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ORIGINAL ARTICLE

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Gliadin increases iNOS gene expression in interferon-γ-stimulated RAW 264.7 cells through a mechanism involving NF-κB

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Abstract Nitric oxide (NO) plays an important role in the pathogenesis of the histological changes seen in coeliac disease. We have investigated the effect of peptic-tryptic digest of gliadin (Pt-G) and gliadin (G) on inducible nitric oxide synthase (iNOS) protein expression in RAW 264.7 macrophages stimulated with interferon-γ (IFN-γ). Pt-G and G enhanced in a concentration and time-dependent manner NO production by IFN-γ-stimulated RAW 264.7 cells. The increase of iNOS protein expression was correlated with NF-κB/DNA binding activity and occurred at transcriptional level. Pyrrolidine dithiocarbamate and N-α-paratosyl-L-lysine chloromethyl ketone, two known inhibitors of NF-κB activation, decreased significantly NO production and iNOS protein expression as well as NF-κB/DNA binding activity. Our results show that the effect of Pt-G and G on enhancement of iNOS protein expression in IFN-γ-treated RAW 264.7 cells is mainly mediated through NF-κB and suggest that blockage of NF-κB activation reduces enhancing effect of gluten on NO production in inflamed mucosa of coeliac patients.

Keywords Coeliac disease · Gliadin · Interferon-gamma · Inducible nitric oxide synthase · Nuclear factor-κB · Interferon regulatory factor-1 · Signal transducer and activator of transcription · Macrophages

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Introduction

Coeliac disease (CD) is an enteropathy caused by permanent intolerance to gluten/gliadin, in genetically susceptible individuals (Sollid 2000; Shan et al. 2002). The disease in its typical form is histologically characterised by a lesion in the upper small bowel (Marsh 1992). The mechanisms by which gluten/gliadin damages the intestinal mucosa of coeliac patients have not been elucidated. Different hypotheses have been proposed to explain the origin of the mucosal damage. Some reports include a direct toxic effect of gliadin on intestinal mucosa (Auricchio et al. 1990; Maiuri et al. 1996). Most observations favour a disregulated immune response to gluten-derived peptides in coeliac patients. Toxic gluten peptides are absorbed across the epithelium, presented in associated with HLA class-II molecules by macrophages in the lamina propria and recognised by gliadin antigen specific $CD4^+$ T cells (Marsh 1992; Sollid 2000). As a result, secreted mediators, including IFN-γ, may cause activation of macrophages, which, in turn, produce pro-inflammatory cytokines contributing to the damage of the mucosal matrix (Przemioslo et al. 1994; Kontakou et al. 1995; Nilsen et al. 1995; Pender et al. 1996). It has been reported that NO may play an important role in the mucosal lesion (Beckett et al. 1998). High levels of nitric oxide products (nitrate/nitrite) in the urine of children with active CD have been correlated with the expression of iNOS in the small intestine (Holmegren Peterson et al. 1998; van Straaten et al. 1999). The promoter region of the iNOS gene has been characterised in different species, including human, rat and mouse (Xie et al. 1993; Chu et al. 1998; Eberhardt et al. 1996). Sequence analysis of promoter revealed the presence of consensus motifs for binding of transcription factors, such as nuclear factor-κB (NF-κB), interferon regulatory factor (IRF)-1 and signal transducer and activator of transcription (STAT)-1α, which are essential for the iNOS induction by IFN-γ (Kamijo et al. 1994; Martin et al. 1994; Weisz et al. 1994; Gao et al. 1997; Kim et al. 1997). It has been shown that in CD both enterocytes and cells with macrophage-like morphology

in the lamina propria are responsible for increased iNOS expression (ter Steege et al. 1997). Recent report has shown that gluten or gliadin and their proteolytic fragments enhanced NO production and iNOS mRNA level in mouse peritoneal macrophages stimulated with IFN-γ (Tuckovà et al. 2000). Moreover, the molecular mechanisms by which gluten/gliadin increases NO production and iNOS expression in intestinal mucosa cells in CD and cultured activated macrophages have not been elucidated. We have investigated the effect of gluten-derived peptides, Pt-G and G, on iNOS protein expression in RAW 264.7 macrophages stimulated with IFN-γ. In the present study, we show that in these cells the enhancing effect of Pt-G and G on iNOS gene induction is mainly mediated through the NF-κB signalling pathway.

Materials and methods

Gliadin and zein digest. Pure bread wheat (*Triticum aestivum*, var. San Pastore) was kindly supplied by the "Istituto Sperimentale per la Cerealicoltura", Rome, Italy. Peptic-tryptic digest of gliadin (Pt-G), purified prolamin fraction, were prepared following a two-step procedure as previously described (De Ritis et al. 1979). Gliadin (100 g) pool was digested in 1 l of HCl 0.2 M (pH 1.8) with 2 g of purified pepsin at 37°C for 2 h. The resultant peptic digest was further digested by addition of 2 g of purified trypsin after pH adjustment to 8.0 with NaOH 2 M. The reaction mixture was vigorously stirred at 37°C for 4 h at pH 8.0. During the entire digestion procedure, the pH was checked periodically and when needed adjusted with HCl or NaOH. At the end of the whole digestion period, the digest was submitted to gel filtration and the peptide fractions eluted after cytocrome c were collected, freeze-dried and stored at –20°C. Zein (Z) from maize (Sigma, Milan, Italy), was extracted from commercial preparations with 70% (v/v) ethanol and freeze-dried. Z (1 g) was dissolved in 10 ml of HCl 0.1 M. Pepsin (20 mg) was added and after digestion for 4 h at 37°C, pH was adjusted to 8.0 by NaOH 5.0 M. The zein was then further digested with 20 mg trypsin at 37°C for 4 h. Inactivation of trypsin was achieved by heating at 90°C for 5 min. Insoluble material was removed by centrifugation at 10,000 *g* for 30 min. The peptic-tryptic digest of zein (Pt-Z) was finally freeze-dried and stored at –20°C.

Cell culture. The mouse monocyte/macrophage cell line RAW 264.7 was cultured at 37°C in humidified 5% CO_2/95% air in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM Hepes and 5 mM sodium pyruvate. The cells were plated in 24 culture wells at a density of 2.5×10^5 cells/ml per well or 10 cm diameter culture dishes at a density of 3×10^6 cells/ml per dish and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and cells were activated with IFN-γ (25 U/ml). Pt-G (50, 100, 200, 400 and 800 µg/ml), G (50, 100, 200, 400 and 800 µg/ml), bovine serum albumin (BSA; 50, 100, 200, 400 and 800 µg/ml), Pt-Z (50, 100, 200, 400 and 800 µg/ml), Z (50, 100, 200, 400 and 800 µg/ml), pyrrolidine dithiocarbamate (PDTC; 0.1, 1 and 10 µM) or N-α-para-tosyl-L-lysine chloromethyl ketone (TLCK; 1, 10 and 100 µM) were added to the cells 5 min after IFN-γ challenge, alone or in combination. The cell viability was determined by using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) conversion assay (D'Acquisto et al. 2001). Briefly, 100 µl MTT (5 mg/ml in complete DMEM) was added and the cells were incubated for an additional 3 h. After this time point the cells were lysed and the dark blue crystals solubilised with 500 µl of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCCC/340) equipped with a 620 nm filter. The cell viability in response to treatment with test compounds was calculated as % dead cells=100–(OD treated/OD control)×100.

Nitrite determination. NO was measured as nitrite (NO^-_2, nmol/10^6 cells) accumulated in the incubation medium after 24, 48 and 72 h. A spectrophotometric assay based on the Griess reaction was used (D'Acquisto et al. 2001). Briefly, Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in phosphoric acid) was added to an equal volume of cell culture supernatant and the absorbance at 550 nm was measured after 10 min. The nitrite concentration was determined by reference to a standard curve of sodium nitrite.

Cytosolic and nuclear extracts. Cytosolic and nuclear extracts of macrophages stimulated for 1, 3, 6, 24, 48 and 72 h with IFN-γ (25 U/ml) in presence or absence of Pt-G (50, 100, 200, 400 and 800 µg/ml), G (50, 100, 200, 400 and 800 µg/ml), PDTC (0.1, 1 and 10 µM) or TLCK (1, 10 and 100 µM) were prepared as previously described with some modification (D'Acquisto et al. 2001). Briefly, harvested cells (3×10^6) were washed two times with ice-cold PBS and centrifuged at 180 g for 10 min at 4°C. The cell pellet was resuspended in 100 µl of ice-cold hypotonic lysis buffer (10 mM Hepes, 10 mM KCl, 0.5 mM phenylmethylsulphonyfluoride, 1.5 µg/ml soybean trypsin inhibitor, 7 µg/ml pepstatin A, 5 µg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle for five or six times and the cytoplasmic fraction was then obtained by centrifugation for 1 min at 13,000 *g*. The supernatant containing the cytosolic fraction was removed and stored at –80°C. The nuclear pellet was resuspended in 60 µl of high salt extraction buffer (20 mM Hepes pH 7.9, 10 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM phenylmethylsulphonyfluoride, 1.5 µg/ml soybean trypsin inhibitor, 7 µg/ml pepstatin A, 5 µg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated with shaking at 4°C for 30 min. The nuclear extract was then centrifuged for 15 min at 13,000 g and supernatant was aliquoted and stored at –80°C. Protein concentration was determined by Bio-Rad (Milan, Italy) protein assay kit.

Electrophoretic mobility shift assay. Double stranded oligonucleotides containing the NF-κB (5′-CAA CGGCAGGGGAATCTCCC-TCTCCTT-3′), IRF-1 (5′-GGAAGCGAAAATGAAATTGACT-3′) and STAT-1α (5′-CATGTTATGCATATTCCTGTAAGTG-3′) recognition sequences were endlabelled with ^{32}P-γ-ATP. Nuclear extracts containing 5 µg protein were incubated for 15 min with radiolabeled oligonucleotides ($2.5–5.0\times10^4$ cpm) in 20 µl reaction buffer containing 2 µg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol. The specificity of the DNA/protein binding was determined by competition reaction in which a 50-fold molar excess of unlabeled wild-type, mutant or Sp-1 oligonucleotide was added to the binding reaction 15 min before addition of radiolabeled probe. In supershift assay, antibodies reactive to p50 or p65 proteins were added to the reaction mixture 15 min before the addition of radiolabeled NF-κB probe. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 1× TBE buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with intensifying screen at –80°C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (Bio-Rad, Milan, Italy) and a computer program (Molecular Analyst; IBM).

Western blot analysis. Immunoblotting analysis of anti-iNOS, anti-IκBα, anti-p50 and anti-p65 was performed on cytosolic or nuclear fraction. Cytosolic and nuclear fraction proteins were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml of bromophenol) in a ratio of 1:1, boiled for 3 min and centrifuged at 10,000 *g* for 5 min. Protein concentration was determined and equivalent amounts (50 µg) of each sample were electrophoresed in a 8% discontinuous polyacrylamide minigel. The proteins were transferred onto nitro-cellulose membranes, according to the manufacturer's instructions (Bio-Rad).

The membranes were saturated by incubation at 4°C overnight with 10% non-fat dry milk in PBS and then incubated with (1:1000) anti-iNOS, anti-IκBα, anti-p50 and anti-p65 for 1 h at room temperature. The membranes were washed three times with 0.05% Triton 100X in PBS and then incubated with anti-rabbit or anti-goat immunoglobulins coupled to peroxidase (1:1000). The immunocomplexes were visualised by the ECL chemiluminescence method (Santa Cruz, Milan, Italy). The membranes were stripped and re-probed with β-actin antibody to verify equal loading of proteins. Subsequently, the relative expression of iNOS in cytosolic fraction was quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer programme (Molecular Analyst, IBM).

Transfection and assay of CAT activity. Cells (1.5×10^5) were seeded overnight in 60 mm culture dishes in complete medium and transfected in triplicate using FuGENE 6 (Roche Molecular Biochemicals) at about 30–50% confluency on the day of the experiment. 4 μg of piNOS CAT1 plasmid (Weisz et al. 1996) and 0.5 μg of the luciferase gene under the control of the cytomegalovirus enhancer (CMV-Luc) were mixed with 6 μl of FuGENE 6 in 100 μl of medium. The mixture was incubated at room temperature for 45 min and then added to cells. After 24 h of transfection the cells were treated with IFN-γ (25 U/ml), G (800 μg/ml), Z (800 μg/ml), PDTC (10 μM). After an additional 24 h the cells were harvested, lysed and luciferase activity was measured in according to the manufacturer's protocol by a luminometer (EG&G Berthold). The samples normalised for luciferase activity were analysed for CAT activity following a previous protocol based on TLC separation of acetylated [14C] chloramphenicol. The amount of acetylated substrate was quantified in a STORM-840 radioactivity detection system.

Statistics. Results were expressed as the means ± SEM of *n* experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA) and Bonferroni-corrected *p*-value for multiple comparison test. The level of statistically significant difference was defined as $p<0.05$. Linear associations between variables were assessed by the use of standard-least-square linear regression. Correlation coefficients (*r*) were presented as measures of linear association for regression relationship. The fold increase was calculated by dividing the combination value by sum of individual values.

Reagents. Recombinant mouse interferon-γ was from Vinci-Biochem (Florence, Italy). ^{32}P-γ-ATP was from Amersham (Milan, Italy). Poly dI-dC was from Boehringer-Mannheim (Milan, Italy). Anti-p50, anti-p65, anti-iNOS, anti-β-actin and anti-IκBα antibodies was from Santa Cruz (Milan, Italy). Phosphate buffer saline was from Celbio (Milan, Italy). Oligonucleotide synthesis was performed to our specifications by Tib Molbiol (Boehringer-Mannheim, Genova, Italy). Non-fat dry milk was from Bio-Rad (Milan, Italy). DL-dithiothreitol, pepstatin A, leupeptin, benzamidine, phenylmethylsulfonilfluoride were from Applichem (Darmstadt, Germany). BSA, MTT, PDTC and TLCK as well as other reagents were from Sigma (Milan, Italy).

Results

Effect of Pt-G and G on nitrite production by RAW 264.7 cells stimulated with IFN-γ for 24 h

The nitrite production by unstimulated cells was undetectable. The stimulation of cells with IFN-γ (25 U/ml) for 24 h resulted in an accumulation of nitrite in the medium. Addition of Pt-G (50, 100, 200, 400 and 800 μg/ml) or G (50, 100, 200, 400 and 800 μg/ml) to the cells increased significantly and in a concentration-dependent manner the nitrite production as compared with IFN-γ alone (Fig. 1).

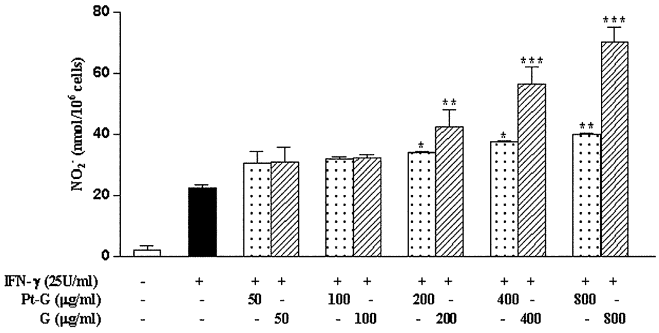


Fig. 1 Concentration-dependent increase of nitrite production by peptic-tryptic digested gliadin (Pt-G) and gliadin (G) in interferon-γ (IFN-γ)-stimulated RAW 264.7 macrophages at 24 h. Data are expressed as mean ± SEM of five experiments in triplicate. *$p<0.05$, **$p<0.001$, ***$p<0.0001$ vs. IFN-γ alone

Table 1 Effect of food antigens on nitrite production in interferon-γ (IFN-γ)-stimulated RAW 264.7 macrophages at 24 h. Results are expressed as nmol/10^6 cells. Data are expressed as mean ± SEM of five experiments in triplicate. *BSA* bovine serum albumin, *Pt-Z* peptic-tryptic digest of zein, *Z* zein

Treatment	Control cells	BSA (800 μg/ml)	Pt-Z (800 μg/ml)	Z (800 μg/ml)
None	2.1±1.2	2.3±0.9	2.2±0.6	2.4±0.8
IFN-γ (25 U/ml)	22.2±1.2	23.1±1.5	20.3±0.9	21.4±1.2

Enhancement of nitrite production by G was greater than that exhibited by Pt-G. In contrast, Pt-G or G added to the cells alone, at the same concentrations, did not elicit the nitrite production (data not shown). BSA, Pt-Z and Z (50, 100, 200, 400 and 800 μg/ml), used as other food antigens, alone or in combination with IFN-γ had no effect (Table 1). All compounds did not affect cell viability (>95%).

Effect of PDTC and TLCK on increase of nitrite production and iNOS protein expression by Pt-G and G in RAW 264.7 macrophages stimulated with IFN-γ for 24 h

Pt-G (800 μg/ml) or G (800 μg/ml), added to the cells together with IFN-γ (25 U/ml), caused an increase of nitrite production (55.4±2.7 and 86.4±3.4, respectively; $n=3$) as compared with IFN-γ alone (36.0±0.9; $n=3$). PDTC or TLCK inhibited significantly the increase of nitrite production induced by Pt-G and G (Fig. 2A, B). PDTC (0.1, 1 and 10 μM) or TLCK (1, 10 and 100 μM) caused a reduction of nitrite production induced by IFN-γ alone (by 31.9±2.1%, 37.5±1.4%, 66.8±1.8% and 40.0±2.35%, 49.0±3.0%, 64.0±2.1%, respectively; $n=3$). Upon stimulation with IFN-γ (25 U/ml) for 24 h, cells expressed high level of iNOS protein expression as compared with untreated cells. Pt-G (800 μg/ml) or G (800 μg/ml), added to the cells together with IFN-γ (25 U/ml), increased iNOS protein expression (by 1.8- and 3.0-fold, respectively). PDTC (10 μM) or TLCK (100 μM) reduced significantly

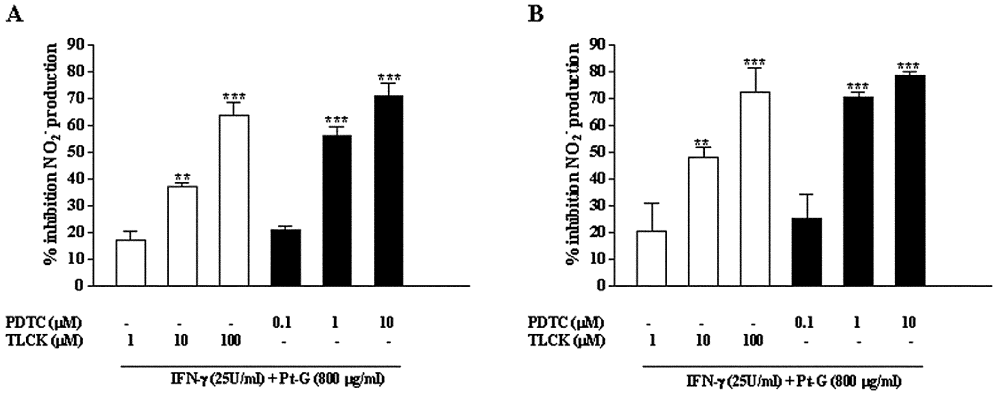


Fig. 2 RAW 264.7 macrophages stimulated with IFN-γ for 24 h in the presence of **A** Pt-G or **B** G were treated with pyrrolidine dithiocarbamate (PDTC) and N-α-para-tosyl-L-lysine chloromethyl ketone (TLCK). Data are expressed as mean ± SEM of three experiments in triplicate. **A** $^{**}p<0.001$, $^{***}p<0.0001$ vs. IFN-γ+Pt-G. **B** $^{**}p<0.001$, $^{***}p<0.0001$ vs. IFN-γ+G

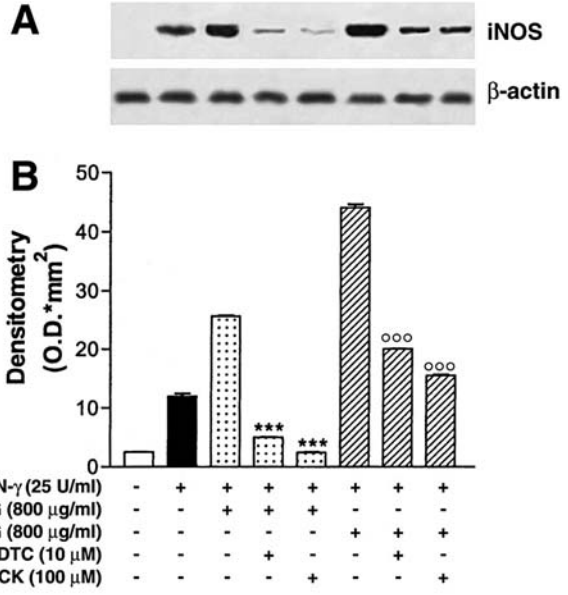


Fig. 3 **A** Representative Western blot of iNOS protein as well as **B** the densitometric analysis show the effect of PDTC and TLCK on enhancing inducible nitric oxide synthase (iNOS) protein expression by Pt-G and G in IFN-γ-stimulated RAW 264.7 macrophages at 24 h. Data in **A** are from a single experiment and are representative of five separate experiments. β-actin expression is shown as a control. Data in **B** are expressed as mean ± SEM of five separate experiments. $^{***}p<0.0001$ vs. IFN-γ+Pt-G; $^{°°°}p<0.0001$ vs. IFN-γ+G

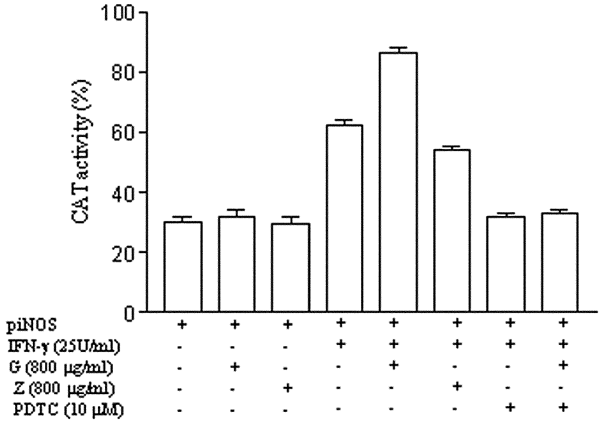


Fig. 4 Effect of G on iNOS promoter activity in IFN-γ-stimulated RAW 264.7 macrophages. The data reported are expressed as mean ± SEM of three transfection experiments

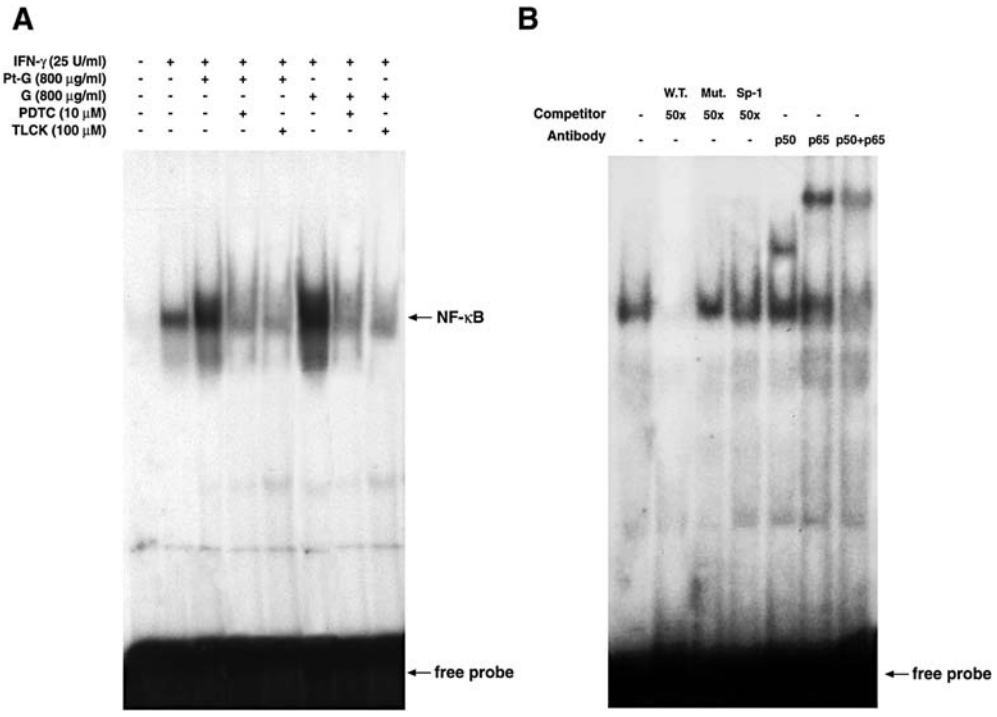
the increase of iNOS protein expression induced by Pt-G (by 80.0±0.1% and 90.0±0.2%, respectively) or G (by 55.1±0.1% and 65.0±0.2%, respectively) (Fig. 3). Pt-G and G alone failed to augment the iNOS protein expression level (data not shown).

Effect of G on iNOS promoter activity in RAW 264.7 cells stimulated with IFN-γ

We examined whether G in association with IFN-γ augmented iNOS gene expression. RAW 264.7 cells were transiently transfected with piNOS CAT1 plasmid. We have tested the promoter activity of the iNOS gene in cells treated with G (800 μg/ml), Z (800 μg/ml), IFN-γ (25 U/ml), PTDC (10 μM) and their combinations. The results normalised by using CMV-Luc as an internal control are reported in Fig. 4 and obtained by quantifying CAT activity in cell extracts. IFN-γ was efficient in enhancing basal transcription of iNOS promoter whereas G and Z alone had no effect. The G+IFN-γ combination treatment led to a higher augmentation of promoter activity compared to IFN-γ alone. This effect was not exhibited by the Z+IFN-γ combination treatment. PDTC inhibited the synergistic induction. Our results indicate that the G increases iNOS expression in IFN-γ-stimulated RAW 264.7 cells through a transcriptional mechanism.

Effect of Pt-G and G on NF-κB/DNA binding activity in RAW 264.7 macrophages stimulated with IFN-γ for 24 h

The effects of Pt-G (800 μg/ml) or G (800 μg/ml) on NF-κB/DNA binding activity in RAW 264.7 macrophages stimulated with IFN-γ (25 U/ml) for 24 h were evaluated by EMSA. A low basal level of NF-κB/DNA binding activity was detected in nuclear proteins from unstimulated macrophages. Conversely, a retarded band was clearly detected following stimulation with IFN-γ. Pt-G and G caused an increase of NF-κB/DNA binding activity (by



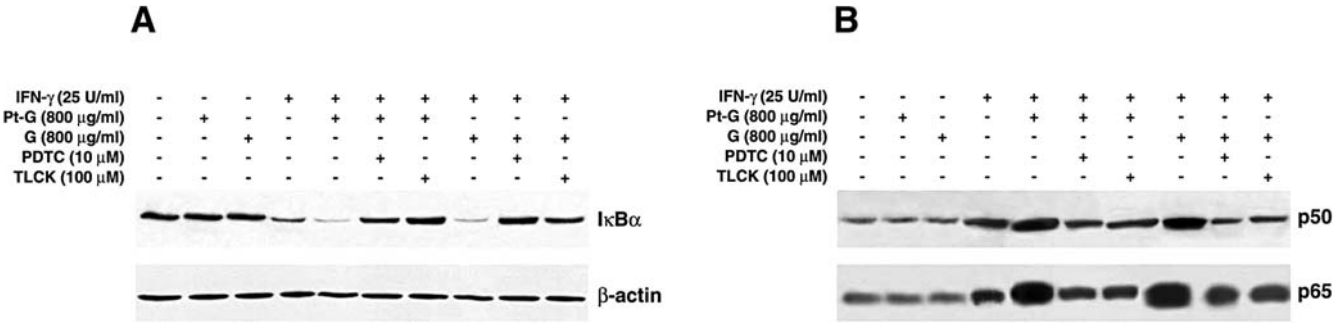


Fig. 5 **A** Representative EMSA shows the effect of Pt-G and G in presence or absence of PDTC and TLCK on nuclear factor-κB (NF-κB)/DNA binding activity in IFN-γ-stimulated RAW 264.7 macrophages at 24 h. Data are from a single experiment and are representative of six separate experiments. **B** Characterisation of NF-κB/DNA complex. In competition reaction nuclear extracts were incubated with radiolabelled NF-κB probe in absence or presence of: identical but unlabeled oligonucleotides (*W.T.*, 50×), mutated non-functional NF-κB probe (*Mut.*, 50×) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50×). In supershift experiments nuclear extracts were incubated with antibodies against p50 (p50) and p65 (p65) 15 min before incubation with radiolabelled NF-κB probe. Data are from a single experiment and are representative of three separate experiments

Fig. 6 Representative Western blot show the effect of Pt-G and G in the presence or absence of PDTC and TLCK on **A** cytosolic degradation of IκBα and **B** nuclear translocation of p50 and p65 in IFN-γ-stimulated RAW 264.7 macrophages at 24 h. β-actin expression is shown as a control. Data are from a single experiment and are representative of four separate experiments

1.3- and 1.6-fold, respectively), which was reduced by PDTC (10 μM; 90.0±4.5% and 90.0±3.8%, respectively) or TLCK (100 μM; 73.0±2.0% and 83.5±1.5%, respectively) (Fig. 5A). The composition of the NF-κB complex was determined by competition and supershift experiments. The specificity of NF-κB/DNA binding complex was evident by the complete displacement of NF-κB/DNA binding in the presence of a 50-fold molar excess of unlabelled NF-κB probe in the competition reaction. In contrast a 50-fold molar excess of unlabeled mutated NF-κB probe or Sp-1 oligonucleotide had no effect on DNA-binding activity. The anti-p50 and anti-p65 antibodies clearly gave rise to a characteristic supershift of the retarded complex. Addition of anti-p50+anti-p65 to the binding reaction resulted in a marked reduction of the intensity of NF-κB specific bands, suggesting that the NF-κB complex contained p50 and p65 dimers (Fig. 5B).

Effect of Pt-G and G on degradation of IκBα and nuclear translocation of NF-κB subunits in RAW 264.7 macrophages stimulated with IFN-γ for 24 h

The presence of IκBα in the cytosolic fraction or p50 and p65 subunits in nuclear fraction was examined by immunoblotting analysis. Unstimulated cells as well as cells treated with Pt-G (800 μg/ml) or G (800 μg/ml) alone expressed high levels of IκBα in the cytosolic fraction and basal levels of p50 and p65 in the nuclear fraction. Stimulation of the cells with IFN-γ (25 U/ml) caused a reduction of IκBα band intensity, which almost disappeared in presence of Pt-G (800 μg/ml) or G (800 μg/ml). PDTC (10 μM) or TLCK (100 μM) prevented IκBα degradation (Fig. 6A). Upon the stimulation with IFN-γ, cells exhibited p50 and p65 high nuclear levels which were increased by Pt-G or G. PDTC or TLCK prevented p50 and p65 nuclear translocation (Fig. 6B).

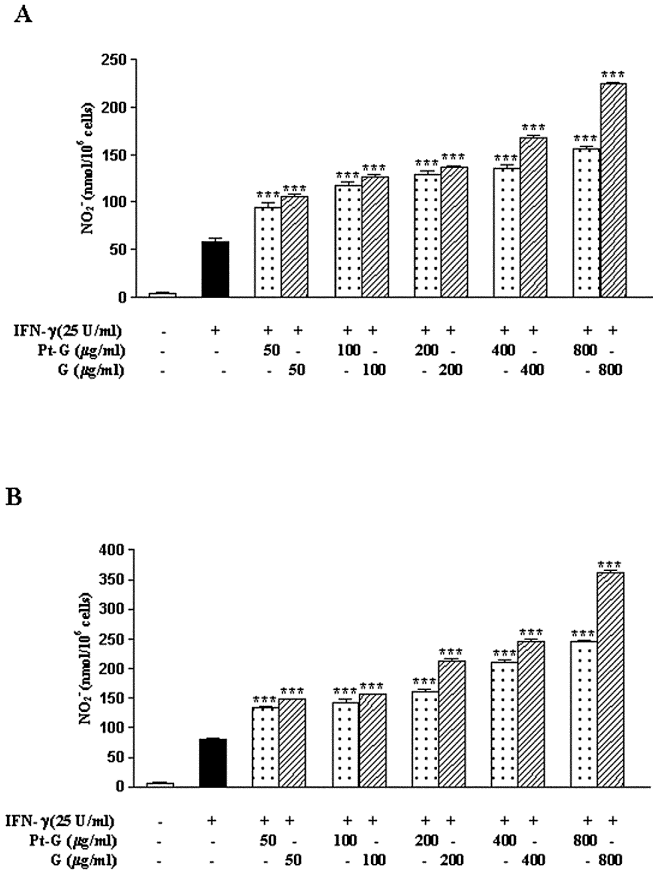


Fig. 7 Concentration-dependent increase of nitrite production by Pt-G and G in RAW 264.7 macrophages stimulated with IFN-γ for **A** 48 h and **B** 72 h. Data are expressed as mean ± SEM of five experiments in triplicate. ***p<0.0001 vs. IFN-γ alone

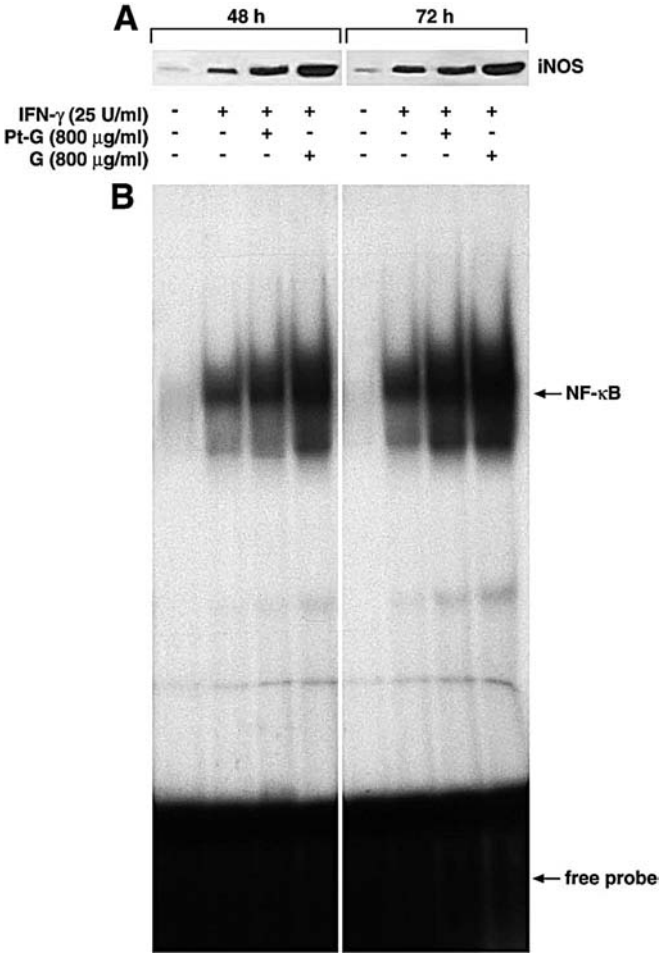


Fig. 8 Representative Western blot and EMSA show the effect of Pt-G and G on **A** iNOS protein expression and on **B** NF-κB/DNA binding activity in IFN-γ-stimulated RAW 264.7 macrophages at 48 and 72 h. Data in **A** are from a single experiment and are representative of four separate experiments. Data in **B** are from a single experiment and are representative of five separate experiments

Effect of Pt-G and G on nitrite production, iNOS protein expression and NF-κB/DNA binding activity in RAW 264.7 macrophages stimulated with IFN-γ for 48 and 72 h

The cells stimulated with IFN-γ (25 U/ml) for 48 and 72 h caused nitrite accumulation which was increased in a significant and concentration-dependent manner by Pt-G (50, 100, 200, 400 and 800 μg/ml) or G (50, 100, 200, 400 and 800 μg/ml) (Fig. 7A, B). Interestingly, Pt-G and G at lower concentrations (50 and 100 μg/ml) enhanced significantly (p<0.0001) the nitrite production. In contrast, Pt-G or G added to the cells alone, at the same concentrations, did not elicit the nitrite production (data not shown). Pt-G (800 μg/ml) or G (800 μg/ml) in combination with IFN-γ also increased both iNOS protein expression (by 1.7- and 2.6-fold, at 48 h; by 1.3- and 1.7-fold, at 72 h, respectively) and NF-κB/DNA binding activity (by 1.4- and 1.8-fold, at 48 h; by 1.3- and 1.6-fold, at 72 h, respectively) as compared with IFN-γ alone (Fig. 8A, B). The increase of iNOS protein expression was correlated with NF-κB/DNA binding activity (r=0.99, *p<0.05, Pt-G; r=0.99, *p<0.05, G) at 24, 48 and 72 h.

Effect of Pt-G and G on IRF-1 and STAT-1α DNA/binding activity in RAW 264.7 macrophages stimulated with IFN-γ: comparison with kinetic analysis of NF-κB DNA/binding activity

We investigated the enhancing effect of Pt-G and G on IFN-γ-induced IRF-1 or STAT-1α DNA binding activity. Nuclear extracts from RAW 264.7 cells treated for 1, 3, 6, 24, 48 and 72 h with IFN-γ (25 U/ml) or both IFN-γ and Pt-G (800 μg/ml) or G (800 μg/ml) were subjected to EMSA using the specific binding elements for each transcription factor. As shown in Fig. 9, a low basal level of IRF-1 and STAT-1α/DNA binding activity was detected in nuclear proteins from unstimulated macrophages. Conversely, a retarded band was clearly detected at 1 h after stimulation with IFN-γ. The addition of Pt-G and G to the cells caused an increase of band intensity up to 24 h for IRF-1 and up to 6 h for STAT-1α. In contrast, NF-κB/DNA binding activity was evident at 3 h and increased up to 72 h. Pt-G and G alone did not change the binding activ-

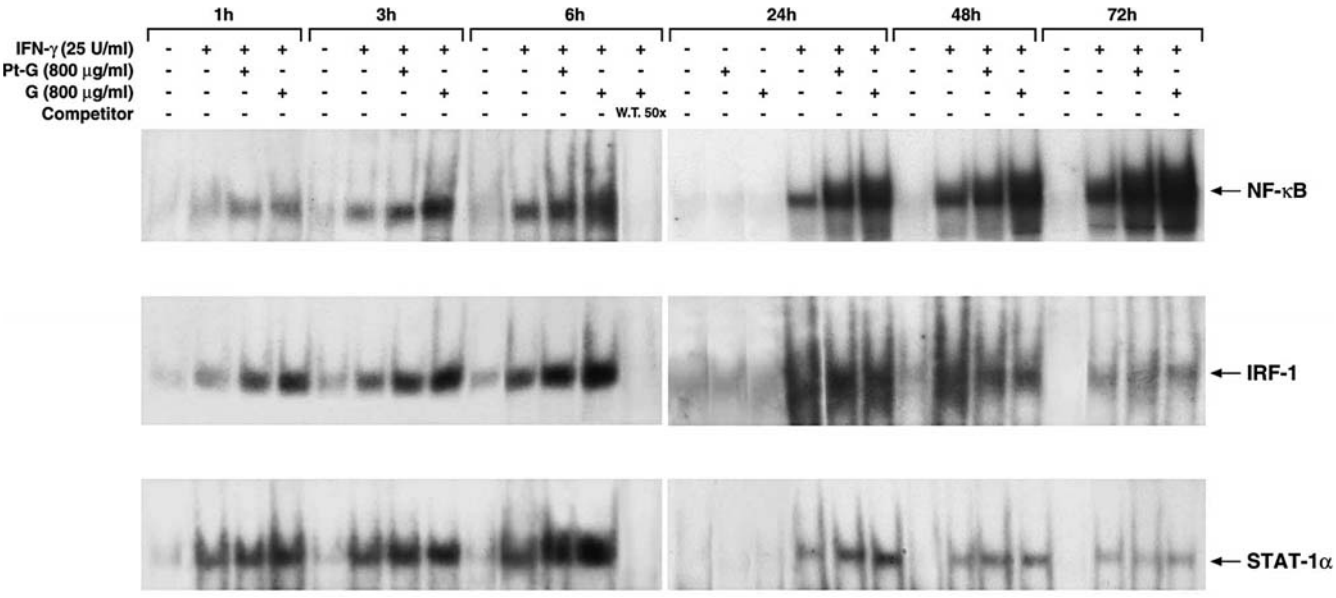


Fig. 9 Kinetic analysis shows the effect of Pt-G and G on IFN-γ-induced NF-κB, IRF-1 and STAT-1α DNA/binding activity in RAW 264.7 macrophages. In competition reaction nuclear extracts were incubated with 50-fold molar excess of each unlabeled probe (*W.T.*, 50×). Data are from a single experiment and are representative of three to six experiments

ity of IRF-1, STAT-1α and NF-κB at time points considered.

Discussion

There is increasing evidence that NO plays important role in the pathogenesis of the histologic changes seen in CD (ter Steege et al. 1997; Beckett et al. 1998; Holmegren Peterson et al. 1998; Beckett et al. 1999; van Straaten et al. 1999). Recently, it has been shown that gluten or gliadin and their proteolytic fragments enhance iNOS mRNA level and NO production by mouse peritoneal macrophages stimulated with IFN-γ (Tuckovà et al. 2000, 2002). The molecular mechanisms by which gluten/gliadin induces iNOS expression and NO production by intestinal mucosa cells in CD and cultured activated macrophages have not been investigated. Here, we report that Pt-G and G were able to increase significantly and in a concentration-dependent manner NO production by RAW 264.7 macrophages stimulated with IFN-γ for 24 h. The increase of NO production by Pt-G and G was associated with an increased expression of iNOS protein compared with IFN-γ alone. In addition, we found that Pt-G and G increased NF-κB/DNA binding activity, IκBα cytosolic degradation and nuclear translocation of p50 and p65 subunits. It is particularly interesting to observe that NF-κB/DNA binding activity as well as iNOS protein expression and NO production were ulteriorly increased by Pt-G and G at 48 and 72 h. In addition, Pt-G and G also at lower concentrations were able to increase significantly NO production. G exhibited greater effects in comparison with Pt-G. These results show that the type and concentration as well as time exposure of gluten derived-peptides induce IFN-γ-stimulated RAW 264.7 cells to produce high levels of NO and suggest a direct toxic action of gliadin in CD. However, Pt-G and G increased iNOS protein expression and NF-κB/DNA binding activity compared with IFN-γ alone. In addition, the G + IFN-γ combination treatment also led to a higher augmentation of iNOS promoter activity compared to IFN-γ alone, suggesting that a synergistic effect of G on IFN-γ-induced iNOS expression occurs at the transcriptional level. Moreover, the NF-κB activation inhibitors, PDTC and TLCK, reduced significantly these effects, thereby indicating that Pt-G and G may modulate iNOS gene expression as co-signal with IFN-γ in RAW 264.7 cells through NF-κB activation. Besides NF-κB, we also investigated the involvement of IRF-1 and STAT-1α in the increase of iNOS expression induced by Pt-G and G in IFN-γ-stimulated RAW 264.7 cells. Interestingly, the kinetics of IRF-1 and STAT-1α activation are rapid and decrease compared with NF-κB activation, which is persistent. It has been reported that STAT-1α and IRF-1 can cooperate with NF-κB to promote synergistically transcriptional activity (Drew et al. 1995; Ohmori et al. 1997; Teng et al. 2002). Although the effect of Pt-G and G on IFN-γ-induced iNOS gene expression seems to depend on the enhanced activity of all three transcription factors, a major role for NF-κB cannot be ruled out. Further studies will be addressed to investigate whether this effect is mediated at the molecular level by a cooperation between transcription factors. Taken together our results show that Pt-G and G cause a time and concentration-dependent increase of IFN-γ-induced NF-κB/DNA binding as well as molecular events downstream of NF-κB activation and suggest a role for gluten/gliadin in maintaining activated NF-κB in infiltrating lamina propria cells of inflamed mucosa of coeliac patients. In CD, it is widely accepted that specific cellular and humoral factors are implicated in morphological and functional changes follow-

ing gluten challenge in intestinal mucosa (Auricchio et al. 1990). Nevertheless, some reports shown the involvement of non specific immune reaction, caused by direct interaction of gluten of innate immune system (Auricchio et al. 1990; Maiuri et al. 1996; Tuckovà et al. 2000). Gliadin is a lectin that is able to bind glycosylated residues, called "lectinic binding sites" and expressed on various cells, by a non-covalent bound (Kolberg and Sollid 1985; Damjanov 1987; Amore et al. 1994). Other studies have shown that gliadin is able to associate with many proteins, mainly by hydrophobic interactions (Farré Castany et al. 1995; Pittschieler et al. 1994; Tuckovà et al. 2000). It has been reported that different cell types in response to pro-inflammatory stimuli, including IFN-γ, induce the expression of cell surface molecules capable of acting as a potent triggers of intracellular activation signals (Marzio et al. 1997). Since Pt-G and G alone had no effect, it could be hypothesised that IFN-γ induces the expression of macrophage surface molecules able of interacting with Pt-G or G and triggering signal transduction pathway. Moreover, gliadin and other lectin fractions of gluten have been shown to be potent modulators of leukocyte function, enhancing chemotaxis and generation of reactive oxygen species (Roccatello et al. 1990; Amore et al. 1994; Rivabene et al. 1999). Several evidences suggest that NF-κB activation may also be under the control of oxidant/antioxidant balance (Flohè et al. 1997). The antioxidant PDTC is thought to inhibit NF-κB activation by depleting the cells of oxygen radicals, whereas TLCK acts by inhibiting proteasome function and hence IκBα degradation (Sherman et al. 1993; Kim et al. 1995; Epinat and Gilmore 1999). We found that Pt-G and G enhanced IκBα degradation, which was prevented by either PDTC or TLCK suggesting that these compounds inhibit NF-κB/DNA binding activity by stabilising IκBα in IFN-γ-stimulated RAW 264.7 macrophages. Thus, it is conceivable that Pt-G and G in combination with IFN-γ increase NF-κB activation through an oxidant mechanism. Moreover, our results show that PDTC and TLCK are able to reduce the increased iNOS protein expression and NO production by activated macrophages suggesting that NF-κB is responsible for the synergistic effect of Pt-G and G together IFN-γ on NO production. High levels of NO are present in serum and urine of children with CD and correlated with an increased iNOS expression in the small intestine (ter Steege et al. 1997; Beckett et al. 1998; Holmegren Peterson et al. 1998; Beckett et al. 1999; van Straaten et al. 1999). However, the molecular mechanisms by which NO induces, directly or indirectly, injury of the small-intestine in coeliac patients are not clear. Excessively produced NO is known to act as a free radical and cause tissue damage (Liu and Hotchkiss 1995). In conclusion, our study provides evidence that the effect of Pt-G and G on iNOS protein expression in IFN-γ-treated RAW 264.7 cells is mediated through NF-κB and suggests that blockage of NF-κB activation reduces enhancing effect of gluten on NO production in inflamed mucosa of coeliac patients.

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