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RESEARCH ARTICLE

Conjugated linoleic acid protects against gliadin-induced depletion of intestinal defenses

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Scope: The involvement of oxidative stress in gluten-induced toxicity has been evidenced in vitro and in clinical studies but has never been examined in vivo. We recently demonstrated the protective activity of conjugated linoleic acid (CLA), which functions by the activation of nuclear factor erythroid 2-related factor2 (Nrf2), a key transcription factor for the synthesis of antioxidant and detoxifying enzymes (phase 2). Here, we evaluate the involvement of nuclear factor erythroid 2-related factor2 in gliadin-mediated toxicity in human Caco-2 intestinal cells and in gliadin-sensitive human leukocyte antigen-DQ8 transgenic mice (DQ8) and the protective activity of CLA.

Methods and results: Gliadin effects in differentiated Caco-2 cells and in DQ8 mice, fed with a gliadin-containing diet with or without CLA supplementation, were evaluated by combining enzymatic, immunochemical, immunohistochemical, and quantitative real-time PCR (qRT-PCR) assays. Gliadin toxicity was accompanied by downregulation of phase 2 and elevates proteasome-acylpeptide hydrolase activities in vitro and in vivo. Notably, gliadin was unable to generate severe oxidative stress extent or pathological consequences in DQ8 mice intestine comparable to those found in celiac patients and the alterations produced were hampered by CLA.

Conclusion: The beneficial effects of CLA against the depletion of crucial intestinal cyto-protective defenses indicates a novel nutritional approach for the treatment of intestinal disease associated with altered redox homeostasis.

Keywords:
Conjugated linoleic acid / Gluten toxicity / Nrf2-mediated defenses / Proteasome-acylpeptide hydrolase activity

1 Introduction

Celiac disease (CD) is a chronic inflammatory pathology of the small intestine, resulting from a complex interplay between environmental and genetic factors [1]. Indeed, the main wheat gluten protein (gliadin) and related proteins from rye and barley represent the environmental factors responsible for the immunotoxic response in CD patients [2]. During the past few years, significant progress has been made in clarifying the different factors that contribute to the pathogenesis of CD. In addition, the presence of human

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Abbreviations: APEH, acylpeptide hydrolase; **CD,** celiac disease; **CLA,** conjugated linoleic acid; **GCL,** γ-glutamylcysteine ligase; **GFD,** gluten-free diet; **GSHtot,** total thiols; **GSSG,** glutathione disulfide; **GST,** glutathione *S*-transferase; **HO-1,** heme oxygenase-1; **IAP,** intestinal alkaline phosphatase; **MGFD,** Modified GFD; **NQO1,** NAD(P)H:quinone oxidoreductase; **Nrf2,** nuclear factor erythroid 2-related factor 2; **PC,** protein-bound carbonyls; **pt-glia,** peptic–triptic digest of gliadin; **StD,** standard diet; **tTG,** tissue transglutaminase

leukocyte antigen (HLA) class II DQ2 and/or DQ8 [3] has been linked to the events leading to gluten toxicity and intestinal damage; however, their presence is necessary but not sufficient for the development of such disease [4]. Transgenic mice expressing human leukocyte antigen-DQ8 in the absence of endogenous mouse class II genes, nontransgenic for human CD4 [5] (DQ8 mice) are widely used for investigating gluten sensitivity [6–8], but in the absence of immunization, gluten is generally well tolerated by the intestine of both DQ8 or DQ2 mice as oral tolerance is still operative [8, 9]. However, despite the large amount of data on immunological factors, the biochemical mechanisms underlying gluten toxicity are not completely understood.

Among the different mechanisms, oxidative stress has been implicated in the pathophysiology of CD by clinical evidence [10] and by in vitro studies [11] but the underlying mechanisms are still unclear. Indeed, oxidants and/or a defective antioxidant defense play a crucial role in the generation of the oxidative stress implicated in the pathogenesis of inflammatory diseases. The epithelium of the small intestine is a dynamic system that is continuously renewed by a differentiation process, and redox status has an important role in these stages. Moreover, owing to the constant exposure to potentially noxious substances, intestinal mucosa is endowed with efficient defenses to preserve cellular integrity and tissue homeostasis. Among these defenses, the nuclear factor erythroid 2-related factor2 (Nrf2) transcription factor has been recognized as the key regulator [12], and its activation, via the modification of cysteine residues of Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm, triggers Nrf2 release from the Keap1/Nrf2 complex. The ensuing translocation of Nrf2 into the nucleus activates the transcription of a battery of genes coding for antioxidant and detoxifying proteins (phase 2 enzymes: γ-glutamylcysteine ligase, GCL; glutathione *S*-transferase, GST; glutathione peroxidase, GSHPx; NAD(P)H:quinone oxidoreductase, NQO1; and heme oxygenase-1, HO-1) [13].

Besides the Nrf2 pathway, several proteases have been reported to play a detoxifying role by degrading oxidatively damaged cytosolic proteins. The ubiquitin–proteasome pathway plays a key role in a broad array of cellular processes (cell cycle, apoptosis, and differentiation) [14], and the proteolytic activity of the proteasome, a major multicomponent enzymatic system, controls and regulates the accumulation of potentially cytotoxic protein aggregates in an ATP- and ubiquitin-independent manner [15]. Additionally, acylpeptide hydrolase (APEH), a novel serine-peptidase enzyme belonging to the prolyl-oligopeptidase family, was recently demonstrated to contribute to the proteasome-mediated elimination of potentially cytotoxic proteins [16].

The potential protective effect displayed by n-3 PUFA on inflammatory bowel disease has been recently reviewed [17] and similarly to n-3 PUFA, conjugated linoleic acid (CLA) has been recognized to promote beneficial effects in animal models of several pathologies, including inflammatory, autoimmune diseases [18], and experimentally induced colitis [19]. In these studies, the modulatory activity of n-3 PUFA and CLA on the peroxisome proliferator-activated receptor γ-NF-κB pathway was demonstrated to contribute to the anti-inflammatory activities of these compounds. In addition, our recent data showing the ability of CLA to enhance Nrf2-mediated defenses in vitro and in vivo raises the possibility that CLA can also exert its protective effects through this mechanism of action [20].

The major objective of this study was to evaluate the detrimental effects of gluten on intestinal antioxidant and detoxifying defenses in vitro and in vivo and to investigate the protective effects of CLA against gluten-induced toxicity in the small intestine of DQ8 mice.

2 Materials and methods

2.1 Reagents

An isomeric mixture of CLA (38.5% t10, c12, 37.4% c9, t11), known as TonalinTM, was from Natural. *N*-Suc-LLVY-AMC, BSA, α-lactalbumin, and other chemicals of the highest purity were from Sigma-Aldrich (St. Louis, MO, USA) or Calbiochem (La Jolla, CA, USA).

2.2 Cell culture and in vitro experiments

The Caco-2 cell line was obtained from the American Type Culture Collection (ATCC, Gaithersburg, MD, USA) and was studied between passages 16 and 35. This is a well-described cell line that spontaneously differentiates and becoming morphologically and functionally similar to enterocytes, and therefore differentiated cells. Unless otherwise specified, in the present study, cells after 21 days postseeding (differentiated) were incubated for 48 h at 37°C with 1 mg/mL of a peptic–tryptic digest of gliadin (pt-glia) or with the same amount of α-lactalbumin (control) prepared according to a published protocol [21].

2.3 In vivo experiments

Transgenic DQ8 mice were bred and maintained in pathogen-free conditions at our animal facility and used at the age of 6–12 wk. All procedures for the use of laboratory animals met the guidelines of the Italian Ministry of Health (permission accreditation no. 164/99-A). Animals were from a colony reared for several generations on a gluten-free diet (GFD) (Altromin-MT-mod, Rieper S.p.A. Italy).

Gluten toxicity in vivo was initially studied in animal groups ($n = 9$ each) fed for 3 wk with a standard diet (StD) or with a GFD. As the StD and GFD differed in both soy and

wheat proteins, we subsequently used a modified GFD (MGFD), adding only wheat proteins to the GFD to specifically analyze the in vivo toxicity of gluten (Supporting Information Table 1). Four groups of female DQ8 mice ($n = 9$ each) were used, and two of these groups were switched to MGFD for 3 wk. Starting from 2 days before the change of diet and during the trial, two groups of animals (one fed with GFD and the other with MGFD) were intraorally administered 20 mg of CLA/day (five times/wk). At the end of this time, the mice were sacrificed. The amount of CLA administered, upon normalization to the body surface area [22], corresponded to a dose (4.9 g/day) comparable to that used in the clinical trials [23]. All mice were maintained under strict pathogen-free conditions and had free access to drinking water.

2.4 Protein extract and brush border membrane vesicle preparation

Cytosolic and nuclear extracts from Caco-2 cells and intestinal tissue were prepared by using the previously published protocols [20] and, if not used immediately, the prepared extracts were frozen on dry ice and stored in aliquots at −80°C. Before their use, protein concentration was determined by protein assay (Bio-Rad). Brush border membrane vesicles were prepared from small intestine samples (approx., 10 mg) accordingly to a published method [24].

2.5 Proteasome and APEH assays

APEH activity was measured using the substrate, acetyl-A-pNA (Bachem). The reaction mixture (1 mL) containing the appropriate amount of cell extract in 50 mM Tris-HCl buffer, pH 7.5, was preincubated at 37°C for 2 min. Then, 1 mM acetyl-A-pNA was added, and the release of *p*-nitroanilide $\varepsilon 410 = 8800\ M^{-1}\,cm^{-1}$) was measured following the absorbance increase at 410 nm on a Cary 100 SCAN (VARIAN) spectrophotometer equipped with a thermostated cuvette. APEH activity is expressed in IU.

The synthetic fluorescent substrate, *N*-Suc-LLVT-AMC, was used for the measurement of the chymotrypsin-like activity of the 20S proteasome at a final concentration of 80 μM. The reaction mixture (0.9 mL) containing partially purified proteasome was preincubated (as above) in buffer. *N*-Suc-LLVT-AMC was added, and the release of fluorescent product (7AMC: 7-amino 4-methylcoumarin) was monitored for 5 min by a fluorimeter (Perkin–Elmer LS 50B) ($\lambda_{Ex} =$ 380 nm and $\lambda_{Em} = 460$ nm).

Partial purification of the proteasome from Caco-2 and/or individual mouse small intestinal protein extracts (0.7 mg) was carried out by gel filtration chromatography on a Superdex 200 column connected to a SMART system and eluted at 0.1 mL/min in 50 mM Tris-HCl, 0.1 M NaCl, pH 7.5. Active fractions, for both APEH and proteasome, were separately collected and used for further analysis. A typical chromatogram is shown in Supporting Information Fig. 1.

2.6 RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from the small intestine of DQ8 mice that were fed with GFD or MGFD and with or without the CLA supplement by using the MELT^{TM} Total Nucleic Acid Isolation System (Ambion). Total RNA was then reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). A total of 50 ng of reverse-transcribed complementary DNA was amplified by quantitative real-time PCR (qRT-PCR) on an iCycler iQTM (Bio-Rad) using 300 nM gene-specific primers, iQTM SYBR Green Supermix (Bio-Rad) and the following PCR conditions: 1 cycle at 95°C for 10 min (denaturation), 95°C for 15 s (amplification) and 40 cycles of 60°C for 30 s, and 72°C for 30 s. The $MgCl_2$ concentration used was 3 mM for GAPDH and GCL and 6 mM for NQO1. The expression level of GAPDH was used as an internal control. Raw cycle threshold values (C_t values) obtained for GCL and NQO1 were subtracted from the C_t value obtained for GAPDH transcript levels. The final graphical data were derived from the $2^{-\Delta\Delta Ct}$ formula, where $\Delta\Delta C_t = (C_{t,\ target} - C_{t,\ GAPDH})_{sample} - (C_{t,\ target} - C_{t,\ GAPDH})_{control}$, where "sample" mice are those fed with MGFD with or without CLA or with GFD and CLA, and "control" mice are those fed with GFD. The primers utilized were as follows: GCL, 5′-CAAAGGCAGTCAAATCTGGTG-3′ and 5′-TGGAGCAGCTGTATCAGTGG-3′; NQO1, 5′-TTCTCTGGCCGATTCAGAGT-3′ and 5′-TCTGGTTGTCAGCTGGAATG-3′; and GAPDH, 5′-TAGACTCCACGACATACTCAGCA-3′ and 5′-GTCGGTGTGAACGGATTTG-3′.

2.7 Immunohistochemistry and microscopic evaluation

The preparation of proximal jejunum fragments and the subsequent analysis (morphometrical, immunohistochemical and cell apoptosis determination) were carried out according to the published protocols [7].

2.8 Statistical analysis

Values are presented as the mean ± SD. Statistical analysis was performed with GraphPad Instat 3 software (San Diego, CA, USA). Groups were compared by the Student's *t*-test, and $p < 0.05$ was considered as significant. Correlation analysis was performed using the Statistical Package for Social Sciences (SPSS version 8.0; SPSS, Chicago, IL, USA).

3 Results

3.1 Redox status and detoxifying enzyme activities are influenced by the differentiation status of Caco-2 cells

The majority of in vitro investigations aimed at the estimation of the pro-oxidant activity of gliadin have been carried out using undifferentiated cultures [11]. To investigate the variation of several antioxidant and detoxifying molecules at different stages of cell differentiation, total thiols (GSHtot), glutathione disulfide (GSSG) concentration, phase 2 enzymes (γ-GCL, GST, and NQO1), proteasome, and APEH activities were assayed in Caco-2 cells at different stages of differentiation, as evaluated by following the intestinal alkaline phosphatase (IAP) activity. As expected, significantly higher GST activity ($p<0.005$) was found in differentiated Caco-2 cells as compared with undifferentiated cultures (Supporting Information Fig. 2A). By contrast, intracellular GSHtot, but not GSSG content, progressively declined during differentiation (Supporting Information Fig. 2B). Similarly, a reduction in both NQO1 and GCL activities occurred during this process ($p<0.01$) (Supporting Information Fig. 2C). Proteasome activity exhibited a strong increase during the proliferative phase, with a maximum reached in confluent cultures followed by a progressive reduction thereafter. A similar trend was observed for APEH specific activity, but the maximum was reached 7 days after confluence. Higher specific activity of APEH was found in differentiated cells as compared with undifferentiated ones (Supporting Information Fig. 2D).

3.2 The pro-oxidant activity of gliadin reduces cell viability and detoxifying enzyme activities but not tissue transglutaminase expression

The influence of cell differentiation status on susceptibility to gliadin-induced oxidative stress was investigated. Intracellular GSHtot content was measured in preconfluent (6 days after plating), differentiating and in differentiated cells (14 and 21 days after plating, respectively) that were incubated for 48 h with increasing concentrations of pt-glia. The results showed a dose-dependent decline in intracellular GSHtot in comparison to untreated cells (control) that was independent of the differentiation stage (Fig. 1A).

To determine the effects of pt-glia exposure on differentiated Caco-2 cell viability, caspase-3 activity and the amount of lactate dehydrogenase (LDH) released in the media were measured upon 48 h of incubation with 0.5 or 1 mg/mL of pt-glia. Treatment with the highest concentration produced a significant pro-apoptotic effect when compared with cells exposed to an equal amount of a peptic–tryptic digest of α-lactalbumin (control) ($p<0.01$) (Fig. 1B). Similarly, increased levels of protein-bound carbonyls (PC) and a marked reduction of IAP activity ($p<0.05$) resulted from pt-glia treatment (Fig. 1C). In

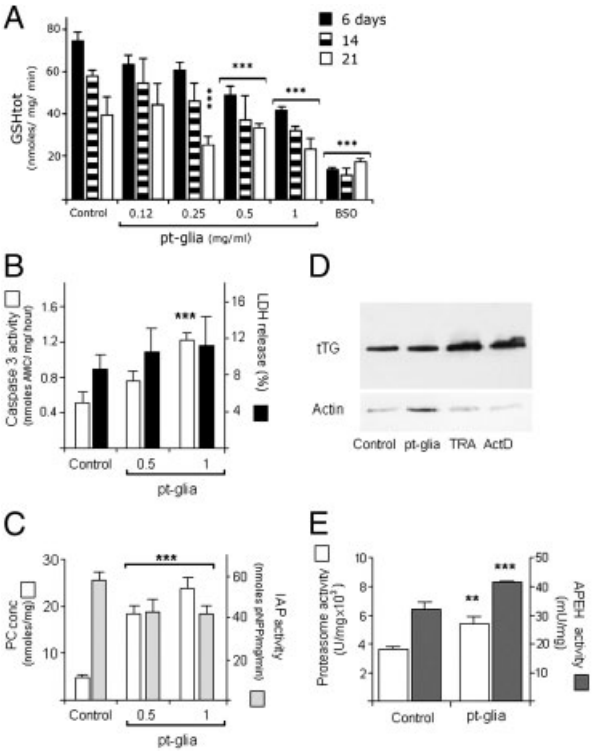

Figure 1. Gliadin exposure enhances apoptosis, PC accumulation, proteasome–APEH activities, and reduces IAP activity in Caco-2 cells (A). Caspase-3 activity and lactate dehydrogenase release were measured in Caco2 cells following 48 h of incubation with gliadin (B). PC levels and IAP activity were measured in differentiated cells exposed to 0.5 or 1 mg/mL of pt-glia (C). Representative Western immunoblot of tTG expression in protein extracts from differentiated Caco-2 cells incubated with 1 mg/mL of gliadin or treated with tTG activators (100 μM trans-retinoic acid: TRA or 50 μM actinomycin D: ActD) for 24 h. Actin was used as loading control (D). The chymotrypsin-like activity of the proteasome and APEH-specific activities were measured in differentiated cultures incubated with 1 mg/mL of pt-glia (E). Results are expressed as the mean ± SD. ***, **Significantly different ($p<0.005$ or <0.01) from controls.

contrast, no difference in tissue transglutaminase (tTG) expression was noted between cells incubated for 48 h with pt-glia and controls although treatment with known tTG activators produced a noticeable increase in tTG levels (Fig. 1D). Figure 1E shows the effect of pt-glia on proteasome and APEH activities. In agreement with the accumulation of PC proteins, a significant enhancement of both specific activities was observed (from 18.4 ± 0.2 to 27.0 ± 1.9 U/mg $\times 10^3$; $p = 0.002$ and from 32.0 ± 2.8 to 41.0 ± 3.2 mU/mg; $p = 0.012$ for proteasome and APEH activity, respectively).

3.3 Gliadin downregulates Nrf2-activated defenses in Caco-2 cells

GSH synthesis and its export from the cell are the main strategies to control GSH intracellular content [25]. To

examine the mechanism by which gliadin perturbs the redox status GSHtot and GSSG concentrations were measured in the cytoplasm and in the culture media of cells exposed to pt-glia for 48 h. Incubation with pt-glia decreased intracellular GSHtot ($p<0.001$) without affecting GSSG content (Fig. 2A). Moreover, a significant decline in GSHtot and GSSG concentrations was also found in the culture media when compared with the control (from 2.85 ± 0.53 to 1.82 ± 0.14 nmol/mg/min; $p=0.0021$ and from 1.47 ± 0.22 to 0.72 ± 0.42 nmol/mg/min; $p=0.014$, respectively) (Fig. 2B).

To further investigate the mechanism underlying the ability of gliadin to reduce intracellular GSHtot levels, we measured the effect of pt-glia treatment on the activity of GCL, the rate-limiting enzyme in GSH synthesis. As shown in Fig. 2C, a significant reduction in enzyme activity (from 8.15 ± 1.45 to 4.6 ± 1.74 nmol NAD/mg/min; $p<0.005$) was associated with the decrease of Nrf2 levels (57 kDa) in the nuclear extracts and the accumulation of the GCL caspase-cleaved form (60 kDa) in the cytoplasm of pt-glia-treated cells as compared with controls (Fig. 2C, inset). To further confirm the involvement of the Nrf2-ARE pathway, the effects of pt-glia exposure (1 mg/mL for 48 h) on NQO1 and GST activities was also investigated. The observed reduction in the enzymatic activities of both ($p=0.0008$ and 0.0052 for GST and NQO1, respectively) (Fig. 2D) confirms the gliadin ability to downregulate Nrf2-mediated defenses in vitro. The probable role of gliadin-mediated oxidative stress in the reduced activity of IAP was next examined. GSHtot concentration and IAP activity were measured in cells exposed to pt-glia and supplemented with or without buthionine sulfoximine (BSO), a specific inhibitor of GCL or with the antioxidant butylated hydroxyanisole (BHA). Besides the expected decrease in GSHtot and IAP activity produced by gliadin exposure, the two-fold reduction of IAP activity (following buthionine sulfoximine treatment) together with its significant increase in cells incubated with BHA ($p<0.05$) (Supporting Information Fig. 3) indicated the role of intracellular redox status in the regulation of IAP activity.

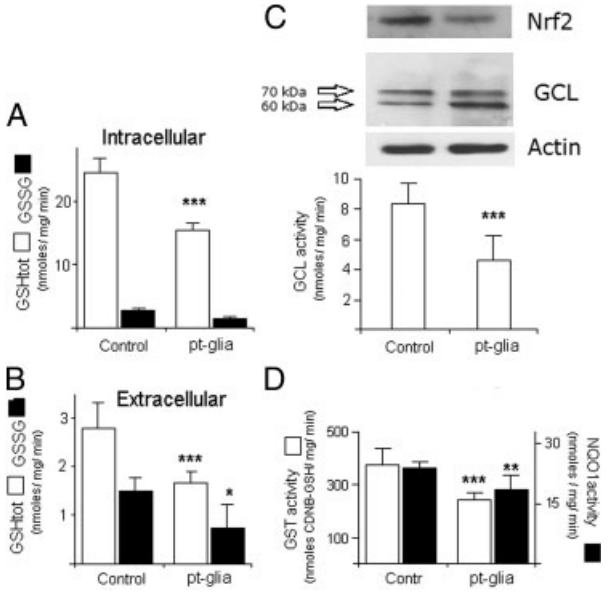

Figure 2. Gliadin exposure reduces intracellular phase 2 enzyme activity in vitro. GSHtot and GSSG concentrations were measured in differentiated Caco-2 cells (A) or in the culture medium following 48 h of incubation with 1 mg/mL of pt-glia, (B) GCL activity and (C) representative Western immunoblot showing GCL expression in differentiated cells following 48 h of exposure to 1 mg/mL of pt-glia. Actin was used as loading control (C, inset). GST and NQO1 activities were evaluated in Caco-2 cells exposed to 1 mg/mL of gliadin for 48 h. (D). Results are expressed as the mean $\pm$ SD. ***, **, *Significantly different ($p<0.005$, <0.01 or <0.05) from controls.

3.4 Gluten intake reduces Nrf2-activated defenses without pathological consequences in DQ8 transgenic mice

To identify possible deleterious effects of gluten intake on animal and intestinal redox status, detoxifying defenses and morphological alterations were examined in DQ8 mice following 3 wk of treatment with StD. The gluten intake did not influenced intestinal tTG expression (Fig. 3A) or caspase-3 activity in the small intestine (Fig. 3B). Similarly, gluten intake only produces minimal alteration of animal redox status as shown by the negligible variation in GSHtot levels (Fig. 3B, lower panel) or PC levels in blood serum sera (data not shown). In contrast, StD intake produced a significant alteration in GST activity and PC content in the intestines of these mice ($p<0.001$) (Fig. 3A). Interestingly, when the GSHtot content of individual mice was plotted against IAP values, a significant positive correlation between these levels was apparent ($r=0.66$; $p=0.036$) (Fig. 3B, upper panel). Notably, the lower intestinal GSHtot content, decreased GSHPx, and IAP activities measured in StD-treated animals compared with mice fed with GFD ($p=0.0004$, 0.025, and 0.0007, respectively) were not associated with some typical pathological alterations of CD (morphological change or increased number of CD3+ lymphocytes) (Table 1).

3.5 Biopsies from CD patients exhibit high oxidative stress conditions

Next, to compare the alterations produced by gluten intake in the small intestine of DQ8 mice to those occurring in CD, tTG expression, PC amount, GST, IAP, and proteasome-APEH specific activities were examined in human biopsies from CD patients. Intestinal biopsies of healthy volunteers were used as controls. Significantly lower GST ($p<0.001$) and proteasome–APEH activities ($p=0.035$ and $=0.023$, respectively) were detected in CD samples as compared with

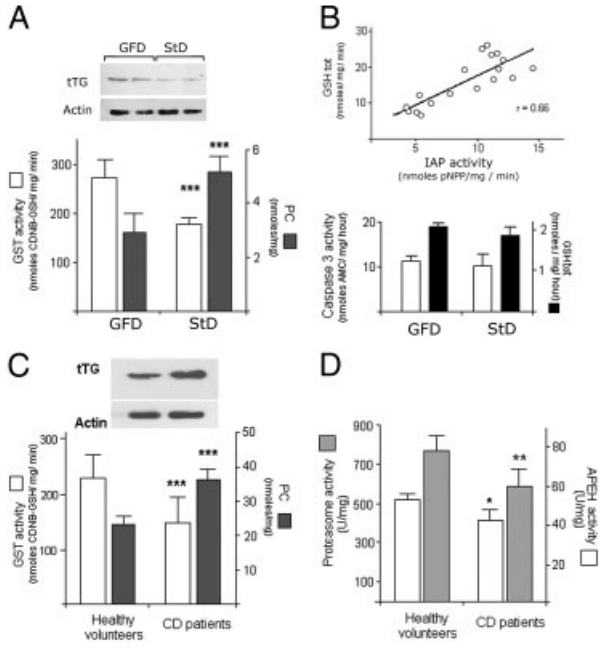

Figure 3. Elevated oxidative stress is observed in CD patient biopsies in comparison to the small intestine of gluten-fed DQ8 transgenic mice. PC accumulation, GST activity (A), and caspase-3 activity were measured in the small intestine of DQ8 mice fed for 3 wk with GFD or StD. (B). Representative Western immunoblot of tTG expression in the intestine of mice receiving different treatments. Actin was used as loading control (A, inset). PC accumulation, GST activity, and (C) proteasome–APEH-specific activities were measured in human biopsies from healthy or CD patients (D). Representative Western immunoblot showing tTG expression in human intestinal proteins. Actin was used as loading control (C, inset). Results are expressed as the mean ± SD from triplicate analyses. ***, **Significantly different ($p < 0.005$ or < 0.01) from GFD-fed mice.

Table 1. The effects of GFD or gluten-containing diet (StD) intake on DQ8 mice intestine

	GFD	StD
GSHtot (nmol/mg/min)	19.2 ± 2.1	9.6 ± 1.1***
GPx (nmol/mg/min)	121.5 ± 31.2	90.4 ± 28.5*
IAP (nmol pNPP/μg/min)	12.5 ± 1.6	8.5 ± 1.3***
Sucrase isomaltase (U/mg/h)	2.0 ± 1.5	2.2 ± 1.0
Height of intestinal villi (μm)	670 ± 23	640 ± 46
$CD3^+$ (cells/mm)	20.5 ± 0.7	20.8 ± 3.5

Results are given as mean ± SD from triplicate analysis.
***,*Significantly different ($p < 0.005$ or < 0.05) from controls.

those from healthy volunteers (Fig. 3C and D). As expected, a marked increase in tTG expression and PC content was found in CD patients when compared with healthy individuals (Fig. 3C) and a conspicuous reduction of IAP activity was also observed in CD patients (data not shown).

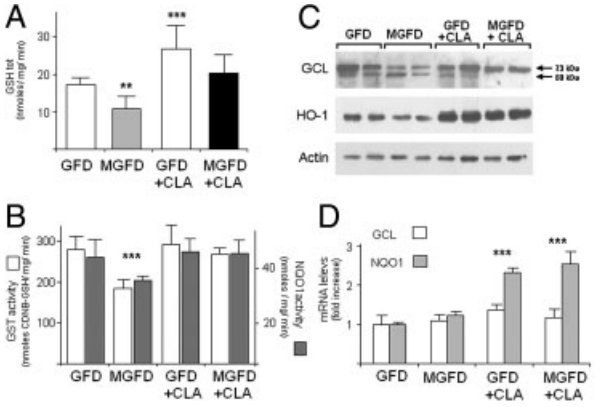

Figure 4. CLA impairs the gluten-mediated decrease in antioxidant/detoxifying defenses in vivo. Small intestine samples from DQ8 mice fed with GFD or MGFD and with or without the CLA supplement were examined for their GSHtot content (A) and GST and NQO1 activities (B). Results are expressed as the mean ± SD from triplicate analyses. ***, **Significantly different ($p < 0.005$ or < 0.01) from GFD-fed mice (C). Representative Western immunoblot of the intestinal expression of GCL and HO-1 from differently treated DQ8 mice. Actin was used as loading control (C). mRNA levels of GCL and NQO1 from the intestines of DQ8 mice receiving different treatments. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G6PD) was used as control, and results were normalized by taking the mRNA levels of GFD in mice to be 1 (D).

3.6 CLA treatment impairs the gluten-mediated decrease in intestinal defenses

To specifically analyze the effect gluten intake in DQ8 mice, the animals were fed with a diet which differed from GFD only for the addition of wheat proteins (MGFD). Animals receiving GFD were used as controls. Feeding with MGFD reduced intestinal GSHtot content ($p = 0.0012$) (Fig. 4A) and GST and NQO1 activities ($p = 0.0031$ and 0.001, respectively) (Fig. 4B), as well as HO-1 and GCL (full-length form, 70 kDa) expression (Fig. 4C). Remarkably, CLA administration in MGFD mice resulted in a significant enhancement of GSHtot content (Fig. 4A) ($p = 0.0002$) elevated the expression of the HO-1 and GCL proteins (full-length form, 70 kDa) (Fig. 4C) and resulted in increased mRNA levels of GCL and NQO1 (Fig. 4D). As expected, MGFD treatment was associated with significant increases in PC levels ($p = 0.0003$) (Fig. 5A), a two-fold decrease in IAP activity (0.0007) (Fig. 5B) and enhanced proteasome–APEH specific activities when compared with controls ($p = 0.003$ and < 0.001, respectively) (Fig. 5C, 5D). To evaluate the Nrf2 involvement in gluten-mediated toxicity, CLA was used as Nrf2 inducer. Noticeably, CLA administration in MGFD-treated animals restored PC and GSHtot levels (Figs. 4A and 5A) and reinstated intestinal protective enzymes activity that had been perturbed by gluten (Figs. 4B and 5B–D). Finally, when individual average values of proteasome activity were plotted against APEH or PC levels,

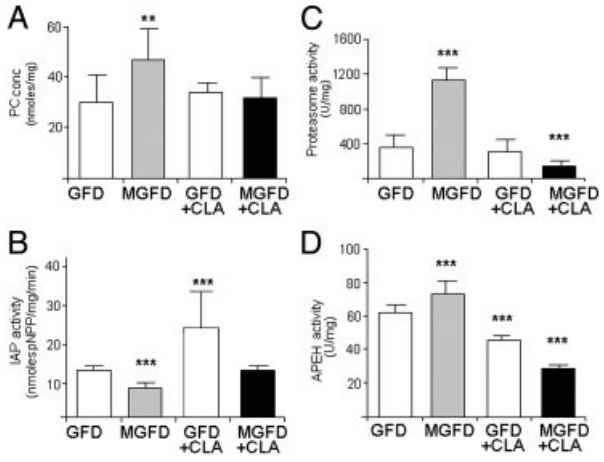

Figure 5. CLA protects against gluten-mediated oxidative stress in vivo. Duodenum samples from DQ8 mice fed with GFD or MGFD and with or without the CLA supplement were examined for (A) their PC concentration and (B) IAP, (C) proteasome and (D) APEH activities, which were measured in the mice receiving different treatments. Results are presented as the mean ± SD from triplicate analyses. ***, **Significantly different ($p < 0.005$ or < 0.01) from controls.

a significant positive correlation was found ($r = 0.935$, $p = 0.0016$ and $r = 0.926$; $p = 0.0001$, respectively).

4 Discussion

The present study confirms the in vitro pro-oxidant activity of gliadin on differentiated Caco-2 cells and demonstrates, for the first time, its in vivo ability to downregulate crucial intestinal defenses. Furthermore, the reported results verify CLA-induced enhancement of the Nrf2 pathway and the central role of this mechanism in the mediation of intestinal protection.

The lower GSHtot content, together with the variation in proteasome–APEH activities during the active metabolic phase, is consistent with a reduced GSH requirement during differentiation and with cellular needs for increased degradation activities during cell proliferation [25]. The decrease of NQO1 activity, which plays a protective effect toward the formation of highly reactive toxic compounds [26], is consistent with the association of cell differentiation with the decline of detoxifying ability [27]. GST enhancement during Caco-2 differentiation is not unexpected and indicates the development of chemoresistance [28].

Gliadin toxicity on redox homeostasis in differentiated Caco-2 cells is consistent with that reported on undifferentiated culture [11] but, in our hands, gliadin treatment only produces a small proapoptotic activity and it is unable to alter tTG expression. This is an ubiquitous intracellular enzyme and its enhanced activity/expression represents an active cellular response to oxidative stress [29]. In particular, the lack of in vitro effect of gliadin on tTG expression is apparently in contrast with the literature data [30, 31] although expression found in biopsies of CD patients is in good accordance with the literature [32]. Bearing in mind that enhanced activity/expression of tTG represents an active cellular response to oxidative stress [29], it is thus likely that chemoresistance development, in differentiated culture might be responsible for the reduced susceptibility to gliadin toxicity. Moreover, the lack of influence of dietary gluten on tTG expression and on the levels of typical pathological markers in mice intestine is consistent with data, indicating that gluten is well tolerated by DQ8 mouse [8]. PC accumulation is a characteristic feature of aging and of a number of pathologies, including inflammatory bowel disease [33] and little is known about the influence of gluten on PC yield. Here, we provide evidence that gliadin raises PC levels in both in vitro and in vivo models and our in vivo data showing that noxious consequences produced by gluten intake are unable to affect the animal redox status are in agreement with the hypothesis that dietary gluten is insufficient to trigger oxidative stress conditions comparable to those found in CD patients [34].

The relevance of Nrf2-mediated defenses in intestinal protection was recently reviewed [14], and among the phase 2 enzymes, GCL has been recognized as the rate-limiting enzyme in GSH synthesis. The alteration of GSH levels may have deleterious effects on both organ homeostasis and disease progression [25]. We confirm the ability of gliadin to decrease intracellular GSHtot content and to block the activity of Nrf2-activated enzymes in vitro [11] and we demonstrate for the first time the ability of gluten to downregulate phase 2 enzyme activity/expression.

The antidifferentiation effect of pt-glia ability was deduced by its ability to affect IAP [35], which was recently demonstrated to play an important role in detoxifying bacterial lipopolysaccharide [36]. The negative correlation between intracellular GSHtot and IAP activity, consistently with data on the inhibitory effect of oxidative stress [37, 38] indicates the modulatory role of Nrf2/ARE pathway on IAP functioning rather than the result of histological injury [39].

Our study demonstrates, for the first time, the in vitro and in vivo ability of gluten to increase the enzymatic activity of the proteasome–APEH system. The marked reduction in proteasome–APEH function detected in intestinal biopsies from untreated CD patients, together with decreased IAP levels, could mirror the severe oxidative stress conditions associated with mucosal lesions in CD; however, further correlation studies on a larger population of CD patients are required.

The ability of CLA to attenuate the oxidative stress cascade has been shown previously [18, 20, 40, 41]. Consequently, to examine the mechanism whereby gluten downregulates intestinal defenses, it was used as an in vivo inducer of Nrf2 pathway. Notably, the association between the increase of cytoprotective defenses with the inhibition of gluten-mediated toxicity in the mouse intestine substantiates the beneficial effects of CLA on the Nrf2 pathway

[20, 40] and supports the relevance of Nrf2 activation against toxic insults in experimental colitis [42]. Taken together, these data indicate the possible use of CLA for the management of intestinal pathologies associated with the depletion of antioxidant/detoxifying defenses. Our results are in good accordance with the previous studies demonstrating the chemo-protective effects of supplementation with mixed CLA isomers on animal [19, 43, 44]. However, since the biological effects of the CLA mixture are likely due to the separate action of its components, further studies are necessary to determine the role of individual isomer in the protective effects against gluten toxicity.

Possible mechanisms by which gluten downregulates intestinal antioxidant/detoxifying may include either indirect modulation of the Nrf2-pathway via the proteasome–APEH system or direct inhibition of Nrf2. However, based on the negligible effects of dietary gluten on intestinal mRNA levels of phase 2 enzymes, the latter hypothesis can be excluded. In addition, bearing in mind that Nrf2 is a known proteasome substrate [45], the hypothesized role played by an increased activity of proteasome–APEH function on the decreased Nrf2-mediated protection upon gluten exposure is supported by data from CLA-supplemented animals. In addition, the ability of CLA to enhance the activity/expression of phase 2 enzymes and to restore proteasome–APEH activity levels is consistent with the reported effects of Nrf2 activation [46, 47]. In fact, it is likely that the release of Nrf2 from Keap1 inhibitor, triggered by CLA, promotes the escape of the Nrf2 protein from proteasomal degradation, thus protecting intestinal cells from gluten-mediated toxicity.

In conclusion, the ability of dietary gluten to produce deleterious effects on several crucial intestinal defense mechanisms, but not the pathological signs associated with CD, is consistent with the hypothesis that gluten exposure may represent only a predisposing factor for further undetermined insults. Moreover, we have identified a novel mechanism by which gluten perturbs several pivotal intestinal defenses and we have discovered the potential therapeutic efficacy of CLA against gluten-mediated toxicity (Supporting Information Fig. 4).

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