Involvement of Parkinson's disease protein 7, peroxisome proliferator-activated receptor gamma and thymic stromal lymphopoietin in childhood celiac disease.

Doctoral thesis

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Introduction

Celiac disease (CD) is a chronic autoimmune enteropathy caused by exposure to dietary gluten in genetically predisposed individuals. Ingestion of gluten leads to immune-mediated damage of the small intestine, resulting in destruction of proximal small intestinal villi, Lieberkühn's crypt hyperplasia, and lymphocyte infiltration. Beside innate and adaptive T cell-mediated immune responses, oxidative stress processes are also known to play an important role in the development of gliadin-induced toxicity. Some α-gliadin peptides are able to internalize into intestinal epithelial cells by endocytosis, they accumulate in lysosomes and increase the levels of oxygen- and nitrogen-derived free radicals. Oxidative stress affects cell proliferation, apoptosis and cell viability. Several studies have demonstrated that gliadin peptides may also directly affect intestinal cell structure and function by modulating gene expression. The increased levels of reactive oxygen species are involved in the reduced degradation of tissue transglutaminase (tTG) by the ubiquitin-proteasome system, thus leading to increased tTG protein levels in the mucosa, which leads to down-regulation of the peroxisome proliferator activated receptor gamma and a derangement of the appropriate control of inflammation.

Parkinson's disease 7 (PARK7) was originally identified as an oncogene in 1997, and it has been shown to be expressed in lung, pancreatic, and ovarian carcinomas. Mutations of the gene were also reported to be associated with autosomal recessive early-onset Parkinson's disease (hence the name PARK7). Altered expression or function of PARK7 has been documented in several other abnormalities including type-2 diabetes or infertility, but at the beginning of our study there were no data available about its potential involvement in gastrointestinal diseases, such as celiac disease.

Based on currently available literature data, PARK7 is a redox-sensitive protein with diverse biological functions and a

cytoprotective effect against increased oxidative stress. Its increased expression and antioxidant role in response to a variety of oxidative stress stimuli has been demonstrated in a variety of cell types and tissues. As a peroxiredoxin-like peroxidase it neutralizes reactive oxygen radicals and as a signal molecule that senses the redox state of cells, it protects cells from oxidative stress.

PARK7 was shown to be an upstream activator of hypoxia-inducible factor (HIF)-1α, a main regulator of cellular response to hypoxia. Expression of PARK7 was shown to be critical for the phosphatidylinositide 3-kinase (PI3K)–protein kinase B (Akt)–mammalian target of rapamycin (mTOR) pathway, essential in sustaining the stability and activity of HIF-1. Previously, we have demonstrated elevated levels of HIF-1α in the duodenal mucosa of children with CD compared to controls or children maintained on GFD. Moreover, in biopsy specimens of CD patients, we found an increased expression of ecto-5-prime nucleotidase, trefoil factor-1 and multidrug resistance gene 1, which are all HIF-1-regulated genes and play a well-known role in the maintenance of the epithelial barrier.

Furthermore, recent data suggest the contribution of PARK7 to the regulation of innate immunity. Toll like receptors (TLRs) play a crucial role in the activation of innate and adaptive immunity either by activation of antigen-presenting cells or by co-stimulation of T-cells. Previously, in line with others, we demonstrated an increased expression of TLR4 in the duodenal mucosa of children with CD, suggesting its importance in the disease. PARK7 is able to selectively affect the TLR4-induced responses. Loss of PARK7 may depress the lipopolysaccharide (LPS)-activated TLR4–apoptosis signal-regulating kinase 1 (ASK1)–p38 mitogen-activated protein kinase (MAPK) signaling pathway, leading to the induction of inducible nitric oxide synthase (iNOS) and consequently to excessive production of nitric oxide (NO). Earlier studies suggested that increased production of NO in the intestine can lead to the formation of peroxynitrite, which was shown to induce the apoptosis

of enterocytes, thereby contributing to the epithelial injury. It is known, that the activity of the constitutively expressed (iNOS) in human duodenal enterocytes is increased in the intestinal mucosa of untreated celiac patients, which is partially corrected by gluten-free diet (GFD). We hypothesized that PARK7 may have an effect on iNOS- and TLR4- mediated functions, thus potentially influencing the pathomechanism of celiac disease.

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-dependent transcription factor belonging to the type II. nuclear receptor family. Due to its ability to negatively regulate the expression of proinflammatory genes, protective effects of PPARγ have been demonstrated in several immune-mediated diseases. To the best of our knowledge, this is largely due by inhibiting the NF-κB transcription factor and its target genes such as tumor necrosis factor (TNF)-α and interleukin (IL)-15 or interferon(IFN)-γ.

Previously it has been suggested that gliadin peptides induce oxidative stress and promote tTG-mediated cross-linking, ubiquitination, and proteasomal degradation of PPARγ in duodenal intestinal epithelial cells and mucosa of adult patients with CD. Moreover, in intestinal samples from gluten-sensitive patients, the levels of several proteins that may be associated with the PPAR signaling pathway were found to be reduced. While investigating the effects of gluten exclusion from the diet, overexpression of PPARγ was demonstrated in mice maintained on GFD compared to animals fed on a gluten containing high-fat diet. The PPARγ agonist mesalazine has been shown to decrease gluten induced cytokine response *in vitro* in celiac mucosal biopsies and its effect was comparable to GFD in reducing oxidative burst. Although our knowledge is limited, the severity of epithelial damage seen in celiac disease may be inversely proportional to PPARγ levels.

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine identified from the supernatant of a mouse thymic stromal cell line as a factor that stimulates the growth of T and B lymphocytes. As an immunoregulator, TSLP is primarily expressed

by epithelial cells in the skin, intestines, lungs, and seems to be involved in the regulation of inflammatory processes occurring at the barrier surfaces. While under normal conditions, TSLP promotes the development of tolerance to the commensal flora and the maintenance of normal barrier homeostasis by modulating dendritic cell function, in some pathological conditions, the accumulation of its long isoform has a distinctly proinflammatory pattern. For example, in eosinophilic esophagitis or ulcerative colitis, epithelial cells overexpress TSLP. TSLP expression may be partially regulated by PPARγ, as in an animal model of atopic dermatitis it has been shown that a synthetic PPARγ agonist (rosiglitazone) significantly decreases TSLP expression. At the beginning of our studies, the role of TSLP in celiac disease was completely unknown.

Aims

Based on the aforementioned, the aim of our research was to investigate the possible roles of PARK7 and PPARγ /TSLP in the pathology of treated and untreated celiac disease in childhood.

I. <u>Investigation of the role of PARK7 in celiac disease.</u>

The cytoprotective, antioxidant, antiapoptotic, and immunoregulatory effects of PARK7 have been described in several organs and diseases but have not yet been studied in inflammatory bowel diseases, such as CD, therefore we aimed to investigate PARK7 in childhood celiac disease. My objectives were the followings:

1. Can the presence of PARK7 be detected in the duodenum at mRNA and protein levels? How does PARK7 expression change in celiac disease?

2. Where is PARK7 localized in the duodenal mucosa of celiac and healthy children?
3. How does GFD affect PARK7 mRNA expression and protein amounts?

II. Investigation of the role of PPARγ and TSLP in celiac disease.

The beneficial effects of the anti-inflammatory PPARγ have been studied in a number of diseases. It has been described that it can modulate the expression of many molecules thus influence the amount of TSLP protein, which also regulates the homeostasis of barrier integrity and is also known as an inflammatory modulator. Previous studies showed that PPARγ levels were reduced in adult celiac disease, however, its expression in childhood celiac disease has not yet been studied. The expression of TSLP was completely unknown at the beginning of our studies, so we aimed to investigate PPARγ, TSLP, and TSLPR in childhood celiac disease.
My objectives were the followings:

1. How are PPARγ, TSLP, and TSLPR expressed in the duodenal mucosa of newly diagnosed, therapy-naïve celiac children at mRNA and protein levels, respectively?
2. Which cells express PPARγ and TSLP in the duodenal mucosa of celiac and healthy children?
3. How does GFD affect the expression of PPARγ, TSLP, and TSLPR?

Methods

Duodenal biopsy samples from 19 children (6 boys, 13 girls; median age 8 years, range 2–15 years) with newly diagnosed CD, 5 and 6 children (PARK7 and PPARγ/TSLP studies) (2 and 3 boys, 3

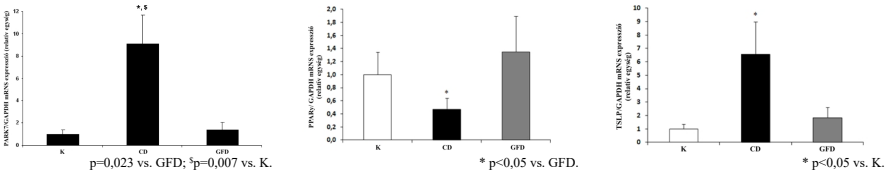
girls; median age 10 years, range 5–16 years) maintained on GFD and 10 controls (7 boys, 3 girls; median age 5.25 years, range 0.33–14 years) were collected. Neither age- nor sex-related differences were observed between the groups (p=NS). Biopsy samples were taken from the duodenal bulb and distal part of the duodenum at routine upper endoscopy. CD was diagnosed by the criteria of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN). Controls were patients referred with chronic abdominal pain, growth retardation, and diarrhea, and an upper gastrointestinal endoscopy was part of their diagnostic procedure. Their duodenal biopsy specimens showed normal appearance and histology. Written informed consent was obtained from parents or caregivers of each participant prior to the procedure. Expression of PARK7, PPARγ and TSLP mRNA was determined by real-time PCR, relative protein amounts were determined by Western-blot analysis, and tissue localizations were determined by immunofluorescent staining. The study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (TUKEB 124/2009).

Results

1. Expression of PARK7, PPARγ, and TSLP mRNA was determined by real-time PCR in biopsies from newly diagnosed coeliac disease (CD) patients, treated patients on gluten-free diet (GFD), and control (K) non celiac children. The mRNA expression of PARK7 and TSLP increased significantly, the mean value of PPARγ mRNA expression showed a decreasing trend in the duodenal mucosa of coeliac children compared to the controls.

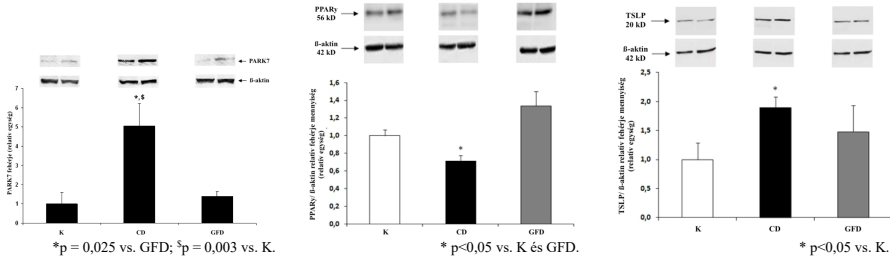
Following gluten-free diet, PARK7 mRNA expression decreased significantly, TSLP mRNA expression showed a decreasing trend, and PPARγ mRNA expression increased significantly. There was no

significant difference between the values of the controls and the GFD group.



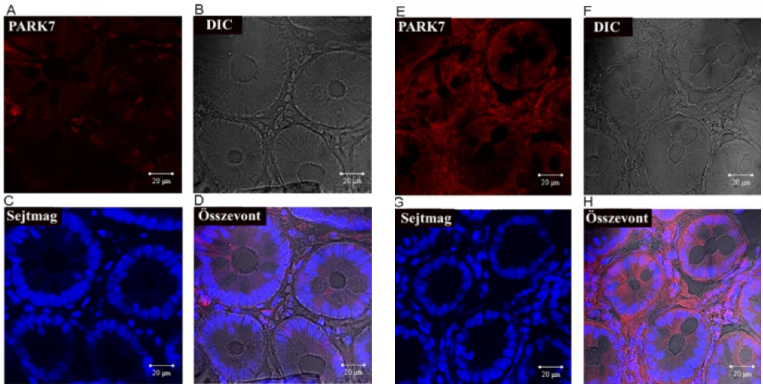
Expression of Parkinson's disease protein 7 (PARK7), peroxisome proliferator activated receptor γ (PPARγ) and thymic stromal lymphopoietin (TSLP) mRNA in the duodenal mucosa of control (K), newly diagnosed coeliac disease patients (CD) and children on gluten-free diet (GFD).

2. The relative amounts of PARK7, PPARγ, and TSLP were determined by Western-blot analysis. Specific signals around 20 kDa, 56 kDa, and 20 kDa were detected on the membranes, corresponding to the molecular weights of PARK7, PPARγ, and TSLP, so our measurements were specific. The amounts of PARK7 and TSLP proteins in the duodenal mucosa of children with celiac disease were significantly increased, and the amount of PPARγ protein was significantly decreased compared to the control group. In children on a gluten-free diet, the amount of PARK7 protein decreased significantly, the amount of TSLP protein showed a decreasing trend, and the amount of PPARγ protein was significantly higher compared to newly diagnosed cases of celiac disease. No significant difference was found between the duodenal protein levels of children on a gluten-free diet and controls. And also, no significant differences in mucosal TSLPR protein was noticed between the studied groups.



Relative protein levels of Parkinson's disease protein 7 (PARK7), peroxisome proliferator activated receptor γ (PPARγ), and thymic stromal lymphopoietin (TSLP) in the duodenal mucosa of control (K), newly diagnosed celiac disease patients (CD) and children on gluten-free diet (GFD).

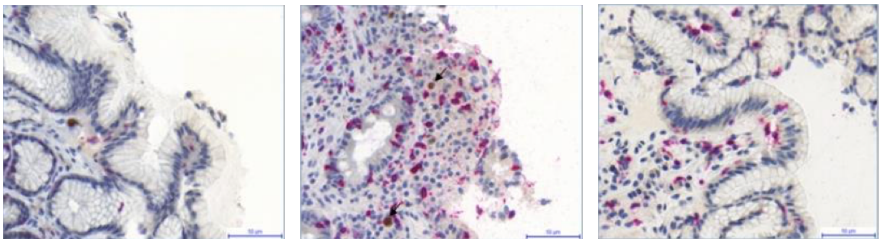
3. Tissue localizations were determined by immunofluorescence staining. Strong PARK7 immunopositivity was observed in duodenal crypt enterocytes and lamina propria from untreated celiac children. The presence of PARK7 was detected in both the nucleus and the cytoplasm. However, only weak PARK7 immunoreactivity was observed in the duodenal mucosa of control children.



Representative images of histological sections from the duodenal mucosa of control (A-D) and untreated celiac children (E-H) showing the tissue

localization of Parkinson's disease protein 7 (PARK7). Immunofluorescence staining was performed with anti-PARK7 primary and Alexa Fluor 568 conjugated secondary antibody (red). The nuclei are stained blue (Hoechst 33342). Weak PARK7 immunoreactivity was observed in the duodenal mucosa of the control group (A–D). In contrast, in the duodenal mucosa of untreated celiac children, strong PARK7 immunoreactivity is seen in crypt enterocytes and the lamina propria (E-H). PARK7 is present in both the nucleus and the cytoplasm. The images were taken with a confocal scanning laser microscope.

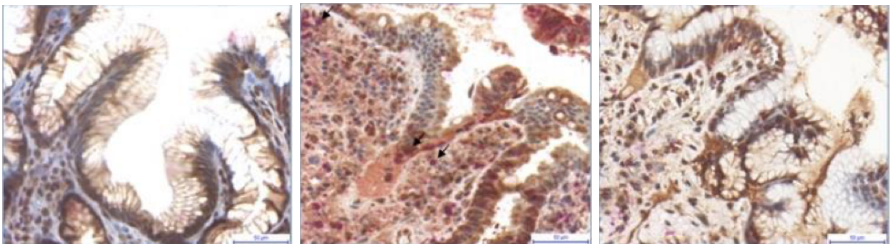
PPARγ and T cell marker CD3 double immunostaining revealed PPARγ-positive T lymphocytes in the duodenal mucosa of celiac children. In addition to T cells, non-CD3-positive immune cells as well as enterocytes also showed positive PPAR staining.



Localization of peroxisome proliferator activated receptor γ (PPARγ, brown) and T cell-specific CD3 (red) in the duodenal mucosa of control (a), newly diagnosed celiac disease patient (b), and children on a gluten-free diet (c). Immunohistochemistry was performed by PPARγ and CD3 double immunostaining to detect the presence of PPARγ-positive T lymphocytes in the duodenal mucosa of newly diagnosed celiac children (black arrows). In samples from control children and gluten-free diet, many non-CD3-positive immune cells and enterocytes also showed PPARγ-positive staining. 200x magnification.

In the duodenal mucosa of celiac children, TSLP-positive T lymphocytes were detected by TSLP/CD3 double staining. In addition to CD3-positive cells, cytoplasmic TSLP positivity was

observed in other intraepithelial and in stromal inflammatory cells as well as in enterocytes.



a. b. c.

Localization of thymic stromal lymphopoietin (TSLP, brown) and the T cell marker CD3 (red) in the duodenal mucosa of control (a), newly diagnosed celiac disease patient (b), and children on a gluten-free diet (c). Immunohistochemistry was used to determine the duodenal localization of TSLP. TSLP-positive T lymphocytes (black arrow) were detected in the duodenal mucosa of newly diagnosed celiac patients, while cytoplasmic TSLP positivity was also observed in other intraepithelial and connective tissue inflammatory cells as well as enterocytes.

Conclusions

Summarizing our results, we showed that PARK7 mRNA expression was increased and protein levels were elevated in the duodenal mucosa of celiac children. Based on our findings, we can conclude that the PARK7 molecule may play a role in the pathomechanism of celiac disease, and reduce the intestinal damage caused by the disease. By regulating HIF-1α and TLR4, and by stabilizing Nrf2 and enhancing its transcriptional activity, it may be involved in maintaining intestinal barrier functions, regulating inflammatory and immune processes, and inhibiting intestinal cell apoptosis. Although further studies are needed to elucidate the exact role of PARK7 in celiac disease, it may be a therapeutic target in the future, presumably due to its protective effects.

Simultaneous decreases in PPARγ levels and increased TSLP expression in the duodenal mucosa of newly diagnosed celiac children suggest their role in the induction and/or maintenance of gluten-induced inflammation. Previous studies in various experimental models have shown that increased PPARγ levels have an anti-inflammatory effect, so we hypothesize that down-regulation of PPARγ in celiac disease may contribute to the chronic inflammation. The normalization of PPARγ levels and concomitant decrease in TSLP expression observed with a gluten-free diet supports the hypothesis that gluten intake may affect the PPARγ/TSLP/TNF-α IL-15, INF-γ, axis through PPARγ degradation and increased TSLP expression. We hypothesize that beside gluten-free diet, through PPARγ activation and regulation of TSLP-stimulated inflammation, synthetic PPARγ agonists may also have therapeutic potential in the treatment of gluten-sensitive enteropathies. To confirm our hypothesis and to establish the specificity of our results for celiac disease, further studies involving inflammatory controls are needed.

Bibliography of the candidate's publications

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