Food Chemistry 176 (2015) 167–174

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Responses of peripheral blood mononucleated cells from non-celiac gluten sensitive patients to various cereal sources

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ARTICLE INFO

Article history:
Received 28 August 2014
Received in revised form 25 November 2014
Accepted 15 December 2014
Available online 23 December 2014

Keywords:
Wheat proteins
Non celiac gluten sensitivity
CXCL10
Ancient grains

ABSTRACT

Non-celiac gluten sensitivity (NCGS) is still an undefined syndrome whose triggering mechanisms remain unsettled. This study aimed to clarify how cultured peripheral blood mononucleated cells (PBMC) obtained from NCGS patients responded to contact with wheat proteins. Results demonstrated that wheat protein induced an overactivation of the proinflammatory chemokine CXCL10 in PBMC from NCGS patients, and that the overactivation level depends on the cereal source from which proteins are obtained. CXCL10 is able to decrease the transepithelial resistance of monolayers of normal colonocytes (NCM 460) by diminishing the mRNA expression of cadherin-1 (CDH1) and tight junction protein 2 (TJP2), two primary components of the tight junction strands. Thus, CXCL10 overactivation is one of the mechanisms triggered by wheat proteins in PBMC obtained from NCGS patients. This mechanism is activated to a greater extent by proteins from modern with respect to those extracted from ancient wheat genotypes.

1. Introduction

The consumption of wheat-based products is estimated to be very high in most Western countries (i.e. Europe, United States) and in continuous increase in Eastern countries as a consequence of a shift toward a Western lifestyle (Rubio-Tapia et al., 2009; Van den Broeck et al., 2010). Nowadays, most of the wheat products we consume are made from modern wheat varieties bred after the "Green Revolution" (in the 1970s). The introduction of dwarf genes led to the development of short straw lines with substantial gains in productivity and technological quality. The variation in dough rheology and bread-making performance among wheat varieties is largely determined by the differences in protein content and quality composition, depending on specific gliadins and high molecular weight glutenin subunits (Sliwinski, Kolster, Prins, & van Vliet, 2004). Not dwarf wheat genotypes, bred after the "Green Revolution" are defined as old. Generally, old wheat varieties have weaker rheological properties than the modern ones, as demonstrated by alveographic analyses (Guarda, Padovan, & Delogu, 2004). In recent years, scientists have shown increasing interest in investigating nutritional differences among wheat varieties, old genotypes and ancestors due to their diverse nutrient and phytochemical composition. Research studies concerned dietary fiber content (Marotti et al., 2012), phenolics and terpenoid composition (Di Silvestro et al., 2012; Shewry et al., 2011), but strong efforts recently focused on the study of gluten proteins, a major cause of celiac disease (CD) and gluten-related pathologies (Carroccio et al., 2011; Molberg et al., 2005; van den Broeck et al., 2010).

From an evolutionary point of view, gluten proteins were absent from the diet of hunter gatherers (Schnorr et al., 2014) and were introduced in human nutrition only about 10,000 years ago. This may explain the lack of a complete adaptation of humans to the ingestion of gluten proteins. Nevertheless, different studies demonstrate that the prevalence of CD has increased over the last 60 years (Lohi et al., 2007; Rubio-Tapia et al., 2009). Moreover, the increasing number of patients worldwide who are sensitive to

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http://dx.doi.org/10.1016/j.foodchem.2014.12.061
0308-8146/

dietary gluten but lack evidence of celiac disease or wheat allergy has contributed to the identification of a new gluten-related syndrome known as non-celiac gluten sensitivity (NCGS) (Volta, Caio, Tovoli, & De Giorgio, 2013). Double-blind placebo-controlled trials have confirmed that gluten proteins are involved in the development of this syndrome (Biesiekierski et al., 2011; Carroccio et al., 2012). The molecular mechanisms of NCGS remain for the most part unknown, even if it is widely accepted that the innate immune system plays a major role in the onset of NCGS (Catassi et al., 2013; Sapone et al., 2011). Even if the prevalence of NCGS in the western population is still debated, it seems clear that in recent years it has rapidly increased in adults (Sapone et al., 2012; Volta et al., 2014) and in children (Francavilla et al., 2014). Recently, modern wheat strains have been implicated in NCGS (de Lorgeril & Salen, 2014).

The present study evaluated the *in vitro* chemokine response of PMBC from NCGS patients to different cereals (including modern and ancient wheat genotypes) aiming to identify a possible marker for NCGS.

2. Materials and methods

2.1. Cereal samples

The investigated cereal samples consisted of a modern common wheat (*Triticumaestivum* L.) "Manitoba" variety, a modern durum wheat (*Triticumturgidum* spp. *durum*Desf. Husn.) "Claudio" variety, an old Italian durum wheat (*Triticumturgidum* spp. *durum*Desf. Husn.) "SenatoreCappelli" variety, an accession of KAMUT® khorasan wheat (*Triticumturgidum* spp. *turanicum*Jakubz), and a sample of rice (*Oryzasativa* L.) gluten-free flour as a negative control. KAMUT® is a registered trademark of KAMUT International Ltd. and KAMUT Enterprises of Europe bvba. Manitoba and rice samples were purchased at local supermarkets. The flours of Claudio, SenatoreCappelli and KAMUT® khorasan were obtained from the wheat germplasm of the Department of Agricultural Science of the University of Bologna (Italy) and were organically grown. Grains were milled using a stone mill (100% flour extraction).

2.2. Protein extraction

Proteins were extracted using the procedure described by Osborne (1907) and subsequently modified by Lookhart and Bean (1995) and van den Broeck et al. (2009). Proteins were extracted from three seed replicates for each accession. Briefly, 100 mg of flour was treated with 500 μl of distilled water (30 min, 160 rpm) vortexing for 1 min at 10-min intervals for the extraction of albumins. Samples were than centrifuged at 7000 rpm for 5 min and the supernatant collected. Albumin extraction was repeated with 400 μl of distilled water (10 min, 160 rpm), centrifuged and the second extract collected. The pellet was treated with 400 μl of NaCl 0.5 M (30 min, 160 rpm) vortexing for 1 min at 10-min intervals for the extraction of globulins. After centrifugation (3500 rpm, 5 min), the supernatant was collected and the globulin extraction repeated (400 μl of NaCl 0.5 M; 10 min, 160 rpm) to obtain the second globulin extract. Subsequently, the remaining pellet was used for the extraction of prolamins. Four hundred microliter of 50% (v/v) aqueous isopropanol were added to the test tube to allow the solubilization of gliadins (30 min, 160 rpm). After centrifugation (3500 rpm, 15 min), the first gliadin extract was collected and the extraction was repeated (10 min, 160 rpm). Glutenins were extracted using a solution of 50% (v/v) aqueous isopropanol containing 1% (w/v) DL-dithiothreitol for 30 min at 160 rpm and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and the extraction repeated once. For the *in vitro* tests, all the extracts were pooled to obtain a total protein extract for each cereal variety.

2.3. Protein electrophoretic profiles (SDS–PAGE)

The protein profiles of the cereal samples investigated were obtained using the Bolt® Mini Gel Tank (Invitrogen™, Life Technologies) compatible with the Bolt® Bis–Tris Plus precast gels (10%). Electrophoretic patterns were obtained from three replicates for each cultivar. For the electrophoresis analysis the first supernatants of albumins, globulins and glutenins, and the second supernatant of gliadins were used. Extracts (30 μl) were diluted with 15 μl Bolt™ LDS sample buffer, 6 μl Bolt™ reducing agent, 9 μl distilled water and denatured at 70 °C for 10 min. The Bolt™ MOPS SDS running buffer was used for the run at 165 V (25 min). The protein patterns were compared with the Mark12™ unstained standard (2.5–200 KDa) for albumins and globulins, and the SeeBlue® Plus2 pre-stained standard (4–250 KDa) for gliadins and glutenins. The protein profiles were elaborated as presence/absence of the observed subunits for each sample.

2.4. Protein content and quantification of allergenic epitopes

The total protein content was measured using the Kjeldahl procedure ($N \times 5.7$) (AACC, 1983). Wheat flours were tested for the content of gluten allergenic epitopes using the RIDASCREEN®Gliadin competitive ELISA (R-Biopharm AG, Darmstadt, Germany). Flour samples were treated with the RIDA® Extraction Solution (R7099) to enable the solubilization of the gliadin fraction. The test is based on the antigen–antibody reaction between gluten epitopes and the R5 antibody. The absorbance values were obtained at 450 nm using a microplate reader (Multiskan EX 1.1 MTX Lab Systems, Virginia, USA). Results were compared with those of gliadin standard (5–80 ppb) and elaborated using the software RIDA®SOFT Win.net (R-Biopharm AG, Darmstadt, Germany). Epitope contents were expressed as percentage of total protein amount.

2.5. Patients

The study population included 48 NCGS patients (females 39, males 9, median age 41 years, range 20–67 years), diagnosed as having NCGS after a thorough evaluation in the tertiary referral centers of the SpedaliCivili (Brescia, Italy) and S.Orsola-Malpighi Hospital (Bologna, Italy). All these patients complained of one or more gastrointestinal (bloating, abdominal pain, diarrhea/constipation, nausea, epigastric pain, gastro-esopagheal reflux, aphthous stomatitis) and extra-intestinal (tiredness, headache, joint/muscle pain, arm numbness, 'foggy mind', dermatitis/skin rash, anxiety, depression, anemia) symptoms/manifestations with an early onset (a few hours or days) after gluten ingestion. As extensively stated in the literature (Catassi et al., 2013; Sapone et al., 2012), a fundamental prerequisite for NCGS diagnosis is the exclusion of both CD and wheat allergy (WA) on a gluten-containing diet. CD was ruled out in all 48 enrolled NCGS patients by negativity for anti-tissue transglutaminase (anti-tTG) and anti-endomysial antibodies (EmA) and the absence of villous atrophy in the duodenal biopsy, whereas WA was excluded by the negativity for specific IgE antibodies to wheat and/or skin prick tests. NCGS patients were put on a gluten-free diet obtaining a complete remission or a significant improvement of both gastrointestinal and extra-intestinal symptoms, thus confirming the clinical suspicion of NCGS. Thirty healthy volunteers, age- and sex-matched with the NCGS group, were enrolled among students and medical staff of the two referral centers (Bologna and Brescia). A blood sample was obtained from both NCGS patients and healthy volunteers. Before undergoing