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Abnormal anandamide metabolism in celiac disease

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Abstract

The endocannabinoid system has been extensively investigated in experimental colitis and inflammatory bowel disease, but not in celiac disease, where only a single study showed increased levels of the major endocannabinoid anandamide in the atrophic mucosa. On this basis, we aimed to investigate anandamide metabolism in celiac disease by analyzing transcript levels (through quantitative real-time reverse transcriptase–polymerase chain reaction), protein concentration (through immunoblotting) and activity (through radioassays) of enzymes responsible for anandamide synthesis (*N*-acylphosphatidyl-ethanolamine specific phospholipase D, NAPE-PLD) and degradation (fatty acid amide hydrolase, FAAH) in the duodenal mucosa of untreated celiac patients, celiac patients on a gluten-free diet for at least 12 months and control subjects. Also, treated celiac biopsies cultured *ex vivo* with peptic–tryptic digest of gliadin were investigated. Our *in vivo* experiments showed that mucosal NAPE-PLD expression and activity are higher in untreated celiac patients than treated celiac patients and controls, with no significant difference between the latter two groups. In keeping with the *in vivo* data, the *ex vivo* activity of NAPE-PLD was significantly enhanced by incubation of peptic–tryptic digest of gliadin with treated celiac biopsies. On the contrary, *in vivo* mucosal FAAH expression and activity did not change in the three groups of patients, and accordingly, mucosal FAAH activity was not influenced by treatment with peptic–tryptic digest of gliadin. In conclusion, our findings provide a possible pathophysiological explanation for the increased anandamide concentration previously shown in active celiac mucosa.

Keywords: Duodenal mucosa; Endocannabinoid; FAAH; Gliadin; NAPE-PLD

1. Introduction

The endocannabinoid system comprises specific G-protein-coupled receptors, a variety of endogenous ligands including amides and esters of long-chain polyunsaturated fatty acids — among which anandamide (also known as *N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol are the main endogenous cannabinoid receptor agonists — and a machinery dedicated to endocannabinoid synthesis and degradation [1,2]. Besides their effects on the central nervous system and gastrointestinal motility [3], endocannabinoids are supposed to modulate the immune response [4], and their involvement in autoimmune and chronic inflammatory conditions has been recently proposed [5,6]. The demonstration of an abnormal expression/metabolism of endocannabinoids in various models of colitis and in patients with inflammatory bowel diseases has suggested that a dysregulation of the endogenous cannabinoid system might contribute to chronic intestinal inflammation [7,8]. Nevertheless, there is still little evidence on the contribution of endocannabinoids in celiac disease, a chronic inflammation of the small bowel induced in genetically susceptible individuals by an environmental irritant, gluten, with the possible participation of other environmental cofactors [9]. In particular, there is only one study showing higher AEA levels in the duodenal mucosa of active celiac patients in comparison to treated celiac patients and controls [10]. In the present study, we aimed to explore AEA metabolism by investigating mRNA, protein and activity of enzymes responsible for the synthesis (*N*-acylphosphatidyl-ethanolamine specific phospholipase D, NAPE-PLD) [11] and degradation (fatty acid amide hydrolase, FAAH) [12] of AEA in the duodenal mucosa of untreated celiac patients, celiac patients on a gluten-free diet and control subjects. Moreover, through

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ex vivo experiments, we investigated the effect of the peptic–tryptic digest of gliadin (PT-gliadin) on AEA metabolism in organ culture biopsies taken from treated celiac patients.

2. Materials and methods

2.1. Materials

AEA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]AEA (60 Ci/mmol) and *N*-[^{3}H]arachidonoylphosphatidyl-ethanolamine ([^{3}H]NArPE, 200 Ci/mmol) were from ARC (St. Louis, MO, USA). Rabbit anti-NAPE-PLD polyclonal antibody was from Novus Biologicals Inc. (Littleton, CO, USA). Rabbit anti-FAAH, anti-β-actin polyclonal antibodies and secondary antibodies conjugated to horseradish peroxidase were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

2.2. Patients and tissues

Biopsies were collected from the second part of the duodenum of 10 patients affected by uncomplicated untreated celiac disease (four males and six females, mean age 34.6 years, range 19–45). The diagnosis was based on positivity of serum antiendomysial antibodies associated with the typical histopathological lesions, namely, villous atrophy, increased intraepithelial lymphocyte infiltration and crypt hyperplasia. Four patients showed a Marsh IIIc lesion, and six showed a Marsh IIIb lesion. Duodenal biopsies were also collected from 10 patients affected by uncomplicated celiac disease on a gluten-free diet for at least 12 months (two males and eight females, mean age 35.6 years, range 22–66), all negative for serum antiendomysial antibodies, and with a substantially normal duodenal mucosal architecture. Finally, duodenal biopsies were also collected from 11 subjects (three males and eight females, mean age 48.5 years, range 21–70) undergoing upper endoscopy for functional dyspepsia, negative for antiendomysial antibodies and with normal histology. Informed consent was obtained in all cases, and the study was approved by the Ethical Committee of University of Brescia. Some of the biopsies were processed for routine histology, some were used for organ culture experiments, and others were directly snap frozen and stored at −70°C.

2.3. Organ culture

Biopsy specimens, placed on grids in the central well of an organ culture dish, were cultured in an airtight container with 95% O_2/5% CO_2 at 37°C in the absence or presence of 1 mg/ml PT-gliadin (Frazer III fraction; Sigma-Aldrich, Poole, UK) added to the medium containing RPMI-1640 medium (Gibco, Invitrogen, Paisley, UK) supplemented with 10% HL-1 (Lonza BioWhittaker, Verviers, Belgium), 100 U/ml penicillin and 100 mg/ml streptomycin [13]. After 24-h culture, biopsies were snap frozen and stored at −70°C.

2.4. Quantitative real-time reverse transcriptase–polymerase chain reaction (qRT-PCR) analysis

RNA was extracted from CD specimens using the RNeasy extraction kit (Qiagen, Crawley, UK), as suggested by the manufacturer. Quantitative RT-PCR assays were performed using the SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA). The target transcripts were amplified by means of an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA, USA), using the primer reported in Table 1. β-Actin was used as housekeeping gene for quantity normalization. One microliter of the first strand of cDNA product was used for amplification (in triplicate) in 25-μl reaction solution containing 12.5 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA) and 10 pmol of each primer. The following PCR program was used: 95°C for 10 min and 40 amplification cycles at 95°C for 30 s, 56°C for 30 s and 72°C for 30 s.

2.5. AEA metabolism

The synthesis of [^{3}H]AEA through the activity of NAPE-PLD (E.C. 3.1.4.4) was assayed in tissue homogenates (100 μg/test), using 100 μM [^{3}H]NArPE [14], by means of reversed-phase high-performance liquid chromatography, as reported [15]. The hydrolysis of [^{3}H]AEA by FAAH was assayed in tissue homogenates (50 μg/test) by measuring the release of [^{3}H]ethanolamine, as reported [16].

2.6. Western blotting

Western blotting was performed according to standard procedures [17]. In brief, tissue samples were lysed in ice-cold lysis buffer (10 mM EDTA, 50 mM pH 7.4 Tris-HCl, 150 mM sodium chloride, 1% Triton-X-100, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 mg/ml leupeptin and 2 mg/ml aprotinin), and the amount of protein was determined by the Bio-Rad Protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK). An equal amount of protein (20 μg) was loaded onto 10% sodium dodecyl sulfate–polyacrylamide gels and blotted onto polyvinylidene fluoride sheets (Amersham Biosciences, Piscataway, NJ, USA) under reducing conditions. Membranes were blocked with 10% nonfat dried milk and 5% bovine serum albumin for 2 h and then incubated with rabbit anti-NAPE-PLD (diluted 1:100), rabbit anti-FAAH (diluted 1:300) or mouse anti-β-actin (1:1000 dilution) antibodies. Membranes were rinsed and incubated with the appropriate horseradish-peroxidase-conjugated secondary antibody (diluted 1:2000) in blocking solution. Detection was performed using West Dura Chemiluminescence System (Pierce, Rockford, IL, USA). Protein expression levels were quantified by densitometric analysis using the ImageJ software after normalization with β-actin.

Table 1
Oligonucleotide sequences in qRT-PCR for NAPE-PLD, FAAH and β-actin detection

mRNA target	Forward	Reverse
NAPE-PLD	5′-TTGTGAATCCGTGGCCAACATGG-3′	5′-TACTGCGATGGTGAAGCACG-3′
FAAH	5′-CCCAATGGCTTAAAGGACTG-3′	5′-ATGAACCGCAGACACAAC-3′
β-Actin	5′-TGACCCAGATCATGTTTGAG-3′	5′-TTAATGTCACGCAGATTTCC-3′

2.7. Enzyme-linked immunosorbent assay (ELISA)

Interferon (IFN)-γ in organ culture supernatants was measured using the IFN-γ ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

2.8. Statistical analysis

Results are expressed as mean±S.E.M., and each experiment was performed in triplicate. Data were analyzed by means of Prism 5 program (GraphPad Software, San Diego, CA, USA) using the one-way analysis of variance followed by Bonferroni's *post hoc* analysis. A level of $P<.05$ was considered statistically significant.

3. Results

3.1. Mucosal NAPE-PLD and FAAH mRNA

The presence of AEA metabolizing enzymes was tested at the transcriptional level by qRT-PCR analysis in biopsies collected from the duodenum of untreated celiac patients, treated celiac patients and control subjects (Fig. 1A–B). As shown in Fig. 1A, NAPE-PLD mRNA levels in the active celiac mucosa were significantly higher (up to threefold) in comparison to control mucosa ($P<.05$) and treated celiac mucosa ($P<.01$). However, no difference in NAPE-PLD mRNA levels was observed between control mucosa and treated celiac mucosa. Conversely, the expression of FAAH mRNA did not significantly differ in the three groups of patients (Fig. 1B).

3.2. Mucosal NAPE-PLD and FAAH protein expression and activity

The translation of NAPE-PLD and FAAH mRNAs into the corresponding proteins was assessed by Western blotting analysis (Fig. 2A–B). The densitometric analysis showed that NAPE-PLD protein expression had a

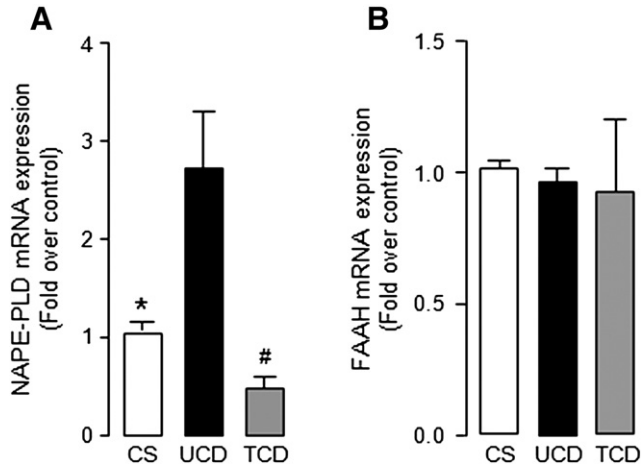

Fig. 1. Quantitative RT-PCR analysis of NAPE-PLD (A) and FAAH (B) in the duodenal mucosa of untreated celiac patients (UCD), treated celiac patients (TCD) and control subjects (CS). *$P<.05$ UCD vs. CS. #$P<.01$ UCD vs. TCD.