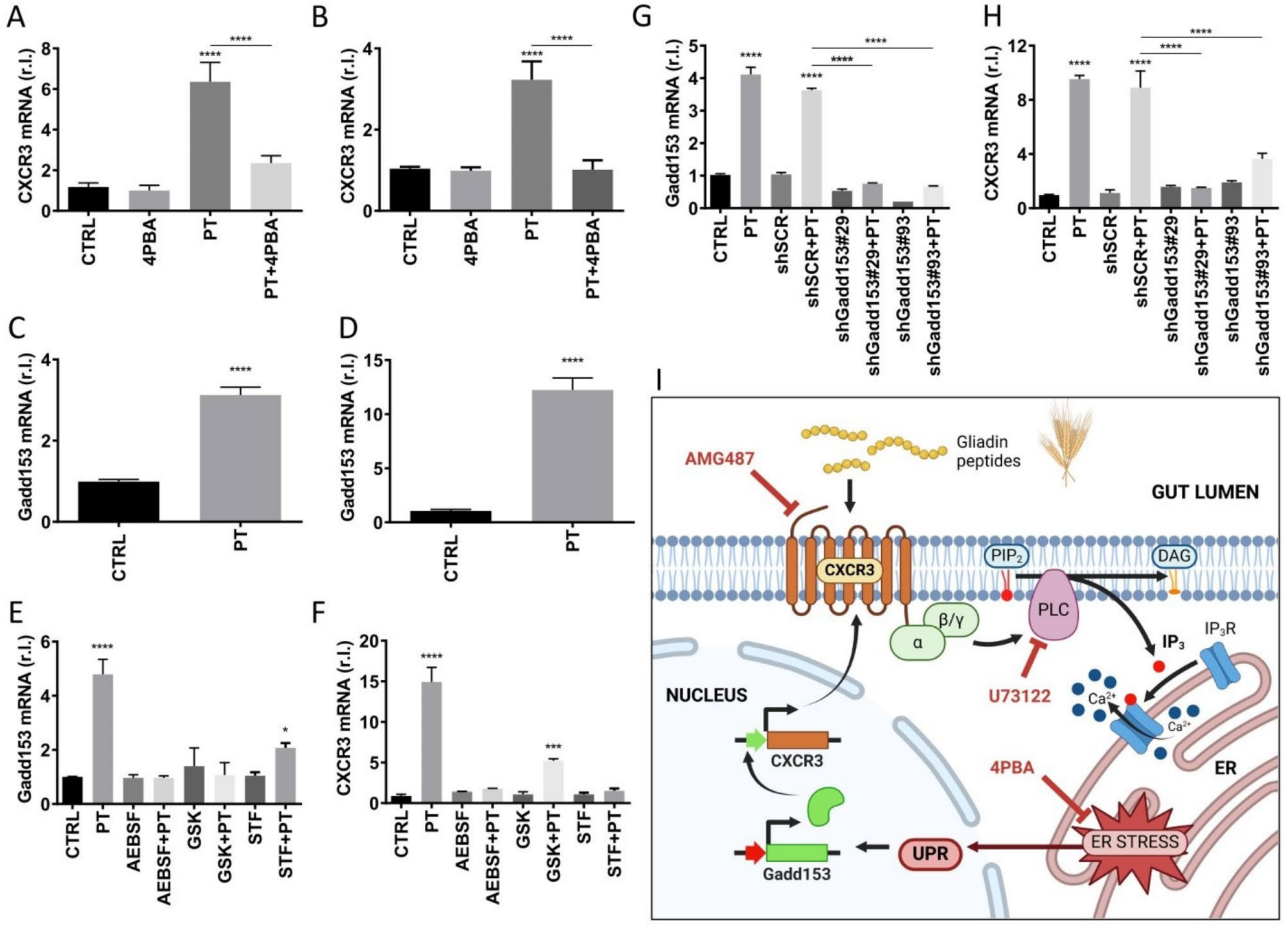

Fig. 4 CXCR3 is required to deregulate TJ proteins expression. Gadd153 enhances the expression of CXCR3, under PT stimulation. The expression of CXCR3 was evaluated in both CaCo-2 cells (**A**) or s.i. (**B**) unexposed (CTRL) or exposed to PT (1 mg/mL, 9 h, in CaCo-2; 5 mg/mL, 16 h, in GEVS) alone or in combination with 4PBA (3 M and 5 M, respectively) by qPCR. The expression of Gadd153 was evaluated in both CaCo-2 cells (**C**) or s.i. (**D**) unexposed (CTRL) or exposed to PT (1 mg/mL, 9 h, in CaCo-2; 5 mg/mL, 16 h, in GEVS) by qPCR. CaCo-2 cells were unexposed (CTRL) or exposed 9 h to PT (1 mg/mL), AEBSF (500 M), GSK2606414 (GSK, 5 M), or STF-083010 (STF, 60 M) alone or in combination, and the expression of Gadd153 (**E**), and CXCR3 (**F**) was evaluated by qPCR. CaCo-2 cells were transfected with vectors carrying two shRNA specific sequences (shGadd153#29; shGadd153#93; using a scrambled sequence as a control, shCR), and the expression of both Gadd153 (**G**) and CXCR3 (**H**) was evaluated by qPCR, in cells unexposed (CTRL) or exposed to PT (1 mg/mL, 9 h). A schematic representation of the mechanism by which gliadin peptides regulate the expression of CXCR3 is shown (I). Data are representative of three independent experiments performed in triplicate. Histograms represent mean ± s.d.; ****$p < 0.0001$ (vs. control); ••••$p < 0.0001$

indicate that Gadd153 expression is induced immediately after PT exposure (Fig. 4C-D).

Although it is generally accepted that Gadd153 is a downstream TF of the UPR program, there is still debate as to whether all three major TFs ATF4, ATF6 and XBP1s are equally required for its transcriptional regulation. To clarify this point, we inhibited the activity of each of the three branches of the UPR individually and examined the expression of Gadd153 after PT exposure. The data presented in Fig. 4E clearly show that the activity of the three axes is required for the regulation of Gadd153 expression. Interestingly, we found that PT-dependent upregulation of CXCR3 was also impaired under the same experimental conditions, further supporting a possible role of Gadd153 (Fig. 4F). Finally, expression of the latter TF was inhibited by transfection of CaCo-2 with vectors carrying two different shRNA-specific sequences (shGadd153#29; shGadd153#93; using a scrambled sequence as a control, shSCR), and expression of Gadd153 (Fig. 4G) and CXCR3 (Fig. 4H) was evaluated by qPCR in cells exposed to PT (1 mg/mL, 9 h) or not (CTRL). Our data confirm a complete abrogation of CXCR3 expression in cells lacking Gadd153 upon PT exposure.

Overall, these data show that gliadin exposure increases the expression of CXCR3 through ER stress-dependent increased expression of Gadd153 (Fig. 4I).

Discussion

The intestinal epithelium plays a crucial role in regulating the permeability of the gut and control the exchange of substances between the body and the external environment represented by the intestinal lumen [1]. Tight junctions between the components of this epithelium, especially the IECs, form a barrier that prevents the uncontrolled passage of water, solutes, and microorganisms [22]. However, certain pathological conditions disrupt the function of the intestinal barrier, often due to impaired tight junction (TJ) function/expression and/or death of IECs. This disruption can lead to tissue inflammation and trigger activation of the immune system. This includes chronic gut inflammation as occurs in diseases such as ulcerative colitis, Crohn's disease, and celiac disease [23]. In these autoimmune diseases, inappropriate activation of the immune system contributes to pathogenesis, and exacerbate tissue damage. Although the gut is the primary site affected, these diseases also manifest extra-intestinally. For example, CD can lead to complications ranging from skin and endocrine disorders to liver dysfunction and neuropsychiatric symptoms [24].

While the role of the immune system in the pathogenesis of CD is well-documented, the specific contribution of the intestinal epithelium is less clear. New evidence suggests that this tissue is not only the primary site of damage but may also play an active role in the initiation of the disease [25]. The exact mechanisms by which initial tissue inflammation triggers inappropriate activation of the immune system, leading to the onset of a self-perpetuating autoimmune syndrome, are not yet fully understood. The specific genetic HLA determinants (DQ2 and DQ8), which have a very high affinity for gliadin peptides and TG2-gliadin epitopes, while undeniably important, are not sufficient to fully explain the autoimmune features of celiac disease [25]. Therefore, the early events in the disease's pathogenesis, such as the interaction between the pathophysiological triggers and the intestinal epithelial cells, seem to play a potentially crucial role.

The study presented here investigates into the molecular mechanisms by which gliadin peptides contribute to the pathogenesis of CD by inducing endoplasmic reticulum (ER) stress in intestinal epithelial cells. We gained new insights into the role of the chemokine receptor CXCR3, its activation by PT, and the downstream signaling events that lead to cellular dysfunction, tissue inflammation, and disrupted intestinal permeability. Indeed, we identified the PLC/IP3/IP3R signaling pathway as a crucial mediator of calcium release from the ER, which subsequently induces ER stress in IECs. The results demonstrate that PT exposure leads to the activation of phospholipase C (PLC), which generates inositol 1,4,5-trisphosphate (IP3). Binding of IP3 to its receptor, IP3R, triggers the release of calcium ions from the ER into the cytosol, a process that contributes to the accumulation of unfolded proteins and activation of the unfolded protein response (UPR) [5]. Remarkably, inhibition of PLC prevents both the PT-induced calcium release and the subsequent induction of ER stress markers and TG2. These findings highlight the active role of the PLC/IP3/IP3R signaling pathway in triggering ER stress, and underscore its central role in PT-induced cellular dysfunction which is not only a consequence of stress.

CXCR3, a G protein-coupled receptor (GPCR), has been shown to be activated by PT, leading to activation of PLC and initiation of the calcium signaling cascade. Inhibition of CXCR3 effectively blocked PT-induced calcium signaling, ER stress, and upregulation of pro-inflammatory cytokines. These results emphasize the importance of CXCR3 in mediating the inflammatory and pathological effects of gliadin in CD. In addition, e*x vivo* experiments using gut tissues from a CD mouse model confirmed that inhibition of CXCR3 prevents PT-induced changes in ER stress, tight junction protein expression, and cytokine production, further confirming the receptor's role in the disease process.

Moreover, gliadin peptides not only induce ER stress but also enhance the expression of CXCR3 itself. This positive feedback loop involves the activation of Gadd153/CHOP, a transcription factor that is

upregulated during ER stress. PT-induced ER stress leads to increased Gadd153 expression, which, in turn, promotes the expression of CXCR3 on the cell surface. This increase in CXCR3 expression by the UPR signaling pathway is important as it suggests that CXCR3 may not only act as a receptor for gliadin but also be a regulated factor in the inflammatory response. The findings that inhibition of the UPR or silencing of Gadd153 expression prevents both CXCR3 upregulation and the subsequent ER stress response underscore the central role of this feedback mechanism in the pathogenesis of CD.

The fact that PT does not induce ER stress in cells that do not express CXCR3 is crucial evidence that the ER stress response is dependent on the presence of CXCR3. Thus, ectopic expression of CXCR3 efficiently reproduces the PT-induced ER stress response, confirming CXCR3 as a necessary component of the signaling pathway leading to cellular dysfunction. The ability of PT to trigger a pro-inflammatory response in IECs but not in cells lacking CXCR3 underscore the specificity of this signaling pathway in the context of CD.

Conclusion

These findings have important implications for understanding the pathophysiology of celiac disease. The identification of the CXCR3/PLC/IP3/IP3R calcium signaling axis as a critical mediator of ER stress provides new insights into the molecular processes that contribute to the inflammation, intestinal permeability, and autoimmune responses characteristic of CD. By clarifying the role of CXCR3 and calcium signaling in the disease process, this study opens new avenues for potential therapeutic strategies. Interfering with the PLC/IP3/IP3R signaling pathway could be a means to modulate inflammatory and ER stress responses in CD patients, and provide an alternative to the current gluten-free diet.

In summary, the research presented here provides a comprehensive understanding of how gliadin peptides trigger ER stress and inflammatory responses in IECs via the CXCR3/PLC/IP3/IP3R signaling pathway (Fig. 5). The results highlight the complex interplay of receptor signaling, calcium dynamics, and UPR in driving the pathogenesis of celiac disease. Future studies will be required to explore the broader implications of these findings, including the potential for therapeutic interventions targeting the PLC/IP3/IP3R or ER stress signaling pathway to treat celiac disease and related autoimmune disorders.

Materials and methods

Cells

CaCo-2 and HEK293T cells were grown in DMEM (Merck) plus 10% FBS (EuroClone), 2 mM glutamine (Merck), and 1% penicillin/streptomycin (Merck). Cells were treated as reported in Supplementary Table 1 [26].

Gut-ex-vivo system (GEVS)

The small intestines (s.i.) from 13-day-old Balb/c mice, which had been fed a gluten-free diet for at least three generations [1, 19, 20], were freshly resected and cultured in a silicone-based Gut-Ex-Vivo System (GEVS), as previously described [21–24]. All procedures were approved by the local Ethics Committee for Animal Welfare (DB064.N.TMC) and adhered to European Community regulations for animal use in research (2010/63/EU).

Tissue cultures and treatments

Each intestine was infused with serum-free tissue culture medium (IMDM), supplemented with 20% KnockOut serum replacement (Gibco), 2% B-27, 1% N-2 supplements (Gibco), 1% L-glutamine, 1% NEAA (Gibco), 1% HEPES (EuroClone), and stimulated as outlined in Supplementary Table 2 [19].

Gliadin peptides (PT) preparation

Wheat gliadin (Sigma) was prepared as previously described and stored at -20 °C [27].

Quantitative PCR (qPCR)

Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific) [21]. cDNA was synthesized with the ExcelRT™ Reverse Transcription Kit (Smobio) using 2 µg of RNA, and fluorescently labeled PCR products were generated using the ExcelTaq™ 2XFastQ-PCR Master Mix (Smobio) [28]. Relative quantitation of gene expression was calculated using the accurate Ct (threshold cycle) method [29]. Supplementary Table 3 shows the primer pairs used in this work. Mouse GAPDH or human L34 were used as housekeeping genes [29].

Western blotting analysis

Tissues and cells were lysed using Cell Lytic buffer (Merck) supplemented with a protease inhibitor cocktail (Merck), 1 mM Na3VO4, and 10 mM NaF (Merck). Protein samples (20 µg each) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad) via electroblotting. The antibodies were diluted in 5% non-fat dry milk (Santa Cruz) in PBS with 0.1% Tween-20 and incubated overnight at 4 °C (see Supplementary Table 4) [21].

HEK293T cell transfection with CXCR3A

The sequence of isoform A of CXCR3 (NM_001504.2) was optimized (Eurofinsgenimic) and cloned in pcDNA3.1/Hygro(+) vector (XbaI/HindIII). 2.5 µg of plasmid was transfected into HEK293T, Lipofectamine®2000 (Invitrogen). Stable clones expressing CXCR3A were selected by 0.2 mg/mL Hygromycin (InvivoGen).

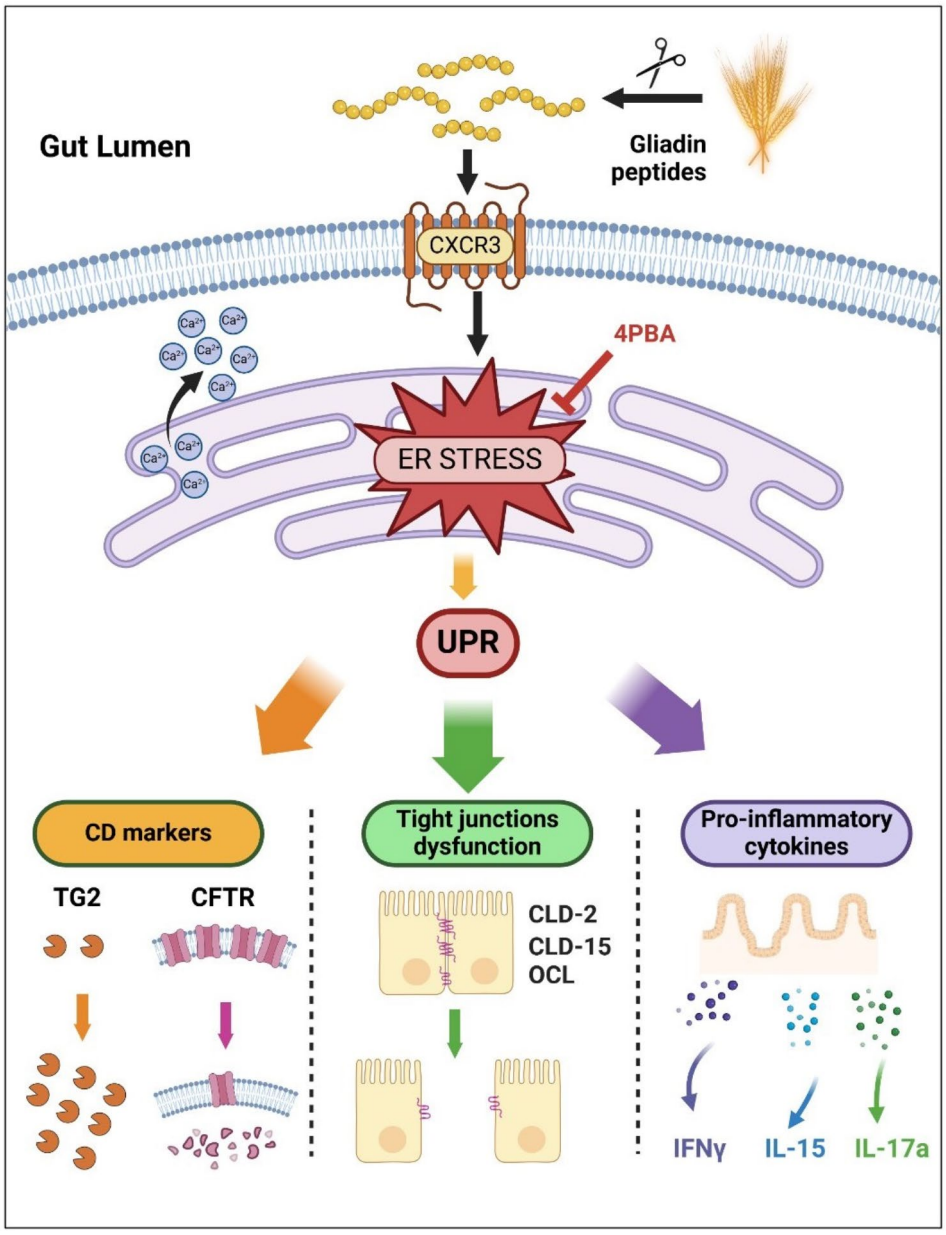

Fig. 5 The CXCR3/PLC/IP_3/IP_3R signaling pathway stimulated by PT. Schematic representation of the signaling pathway stimulated by the binding of gliadin peptides (PT) to the IEC membrane receptor CXCR3. CXCR3 is a GPCR that activates PLC enzymatic activity through the involvement of a G protein. Active PLC cleaves PIP_2 into DAG and IP_3. The latter binds to its specific receptor, the IP_3R calcium channel located in the ER membranes. This induces the efflux of calcium from the ER into the cytosol, altering the redox status of the organelle, which impairs its folding capacity and triggers the unfolded protein response (UPR). As a result, downstream effects include dysregulation of TG2 and CXCR3 gene expression, disruption of gut permeability, and the production of pro-inflammatory cytokines, which are key features of celiac disease (CD)

RNA interference (shRNA/siRNA)

1 µg of pLKO vecrtors (shGadd153#29, shGadd153#93, or shSCR – scrambled seq) were transfected into 25×10^4 cells/well, by using the jetPTIME reagent (Polyplus). qPCR was performed after 48 h post-transfection [20].

Tissue viability

Tissue viability was evaluated through AlamarBlue staining (Thermo Fisher Scientific Fisher), as previously described [22].

Tissue permeability

A 0.1 mg/mL solution of FITC-dextran (FITC D4000, Merck) was added to the infusion medium, and fluorescence was measured in 100 µL of the outer medium from

each sample using a TECAN (SPARK) instrument. The concentration of FITC-dextran was determined by referencing a standard curve (0–100 µg/mL).

ELISA

ELISA test was performed on tissue samples lysates using commercially available ELISA kits (R&D Systems) for mouse IL-15, IL-17 A, IFN-γ (R&D System), as previously described [22].

Calcium assay

CaCo-2 cells were seeded onto 24 mm round coverslips (3×10^4 cells), and loaded with 2.5 µM Fura-2/AM (Life Technologies) in the presence of 0.005% Pluronic F-127 (Life Technologies) and 10 µM sulfinpyrazone (Merck) in KRB solution (125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 2 mM $CaCl_2$, 20 mM HEPES, pH 7.4). After 30 min (in the dark at RT) cells were washed with KRB and allowed to de-esterify, 30 min. Next, coverslips were mounted on a Leica DMI6000B microscope (with an S Fluor 40×/1.3 objective and a Polychrome V monochromator (Till Photonics)). The cells were alternately excited at 340 and 380 nm and fluorescence were collected by a Hamamatsu cooled CCD camera (Hamamatsu Photonics) and recorded using MetaFluor software (Molecular Devices). For comparison of Ca^{2+} dynamics, measured as an amplitude of Ca^{2+} increase from the baseline level, Fura-2 ratio values were normalized using formula (Fi-F0)/F0 [referred to as Normalized (Norm.) Fura Ratio].

Statistical analysis

All experiments were performed in triplicate, repeated at least three times, and statistical analysis was performed using GraphPad_Prism_6. The student-t test or ANOVA was used to determine statistical significance. A p-value equal to or less than 0.05 was considered significant. Histograms represent mean ± SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (vs. controls); • $p < 0.05$; •• $p < 0.01$; ••• $p < 0.001$; •••• $p < 0.0001$.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13062-025-00633-y.

Supplementary Material 1

Supplementary Material 2

Acknowledgements

This study is not a clinical trial. Therefore, we now included: 'Clinical trial number: not applicable' in the manuscript.

Author contributions

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and comsent to participate

All procedures were approved by the local Ethics Committee for Animal Welfare (DB064.N.TMC) and conformed to the European Community regulations for animal use in research (2010/63 UE).

Competing interests

The authors declare no competing interests.

Clinical trial number

Not applicable.

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