Gliadin stimulates human monocytes to production of IL-8 and TNF-α through a mechanism involving NF-κB

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Abstract Wheat gliadin is the triggering agent in coeliac disease. In this study, we documented that proteolytic fragments of gliadin, in contrast to other food antigens, induced interleukin (IL)-8 and tumour necrosis factor-α (TNF-α) production and significantly increased interferon (IFN)-γ-induced cytokine secretion in human monocytic line THP-1 cells. Stimulation with gliadin resulted in elevated phosphorylation of the IκBα molecule and increased NF-κB/DNA binding activity that was inhibited by sulfasalazine, 1,1-tosylamido-2-phenylethyl chloromethyl ketone and pyrrolidine dithiocarbamate (PDTC). The activation pathway was shown to be independent of the CD14 molecule. Less mature U-937 monocytes responded to gliadin stimulation by low IL-8 secretion, TNF-α production was not detectable. We propose that gliadin-induced activation of monocytes/macrophages can participate in mechanisms leading to the impairment of intestinal mucosa in coeliac patients.

Keywords: Monocyte; Gliadin; Interleukin-8; Tumour necrosis factor-α; NF-κB; Innate immunity

1. Introduction

Coeliac disease, a chronic inflammation of small intestine, develops in genetically susceptible individuals because of intolerance to wheat gluten and related prolamins. Gluten, a complex mixture of gliadins and glutenins, is usually digested in the gastrointestinal tract by various enzymes of human and bacterial origin, mainly by pepsin in stomach.

Gliadin-peptide-specific CD4+ α/β T lymphocytes present in jejunal mucosa seem to be central in the immunopathology of the disease. After challenge, they produce increased levels of inflammatory cytokines of T-helper 1 profile (mainly interferon-γ, IFN-γ) and other mediators that can activate other cell types including cells of innate immunity. Macrophages and dendritic cells in intestinal mucosa play a role as antigen presenting cells and could be activated by various stimuli to the production of inflammatory cytokines (such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1 and IL-6), chemokines (IL-8, monocyte chemotactic protein-1 [MCP-1]) and reactive oxygen and nitrogen intermediates [1-4].

Although the vast majority of bacterial and food components do not elicit intestinal inflammation, it is becoming increasingly recognised that pathogens that cause acute inflammation do activate the NF-κB pathway, resulting in a regulation of genes encoding proinflammatory cytokines, chemokines and adhesion molecules. The NF-κB family of proteins consists of homo- and heterodimeric subunits of the Rel family, including p50 and p65. The activity of NF-κB is regulated by a family of IκB inhibitor proteins (IκB α, β, γ and ε), which sequester NF-κB in the cytoplasm. Upon stimulation, IκB is phosphorylated, ubiquitinated, and subsequently degraded by the proteasome complex. Degradation of IκB allows NF-κB to translocate to the nucleus, bind to its specific promoter elements and activate gene transcription [5].

In recent years, the mechanism of activation of monocytes, intestinal epithelial cells or macrophages and the involvement of upregulated membrane expression and secretion of molecules such as HLA-DR, CD95/Fas, intercellular cell adhesion molecule-1 (ICAM-1), IL-15 or reactive nitric oxide (NO) radicals in coeliac disease immunopathogeny became a topic of intensive studies [6-10].

We have shown that wheat gliadin and its peptic fragments have the unique ability, in contrast to other food proteins, to activate the mouse peritoneal macrophages to the production of TNF-α, IL-10, RANTES and an inducible form of NO synthase. Gliadin fragments active in these processes were isolated and identified [3,4].

The aim of the present study was to investigate whether food proteins such as gliadin, soya protein or ovalbumin can also activate human monocytic cell lines (THP-1 and U-937 differing in surface marker expression) to cytokine and chemokine production and whether this activity could be modulated by IFN-γ, the main cytokine in coeliac disease. We also analysed the potential role of CD 14 receptor and NF-κB family members in the activation pathway.

2. Materials and methods

2.1. Food proteins

Ovalbumin and soya proteins (Sigma, St. Louis, MO) were diluted to 1 mg/ml concentration in incomplete RPMI-1640 medium (Endo-toxin tested, Sigma), centrifuged (5000 g, 10 min) and supernatants were stored at –20 °C. The stock solution of crude gliadin 5.0 mg/ml in

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50 mM HCl was prediluted in RPMI-1640 medium, centrifuged (10 000 × g, 10 min), the sediment was dissolved in HCl solution, the concentration of soluble molecules was measured and the final dilution calculated.

Pepptic fragments of proteins were prepared using the pepsin–agarose gel digestion [6, 7]. Bovine胰蛋白酶, OH, USA], 7 ml of protein (10 mg/ml) in 0.1 M HCl, pH 1.5, was incubated with 5 ml of pepsin–agarose gel (45 min, 37 °C). Removing the gel by centrifugation (1500 × g, 10 min) stopped enzymatic cleavage. The supernatants were then centrifuged (12 000 × g, 10 min) and soluble protein fragments divided into aliquots and frozen at −20 °C.

The gliadin 33-amino acid (AA) peptide [11], gliadin peptide p31–43 [6], and B peptide [4] were synthesised using the Fmoc/tBu protection strategy on aminoethyl copoly (styrene-1% divinylbenzene) resin with Knorr linker. After cleavage from the resin, the peptides were purified using high-performance liquid chromatography and characterised by AA analysis and liquid chromatography/mass spectrometry (System Waters 2690 Separation Module and Waters 2487 Dual A, Absorbance Detector, connected to a Micromass Platform L.C.). Potential presence of lipopolysaccharide (LPS) was tested using the E-toxate test (Sigma). The LPS level in all reagents used in the study was below the detection limit.

2.2. Cell lines and their activation

THP-1 and U-937 cells (GCMCC, Braunschweig, Germany) were cultured as described earlier [12]. Cells in concentration of 1 × 10^6/ml were exposed to gliadin or its pepptic anphospho-(10–500 μg/ml) alone or together with human IFN-γ (150 U/ml; R&D System, Minneapolis) or to LPS (Salmonella typhimurium, 1–10 μg/ml, Sigma) for 24 h. THP-1 cells were also incubated with soy protein, ovalbumin or their pepptic fragments (100–500 μg/ml) or synthetic gliadin peptides [33 AA gliadin peptide, B peptide and p31–43 peptide (200 μg/ml)] alone and/or with IFN-γ (150 U/ml). Alternatively, the cells were preincubated with IFN-γ for 2 or 24 h, washed twice with PBS and stimulated for additional 24 h with gliadin digest alone or together with IFN-γ. In some experiments, THP-1 cells were preincubated for 1 h at 37 °C with mouse anti-CD14 monoclonal antibody (mAb) MEM-18 or with isotype control IN-OS mouse anti-insulin mAb (both mAb provided by Prof. V. Horejš); the gliadin fragments or LPS were then added to cultured cells in the presence of anti-CD14 mAb or IN-OS mAb for 24 h. Furthermore, NF-κB inhibitors sulfasalazine (0.1–2.0 μM), pyrrolidine dithocarbamate (PDTC) (0.1–10 μM) and t-1-tosylamido-2-phenethyl chloromethyl ketone (TPCK) (1.0–25.0 μM) (Sigma) were added to cultured cells for 30 min, and then gliadin digest was added for 24 h.

2.3. Enzyme-linked immunosorbent assay

The level of IL-8 and TNF-α was determined in cell culture supernatants collected after 6, 24 and 48 h of cultivation by enzyme-linked immunosorbent assay DuoSet kit (R&D System, Minneapolis) according to the manufacturer’s instructions.

2.4. Western blot analysis

THP-1 cells were stimulated for 30 min with gliadin digest (200 μg/ml), IFN-γ (150 U/ml) or LPS (1 μg/ml), rinsed in cold 1 μM NaVO₄ in Tris buffered saline, lysed in iced-cold lysis buffer (25 mM Tris–HCl, pH 7.4, 1 mM DTT, 1 mM NaVO₄ and 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 10 mM NaF and 1 mM PMSF) for 30 min at 4 °C and centrifuged (14 000 × g, 15 min, 4 °C). Supernatants were assayed for protein by the BCA protein kit (Pierce, USA). The same amount of protein from each lysate (30 μg) was subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) [13] and transferred onto nitrocellulose membranes. Phosphorylated IκB protein was detected after incubation with rabbit anti-phospho-IκB Abs (1:1000 final dilution) (Cell Signaling, USA). The membrane was then incubated with goat anti-rabbit horseradish peroxidase-conjugated Abs (1:2500 final dilution) (Cell Signaling, USA). Immunoreactive bands were visualised by ECL detection kit (Amersham, UK) and quantified (1:2500 final dilution) (Cell Signaling, USA). Immunoreactive bands were visualised by ECL detection kit (Amersham, UK) and quantified

2.5. Preparation of cell nuclear extract and colorimetric NF-κB assay

Nuclear extracts were prepared from THP-1 cells stimulated for 90 min with gliadin digest (500 μg/ml) alone or together with IFN-γ (150 U/ml), sulfasalazine (2 mM), PDTC (0.5 μM) and TPCK (5 μM) using a Nuclear extract Kit (Active Motif, USA). NF-κB DNA binding activity was detected using a TransAM NF-κB family transcription factor assay kit (Active Motif, USA) according to the manufacturer’s protocol. Briefly, microwells coated with a double-stranded oligonucleotide containing the NF-κB consensus sequence were incubated with the nuclear extract for 1 h at room temperature and washed three times with washing buffer. The captured active transcription factor was incubated for 1 h with Ab specific for p65 or p50 NF-κB subunit, then for 1 h with anti-rabbit IgG coupled-horseradish peroxidase and after washing exposed to developing solution for 10 min. The optical density was measured at 450 nm using a Titertec Multiscan MCC/340 (Flow Lab., Irvine, Scotland).

2.6. Statistical analysis

Data are presented as arithmetic means of at least three independent experiments ± S.E.M. Statistical analysis was performed by Student–Newman–Keuls multiple range test and Student’s t-test. P values smaller than 0.005 were considered to be significant.

3. Results and discussion

3.1. Cytokine production by human monocytic cell lines stimulated with gliadin

Since the phenotypic and functional characteristics of human jejunal macrophages have not been precisely determined in healthy and diseased conditions, the effect of food proteins was tested using human cell lines THP-1 and U-937. THP-1 and U-937 cells represent different stages of monocytic/macrophage maturation. Unlike the polymorph U-937 cells (CD 14+, CD 68+), THP-1 cells (CD 14+, CD 68+) possesses a normal complement of chromosomes and resemble primary monocoyte-macrophage-derived macrophages in terms of inducible functions [14].

The crude gliadin had a very low direct effect on the activation of THP-1 cells evaluated by IL-8 and TNF-α production, while gliadin proteolytic fragments (100–500 μg/ml) elicited a significant secretion of both cytokines that was enhanced on its joint administration with IFN-γ (150 U/ml). Soya protein and ovalbumin, treated similarly to gliadin, had no effect on IL-8 and TNF-α production either when applied alone or in combination with IFN-γ (Fig. 1).

Since joint administration of IFN-γ with gliadin fragments enhanced the gliadin-induced production of IL-8 and TNF-α, we investigated the effect of IFN-γ prestimulation (Fig. 2). Interestingly, prestimulation of THP-1 cells with IFN-γ (150 U/ml) for 2 or 24 h prior to the addition of gliadin fragments resulted also in a higher secretion of IL-8 and TNF-α with respect to non-prestimulated cells (Fig. 2A and B). The production of IL-8 and TNF-α was even higher when gliadin fragments were applied to prestimulated cells simultaneously with IFN-γ. In contrast to THP-1 cells, the response of U-937 cells to gliadin fragments was very low and these cells produced significantly increased amount of IL-8 only when gliadin was added to the cells prestimulated for 24 h with IFN-γ (Fig. 2C). The TNF-α production after gliadin challenge was not detectable (data not shown).

To complete the time schedule, the IL-8 and TNF-α secretion by THP-1 cells was measured also 6 and 48 h after triggering with gliadin fragments (200 μg/ml) alone or along with IFN-γ (150 U/ml). IL-8 production increased in time, reaching a maximum value after 48 h of cultivation. TNF-α secretion was elevated during the first 24 h. Extension of the cultivation to 48 h did not elevate cytokine secretion in response to gliadin fragments and increased spontaneous release of TNF-α by cells was observed (Fig. 3).
For comparison, we tested the stimulatory capacity of 33-AA peptide derived from α-gliadin (resistant to digestive enzymes), described as an inducer of gliadin-specific T cells [11], synthetic dodecapeptide B (FQQPQQQYPSSQ), a potent mouse macrophage-stimulating peptide [4] and the gliadin peptide (p31-43) that stimulates innate response in cultivated coeliac biopsies [6]. The IL-8 production by THP-1 cells in response to the 33-AA peptide reached only about 20% and to the B peptide about 60% of the response detected with the whole gliadin digest, while p31-43 gliadin peptide exerted the same stimulatory capacity as the gliadin digest. On evaluating TNF-α production, the B peptide reached about 10% and p31-43 about 40% of the response detected with the gliadin digest, while 33-AA peptide was unable to induce TNF-α secretion even in combination with IFN-γ. Similarity in the synergistic effect of IFN-γ and gliadin-derived peptide B, p31-43 and/or the whole gliadin digest on cytokine secretion was observed (data not shown). These results indicate that gliadin-derived peptides differ in their capacity to activate human monocytes and/or macrophages; however, the whole gliadin digest was found to be the most potent activator of these cells.

3.2. The CD14 molecule is not involved in the activation of THP-1 cells by gliadin

The response of THP-1 and U-937 cells to LPS (1 μg/ml), known to activate monocytes/macrophages, was compared with activation by gliadin and/or IFN-γ. When stimulated with LPS, THP-1 cells produced significantly higher levels of IL-8 (558.7 ± 23.7 pg/ml) and TNF-α (107.3 ± 13.0 pg/ml) than U-937 cells (IL-8 = 322.6 ± 16.1 pg/ml; TNF-α = 33.5 ± 5.2 pg/ml). The differences in response of THP-1 and U-937
Fig. 3. The kinetics of IL-8 (A) and TNF-α (B) production by THP-1 cells. Cytokine production was determined 6, 24 and 48 h after addition of gliadin fragments and/or IFN-γ to cells directly or after IFN-γ preincubation (for 2 or 24 h). Data obtained from at least three experiments are expressed as means ± S.E.M.; differences between values at individual time intervals were calculated by Student’s t-test. * P < 0.05, ** P < 0.01, *** P < 0.001.

monocytes were also documented by other authors using Escherichia coli LPS and purified Shiga-like toxins [15,16].

Since the more pronounced response of THP-1 cells seemed to be in accordance with the higher expression of CD14 molecule, we examined the effect of anti-CD14 mAb on cytokine production. Addition of anti-CD14 mAb to THP-1 cells substantially reduced the IL-8 production induced by LPS, but had no effect on the activation by gliadin digest (Table 1). This observation throws doubt on the potential role of CD14 molecule in the activation of cells with gliadin.

3.3. Gliadin-induced IL-8 secretion is mediated via NF-κB pathway

Stimulation of THP-1 cells with gliadin digest (500 μg/ml) and/or with IFN-γ (150 U/ml) resulted in a marked increase of the binding activities of NF-κB subunits p50 and p65, more pronounced in the case of p50 subunit. To confirm the role of NF-κB in gliadin-induced activation of THP-1 cells, we examined the effects of NF-κB inhibitors: sulfasalazine, PDTC and TPCK. Sulfasalazine inhibits phosphorylation of IκB [17]. PDTC functions as a NF-κB inhibitor by blocking the dissociation of the NF-κB/IκB complex [18] and TPCK plays a role as proteasome inhibitor; it can therefore inactivate NF-κB by preventing the degradation of IκB [19]. Treatment with 0.5 μM PDTC and 2 mM sulfasalazine inhibited substantially p65 but only slightly p50 binding activity; 5 μM TPCK reduced the p50 binding capacity more efficiently than that of p65 (Fig. 4).

Moreover, sulfasalazine markedly suppressed, in a dose-dependent fashion, gliadin-stimulated IL-8 secretion by THP-1 cells, producing 15% inhibition at 0.1 mM and 90% inhibition at 2 mM concentration. The effect of PDTC was first evident at 0.1 μM concentration. TPCK produced 32% inhibition at 1 μM and completely abolished IL-8 production at 25 μM concentration (data not shown).

Since NF-κB activation involves the phosphorylation and subsequent degradation of IκB complex [18], we assayed the phosphorylation of IκBα by Western blot analysis. Fig. 5 shows that the treatment of THP-1 cells with gliadin alone or together with IFN-γ increased phosphorylation of IκBα, when compared with unstimulated cells. Taken together, these results suggest that NF-κB molecule containing p50 and p65 subunits is involved in the activation pathway triggered by gliadin fragments in human monocytes.

In summary, our results document for the first time the direct effect of gliadin on human monocytes. In contrast to soya

Table 1

<table>
<thead>
<tr>
<th>IL-8 secretion (pg/ml)</th>
<th>mAb added</th>
<th>+ MEM-18 (10 μg) (% of blockade)</th>
<th>+ IN-05 (10 μg) (% of blockade)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS 10 μg</td>
<td>1030.3 ± 130.0</td>
<td>324.4 ± 76.8 (70)</td>
<td>1038.4 ± 56.6 (0)</td>
</tr>
<tr>
<td>Gliadin 500 μg</td>
<td>287.5 ± 21.2</td>
<td>273.1 ± 11.0 (5)</td>
<td>300.5 ± 20.2 (0)</td>
</tr>
<tr>
<td>Cells</td>
<td>98.0 ± 11.8</td>
<td>100.2 ± 10.5 (0)</td>
<td>98.2 ± 15.6 (0)</td>
</tr>
</tbody>
</table>

Note. Data are shown as means ± S.E.M. MEM-18, anti-CD14 monoclonal antibody IN-05, anti-insulin control monoclonal antibody.
protein and ovalbumin, gliadin and its fragments stimulated human monocytes to increased IL-8 and TNF-α production. IFN-γ, the main cytokine produced by gluten-specific T cells of coeliac patients, exerted a costimulatory effect on chemokine and cytokine production. Moreover, the data from our ongoing experiments show that gliadin peptides also activate human peripheral blood monocytes and that the production of IL-8 and TNF-α significantly differs in cells isolated from healthy donors and coeliac patients. The analysis of factors affecting the responsiveness is under study. Based on these findings, it could be suggested that increased secretion of IL-8 (a chemokine attracting immune cells to inflamed tissue) and TNF-α (cytokine involved in the activation of metalloproteinas digesting tissue components) produced by activated monocytes and/or macrophages could be involved in the impairment of coeliac intestinal mucosa.

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References

Fig. 5. Representative Western blot shows the effect of gliadin fragments, IFN-γ or LPS (positive control) on phosphorylation of IκBα in THP-1 cells (p-IκBα formation) (A) and its densitometric evaluation presented as mean AUC (arbitrary units) ± S.E.M. of three independent experiments, ** P < 0.01 (B).