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Prolamins of maize and wheat differentially affect intestinal cells both in biopsies of celiac patients and CACO-2 cell line

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ABSTRACT
We study the effect of maize zeins on cellular immune response as compared to that of wheat gliadins for exacerbating celiac disease due to a hypothetical similar response in some patients. *In vitro* activation of celiac duodenal mucosa with gliadin or zein immunoreactive peptides and pro-inflammatory response of CACO-2 cells to digested gliadin or zein fractions were evaluated as indicators of adaptive and innate response, respectively. In 3/5 biopsies, zein increased production of IFN-γ, whereas gliadin has done it in all the patient biopsies. In CACO-2 cells, two zein fractions (3–5 and 1–3 kDa), similar to gliadin fractions, stimulated the production of IL-8, p38 MAPK, COX2, and release of ZO-1 as compared to medium alone. The 3–5 kDa zein fraction increased permeability in cell monolayers, although less than gliadin. Zein peptides are immunogenic for some patients and induce a similar innate response, but to a lesser extent than gliadin peptides.

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celiac disease; cell immune response; maize prolamins; zeins

1. Introduction

Celiac disease (CD) is an autoimmune-mediated enteropathy in genetically predisposed individuals exposed to dietary wheat gluten (Ludvigsson et al., 2012). The poor digestion of gluten proteins by intestinal proteases originates several toxic and immunogenic peptides. Toxic peptides are capable of stimulating the innate immune response that precedes the abnormal adaptive immune response in CD. The internalization of toxic peptides intra or inter-enterocytes can trigger a series of events that lead to membrane disruption and cell apoptosis (Di Sabatino & Corazza, 2009). Then, the immunogenic peptides enter the lamina propria and are deamidated by the tissue transglutaminase (tTG), captured by dendritic cells and presented to gluten-sensitive Th1 cells in the context of human leukocyte antigen (HLA)-DQ2/8 (Green & Cellier, 2007).

A gluten-free diet is the only treatment for CD, where in addition to wheat, barley and rye must be avoided. One of the more common wheat substitutes is maize as a principal ingredient for different gluten-free foodstuffs. However, some unexpected responses to

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maize proteins have been found in some CD patients after oral challenge (Accomando, Albino, Montaperto, Amato, & Corsello, 2006); as humoral IgA immunoreactivity (Cabrera-Chávez, Rouzaud-Sánchez, Sotelo-Cruz, & Calderón de la Barca, 2008); and other *in vivo* and *in vitro* findings previously reviewed (Ortiz-Sánchez, Cabrera-Chávez, & Calderón de la Barca, 2013). Hypothetically, maize prolamins could be harmful for a very small group of CD patients, especially those that are nonresponsive, and if it is confirmed, they should follow a gluten-free diet that also excludes maize foodstuffs. The principal information about maize in CD comes from casual results by using its proteins as "negative" controls in studies of CD immune response. There are no experiments intended to directly prove the immune response to maize proteins in CD patients.

Because of the wide use of maize in gluten-free foods, it is important to study the effect of its prolamins (called zeins) on innate and adaptive immune response as compared to those elicited by wheat gliadins for exacerbating CD. To achieve this objective, duodenal bulb biopsies of CD patients were challenged with immunogenic zein and gliadin peptides. Also, CACO-2 cells were stimulated with fractionated pepsin–trypsin (PT)-digested gliadins and zeins in order to elucidate their toxic effect, by measuring their ability to affect the permeability and induce pro-inflammatory markers.

2. Materials and methods

2.1. *Patients*

Biopsies were taken from duodenal bulb of each five patients undergoing upper endoscopy at the Hospital Infantil del Estado de Sonora (HIES) in Hermosillo, Mexico. The study was approved by the ethical committee of the HIES and all samples were taken under a parent's informed written consent. Whole blood was also extracted from each patient for genomic DNA extraction using the QIAamp DNA Blood Mini Kit (QIAGEN, USA) and genotyping of HLA-DQ2/DQ8 was done by real-time Polimerase Chain Reaction (Step One Plus, Applied Biosystems) using specific primers (Olerup, Aldener, & Fogdell, 1993). Serum anti-gliadin IgG, anti-gliadin IgA and anti-tTG IgA antibodies were analyzed by a direct enzyme-linked immunosorbent assay (ELISA), as previously reported (Cabrera-Chávez, Rouzaud-Sánchez, Sotelo-Cruz, & Calderón de la Barca, 2009). Briefly, microplates were coated with 5 µg/mL gliadins or guinea pig liver transglutaminase (Sigma-Aldrich, St Louis, MO) in coating buffer overnight at 4°C. The plates were blocked for 1 h with 3% gelatin and then incubated for 4 h at 25°C with human serum samples in incubation buffer. After washings, plates were incubated for 1 h with horse radish peroxidase-conjugated anti-human IgA antibodies (DAKO, Carpinteria, CA), at 25°C. Peroxidase activity was developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma-Aldrich). Absorbance at 450 nm was read (Microplate reader, Bio-Rad, Hercules, CA).

2.2. *Peptide preparation*

The immunogenic peptides α-gliadin 33-mer (LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF; Molecular Weight (MW) = 3914.51 Da) (Shan et al., 2002), α-zein 34-mer (LQQAIAASNIPLSPLLFQQSPALSLVQSLVQTIR; MW = 3646.32 Da) (Cabrera-Chávez et al., 2012), and the toxic peptide α-gliadin p31–49 (LGQQQPFPPQQPYPQPQPF;

MW = 2222.51 Da) (Maiuri et al., 2003; Matysiak-Budnik et al., 2003), were supplied by United Biosystems (USA) with purities of 97.54%, 95.66% and 95.18%, respectively. The immunogenic peptides were used in the experiments at 1 mg/mL and the toxic peptide at 50 µg/mL, as it has been seen in previous work (Capozzi et al., 2013; Maiuri et al., 2003; Picarelli et al., 2010).

2.3. *Duodenal biopsies, tissue culture and cytokine assays*

Five duodenal bulb fragments were collected from each patient. Two fragments were used for routine histology and three were placed on culture plates and cultured in Dulbecco's Modified Eagle's Medium (D-MEM) containing 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL gentamicin (Gibco, USA) at 37°C in a 5% CO_2 atmosphere. One fragment was used for α-gliadin 33-mer challenge, other for α-zein 34-mer challenge, both peptides at a concentration of 1 mg/mL, and the third fragment left without challenge. After 24 h, supernatants were collected and frozen at −70°C prior to cytokine evaluation.

ELISA kits were used for IFN-γ (Mabtech) and IL-15 (eBioscience) detection according to manufacturers' recommendations. ZO-1 release in biopsies was determined by ELISA as above for anti-gliadin antibodies detection. Microplates were coated with 100 µL of cell supernatant diluted 1 : 5, after blocking, were incubated for 2 h with rabbit polyclonal anti-human ZO-1 antibodies (Abcam, Cambridge, UK) and detected by incubation for 1 h with goat anti-rabbit IgG-HRP (DAKO, Carpinteria, CA).

2.4. *Cell line culture*

CACO-2 cells [HTB-37™ American Type Culture Collection] were cultured in 9.5 cm^2 surface wells containing D-MEM with 10% FCS, 4 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL gentamicin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 atmosphere. The culture medium was replaced three times a week. Sub-culture was performed at 80% of confluence. All experiments were performed between passages 65 and 78. Monolayers (passages 22–35) were grown on 0.33 cm^2 permeable collagen-coated polytetrafluorethylene filters with 0.4 µm pore size (Corning, Lowell, MA) for 14–21 days until confluency, polarized and differentiated state.

2.5. *Gliadin and zein peptic–tryptic digestion*

Gliadins from wheat and zeins from maize (Sigma Chem Co, St Louis, MO, USA) were subjected to PT digestion, as previously described (Cabrera-Chávez et al., 2008) with some modifications. Briefly, gliadins or zeins were exposed to pepsin from porcine gastric mucosa (3200–4500 units/mg protein, Sigma-Aldrich, USA) at 37°C, pH 2.0 for 4 h, followed by trypsin from porcine pancreas (1000–1500 Benzoyl-L-arginine ethyl ester units/mg solid, Sigma-Aldrich, USA) at 37°C, pH 7.8 for 4 h. After reaction, the incubation was stopped at 80°C for 45 min and then sequentially fractionated in Millipore membranes with four different Molecular Weight Cut-Offs (MWCO: 30, 5, 3 and 1 kDa). Table 1 presents the molecular weights of digested gliadin or zein fractions. Protein fractions were filtered through 0.22 µm pore filter and $NaHCO_3$ was added to a

Table 1. Molecular weight range of protein fractions of PT-digested gliadins and zeins.

Prolamins	Fraction	Protein size (kDa)
Gliadins or zeins	1	≥30
	2	5–30
	3	3–5
	4	1–3

Note: kDa, kilodaltons.

final concentration of 44 mM. Protein concentration was determined by absorbance at 280 nm using a Nano-Drop 2000 UV-Vis Spectrophotometer (ThermoScientific, USA) and used at a concentration of 1 mg/mL for cell stimulation.

2.6. *Cytokine determination*

CACO-2 cells were stimulated separately with fractions 1, 3 and 4 of digested gliadins and zeins, with p31–49 or left untreated for 24 h at 37°C in 5% CO_2 atmosphere. After treatment, cell supernatants were collected and frozen at −70°C until analysis. Production of the pro-inflammatory cytokines IL-6 and IL-8 was measured in cell supernatants by ELISA and quantified using the provided reference standard curve (Affymetrix, eBioscience, San Diego, CA, USA). The experiments were performed twice in duplicate assays.

2.7. *Cell lysis and pro-inflammatory markers by ELISA*

After the former stimulation, CACO-2 cells were placed on ice, washed once in phosphate buffer saline (PBS) and detached with trypsin– ethylenediaminotetraacetic acid (EDTA) for 10 min at 37°C. Whole cell extracts were prepared as described before (Capozzi et al., 2013). Briefly, cells were re-suspended in lysis buffer (20 mM Hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), pH 7.2, 1% Nonidet P-40, 10% glycerol, 50 mM NaF and 1 mM Na_3VO_4). DNA was fragmented by brief sonication and released proteins were recovered after centrifugation of lysates at 12,000 rpm for 15 min at 4°C.

For ELISA, microplates were coated with 100 µL of cell lysate (1:10) and washed, as previously described at the former ELISAs for ZO-1. The coated microplates were incubated for 2 h with rabbit monoclonal anti-phospho-p38 mitogen-activated protein kinases (MAPK) (Thr180/Tyr182) from Cell Signaling Technology, or with rabbit monoclonal anti-ciclooxigenase 2 (COX2) from Abcam (1:1000 dilution). Detection was made by incubation for 1 h with goat anti-rabbit IgG-HRP (DAKO, Carpinteria, CA) and further chromogenic convertion of TMB.

2.8. *Cell monolayer polarization and permeability*

After culture medium replacing with PBS at room temperature, the trans-epithelial electric resistance (TEER) was measured using a silver chloride adjustable electrode (Millicell ERS; Millipore Co., Bedford, MA) adapted to a Fluke multimeter (Everett, WA, USA). TEER values were expressed in ohms (Ω)/cm^2.

The permeability of the CACO-2 cell monolayer was evaluated according to Fiorentino, Levine, Sztein, and Fasano (2014) with some modifications. Bovine serum albumin (BSA) was dissolved in P buffer (10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 10 mM glucose, 3 mM $CaCl_2$, 145 mM NaCl) or P/EDTA buffer (10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 10 mM glucose, 145 mM NaCl, 2 mM EDTA). The apical surface of CACO-2 cell monolayers was stimulated with fractions 3 and 4 of PT-digested gliadin, zein or α-gliadin peptide p31–43 in cell culture medium and incubated at 37°C for 6 h, and then apical an basolateral sides were washed with PBS. In order to measure the paracellular flux, the apical and basolateral cell culture media was replaced with P buffer containing 10 mg/mL BSA or P buffer alone, respectively. P/EDTA buffer containing BSA (10 mg/mL) and P/EDTA buffer were used as positive controls. After incubation for 4 h, BSA in the basolateral media was measured at 280 nm.

2.9. *Statistic analysis*

Cytokine concentrations, as well as absorbance values from ZO-1 release in biopsy supernatants and activation of pro-inflammatory proteins in CACO-2 culture supernatants were calculated from duplicates of two experiments by ELISA. Mean values were compared by ANOVA and statistic significance by the Tukey's test using the statistical software NCSS, version 2001.

3. Results and discussion

The clinical characteristics of participants, all of them CD patients whose biopsies were studied, are presented in Table 2. Patients represent a variety of clinical manifestations with typical and atypical symptoms, alleles of genetic predisposition and positive indexes of antibodies for CD. Ages ranged from 0.9 to 18 years and histology shows villus atrophy. All of the patients presented at least two alleles that encode for HLA-DQ2 or HLA-DQ8 molecules.

3.1. *Prolamin-induced cytokine responses in biopsies*

IFN-γ was measured in the culture supernatants of biopsies following 24 h of culture with immunogenic peptides from gliadins or zeins. Three of the patient biopsies produced an

Table 2. Characteristics of CD patients.

Patient	Age (in years)	Diagnostic	Haplotype or alleles	Positive index for antibodies[a]	Biopsy	Symptoms
1	0.9	CD	DQA1*501, DQB1*0301	ND	Villus atrophy	Seizures and GERD
2	1.5	CD	DQA1*501, DQA1*302	Anti-Gd IgG; anti-tTG IgA	Villus atrophy	Diarrhea, bloating and vomit
3	10	CD	DQB1*0201, DQB1*0301	Anti-Gd IgG; anti-Gd IgA; anti-tTG IgA	Villus atrophy	GERD, abdominal pain and diarrhea
4	18	CD	HLA-DQ2, DQB1*301	Anti-Gd IgG; anti-tTG IgA	Villus atrophy	Gastritis
5	1	CD	DQA1*0501, DQA1*0302	Anti-Gd IgG; anti-Gd IgA	Villus atrophy	Chronic constipation seizures

Notes: ND, not done; DQA1, alpha-chain DQ alleles; DQB1, beta-chain DQ alleles; IgG, G isotype immunoglobulin; IgA, A isotype immunoglobulin; Gd, gliadins; GERD, gastroesophageal reflux disease.
[a]Antibodies were analyzed before biopsies were taken.

IFN-γ increase when stimulated with the 34-mer zein peptide, two of them similar to it of the 33-mer gliadin peptide ($p = 0.05$). A 14-month-old patient who carries the HLA-DQ2 haplotype and suffers from gastritis and chronic diarrhea has not presented CD antibodies or mucosal damage, thus considered non-celiac patient. Nevertheless, its biopsy increased IFN-γ levels when stimulated with gliadins, but the levels were lower than those of biopsies of CD patients (data not shown).

The former results confirm the theoretical immunogenicity of the 34-residue peptide of maize, a product of the PT-digested alpha-zein A20, with glutamine residues situated in favored positions to bind effectively to the HLA-DQ8 molecules (Cabrera-Chávez et al., 2012; Calderón de la Barca & Cabrera-Chávez, 2014). As expected, all the five biopsies produced significant higher ($p = 0.05$) IFN-γ levels in response to gliadins than their respective negative controls (Figure 1(A)). Gluten-reactive CD4+ T cells of CD patients recognize the gliadin immunogenic peptide 33-mer in the HLA-DQ2/8 context and elicit a Th1 type

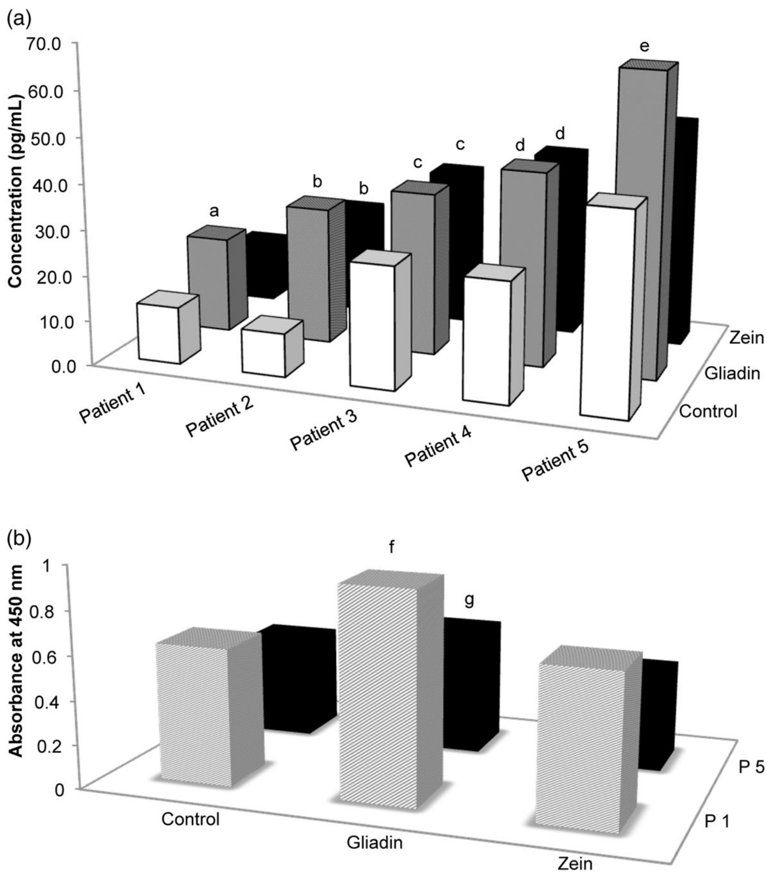

Figure 1. Zein peptides induce increased levels of IFN-γ but no ZO-1 in intestinal biopsies. (A) Patient biopsies stimulated with either gliadin (α-gliadin 33-mer), zein (α-zein 34-mer) or unstimulated were analyzed for IFN-γ production in supernatant by ELISA. (B) Patient biopsies either stimulated with gliadin (α-gliadin 33-mer), zein (α-zein 34-mer) or unstimulated were analyzed for ZO-1 liberation to the supernatant by ELISA. Statistic analysis was made within each patient: a to g $p = 0.05$ versus control. Results are expressed as mean ± SD of duplicate analysis in two different experiments. P1 and P5: patient 1 and patient 5, respectively.

cytokine response, mainly with the production of IFN-γ (Nilsen et al., 1998). This cytokine induces damage on the intestinal mucosa through the activation and release of enzymes such as matrix metalloproteinases in lamina propria mononuclear cells and fibroblasts (Ciccocioppo, Di Sabatino, & Corazza, 2005).

The previous results are consistent with Brottveit et al. (2013), who observed that IFN-γ increased after a short-term gluten challenge in celiac and non-celiac gluten sensitivity patient biopsies. Nevertheless, the induction of pro-inflammatory cytokines by gliadin 33-mer in macrophages does not include IFN-γ (Thomas, Sapone, Fasano, & Vogel, 2006). Hence, dendritic cells or gluten-reactive CD4+ T cells can be mainly responsible for production and secretion of INF-γ, suggesting an adaptive immune response. Although the alpha-gliadin 33-mer is considered to have a higher affinity to the HLA-DQ2 molecule (Qiao et al., 2004), our results suggest that both gliadin and zein peptides elicited an adaptive response regardless of DQ2 or DQ8 haplotype.

No significant ($p = 0.05$) differences were detected in the production of IL-15 from biopsies stimulated with the 34-mer zein (50.13 ± 13.92 pg/mL) or 33-mer gliadin peptides (56.33 ± 5.66 pg/mL) as compared to negative controls (60.81 ± 2.75 pg/mL). IL-15 is the main innate response cytokine overexpressed in untreated CD and can be induced not only by gliadin "toxic" peptides such as p31–42 and p31–49 (Maiuri et al., 2003; Mention et al., 2003) but also immunogenic peptides as the 33-mer, with effect on membrane integrity and permeability (Thomas et al., 2006). Nevertheless, even gliadin 33-mer could not elicit IL-15 in patient biopsies. Our results agree with those of Mention et al. (2003) who found out very low IL-15 secretion in CD intestinal biopsies of active or treated CD patients after 24 h incubation with digested gliadin peptides.

IL-15 is mostly delivered at the enterocytes surface (Meresse et al., 2004), and it can also be secreted in untreated CD patient enterocytes (Di Sabatino et al., 2006). Therefore, the lack of cellular response for producing secreted IL-15 after gliadin 33-mer treatment can be attributable to that all of the five studied patients are currently in gluten-free treatment. Finally, there is a need to evaluate IL-15 expression in biopsy specimens by immunohistochemistry assays, in order to clarify if both assayed maize and wheat peptides have an exclusive immunogenic effect and do not bind to the receptors for cytotoxic peptides in enterocytes.

ZO-1 release was higher in two of the five patients (P1 and P5) biopsies that were stimulated with the 33-mer gliadin peptide ($p = 0.05$), but there was no ZO-1 liberation under the 34-mer zein stimulus (Figure 1(B)). ZO-1 is part of the tigh-junctions (TJ) protein complex, released after membrane disruption (Fasano, 2008). In the same way that IL-15, ZO-1 is also an innate response indicator and it was expected to remain the same after challenged with the immunogenic gliadin peptide or the zein peptide. Nevertheless, the ZO-1 releasing found could be related to a high concentration of IFN-γ, a cytokine which can disrupt the intestinal barrier integrity in murine intestinal cell lines (Zufferey, Erhart, Saurer, & Mueller, 2009) and induce internalization of transmembrane proteins, with the release of ZO-1, a cytosolic plaque TJ protein (Bruewer et al., 2005).

3.2. *Cytokines production by zein PT-prolamin fractions in CACO-2 cells*

Due to most of the immunogenic and toxic peptides are under 5 kDa (Ciccocioppo et al., 2005); we use fractions 3 and 4 of zeins or gliadins for cytokine assays and fraction 1 was used as an irrelevant peptide fraction. To analyze the innate response to the zein peptides, the production of pro-inflammatory cytokines interleukins 6 and 8 (IL-6 and IL-8) was measured in challenged CACO-2 cells. Fractions 3 and 4 of PT-digested zeins stimulated production of IL-8 in a similar way as gliadin fractions 3 and 4 did (Figure 2(A)). Gliadin peptides induced releasing of IL-8 from enterocytes that leads to the recruitment of neutrophils in the lamina propria (Fasano, 2011). An increased level of IL-8 in CACO-2 cells stimulated with PT-digested gliadin was also observed by Capozzi et al. (2013). Zein peptides considered within fractions 3 and 4 (1–5 kDa) can stimulate the production of IL-8, an important pro-inflammatory cytokine induced by oxidative stress in intestinal cells.

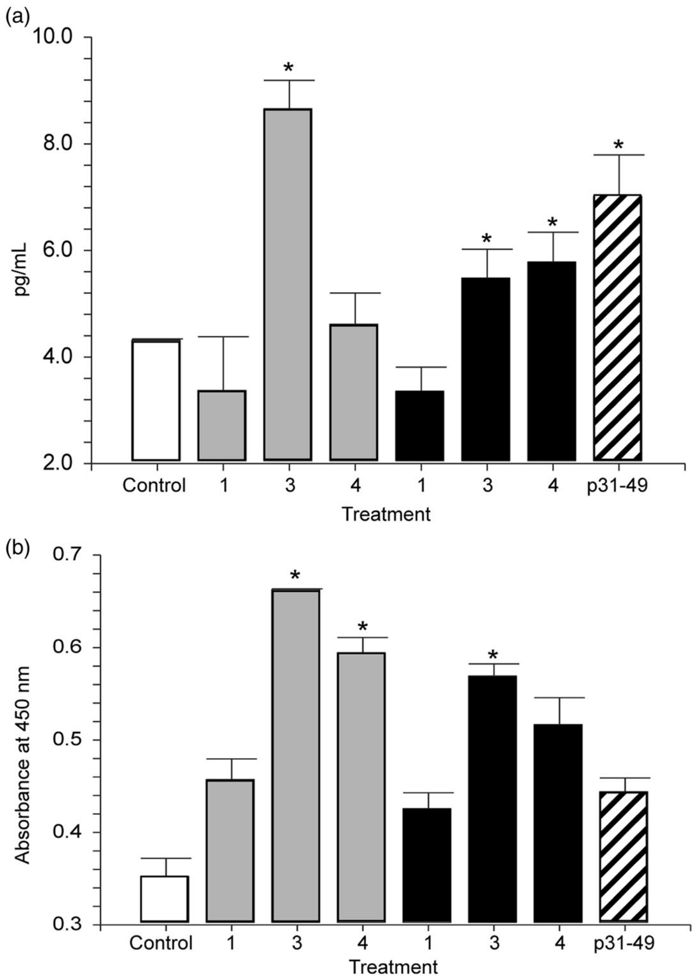

Figure 2. Chemotactic and release of ZO-1 is increased by zein in the same way of gliadin peptides in CACO-2 cells. (A) CACO-2 cells were stimulated with gliadin fractions 1, 3 or 4 (gray columns), zein fractions 1, 3 or 4 (black columns) or toxic peptide *α*-gliadin p31–43 (striped column) for 24 h and were analyzed for IL-8, statistical analysis: $*p = 0.05$ versus unstimulated (control) cells (white column). (B) Liberation of ZO-1 to supernatant by ELISA in the same stimulated CACO-2 cells. Statistical analysis: $*p = 0.05$ versus unstimulated (control) cells (white column). Results are expressed as mean ± SD of duplicate analysis in two different experiments.

Production of IL-6 was not detected for any of the zein nor gliadin PT-digested fractions as well as for the positive control p31–49. A similar result of IL-8 production but not IL-6 was obtained by Fiorentino et al. (2014) after stimulation of CACO-2 cells with *Shigella* antigens. However, Capozzi et al. (2013), observed a small increase in IL-6 in comparison to the IL-8 increase after stimulation of CACO-2 cells with PT-gliadin. Perhaps, we did not detect IL-6 due to the use of PT-digested gliadin fractions, a different control peptide (p31–49 instead of p31–43) or to different sensitivity of the analysis tools. Furthermore, in cells of an isolated medium with a lack of cytokines like IL-1 and tumor necrosis factor, the chemotactic functions in the gliadin-stimulated enterocytes given by IL-8 could be prioritized.

3.3. *Zein PT-prolamin fractions in CACO-2 cells activate MAPK inducing a pro-inflammatory response*

We analyzed phosphorylation of the p38 group of the MAPK (p38 MAPK), by challenging intestinal CACO-2 cells with our fractions in comparison to the gliadin peptide p31–49, an inducer of innate response in CD mucosa (Hüe et al., 2004). We found out that the pro-inflammatory response elicited by fractions 3 and 4 of PT-digested zeins were similar to those of the PT-digested gliadin fractions and to the p31–49 for increasing p38 MAPK, as shown in Figure 3(A).

The p38 MAPK participates in a signaling cascade controlling cellular responses, like inflammation and cell apoptosis, to different cellular stresses (Zarubin & Han, 2005). According to Luciani et al. (2010), gliadin peptide p31–42 is internalized in human intestinal cell lines and induces oxidative stress by the generation of reactive oxygen species. In addition to p31–42, gliadin peptide p31–49 can induce a pro-oxidative environment that induces activation of stress-sensitive signaling pathways. Interestingly, in CACO-2 cells, inhibition of the p38 and NF-$\kappa\beta$ signaling pathways results in the reduction in pro-inflammatory cytokines (Garat & Arend, 2003). On epithelial cells, stress induced by gliadin peptides activates p38 MAPK in order to regulate the actin dynamics during adverse environmental conditions (Guay et al., 1997). Therefore, in the same way as gliadin, maize peptides can activate signaling pathways that induce to enterocyte oxidative stress and a consequent damage to the membrane.

The oxidative stress on CACO-2 cells stimulated with PT-digested zeins also increased the COX2 levels. Figure 3(B) shows that COX2 amounts after stimuli with fractions 3 of PT-digested gliadins, 4 of PT-digested zeins or p31–49 are not different from negative control (unstimulated). However, COX2 levels of fractions 3 and 4 PT-digested zeins were similar to the corresponding fractions of gliadins ($p = 0.05$). It seems that zein peptides induced an oxidative imbalance as well as gliadins. COX2 response is to oxidative damage and converts arachidonic acid to prostaglandins that plays a crucial role in inflammation, although in enterocytes is mainly due to the way of the NF-$\kappa\beta$ activation (Ferretti, Bacchetti, Masciangelo, & Saturni, 2012). However, it has been reported that in mononuclear cells of lamina propria, COX2 is activated through the p38 MAPK pathway when cells were stimulated with the gliadin peptide p31–43 (Maiuri et al., 2003). Increased levels of phosphorylated p38 MAPK and COX2 were also observed by Capozzi et al. (2013) when CACO-2 cells were stimulated with PT-digested gliadins. For all the above, our results suggest an evidence that zein peptides induce an pro-inflammatory

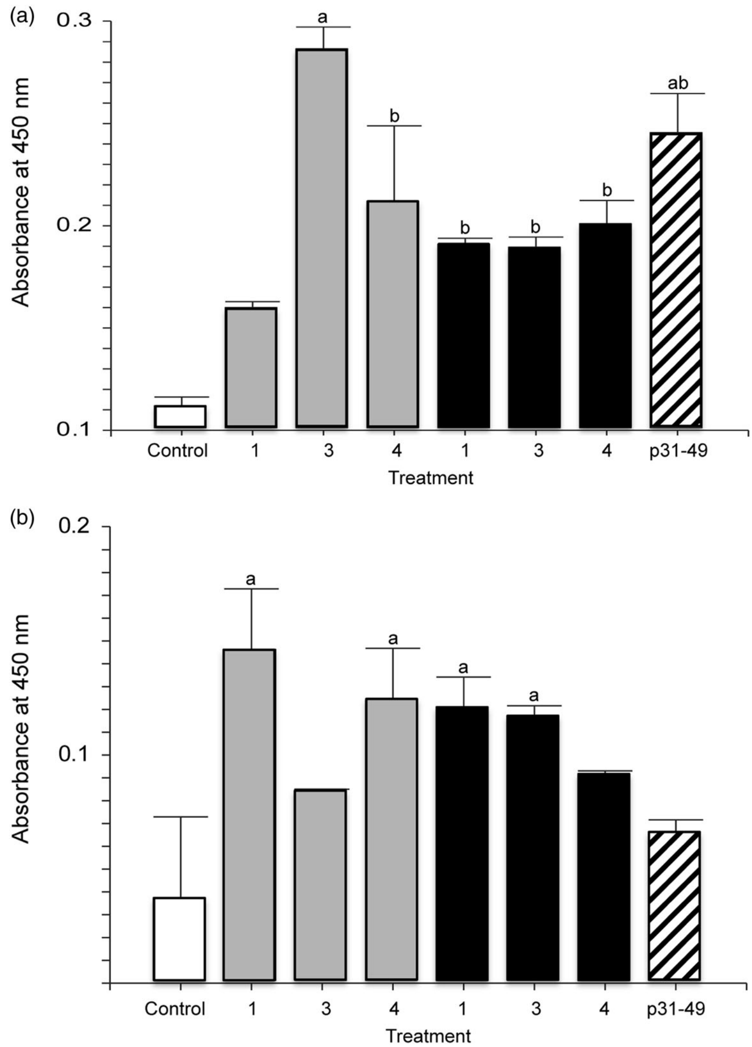

Figure 3. Induction of oxidative response by zein peptides in CACO-2 cells via activation of p38 MAPK and COX2. (A) CACO-2 cells were stimulated with gliadin fractions 1, 3 or 4 (gray columns), zein fractions 1, 3 or 4 (black columns) or toxic peptide *α*-gliadin p31–43 (striped column) for 3 h and were analyzed for pp38 in cell lysate by ELISA. Statistic analysis: $^{ab}p = 0.05$ versus unstimulated (control) cells (white column). (B) Production of COX2 in cell lysates by ELISA in the same stimulated CACO-2 cells. Statistic analysis: $^{a}p = 0.05$ versus unstimulated (control) cells (white column). Results are expressed as mean ± SD of duplicate samples of two experiments.

effect in CACO-2 cells and this mechanism is regulated by the p38 MAPK, which is also involved in damage on cellular integrity.

3.4. *ZO-1 liberation by zein PT-prolamin fractions in CACO-2 cells*

Similar to IL-8, fractions 3 and 4 of PT-digested zein stimulate production of ZO-1 the same way gliadin fractions 3 and 4 do (Figure 2(B)). ZO-1 is an important component of the TJ protein complex. The release of zonulin from the enterocytes is concomitant to the disengagement of ZO-1 from the TJ complex between cells and its consequent disassembly leads to increased membrane permeability. Zonulin increases in culture media of CACO-2 cells when exposed to gliadin peptides (Orlando, Linsalata, Notarnicola, Tutino, & Russo, 2014), additionally, cell apoptosis induced by gliadin (Giovannini et al., 2000) can also increase ZO-1 levels in the culture media and this represents a double marker of membrane permeability and cell destruction simultaneously. Our results show, indirectly by the increase in ZO-1, that maize prolamins, as well as gliadin "toxic" peptide p31–49, can induce liberation of zonulin in CACO-2 cells and affecting membrane integrity.

3.5. *Permeation effects of zein PT-prolamin fractions in CACO-2 cells*

After 18 days, CACO-2 cells had covered the insert membrane and reached constant TEER values. As positive control for well-polarized cells in the inserts, TEER was measured in untreated CACO-2 cells or stimulated with p31–49. TEER values were of 1.18 and 0.38 Ω/cm^2, respectively. Membrane permeation was measured by albumin concentration in the basolateral zone of insert well of the CACO-2 cell layer incubated with fraction 3 of PT-digested zeins or p31–49 peptide (0.107 mg/mL for both). Although the two albumin concentrations were lower than that for fraction 3 of PT-digested gliadins (0.301 mg/mL), no other fraction of gliadins nor zeins induced disruption of the cell monolayer (Table 3). Albumin concentration of basolateral zone of cells with full permeation buffer P/EDTA was 0.636 mg/mL. Gliadin peptide concentrations of 1 mg/mL were also used by Orlando et al. (2014) to alter cellular permeability in CACO-2 for 6 h. Interestingly, in our study, the use of unmarked BSA proved to be a reliable and economic method for testing cell membrane permeability.

4. Conclusions

The zein 34-mer (α-zein 58–91) peptide possesses immunogenic capacity in some patients' biopsies through the production of IFN-γ, a key cytokine in the adaptive response in CD. From the pro-inflammatory and the permeation effects of the fractions of PT-digested zeins on the intestine cell line CACO-2, we inferred that there are toxic peptides in zeins (1–5 kDa). These results suggest that there are innate and adaptive responses to zeins similar, but to a lesser extent than, to gliadins.

Although reaction to maize prolamins in CD patients appears to be a rare event, the confirmation that they play a role in the pathogenesis of CD will be useful information for the follow-up of some non-responsive celiac patients. It is estimated that

Table 3. Permeability effect of PT-digested zeins and gliadins in CACO-2 cells monolayers, albumin corresponds to basolateral concentration after 4 h.

Challenge	Albumin (μg/mL)
Blank	76
α-Gliadin 31–49	107
Gliadin fraction 3	301
Gliadin fraction 4	82
Zein fraction 3	107
Zein fraction 4	23

approximately 10% to 18% of these cases are refractory CD, which represents a more severe CD, with a clear malignity and a less favorable prognosis (Rubio-Tapia & Murray, 2010). Despite the low content of zeins in maize-containing foods compared with that of gliadins in wheat-containing foods, maize could be responsible for persistent mucosal damage in a very limited subgroup of CD patients (Ortiz-Sánchez et al., 2013). However, before considering maize as non-safe for patients who do not respond to a gluten-free diet, an oral challenge needs to be assessed.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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