Research Article

PARK7 Diminishes Oxidative Stress-Induced Mucosal Damage in Celiac Disease

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Coeliac disease (CD) is a chronic, immune-mediated small intestinal enteropathy, accompanied with gluten-triggered oxidative damage of duodenal mucosa. Previously, our research group reported an increased mucosal level of the antioxidant protein Parkinson’s disease 7 (PARK7) in children with CD. In the present study, we investigated the role of PARK7 level on epithelial cell and mucosal integrity of the small intestine. The presence of PARK7 was investigated using immunofluorescent staining on duodenal mucosa of children with CD and on FHs74Int duodenal epithelial cells. To investigate the role of oxidative stress, FHs74Int cells were treated with H2O2 in the absence or presence of Comp23, a PARK7-binding compound. Intracellular accumulation of reactive oxygen species (ROS) was determined by DCFDA-based assay. Cell viability was measured by MTT, LDH, and Annexin V apoptosis assays. Disruption of cytoskeleton and cell adhesion was investigated by immunofluorescence staining and by real-time RT PCR. Effect of PARK7 on mucosal permeability was investigated ex vivo using intestinal sacs derived from control and Comp-23-pretreated mice. Comp23 treatment reduced the H2O2-induced intracellular accumulation of ROS, thus preserving the integrity of the cytoskeleton and also the viability of the FHs74Int cells. Accordingly, Comp23 treatment increased the expression of antioxidants (NRF2, TRX1, GCLC, HMOX1, NQO1), cell-cycle regulators (TP53, CDKN1A, PCNA, BCL2, BAX), and cell adhesion molecules (ZO1, CDH1, VCL, ITGB5) of H2O2-treated cells. Pretreatment with Comp23 considerably decreased the small intestinal permeability. In this study, we demonstrate that PARK7-binding Comp23 reduces the oxidative damage of duodenal epithelial cells, via increased expression of NRF2- and P53-regulated genes. Our results suggest that PARK7 plays a significant role in the maintenance of mucosal integrity in CD.

1. Introduction

Coeliac disease (CD) is a chronic, immune-mediated small intestinal enteropathy triggered by the ingestion of gluten in the genetically predisposed individuals [1]. Degraded gluten peptides and the resulting chronic inflammation induce excessive accumulation of reactive oxygen species (ROS) [2], leading to the injury of the epithelial layer and the subsequent deterioration of mucosal integrity [3, 4].

Parkinson’s disease 7 (PARK7) is a multifunctional molecule, which is primarily investigated in connection with neurodegenerative diseases [5, 6]; however, its possible role has been suggested in other diseases, as well [7]. Previously, our research group reported elevated expression of PARK7 in the duodenal mucosa of therapy-naive children with CD [8], and genome-wide association studies identified PARK7 polymorphisms as a predisposing factor in CD [9] and ulcerative colitis [10].

The cytoprotective effect of PARK7 can be exerted through various mechanisms. Among others, PARK7 is suggested to exert antioxidative defence directly via its enzymatic and chaperon activity; however, PARK7 influences the degree of oxidative damage more likely through its transcriptional regulatory effect. PARK7 as an oxidative sensor stabilizes transcription factors, including NRF2 and P53 in an oxidation-dependent manner. Indeed, in response to
oxidative stress due to its conformation changes, PARK7 releases these transcription factors, allowing them to translocate into the nucleus and to induce the expression of stress-response elements [11, 12]. These mechanisms altogether moderate the oxidative damage of intracellular macromolecules, promote repair processes, and enhance the viability of the affected cells.

In the present study, we aimed to investigate the possible role of PARK7 in the pathomechanism of CD, with particular attention on oxidative damage of intestinal epithelial cells.

2. Methods

2.1. Duodenal Biopsies. Mucosal biopsies of pediatric therapy-naïve CD patients and controls were collected at the 1st Department of Paediatrics, Semmelweis University, Hungary. Written informed consent was obtained from the parents of each participant prior to the procedure, and the study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (TUKEB 58/2013). CD was diagnosed based on the criteria of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition [13]. Controls were referred with chronic abdominal pain, growth retardation, and diarrhoea, and an upper gastrointestinal endoscopy was part of their diagnostic procedure. Biopsy samples were immediately snap-frozen and stored at −80°C until further analysis.

2.2. Ex Vivo Mucosal Permeability Measurement. All experiments were approved by the institutional committee on animal welfare (PEI/001/83-4/2013). Intestinal permeability was measured based on Mateer et al. and Lange et al. [14, 15]. Briefly, 2 cm long intestinal sacs were prepared from the small intestine of untreated (controls) and Comp23 (0.0001, 0.001, 0.01, 0.1, 1 μM) and treated with Comp23 (0.001 μM) overnight, thereafter with H2O2 (1000 μM) for 24 h. For Annexin V apoptosis assay, cells were seeded into 6-well plates at a density of 3 × 10^5 cells/well and treated with Comp23 (0.001 μM) overnight, thereafter with H2O2 (1000 μM) for 24 h, then measured in triplicates (n = 9/treatment group). For real-time RT-PCRs, cells were seeded into 96 well plates at a density of 10^4 cells/well (n = 5 well/treatment group) and treated with Comp23 (0.001 μM) overnight, thereafter with H2O2 (1000 μM) for 24 h. Vehicle-treated cells served as controls in all experiments.

2.4. MTT Cell Proliferation Assay. MTT cell proliferation assay was performed using the Cell Proliferation Kit I (MTT) (Roche Diagnostics) according to the manufacturer’s recommendations. Absorbance was recorded at 570 nm and 690 nm as background using a Hidex Chameleon Microplate Reader using the MikroWin 2000 program.

2.5. LDH Cytotoxicity Assay. Reagents for in vitro LDH cytotoxicity assay [16] were purchased from Sigma-Aldrich. Absorbance was recorded at 570 nm and 690 nm as background in a Hidex Chameleon Microplate Reader using the MikroWin 2000 program.

2.6. Apoptosis Detection Assay. Apoptosis assay was performed using the FITC Annexin V Apoptosis Detection Kit I (BD Pharlmingen) according to the manufacturer’s recommendations. Cells that are negative for Annexin V and PI were referred to as viable, cells that are positive for Annexin V and negative for PI were referred as early apoptotic, and cells that are positive for both Annexin V and PI were referred as late apoptotic cells. Flow cytometry analysis was performed using a FACS Aria cytometer (BD).

2.7. ROS Assay. Intracellular ROS accumulation was measured using 2′,7′-dichlorofluorescein diacetate (DCFDA, Sigma-Aldrich) fluorescent dye [17]. Cells were washed twice with PBS; thereafter, 50 μl of DCFDA solution (5 μM in PBS) was added for each well and incubated for 30 minutes in cell culture incubator. The fluorescence signal was measured for 30 minutes in every 5 minutes after induction of oxidative stress in the Hidex Chameleon Microplate Reader (λ_{exc}: 485 nm, λ_{em}: 535 nm) using MikroWin 2000 program.

2.8. RNA Isolation, Reverse Transcription, and Real-Time RT-PCR. Total RNA was isolated from FHs74Int cells by Genead Total RNA Mini Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan). Equal RNA was reverse-transcribed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Life
Technologies) to generate the first-stranded cDNA. The mRNA expressions were determined by real-time RT-PCR using the LightCycler 480 SYBR Green I Master enzyme mix on a Light Cycler 480 system (Roche Diagnostics, Mannheim, Germany). The nucleotide sequences of the primer pairs are shown in Table 1. The results were analyzed by the LightCycler 480 software version 1.5.0.39 (Roche Diagnostics). The relative mRNA expression was determined in comparison with RN18S as an internal control using the \( \Delta \Delta C_t \) method [18]. Data were normalized and presented as the ratio of their control values.

2.9. Immunofluorescence Staining. The localization of PARK7, ZO-1, and the cytoskeletal actin architecture was investigated by immunofluorescence staining on frozen biopsy samples and FHs74Int cells. After repeated washing

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with PBS, slides were permeabilized with Cytofix/Cytoperm (BD Pharmingen, San Diego, CA, USA) for 15 minutes at RT, washed with Perm/Wash Buffer solution (BD Pharmingen), and incubated with a primary antibody specific for PARK7 (ab18257; rabbit, 1:1000, Abcam, Cambridge, US), ZO-1 (ab96587; rabbit, 1:1000, Abcam), or Alexa Fluor® 546 phalloidin (7.5 units/mL, A22283; Thermo Fisher Scientific) for 1 hour at RT. In case of PARK7 and ZO-1 staining, slides were incubated with antirabbit Alexa Fluor 568®-conjugated secondary antibody (1:1000, A11036; Thermo Fisher Scientific) or antirabbit Alexa Fluor 488®-conjugated secondary antibody (1:1000, A21206; Thermo Fisher Scientific) for 30 minutes at RT. Thereafter, the slides were washed with a Perm/Wash Buffer solution and coverslipped with ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Sections were analyzed with an Olympus IX81 fluorescent microscope system.

2.10. Graphical Analysis of Damaged Cell Ratio. The ratio of phalloidin-stained FHs74Int cells with different cytoskeletal status was analyzed. The images were taken with 10x objective, and each cell from the field of view (n = 400-500/treatment group) was categorized manually into three groups. Thereafter, cells with a healthy cytoskeleton, cells with a damaged cytoskeleton, and burst cells were counted using the ImageJ 1.48 software (The National Institutes of Health, Bethesda).

2.11. Statistical Analysis. The statistical evaluation of data was performed by the GraphPad Prism 6.01 software (GraphPad Software Inc., La Jolla, CA, USA). After testing normality with the Kolmogorov-Smirnov test, the raw data of real-time RT-PCR measurements were analyzed with the Mann–Whitney U test to determine the differences between the corresponding groups. Multiple comparisons of raw data derived from MTT, LDH, Annexin V apoptosis, ROS assays, and ex vivo mucosal permeability measurements were performed using multiple t-test and ordinary two-way ANOVA with Dunnett correction. The ratio of cells with healthy or damaged cytoskeleton after various treatments was compared using the chi-square test. p ≤ 0.05 was considered as statistically significant.

3. Results

3.1. The Presence of PARK7 in Duodenal Epithelial Cells. An increased PARK7 immunopositivity was observed in the epithelial cells of the duodenal crypt and in the lamina propria of duodenal biopsies derived from children with CD compared to controls (Figure 1(a)). A definite PARK7 immuno-positivity was also present in the cytoplasm and nucleus of FHs74Int cells (Figure 1(b)).

3.2. Comp23 Prevented Intracellular ROS Accumulation. The effect of Comp23 on the H2O2-induced ROS accumulation in the FHs74Int duodenal epithelial cells was investigated by DCFDA-based ROS assay (Figures 2(a) and 2(b)). The treatment with H2O2 resulted in an increasing accumulation of ROS in a dose-dependent manner, which was reduced by 0.001 μM Comp23 at each H2O2 concentration. The oxidative stress induced by H2O2 resulted in decreased NRF2, NQO1, and GCLC and elevated HMOX1 mRNA levels, while cotreatment with Comp23 increased the expression of NRF2, HMOX1, NQO1, TRX1, and GCLC (Figure 2(c)).
3.3. Comp23 Protected Cells against Oxidative Stress-Induced Cytotoxicity. The effect of Comp23 treatment on the H$_2$O$_2$-induced cytotoxicity of FHs74Int duodenal epithelial cells was investigated by MTT, LDH, and Annexin V apoptosis assays. The treatment with H$_2$O$_2$ resulted in decreased viability, which was significantly restored by Comp23 at a wide concentration range (Figure 3(a)). Accordingly, the enzyme activity of LDH, released from the injured cells, was significantly lower in the supernatant of Comp23-treated cells (Figure 3(b)). Similarly, the Comp23 treatment significantly reduced the percentage of apoptotic cells in the H$_2$O$_2$-treated group (Figures 3(c) and 3(d)).

The oxidative stress induced by H$_2$O$_2$ resulted in decreased TP53, BCL2, and BAX and elevated PCNA mRNA levels, while cotreatment with Comp23 increased the expression of TP53, PCNA, CDKN1A, BCL2, and BAX (Figure 3(e)).

3.4. Comp23 Improved the Cytoskeletal Status under Oxidative Conditions. The architecture of the actin cytoskeleton of mucosal epithelial cells was visualized by phalloidin staining in duodenal biopsy samples of children with CD and controls and also in FHs74Int epithelial cells (Figure 4(a)). The fluorescence signal was intense and continuous at the apical, basal, and lateral surface of the epithelial cells both in the villi and in the crypts of the control children. However, the healthy architecture of the actin cytoskeleton was disturbed in the mucosal epithelial cells of children with CD, and instead, granular staining appeared in the cytoplasm. The H$_2$O$_2$ treatment disrupted the actin filaments of the FHs74Int cells shrinking them into intracellular dots. In some cells, oxidative stress led to cell death as indicated by the increased number of burst cells (white arrows). Comp23 treatment of the cells largely reduced the signs of

Figure 2: Effect of Comp23 on ROS accumulation in FHs71Int cells. FHs74Int duodenal epithelial cells were treated with different concentrations of H$_2$O$_2$ in the absence or presence of Comp23 (0.001 μM). Scatter plot (a) serves as a representative figure of the time-related fluorescence intensity after treatment with 1000 μM H$_2$O$_2$ with or without Comp23. Violin plot (b) indicates the slope distribution of time-related DCFDA fluorescence intensity change in the given group at different concentrations of H$_2$O$_2$. Relative mRNA expressions (c) at 1000 μM H$_2$O$_2$ concentration were determined by comparison with 18S ribosomal RNA as an internal control. Results are presented as mean ± SD (n = 5). $p < 0.05$ vs. Ø compound at the concerning H$_2$O$_2$ concentration (multiple t-test); *$p < 0.05$ vs. control+Ø compound (Mann-Whitney U test); #{$p < 0.05$ vs. H$_2$O$_2$+Ø compound (Mann-Whitney U test).
Figure 3: Continued.
Oxidative stress and consequent accumulation of damaged intracellular macromolecules [19, 20] are important components of mucosal deterioration and increased intestinal permeability of patients with CD [21]. Recently, Moretti et al. reported that the production of ROS strictly correlates with the severity of intestinal damage; therefore, they recommended the routine determination of serum antioxidant capacity to monitor the efficiency of the gluten-free diet of patients with CD [22].

Previously, our research group demonstrated the increased presence of PARK7 in the small intestinal mucosa of children with CD [8]. PARK7 is an ubiquitously expressed antioxidant protein, in which significance is clearly demonstrated in the pathomechanism of different neurodegenerative diseases [12]. However, our knowledge is limited about the possible role of PARK7 in the preservation of the epithelial layer integrity in the inflamed intestine.

In the present study, in accordance with our previous study [8], we found that PARK7 is abundantly present in the duodenal epithelial cells of therapy-naive children with CD, and also in FHs74Int duodenal epithelial cells (Figure 1).

Therefore, in the following experiments, FHs74Int cells exposed to oxidative stress were used to investigate the role of PARK7 in the maintenance of mucosal integrity. In these experiments, Comp23, a PARK7-binding compound developed by Kitamura et al. [23, 24], was used to enhance the antioxidative function of PARK7. They suggest that Comp23 can prevent the excessive oxidation of PARK7 by binding to its C106 cysteine residue, thereby preserving the antioxidant function of PARK7.

In the first set of experiments, we found that Comp23 decreased the intracellular accumulation of ROS in H$_2$O$_2$-treated FH74Int cells (Figure 2). Our result is in line with previous studies demonstrating that treatment with recombinant PARK7 decreased the oxidative stress-induced ROS accumulation in neuronal cells [25, 26]. Similarly, previous studies also demonstrated that the lack of PARK7 is associated with enhanced ROS accumulation in various cell types proposing the determinative role of PARK7 in the antioxidant defence [27–29].

Investigating the molecular mechanism by which PARK7 may modulate the oxidative stress, we found that Comp23 treatment increased the expression of nuclear factor
Figure 4: Cytoskeletal disruption of intestinal mucosa in celiac disease (CD) and of duodenal epithelial cells. Actin filament structure was investigated by phalloidin (orange) staining in duodenal mucosa of children with CD and controls (a) and in FHs74Int duodenal epithelial cells (b) after treatment with H$_2$O$_2$ (1000 μM) in the absence or presence of Comp23 (0.001 μM). Cell nuclei were counterstained with DAPI (blue). White arrows indicate burst cells. Scale bar: 500 μm, 100 μm, and 50 μm. The percent of intact and damaged cells (c) was determined by graphical analysis. Results are presented as the percentage of total cells in a field of view (n = 400-500/treatment group). * $p$ < 0.05 vs. control+Ø compound; # $p$ < 0.05 vs. H$_2$O$_2$+Ø compound (chi-square test).
Figure 5: Damage of cell adhesion in epithelial cells induced by oxidative stress. Colocalization of actin (red) and zonula occludens 1 (ZO-1, green) was investigated by immunofluorescence staining (a) in FHs74Int duodenal epithelial cells after treatment with \( \text{H}_2\text{O}_2 \) (1000 \( \mu \text{M} \)) in the absence or presence of Comp23 (0.001 \( \mu \text{M} \)). Cell nuclei were counterstained with DAPI (blue). Scale bar: 50 \( \mu \text{m} \). Relative mRNA expressions (b) in FHs74Int cells were determined by comparison with 18S ribosomal RNA as an internal control. Results are presented as mean + SD (\( n = 5 \)). * \( p < 0.05 \) vs. control+Ø compound (Mann-Whitney U test); # \( p < 0.05 \) vs. \( \text{H}_2\text{O}_2+\text{Ø} \) compound (Mann-Whitney U test).
erythroid 2-related factor 2 (NRF2) transcription factor, which is known as one of the major regulators of cellular antioxidant defence. In accordance, we found that Comp23 treatment increased the expression of a multitude of NRF2-dependent antioxidant response genes, including thioredoxin (TRX1), γ-glutamyl cysteine synthetase (GCLC), heme oxygenase (HMOX1), and NAD(P)H:quinone oxidoreductase 1 (NQO1) of the H₂O₂-treated FHs74Int cells (Figure 2).

Our findings, in line with the literature, strongly suggest that Comp23 significantly influences the activation of the PARK7-NRF2 axis thereby influencing the consequence of oxidative stress [30, 31]. Indeed, previously, it has been shown that the activation of the NRF2 pathway attenuates the stress-related apoptosis in numerous cell types [32–34].

In accordance with decreased intracellular ROS accumulation and increased antioxidant preparedness of the epithelial cells, we demonstrated that Comp23 treatment improved the survival of H₂O₂-treated cells (Figure 3). Our finding is in accordance with the results of Kitamura et al., describing for the first time the PARK7-dependent protective effect of Comp23 against H₂O₂-induced cell death in neuroblasts [23, 24]. Investigating the possible molecular mechanism, we found that Comp23 treatment increased the expression of tumor antigen P53 (TP53) transcription factor and its target genes [35], including cyclin-dependent kinase inhibitor 1-p21 (CDKN1A), proliferating cell nuclear antigen (PCNA), apoptosis regulator Bcl-2 (BCL2), and apoptosis regulator BAX (BAX) (Figure 3). These results are in accordance with the previous findings, demonstrating that PARK7 modulates P53-P21 pathway in an oxidation status-dependent manner [36]. P53 is a determinative regulator of the cell cycle, having a dual function during stress response as it prevents intracellular oxidation to ensure cell survival and repair at a low level of damage, but it also can induce apoptosis in case of severe cellular injury [37, 38]. Taken together, our results suggest that the antiapoptotic effect of Comp23 is owing to the combined activation of NRF2 and P53 pathways.

In the next set of experiments, we investigated the role of PARK7 in the maintenance of mucosal integrity of CD patients. Investigating the duodenal biopsies of CD patients, we found that the actin cytoskeleton of epithelial cells is seriously injured. Indeed, we found that the well-contoured structure of the actin network, a characteristic for the healthy cells, collapsed resulting in granular dots in the epithelial cells of inflamed mucosa (Figure 4).

Examining the effect of oxidative stress on FHs74Int cells, we found that similarly to the duodenal epithelial cells of the inflamed mucosa, the H₂O₂ treatment destroyed the filamentous actin network of the cells, resulting in dot-like aggregates (Figure 4). The oxidative damage also decreased the expression of CAMs, including zona occludens 1 (ZO1), CDH1, VCL, and ITGB5, and led to the dissociation of ZO-1 from the cytoskeleton of the cells (Figure 5). Our observation is in line with previous literary knowledge about the oxidative stress-induced structural and functional modification of intracellular macromolecules, including filamentous actin resulting in its depolymerisation and thereby the disruption of the physiological cytoskeleton [39]. Actin filaments are associated with the intracellular localisation of CAMs, including cadherins, tight-junction, and focal adhesion proteins [40, 41], ensuring the stable cell surface and cell-cell adhesion and thereby the maintenance of the mucosal barrier function [42, 43]. Interestingly, we also observed that the Comp23 treatment increased the expression of CAMs and, more importantly, preserved the healthy architecture of the actin cytoskeleton-CAM complexes in the epithelial cells exposed to oxidative stress (Figures 4 and 5).

Therefore, to further investigate the significance of PARK7 in the maintenance of the mucosal integrity, we examined its effect on the permeability of H₂O₂-treated small intestinal tissue preparations ex vivo. According to our previous in vitro experiments, we found that Comp23 greatly decreased the permeability of the small intestinal sacs exposed to oxidative stress (Figure 6), confirming that PARK7 has a determinative role in the control of oxidative stress and in the maintenance of mucosal integrity. The damage of the intestinal barrier leads to increased penetration of luminal noxious molecules, including gliadin or luminal bacteria into the lamina propria of the intestine thereby exacerbating mucosal inflammation of patients with CD [40]. Therefore, the preservation of the intestinal barrier may offer a new therapeutic approach to inhibit intestinal inflammation in CD patients.

5. Conclusion

In summary, we demonstrated the protective effect of the PARK7-binding Comp23 in the duodenal epithelial cells exposed to oxidative stress. We showed that PARK7 induced the expression of stress-response elements, including antioxidant and cell-cycle regulator genes, and normalized the
expression of CAMs in the epithelial cells exposed to oxidative stress (Figure 7). These processes contribute to the maintenance of mucosal integrity as demonstrated by our ex vivo experiment and thereby may moderate the small intestinal inflammation in CD.

Although there are limitations of our study, including the lack of quantitative measurement at the protein level, we hope that our study made a great progress in the understanding of the biological effects of PARK7 in the pathomechanism of CD. Moreover, our present work may contribute not only to the better understanding of the pathomechanism of CD, but also to the development of new therapeutic approaches.

Abbreviations
CAM: Cell adhesion molecule
CD: Celiac disease
DCFDA: Dichlorofluorescein diacetate
FBS: Fetal bovine serum
LDH: Lactate dehydrogenase
PARK7: Parkinson’s disease 7
PBS: Phosphate-buffered saline
ROS: Reactive oxygen species
RT: Room temperature.

Data Availability
The data used to support the findings of this study are included in the article.

Conflicts of Interest
The authors declare no conflict of interest.

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