Effect Of Gluten on Intestinal Mucosal Biology: Studies on Cell Lines and Human Intestinal Biopsies

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ABSTRACT

Background & Aims Little is know about the interaction of gliadin with intestinal epithelial cells and mechanism(s) through which gliadin crosses the intestinal epithelial barrier. Based on our recent discovery of zonulin, a modulator of intestinal tight junctions, and its up-regulation in celiac disease, we elected to establish whether gliadin has any immediate effect on the zonulin pathway. *Methods* Both *ex vivo* human small intestines and intestinal cell monolayers were exposed to gliadin, and zonulin release and changes in paracellular permeability were monitored in the presence and absence of zonulin antagonism. Zonulin binding, cytoskeletal rearrangement, and zonula occludens-1 re-distribution were evaluated by immunofluorescence microscopy. *Results* Zonulin receptor positive IEC6 and Caco 2 cells exposed to gliadin released zonulin in the cell medium with subsequent zonulin binding to the cell surface, rearrangement of the cell cytoskeleton, loss of occludin-ZO1 protein-protein interaction, and increased monolayer permeability. Pretreatment with the zonulin antagonist FZI/0 blocked these changes without affecting zonulin release. When exposed to luminal gliadin, intestinal biopsies from celiac patients in remission expressed a sustained luminal zonulin release and increase in intestinal permeability that was blocked by FZI/0 pre-treatment. Conversely, biopsies from non-celiac patients demonstrated a limited, transient zonulin release which was paralleled by a reduction in intestinal TEER that never reached the level of permeability seen in CD tissues. Chronic gliadin exposure caused a down-regulation of both ZO-1 and occludin gene expression. *Conclusions* Gliadin activates the zonulin system irrespective of the genetic predisposition to autoimmunity, leading to increased intestinal permeability to macromolecules.

BACKGROUND & AIM

- It is known that celiac disease is the result of an inappropriate T-cell mediated immune response against ingested gliadin.
- Little is know about the possible interactions of gliadin (and/or its peptide derivatives) with intestinal epithelia and the mechanism(s) through which it crosses the epithelial barrier to reach the submucosa.
- The upregulation of zonulin, a recently described intestinal peptide involved in tight junctions regulation, seems to be responsible, at least in part, for the increased gut permeability characteristic of the early phase of celiac disease.
- The aim of this study was to investigate the early effects of gliadin on intestinal epithelial mucosa and the structures that dictate mucosal tight junctions competency.
- Our results provide evidence that gliadin activates the zonulin innate immunity pathway, resulting in immediate reduction of intestinal barrier function and passage of gliadin into the subepithelial compartment. This process is dependent on the presence of the zonulin receptor but independent of individual genetic predisposition.

METHODS

INTESTINAL CELL LINES

Intestinal cell cultures

Both human (Caco-2 and T84) and rat-derived (IEC6) intestinal cells

Immunofluorescence microscopy PT-gliadin for 30 minutes incubation. FITC-phalloidin to stain actin filaments.

Anti-ΔG antibodies (Zonulin-specific polyclonal anti-Zonula occludens toxin (Zot) ΔG antibodies)

Direct immunofluorescence and ZO1-localization-migration Intestinal cells were incubated with either PT-gliadin or the negative control PT-casein (1 mg/ml) at increasing time intervals. PBS-exposed monolayers were used as additional controls. FITC-conjugated anti-ZO1 monoclonal antibody.

Caco 2 monolayers experiments Caco2 cells were grown on filter-clusters. Replicates of Caco2 monolayers were incubated at increasing time intervals with either 1mg/mL PT-Gliadin or 1mg/mL PD-casein used as control. Both preparations were added to the mucosal (apical) side of the Caco2 monolayers.

Transepithelial electrical resistance (TEER) measurements The baseline TEER of Caco2 monolayers was measured using a dual planar electrode (Endhom SNAP Evom G WPI analyzer, World Precision Instruments) and expressed in $\Omega.cm^2$. TEER values were measured for each incubation time after the addition of PT-gliadin and PD-casein and corrected for the baseline resistance values.

Measurement of lactulose flux from the apical to baso-lateral side of Caco2 monolayers Lactulose, a probe used to check paracellular permeability, was added to the apical side of all monolayers. Samples were collected from the basolateral side at increasing time intervals and lactulose concentration measured by High Performance Anion Exchange Chromatography (HPAEC).

Sandwich ELISA to quantify the content of zonulin from cell cultures (both cell culture media and subcellular protein fractions)

METHODS

HUMAN INTESTINAL TISSUE

Immunofluorescence microscopy with anti-ΔG antibodies. Samples of small intestinal mucosa were taken from the second/third portion of the duodenum from subjects undergoing upper GI endoscopy.

Sandwich ELISA to quantify the content of zonulin from intestinal segments mounted in the microsnapwell system.

Patients included:

1. subjects with active CD at diagnosis;
2. subjects with CD on treatment with the gluten-free diet from at least two years, and;
3. non-CD GI controls with persistent dyspeptic complaints.

The small intestinal biopsies were oriented under a dissecting microscope with the villi facing upward and mounted onto the modified microsnapwell system. The study was then conducted as described above for the Caco2 monolayers experiments.

Quantitative polymerase chain reaction with TaqMan procedure. Total tissue RNA was extracted from each intestinal fragment (from both CD and non-CD subjects) mounted in the microsnapwell system.

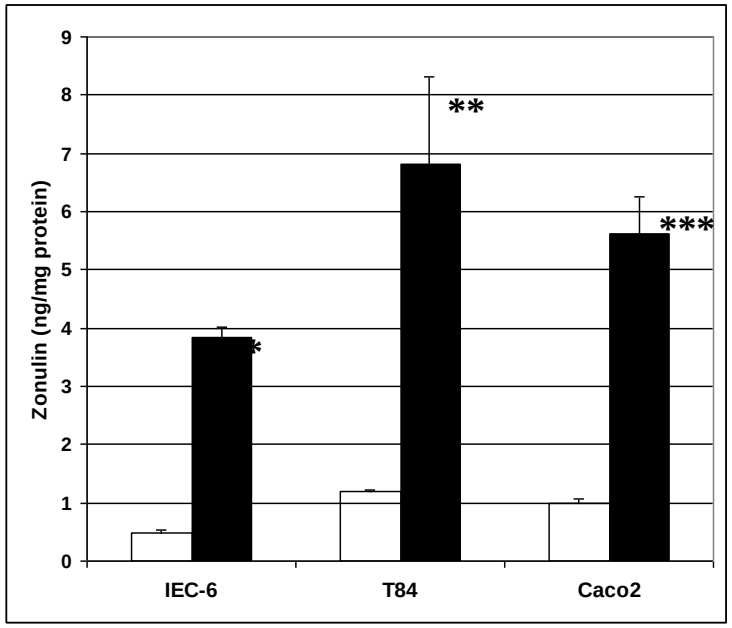
To quantify the occludin and ZO-1 RNA level, primers and probes were designed and used in a multiplex Real Time PCR assay.

Construction of standard plasmid DNA In order to quantify occludin, ZO-1 and TNFα mRNA levels we constructed a recombinant plasmid to produce a standard DNA molecule (prGAPDH).

Intestinal Tissues treatment with RNA inhibitors experiments were performed by pre-treating the tissues with Actinomicyn D and α-Amanitin.

RESULTS: zonulin release (A) and zonulin surface binding (B) in intestinal epitelial cell culture following gliadin exposure

A



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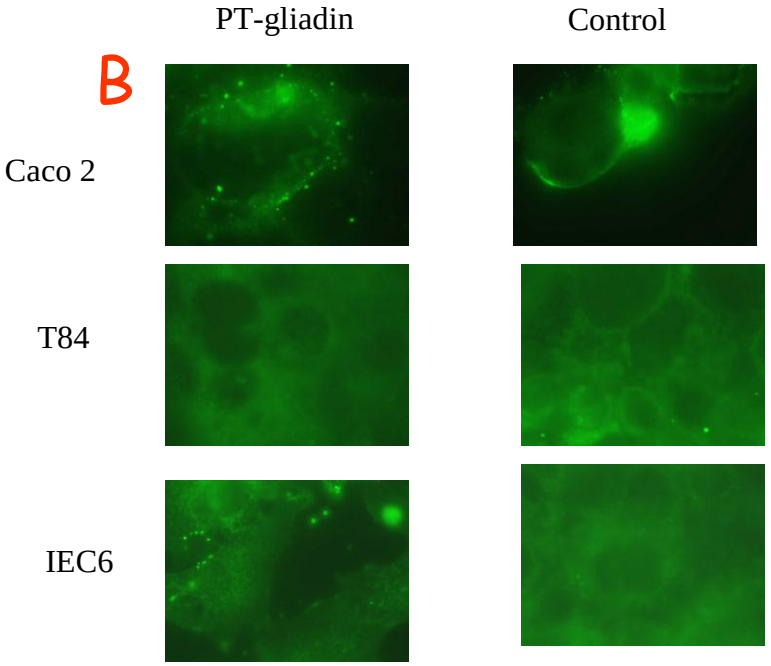


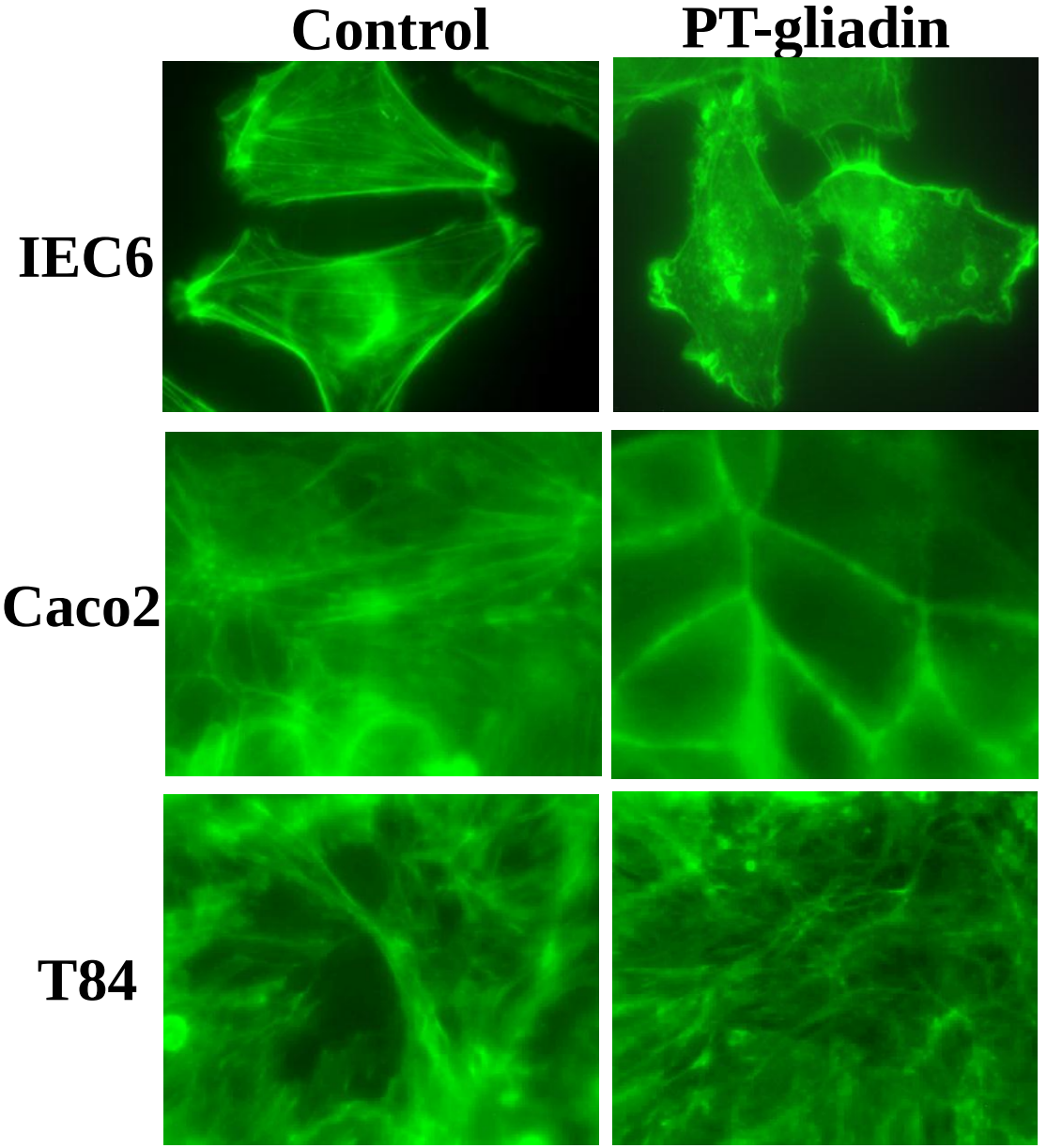
(A) The zonulin release was detected in the medium of PT-gliadin-exposed cells (closed bars) but not in PD-casein-exposed cells (open bars).

(B) Immunoflourescence analysis also revealed that this release was associated with zonulin binding on the surface of zonulin receptor-positive Caco2 and IEC6 cells (see arrows) but not zonulin receptor-negative T84 cells. Magnification=40X; N=4-8 *p<0.0001; **p<0.003; ***p<0.009

RESULTS: effect of gliadin on intestinal epithelial cell cytoskeleton

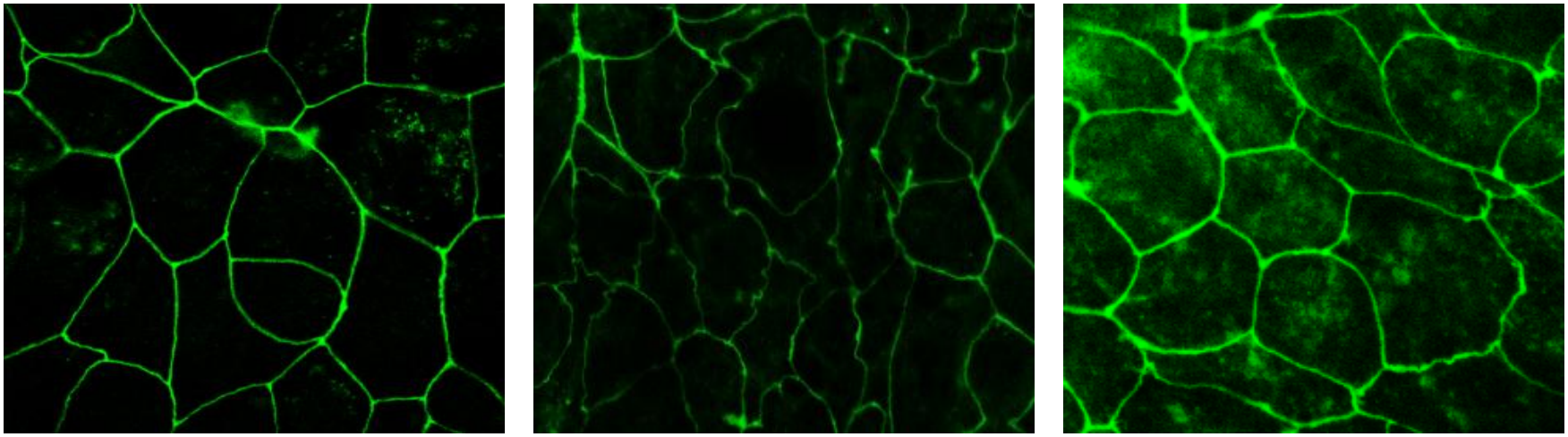
Incubation of both Caco2 and IEC6 cells with PT-gliadin led to a reorganization of actin filaments characterized by their redistribution to the cell subcortical compartment.
F-actin fluorescence pattern remained unchanged in T84 cells exposed to PT-gliadin.

Magnification: 40X.



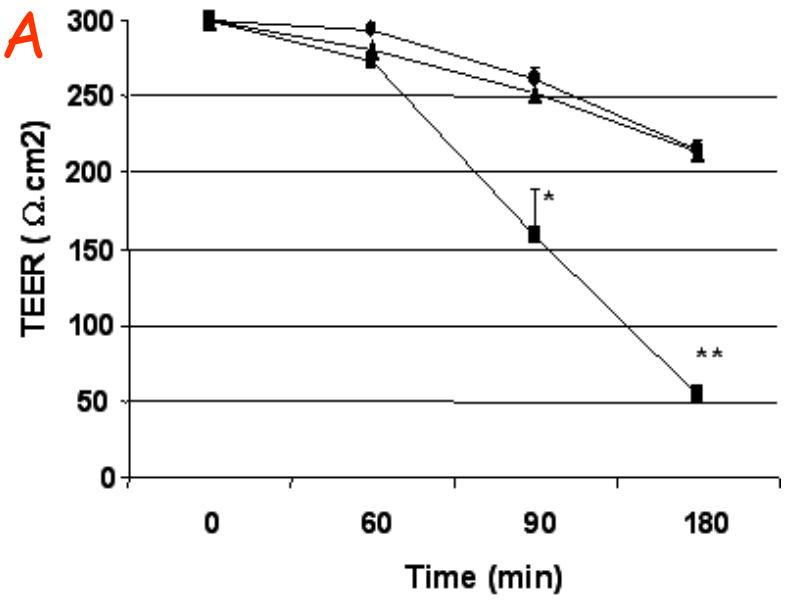
RESULTS: effect of gliadin on the junctional complex protein ZO-1 localization in Caco2 cells

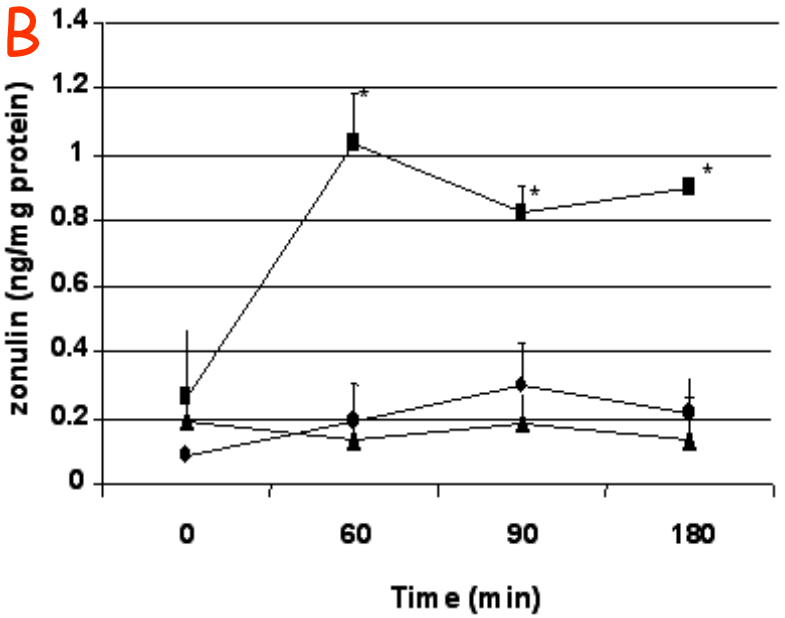
Control **PT-Gliadin** **PT-Casein**



Human intestinal Caco2 cells were exposed to PT-gliadin and immunostained using anti-ZO1 antibodies.
Control monolayers showed the typical ZO-1 localization at the cells' periphery while monolayers exposed to PT-gliadin showed a fluorescent irregular pattern at the edge of the cells due to a redistribution of the ZO1 protein.
No changes in ZO-1 localization were detected in monolayers exposed to PD-casein.

RESULTS: TEER (A) and zonulin release (B) changes in Caco2 monolayers exposed to PT-gliadin



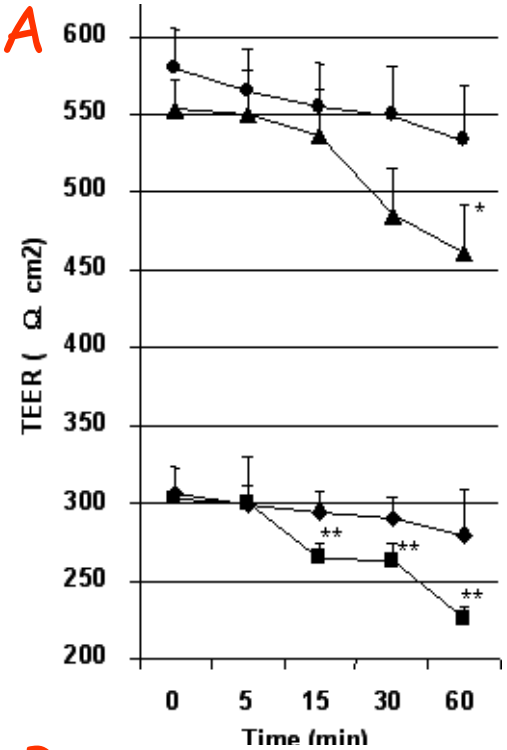


A: Monolayers exposed to PT-gliadin (squares) showed significant TEER decrease (*p<0.01; **p<0.005) starting 90 min post-incubation compared to both media control monolayers (circles) and PT-casein-exposed monolayers (triangles). (

B: Kinetics of zonulin release from Caco2 monolayers exposed to media control (circles), inoculated with PT-gliadin (squares), or casein (triangles). PT-gliadin-exposed monolayers showed a significantly higher zonulin release starting 60-min post-incubation compared to both control and PD-casein-challenged monolayers (*p<0.01; **<0.005).

RESULTS: TEER (A) and zonulin release (B) changes in human intestinal biopsies exposed to PT-gliadin

A



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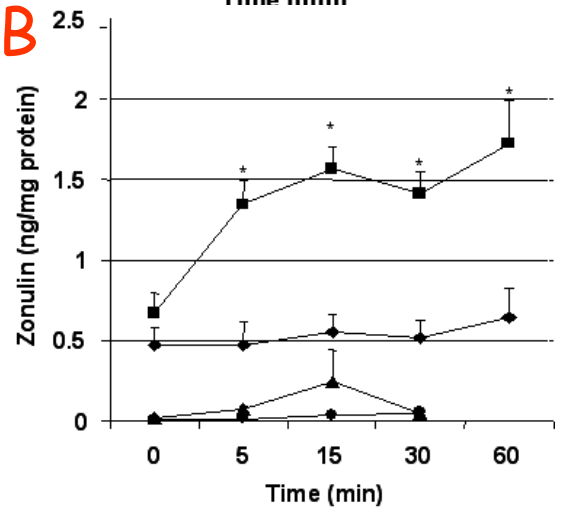
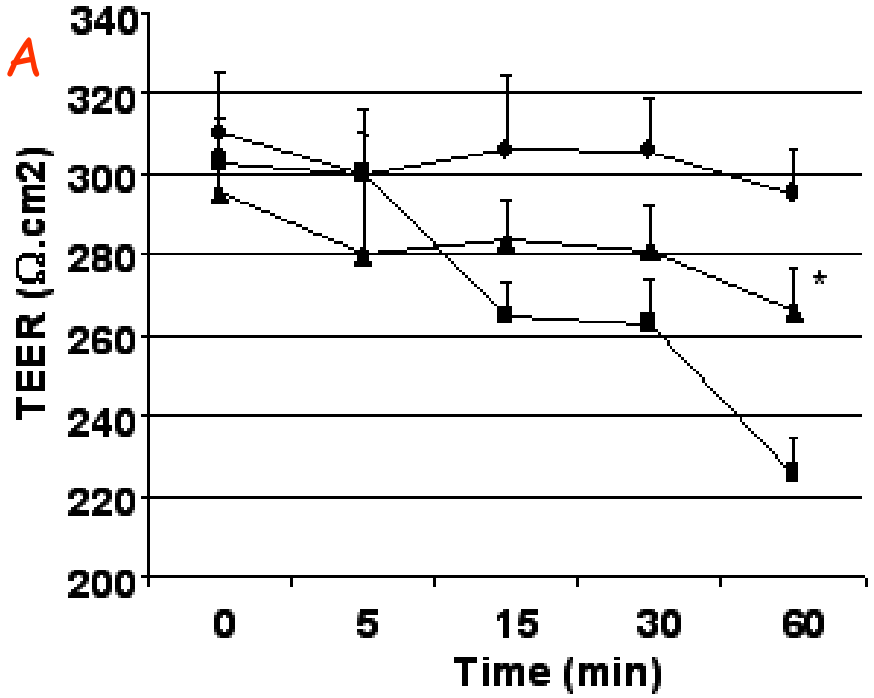
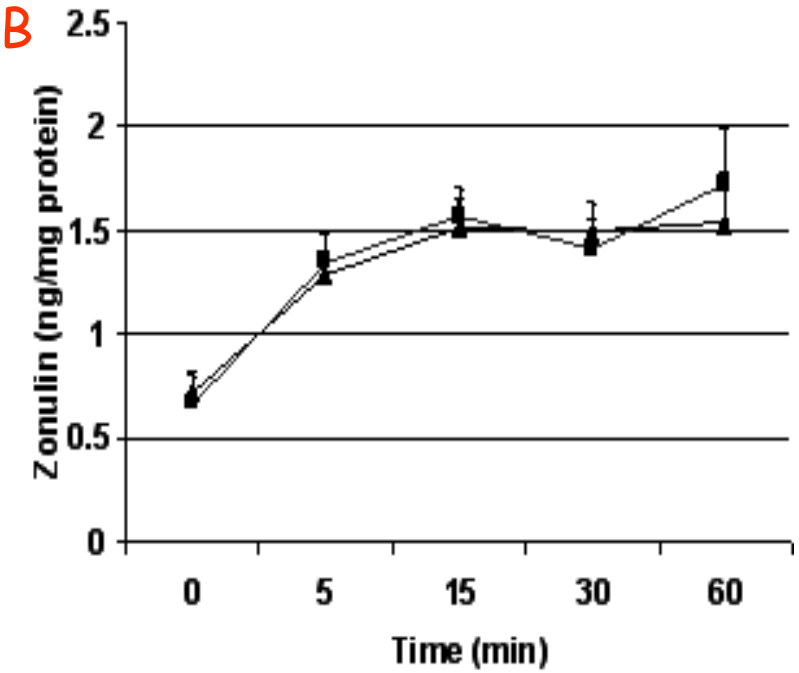


A TEER changes. Tissues obtained from both CD patients in remission and non-CD controls showed a TEER decrement temporally associated to zonulin release. No significant changes were detected in tissues not exposed (diamonds). Conversely, in tissues from non-CD patients a significant decrease in TEER was only observed after 60 min post PT-gliadin incubation (triangles). Again, no changes were detected in tissues exposed to negative control (circles). *p,0.05; **p<0.001 compared to respective negative controls. N=17 CD patients and N=5 non-CD controls.

B Zonulin release. Following exposure to PT-gliadin, tissues obtained from CD patients showed a luminal (squares) but not serosal (diamonds) zonulin release. Conversely, biopsies from non-CD patients showed a transitory zonulin release in the mucosal (triangles) but not serosal (circles) media that reached its peak at 15 min post-PT-gliadin incubation and returned to baseline within 30 min. *p<0.005 compared to serosal zonulin N=17 CD patients and N=5 non-CD controls.

RESULTS: effect of the zonulin inhibitor FZI/0 on gliadin induced TEER (A) and zonulin release (B) changes in duodenal tissues from CD patients in remission

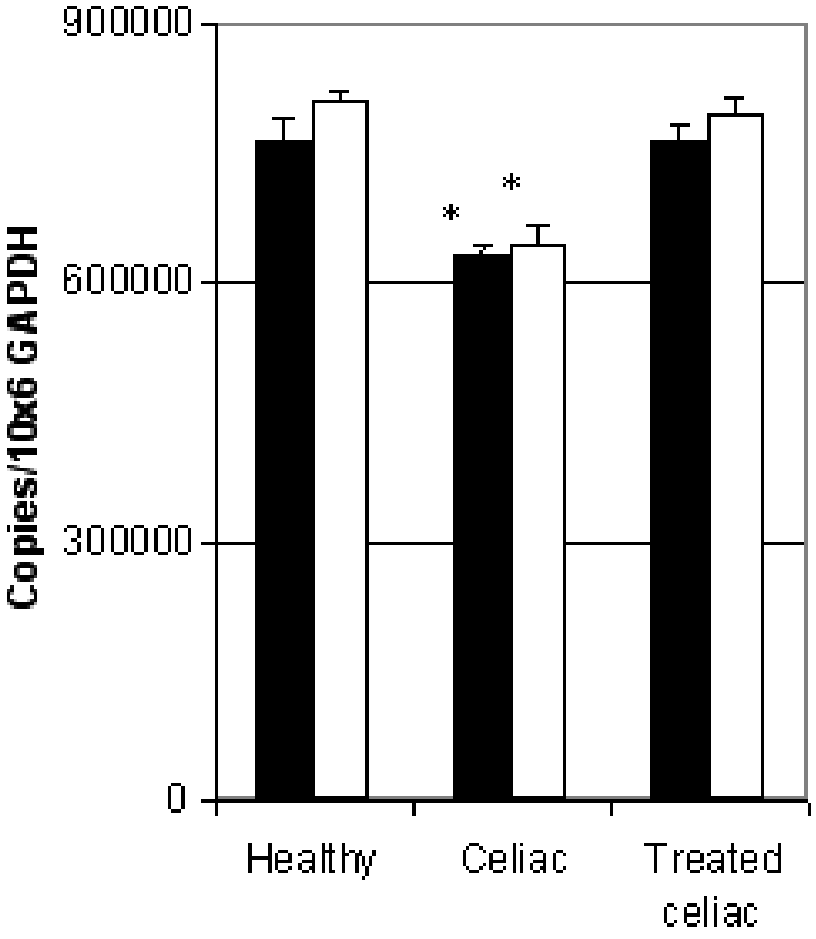




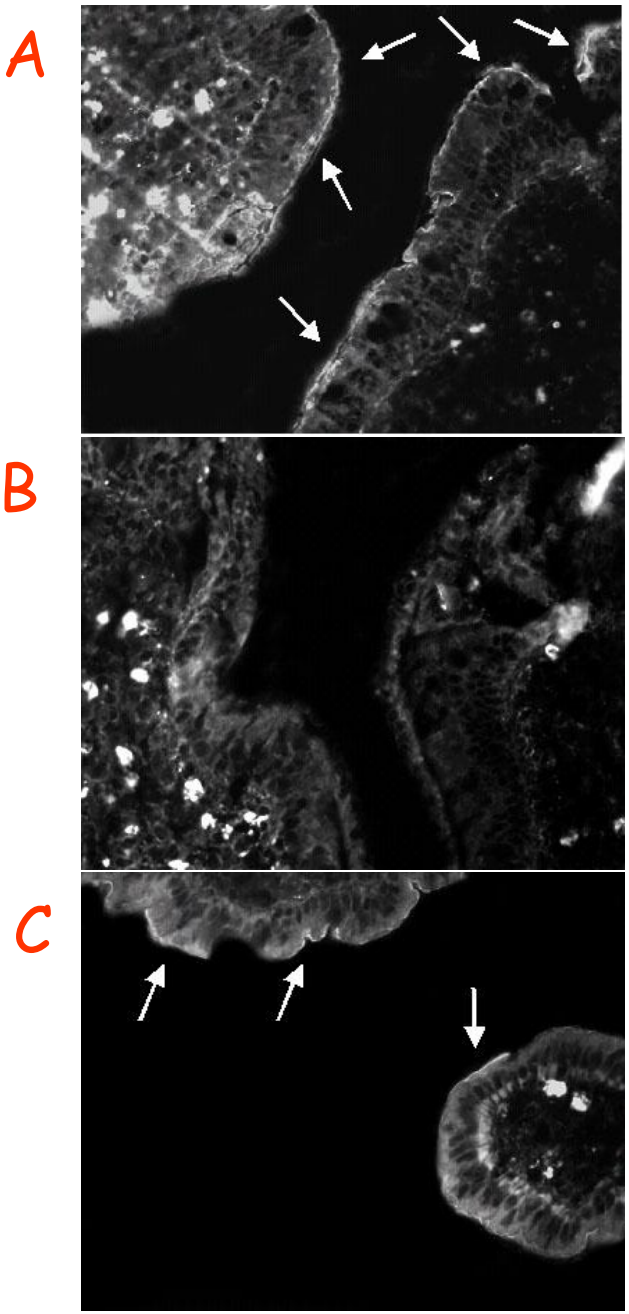
Duodenal tissues from CD patients in remission were exposed to PT-gliadin, either alone or following 15 min pre-incubation with FZI/0.
(A) The TEER decrement induced by PT-gliadin (squares) was prevented by pretreatment with FZI/0 (10 mg/ml) (triangles). PD-casein-treated tissues (circle) are shown as negative controls.
(B) FZI/0 pretreatment (triangles) did not affect the zonulin release by PT-gliadin (squares) (B). *$p<0.02$ compare to PT-gliadin.

RESULTS: ZO-1 and occludin genes expression following chronic exposure to gliadin

Intestinal tissues obtained from CD patients (both during the acute phase of the disease and after remission following a gluten free diet) and from non-CD patients were treated for mRNA extraction following immediately their collection. Quantitative Real Time PCR using specific primers was performed. Both ZO-1 (closed bars) and occludin (open bars) genes expression was suppressed in CD patients during the active phase of the disease compared to non-CD controls. ZO-1 and occludin genes expression reverted to normal values during the disease remission following a gluten free diet. *p<0.01 compared to both controls and treated celiacs.



RESULTS: Zonulin surface binding on intestinal biopsies



(A) Intestinal biopsies from CD patients in remission exposed to PT-gliadin showed a homogeneous zonulin binding on the mucosal surface (arrows) and an increase staining in the submucosa.

(B) No surface staining was observed in tissues exposed to negative control.

(C) Tissues obtained from non-CD patients and exposed to PT-gliadin also showed zonulin surface staining.

CONCLUSIONS

1. Our results suggest that PT-gliadin peptides induce a series of early mucosal events including:

   - zonulin release and binding to receptor-positive cells
   - cytoskeleton rearrangement
   - ZO-1 displacement from the junctional complex
   - tight junctions disassembly

2. Therefore, a direct effect of gliadin on the activation of the zonulin system can be proposed as a driver for antigen access to the GALT via the paracellular route.

3. Pretreatment with the zonulin inhibitor FZI/0 prevents the gliadin-induced TEER decrement, confirming that zonulin is the mediator of these gluten-induced changes and possibly paving the way to treatment alternatives to a gluten free diet.