Biochimica et Biophysica Acta 1852 (2015) 1779–1786

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Gliadin-mediated production of polyamines by RAW264.7 macrophages modulates intestinal epithelial permeability *in vitro*

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ARTICLE INFO

Article history:
Received 16 February 2015
Received in revised form 28 April 2015
Accepted 1 June 2015
Available online 3 June 2015

Keywords:
Celiac disease
Gliadin
Polyamines
Arginase
Intestinal permeability

ABSTRACT

Celiac disease (CD) is an immune-mediated enteropathy sustained by dietary gluten in susceptible individuals, and characterized by a complex interplay between adaptive and innate responses against gluten peptides (PTG). In a recent contribution we have demonstrated that the treatment with PTG induces the expression and activity of arginase in both murine macrophages and human monocytes from healthy subjects, thus suggesting a role for arginine and its metabolites in gluten-triggered response of these cells. Here we further explore this field, by addressing the effects of PTG on polyamine synthesis and release in murine RAW264.7 macrophages, and how they affect epithelial permeability of Caco-2 monolayers. Results obtained show a massive production and release of putrescine by macrophages upon incubation with gluten peptides; this, in turn, causes a decrease in TEER in epithelial cells, indicating that PTG-driven secretion of polyamines by macrophages has a role in the modulation of intestinal permeability *in vitro*. At a molecular level, putrescine production appears referable to the activation of C/EBPβ transcription factor, which is known to be responsible for arginase induction in activated macrophages and is a crucial mediator of inflammation. Whether these pathways are stimulated also *in vivo* deserves to be further investigated, as well as their role in gluten-driven cellular and intestinal defects typical of CD patients.

1. Introduction

Celiac disease (CD) has been recently defined as "a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals" [1].

Actually, gluten peptides enter the body as components of common dietary grains, evade destruction by gastrointestinal proteases and access the lamina propria, where they are deamidated by the enzyme transglutaminase 2 (also referred to as tissue transglutaminase, tTG) [2]. Only in patients with CD, however, this deamidation allows for high-affinity binding to human leucocyte antigen HLA-DQ2 and -DQ8 molecules and subsequently triggers an inflammatory reaction, involving a complex interplay between adaptive and innate responses [3,4]. It is generally accepted, indeed, that CD is a T-cell mediated disease, in which lamina propria infiltrating T lymphocytes release proinflammatory cytokines that lead to profound tissue remodeling in response to gluten ingestion; however, only 2–5% of individuals expressing CD-associated HLAs develop the disease, indicating that additional pathogenetic mechanisms mediated by innate immune activation are involved [5]. To this concern, recent findings indicate that, unlike other food proteins, gluten and its proteolytic fragments, besides inducing a specific immune response, also activate components of innate immunity. For example peptides, such as the P31–43 peptide, are not recognized by T cells but are able to activate an innate immune response, as well as to damage the celiac intestinal mucosa *in vitro* and *in vivo* [6]. How the activation of immune cells ultimately relates to *in vivo* toxicity for celiac patients and, in particular, to the intestinal affection typical of CD, remains, however, thus far unknown.

During active disease, the architecture of the celiac epithelium is massively perturbed, with villous flattening and crypt hyperplasia [7], and the tight junction system appears morphologically altered too [8]. As a result, untreated celiac patients exhibit increased intestinal permeability, which is considered an important contributor to the influx of gluten peptides into the subepithelial lamina propria [3]. However, whether altered intestinal permeability is a primary cause or a consequence of intestinal inflammation is still unclear, as well as the precise mechanisms that control this event.

Several substances are known to be involved in the control of intestinal permeability; among these, a key role has been recently attributed to polyamines [9]. These organic polycations, namely putrescine, spermidine and spermine, play a pivotal role in the regulation of many

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http://dx.doi.org/10.1016/j.bbadis.2015.06.001
0925-4439/

physiological functions at transcriptional, translational and post-translational level [10]. The intracellular content of these molecules is tightly regulated through processes of both synthesis/degradation and uptake/release. As far as polyamine transport is concerned, although the system specifically involved in this event has not been completely elucidated thus far, different putative transport models have been recently proposed, including both endocytosis and active transport operated by membrane transporters as those belonging to solute carrier (SLC) superfamily [11]. When considering polyamine metabolism, on the other hand, their synthesis is known to involve two alternative pathways, via ornithine and agmatine, both leading to the formation of the diamine putrescine, with the other two polyamines deriving from the subsequent attachment of two propylamine groups to putrescine by spermidine and spermine synthetase. However, the role of agmatine in polyamine metabolism in extra nervous mammalian tissues is still under debate, although well-characterized in plants, bacteria, fungi and invertebrates [12]. Therefore, the main factor in determining tissue polyamine levels remains undoubtedly the activity of ornithine decarboxylase (ODC), the rate limiting enzyme in polyamine synthesis from ornithine [10]. Ornithine, in turn, is produced from arginine by arginase, which plays a key role in the synthesis of polyamines under pathological and physiological conditions [13].

The involvement of arginine in the pathogenesis of CD has been thus far indirectly investigated, with reference to its role as the obliged precursor of nitric oxide (NO) via the activity of nitric oxide synthase (NOS) [14]. Several experimental and clinical findings indicate, indeed, that high levels of nitrites, stable derivatives of NO, are present in serum and urine of children with celiac disease and correlate with an increased expression of iNOS in the small intestine; moreover, NOS activity appears increased in patients with untreated CD and is partially corrected by a gluten free diet (GFD) (see [15] for review). Recently, our group has demonstrated that, besides strengthening the stimulatory effects of IFNγ on NO production, the treatment with gluten peptides (PTG) also induces the expression and activity of arginase in both murine macrophages and human monocytes from healthy subject, thus suggesting a role for arginine and its metabolites in gluten-triggered response of these cell models [16]. In the present paper we further explore this field, by addressing the effects of gluten peptides on polyamines synthesis and release in murine RAW264.7 macrophages. The effects of arginine metabolites produced by PTG-treated macrophage on intestinal epithelial permeability have been also evaluated.

2. Materials and methods

2.1. Cell cultures and experimental treatments

RAW264.7 murine macrophages and Caco-2 human colon carcinoma cells were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia-Romagna (Brescia, Italy) and routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C, pH 7.4, in an atmosphere of 5% CO_2.

For the experiments, RAW264.7 cells were seeded onto multi-well trays (2×10^5 cells/ml) and incubated in complete growth medium in the absence or in the presence of 10 ng/ml IFNγ, 1 mg/ml gliadin peptic–tryptic peptides (PTG, obtained as previously described [16]) or both, as indicated. Albumin peptic–tryptic peptides (PTA), obtained from bovine serum albumin (BSA) through the same enzymatic digestion as PTG (see above), were employed as control. When the inhibition of ornithine decarboxylase (ODC) was required, cells were pre-treated for 12 h with 5 mM 2-(difluoromethyl)ornithine (DFMO), before being incubated in fresh complete growth medium, either in the absence or in the presence of 1 mg/ml PTG, for further 24 h. For the experimental treatments under Arg-free conditions, cells were incubated in a modified arginine-free RPMI-1640 supplemented with 10% FBS, either in the absence or in the presence of 1 mg/ml PTG for 24 h. At the end, incubation media were collected and centrifuged for 3 min at 12,000 *g*, so as to pellet and discard dead cells. The supernatants were, then, employed as conditioned media (CM) for the treatment of Caco-2 cultures, or analyzed for nitrite or polyamines content.

Caco-2 cells were seeded on Cell Culture Inserts (12 mm in diameter, pore size 0.4 μm; Falcon) at the density of 3×10^5 cells/ml, with apical and basolateral fluid volumes of 250 and 700 μl, respectively. Monolayers were used after 7 days, when transepithelial electrical resistance (TEER), measured with EVOM epithelial voltmeter (World Precision Instruments, Sarasota, FL, USA), reached values >800 Ω/cm^2, thus indicating a tight monolayer. At this time, either the apical or the basolateral fluids were replaced with Conditioned Medium (CM) from RAW 264.7 cells, while the medium in the other compartment remained unaltered. TEER was monitored at the indicated times and values expressed as the percentage of the initial value adjusted for control cell layers according to the following equation [17]:

$$\Delta_{\mathrm{TEER}}(\%) = \frac{\text{final TEER}_{\text{treated}}}{\text{final TEER}_{\text{control}}} \times \frac{\text{initial TEER}_{\text{control}}}{\text{initial TEER}_{\text{treated}}} \times 100.$$

2.2. NO production

RAW264.7 cells were incubated for 24 h as required by the experimental plan. At the end, the production of NO was assessed through the quantification of nitrite, stable derivatives of NO, in the culture media, as already described [18]. A fluorimetric approach was employed, based on the production of the fluorescent molecule 1-(H)-naphtotriazole from DAN in the presence of nitrite in an acid environment. Fluorescence was determined with EnSpire® Multimode Plate Reader (Perkin Elmer, Monza, Italy). Nitrite production is expressed in nmol/ml of extracellular medium (μM).

2.3. Determination of citrulline content

The intracellular content of citrulline was determined through HPLC analysis with a Biochrom 20 Amino Acid Analyzer (Biochrom, Cambridge, UK) using a High Resolution Column Bio 20 Peek Lithium and the Physiological Fluid Chemical Kit (Biochrom) for elution, as already described for arginine [19]. The column effluent was mixed with ninhydrin reagent, passed through the high temperature reaction coil, and read by the photometer unit. Data are expressed as nmol/mg of protein.

2.4. UHPLC-MS/MS analysis of putrescine production

For the determination of putrescine production, cells were seeded onto 6-well trays and treated as indicated. At the end, extracellular incubation medium was collected, while cell monolayers were rapidly washed with ice-cold PBS and the intracellular pool was extracted through 5 min incubation in EtOH (1 ml/well) at 4 °C. Extracted cells were finally dissolved with 0.5% sodium deoxycholate in 1 M NaOH, so as to determine protein content in each well using a modified Lowry procedure [20].

Intra- and extracellular extract samples were analyzed using a UHPLC (Dionex Ultimate) system equipped with a triple quadrupole (TSQ Vantage) (Thermo Fisher Scientific Inc., San Jose, CA, USA). The RP-C18 column used was an Aeris Peptide, 1.7 μm, 150 mm length (Phenomenex, Torrance, CA, USA); eluent A was water and 0.2% acetonitrile while eluent B was acetonitrile both acidified with 0.1% formic acid. The total flow was maintained at 0.2 ml/min; the gradient started from 0% of B (0–1.8 min) and it increased at 50% (1.8–13.2 min), this condition was maintained for 1.8 min (13.2–14 min), then the column was flashed increasing % of B to 100% (14–14.5 min) for 1.6 min

(14.5–16.1 min) and after that the initial conditions were re-established in 1.4 min. The total run time was 29.5 min.

The compounds of interest (putrescine, spermine and spermidine) were monitored using positive ionization mode (spray voltage = 3200 V), with the capillary temperature at 270 °C, while the vaporizer temperature was kept at 250 °C. The sheath gas flow was 50 units and the auxiliary gas flow was 15 units. All the other parameters, as S-Lens RF amplitude value, were obtained by tuning a methanolic standard solution of the three analytes separately (1000 μg/kg).

The three considered amines were monitored in SRM mode: the transitions utilized were 89.0 m/z → 72.1 m/z (CE = 10 V) and 89.0 → 55.0 m/z (CE = 20 V) for putrescine, 203.1 m/z → 129. 2 m/z (CE = 12 V) and 203.1 → 112.1 m/z (CE = 18 V) for spermine and 146.0 m/z → 72.1 m/z (CE = 15 V) and 146.0 → 112.1 m/z (CE = 14 V) for spermidine.

Calibration curve was performed both in ethanol (for intracellular extracts) and in medium (for extracellular extracts) at the following concentrations: 2.5, 5, 10, 15 and 20 μM. Chromatographic signals were integrated using the Xcalibur Software. Amine production is expressed as nmol/mg of protein for the intracellular content and as nmol/ml for the extracellular medium (μM).

2.5. Western blot analysis

For the analysis of total C/EBPβ expression, RAW264.7 cells, grown on 10-diameter Petri dishes (3×10^6 cells/dish) and treated as indicated, were lysed in RIPA buffer containing a cocktail of protease inhibitors (Complete, Mini, EDTA-free, Roche). Lysates were, then, sonicated for 5 s and centrifuged at 12,000 *g* for 10 min at 4 °C. For the subcellular localization of the transcription factor, nuclear proteins were isolated by means of Nuclear Extract Kit (Active Motif, Vinci-Biochem, Firenze, Italy), according to the manufacturer's instructions. Western blot analysis was performed as described previously [21]. Briefly, 20 μg of proteins were separated by SDS-PAGE (10% acrylamide) and electrophoretically transferred to PVDF membranes (Immobilon-P membrane, Millipore, Milano, Italy). Membranes were incubated for 1 h at RT in Tris-buffered saline solution (TBS; 50 mM Tris–HCl pH 7.5, 150 mM NaCl) containing 5% dried milk, then incubated overnight at 4 °C in TBS added with 3% BSA, 1% casein and anti-C/EBPβ (1:200) purified rabbit polyclonal antibodies (Santa Cruz Biotechnology, DBA, Milano, Italy). LAMIN A/C detected with a polyclonal antibody (1:400, Santa Cruz Biotechnology), or β-tubulin detected with a monoclonal antibody (1:1000, Santa Cruz Biotechnology) were employed as internal standard. Immunoreactivity was visualized with enhanced chemiluminescence (Millipore, Milano, Italy). The densitometric analysis of blots was performed employing Image Quant® software by Molecular Dynamics (GE Healthcare Life Sciences, Milano, Italy).

2.6. qRT-polymerase chain reaction

1 μg of total RNA, extracted with GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) was reverse transcribed and 40 ng of cDNA were amplified as described previously [22], employing forward and reverse primers specific for *cebpb* (GGAGACGCAGCACAAGGT and AGCTGCTTGAACAAGTTCCG), *slc3a2/4F2hc* (GCAGGACGGTGTGGATGG and GTCGCTGGTGGATTCAAGTATG) or *gapdh* (TGTTCCTACCCCCAATGT GT and GGTCCTCAGTGTAGCCCAAG). The expression of the gene of interest under each experimental condition was normalized to that of the housekeeping gene (*gapdh*) and shown relatively to its expression level in control, untreated cells (=1).

2.7. Putrescine transport

For the determination of putrescine influx, RAW264.7 cells seeded on 96-well dishes, were washed with Earle's Balanced Salt Solution (EBSS) containing (in mM) 117 NaCl, 1.8 $CaCl_2$, 5.3 KCl, 0.9 NaH_2PO4, 0.8 $MgSO_4$, 5.5 glucose, 26 Tris–HCl adjusted to pH 7.4 or, for sodium-free determinations, with a modified EBSS, where NaCl was replaced with equimolar *N*-methylglucamine. Cells were then incubated for 30 min in 50 μl of the same solutions containing [1,4-^{3}H(N)]-Putrescine (19 μM, 2 μCi/ml), both in the absence and in the presence of the indicated amino acids (2 mM). The experiment was terminated by two rapid washes (<10 s) in ice-cold urea (300 mM) and cell monolayers were extracted in 50 μl ethanol. The radioactivity in cell extracts was quantified with a Wallac Microbeta Trilux^2 liquid scintillation spectrometer (Perkin Elmer). Extracted cells were then dissolved with 0.5% sodium deoxycholate in 1 N NaOH and protein content was determined directly in each well using a modified Lowry procedure as previously described [18]. Putrescine transport is expressed as nmol/mg of protein/min.

When addressing putrescine efflux, cells, seeded on 96-well plates, were loaded with 50 μM [1,4-^{3}H(N)]-putrescine for 15 min in EBSS. Then, cells were washed twice with EBSS and incubated in 100 μl of the same solution (efflux medium) in the absence or in the presence of 1 mM putrescine or leucine. At the indicated times efflux medium was removed, replaced with fresh medium and counted for radioactivity ($CPM_{efflux\ medium}$) with Wallac Microbeta Trilux^2. Cell monolayers were extracted in 50 μl of ethanol and radioactivity in the extracts was measured, so as to determine putrescine remained within the cells ($CPM_{intracellular}$). The percent of putrescine efflux at each time was calculated according to the formula

$$\frac{CPM_{efflux\ medium}}{CPM_{intracellular} + CPM_{total\ efflux\ media}}, \%.$$

2.8. Statistical analysis

Statistical analysis was done with Prism® 5.0 Graph Pad software, using one-way ANOVA followed by Bonferroni post hoc test. Differences were considered significant when the associated probability value (p) was <0.05.

The effects of drug combination have been studied by means of the CompuSyn software (ComboSyn, Inc., available online at www.combosyn.com), developed by Ting-Chao Chou and Nick Martin according to the original method of the Combination Index (CI) by Chou and Talalay [additive (CI = 1); synergistic (CI < 1); antagonistic (CI > 1)] [23,24].

2.9. Materials

RPMI-1640 and endotoxin-free FBS were purchased from Euroclone (Milano, Italy). Arg-free RPMI1640 (1X) mod. was from Biochrom AG and purchased from VWR International PBI s.r.l. (Milano, Italy), as well as IFNγ. Microtech s.r.l. (Napoli, Italy) provided Falcon plastic disposable. [1,4-^{3}H(N)]-Putrescine (51.8 Ci/mmol) was obtained from Perkin-Elmer (Monza, Italy). Sigma-Aldrich (Milano, Italy) was the source of gliadin, albumin, and, unless otherwise specified, of all the other chemicals.

3. Results

3.1. Gliadin peptides modify arginine metabolism in macrophages

Previous findings obtained in our lab sustained the hypothesis of a role for arginine (Arg) metabolism in the effects exerted by gliadin in macrophages [16]. To further address this issue, we monitored here the production of Arg main metabolites in murine macrophages (RAW264.7 cells) treated for 24 h with gliadin peptides (PTG) or albumin peptides (PTA) employed as negative control, both alone or in the presence of INFγ. Data in Fig. 1 clearly indicate that the treatment with PTG, but not with PTA, caused a modest, not significant increase of intracellular citrulline content, as well as of nitrites concentration in

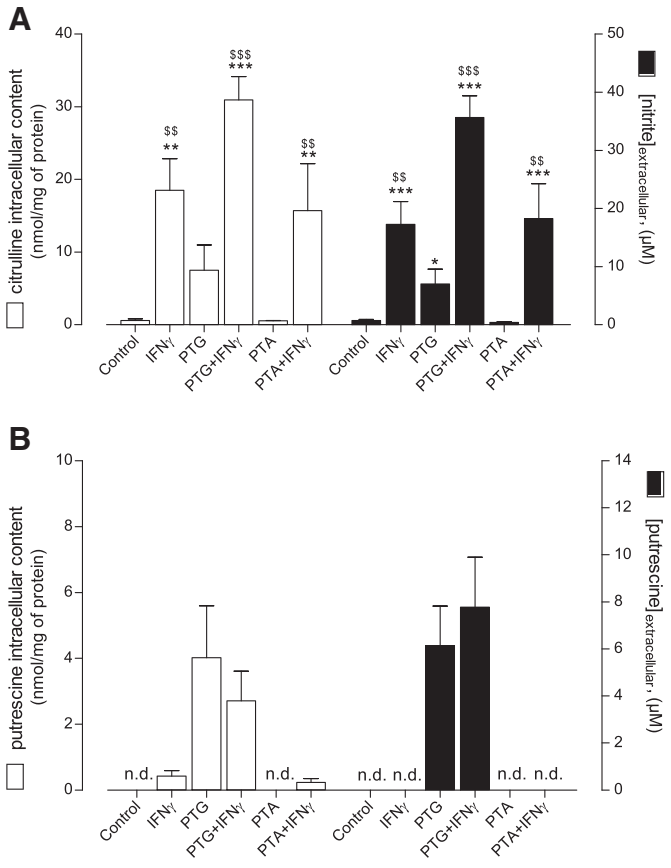

Fig. 1. Effects of gliadin peptides on arginine metabolism. RAW264.7 cells were either untreated (control) or treated with 10 ng/ml IFNγ, 1 mg/ml PTG, 1 mg/ml PTA or the indicated combinations of the compounds in complete growth medium for 24 h. Panel A: The activity of NOS was monitored through the measurement of intracellular citrulline content (empty bars) and the quantization of nitrites in the incubation medium (solid bars), as described in Methods. Panel B: The activation of arginase pathway was monitored by measuring both the intracellular content (empty bars) and the extracellular concentration (solid bars) of putrescine, as described in Methods . Data are means ± SEM of 4 independent determinations. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control, untreated cells; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ *vs.* PTG-treated cells. n.d.: not detectable.

the extracellular medium, hence indicating an at least slight, specific activation of iNOS pathway by gliadin peptides (panel A). When simultaneously added to the incubation medium along with INFγ, PTG stimulated both citrulline and nitrites production in synergism with the cytokine (CI value < 1, according to the Combination Index method for drug combination studies described in Methods), due to a further induction of iNOS activity by PTG, beside that due to the cytokine alone [16]. Under the same experimental conditions, arginase pathway has been also addressed by monitoring the production of polyamines. Panel B of Fig. 1 shows that the treatment with gliadin peptides, alone or in combination with IFNγ, lead to a marked increase in the intracellular content of putrescine and an even more evident accumulation of this polyamine in the extracellular medium, which could not be observed upon treatment with PTA. The simultaneous addition of IFNγ, which *per se* exerted only a modest increase of the intracellular level of putrescine, did not further stimulate the PTG-driven release of the polyamine. No significant production of the other polyamines spermine and spermidine was observed under any of the experimental conditions adopted (data not shown).

3.2. Gliadin-induced arginase pathway is elicited by C/EBPβ

Since increasing evidences identify C/EBPβ as the transcription factor essential for arginase 1 (*Arg1*) expression in macrophages [25,26], we next addressed the involvement of this protein in the PTG-dependent induction of arginase pathway in RAW 264.7 cells (Fig. 2). A significant modulation of C/EBPβ actually occurred upon stimulation of RAW264.7 macrophages with PTG, either alone or combined with INFγ, as indicated by nuclear translocation of the transcription factor (panels A and B) and by the increased expression of both mRNA and protein (panels C and D). In particular, an early (1 h), significant accumulation of C/EBPβ in the nuclear compartment was evident, along with a concomitant reduction in the cytosol (panels A and B) in PTG-treated cells, thus indicating a translocation-mediated activation of the transcription factor. In parallel, a progressive increase of *cebpb* mRNA expression was observed, that reached the maximal values after 8 h exposure (panel C). At this time, also the expression of C/EBPβ protein was significantly higher in total lysates of PTG-treated than in control, untreated cells (panel D), hence suggesting that a regulation of the protein also occurs at transcriptional/translational level.

3.3. Conditioned medium from PTG-treated macrophages affects the integrity of epithelial monolayer

Due to the evident alteration of Arg metabolism induced by PTG, we next hypothesized that the release of specific metabolites by activated macrophages could somehow have a role in the perturbation of the intestinal epithelial integrity, as observed in many clinical events [27]. To verify this hypothesis, RAW264.7 macrophages were treated for 24 h with PTG, IFNγ, or both. After this time, culture media were collected and employed as the incubation medium (Conditioned Medium, CM) for human intestinal epithelial Caco-2 cells grown on Cell Culture Inserts. The integrity of the intestinal cell monolayer under the different experimental conditions was evaluated by monitoring the trans-epithelial electrical resistance (TEER). As shown in Fig. 3, the addition of the different CM to the apical compartment of intestinal cultures did not alter the TEER value of the monolayer (panel A), indicating that the integrity of the epithelial monolayer was preserved under any of the experimental conditions adopted. Conversely, when the medium was added to the basolateral compartment of Caco-2 cultures (panel B), a progressive, evident decrease in TEER was observed in cells incubated with conditioned medium from PTG-treated macrophages, both in the absence (CM-PTG) or in the presence of IFNγ (CM-PTG + IFNγ). The CM-IFNγ alone, however, was ineffective, and the presence of the cytokine did not further strengthen the effect of the peptide. In a parallel experiment, the effect of fresh, not-conditioned growth medium containing PTG, IFNγ or both added to the basolateral compartment was also evaluated (panel C). Since no significant change of TEER was detectable under these latter conditions, we can conclude that the alterations observed in the monostrate permeability of CM-treated Caco-2 monolayers are specifically attributable to the presence in the basolateral compartment of molecules released by macrophages in CM, and not to PTG or INFγ themselves.

3.4. Arginine metabolites have a role in the modulation of epithelial barrier permeability

In light of the results thus far obtained, we next address the potential link among the production of polyamines by PTG-treated RAW264.7 and the observed impairment of the epithelial integrity of Caco-2 cells. To this aim, we adopted two experimental strategies able to prevent the production of polyamines by PTG-treated RAW264.7, *i.e.* the addition of 2-(difluoromethyl)ornithine (DFMO), a well-known inhibitor of the enzyme ornithine decarboxylase (ODC) [28] and the use of an arginine-free medium, so as to eliminate the substrate required by ODC. As expected, the levels of extracellular putrescine, which raised to about 8 μM when RAW264.7 were treated with PTG alone, were undetectable when PTG was added both along with DFMO or in arginine-free medium (Fig. 4, panels A and C). These conditioned media were collected and added to the basolateral compartment of

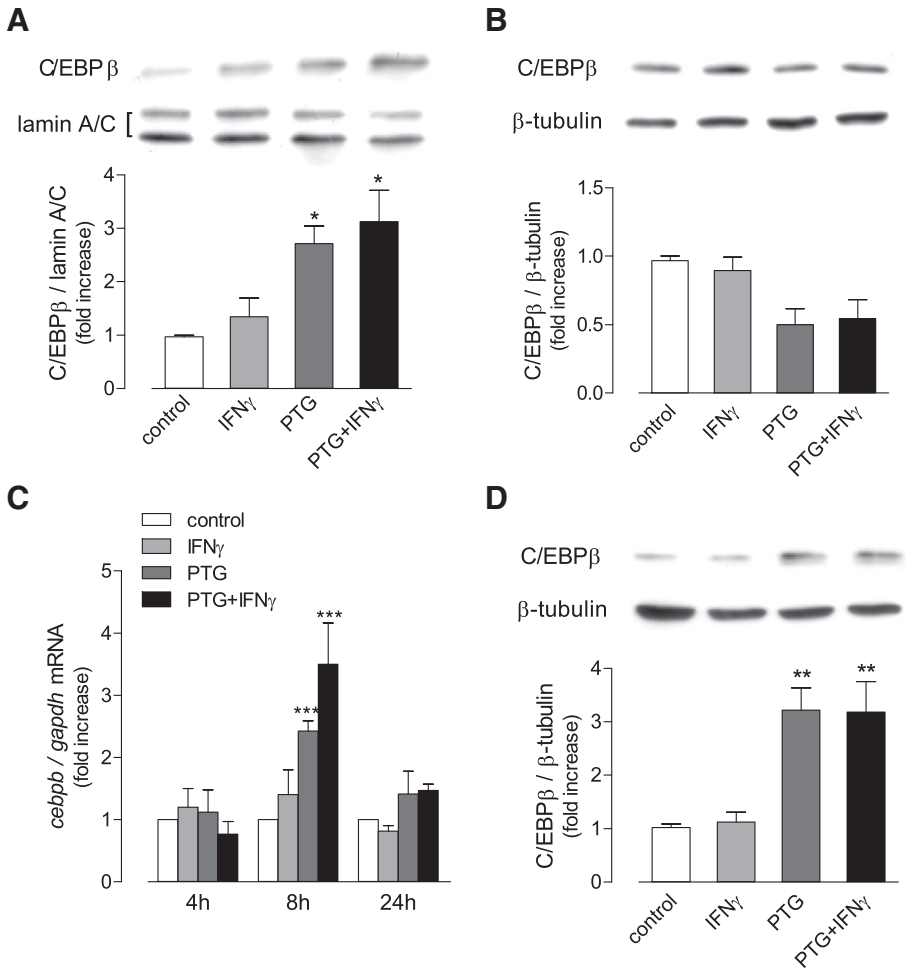

Fig. 2. Involvement of C/EBPβ in PTG-mediated induction of arginase pathway. RAW264.7 cells were either untreated (control) or treated with 10 ng/ml IFNγ, 1 mg/ml PTG or both in complete growth medium. The nuclear (panel A) and cytosolic (panel B) localizations of C/EBPβ protein were investigated as described in Methods, after 1 h exposure to the indicated experimental treatments. The experiments were repeated 3 times with comparable results; representative western blots are shown, along with the densitometric analyses corresponding to the means ± SEM of the repeats. Panel C: The level of *cebpb* mRNA was measured at the indicated times as detailed in Methods. Bars represent means ± SEM of 4 independent determinations, each performed in duplicate. Panel D: The expression of C/EBPβ protein was quantified in total cell lysates, as described in Methods. A representative western blot is shown, while the densitometric analysis reported corresponds to the mean ± SEM of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control, untreated cells.

Caco-2 cultures. Transepithelial electrical resistance was measured at different times as an indicator of epithelial integrity. As shown, neither DFMO (panel C) nor arginine-free medium (panel D) did affect TEER at any time, while CM-PTG caused a marked time-dependent reduction, as previously observed (see Fig. 2). The presence of DFMO (panel C) and the absence of Arg (panel D) in CM, when combined with the treatment

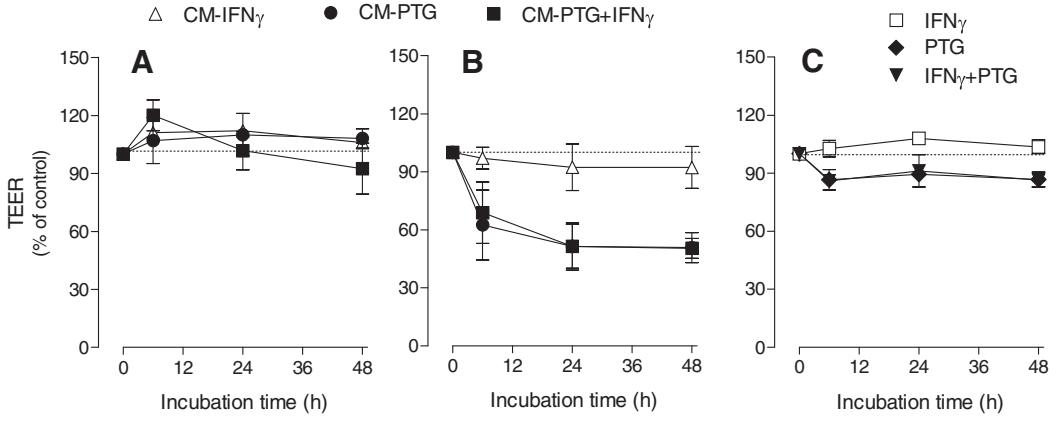

Fig. 3. Effects of RAW264.7 conditioned medium on the epithelial permeability of Caco-2 monolayers. RAW264.7 cells were either untreated (dotted line) or treated with 10 ng/ml IFNγ, 1 mg/ml PTG or both in complete growth medium. After 24 h, conditioned media (CM-IFNγ, CM-PTG or CM-PTG + IFNγ, respectively) were collected and added to the apical (panel A) or to the basolateral (panel B) compartment of Caco-2 intestinal epithelial cell monolayers grown on Cell Culture Inserts. Panel C: Not-conditioned, fresh growth medium containing 10 ng/ml IFNγ, 1 mg/ml PTG or both was added to the basolateral compartment of Caco-2 monolayers. Transepithelial electrical resistance (TEER) was measured in each well at the indicated time, as described in Methods.

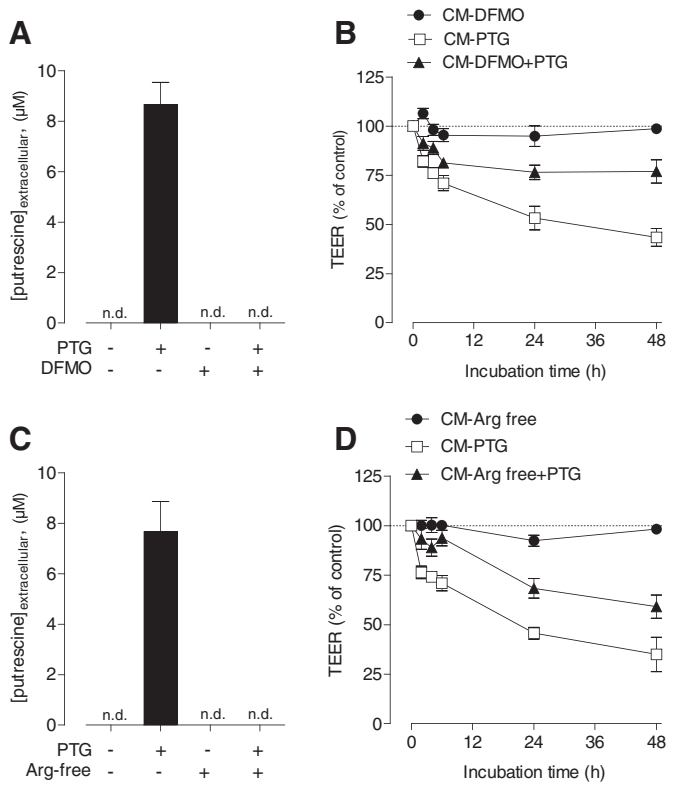

Fig. 4. Role of arginine metabolites in the modulation of the epithelial permeability of Caco-2 monolayers. Panels A and C: RAW264.7 cells were either untreated or treated with 1 mg/ml PTG in complete growth medium in the absence or in the presence of 5 mM DFMO (panel A) or in arginine-free medium (panel C), as indicated. After 24 h, the extracellular concentration of putrescine under each condition was measured as described in Methods. Data are means ± SEM of 4 independent determinations. n.d.: not detectable. Panels B and D: Following exposure to the treatments above, the conditioned media (CM) from RAW264.7 cells were collected and added to the basolateral compartment of Caco-2 intestinal epithelial cell cultures. CM obtained as described in panels A and C were used for the determinations shown in panels B and D, respectively. Transepithelial electrical resistance (TEER) was measured at the indicated times, as described in Methods. Data are means ± SEM of 4 independent determinations.

with PTG, were equally effective in reducing TEER decrease, sustaining the hypothesis that the release of putrescine by PTG-treated macrophages is somehow involved in the increase of epithelial permeability. Actually, after 48 h of incubation, TEER decrease in CM-PTG treated cells was reduced by the presence of DFMO by more than 40%. When considering the absence of arginine, the protective effect was more evident for short-term incubations (2, 4 and 6 h), when TEER decrease was almost negligible. Anyway, even after 48 h in CM-Arg free + PTG, TEER decrease was reduced by more than 30% with respect to cells maintained in CM-PTG.

3.5. 4F2hc is induced by PTG in RAW264.7 macrophages

The system for the transmembrane transport of polyamines in mammalian cells has not been fully defined, yet. However, increasing evidences suggest that some of the known membrane transporters might be involved in polyamines fluxes [11]. Among these, SLC3A2/4F2hc has been identified as a component of the DiAmine eXporter (DAX) which exports putrescine in CHO cells [29]. In light of these evidences, we addressed here the expression of *slc3a2/4F2hc* in RAW264.7 macrophages. As shown in Fig. 5, panel A, a significant induction of *slc3a2/4F2hc* mRNA occurred upon treatment with PTG, thus suggesting an involvement of this transport component in mediating putrescine efflux under our experimental conditions. In an effort to identify the transport system specifically engaged in this process, the transmembrane transport of ^{3}H-putrescine in RAW264.7 cells has been measured under conditions able to discriminate the contribution of the different transporters whose operation requires 4F2hc as heavy chain (panel B). In particular, the reduction of putrescine influx by an excess of the substrate confirmed the contribution of a saturable transport system. Moreover, since the absence of sodium significantly lowered putrescine fluxes with respect to what observed in its presence (control), we can conclude that the transporter involved operates in a sodium-dependent manner (thus excluding the contribution of system L and system x^{-c}). However, neither an excess of extracellular arginine nor of leucine did affect putrescine uptake, hence excluding the involvement of 4F2hc-linked co-transporter system y+L. This result has been also confirmed by measuring the efflux of the polyamine. As shown in panel C, indeed, the presence of leucine in the extracellular medium did not trans-stimulate putrescine efflux, as it would have been expected for system y+L; the efflux was neither affected by extracellular putrescine itself.

4. Discussion

In celiac disease (CD) an evident increase of mucosal permeability is observed since the early phases of the pathological onset, and associates with the small-intestinal inflammation and damaged morphology [30,31]. However, the precise mechanisms that lead to compromised intestinal barrier function in CD are still under debate, although a key role

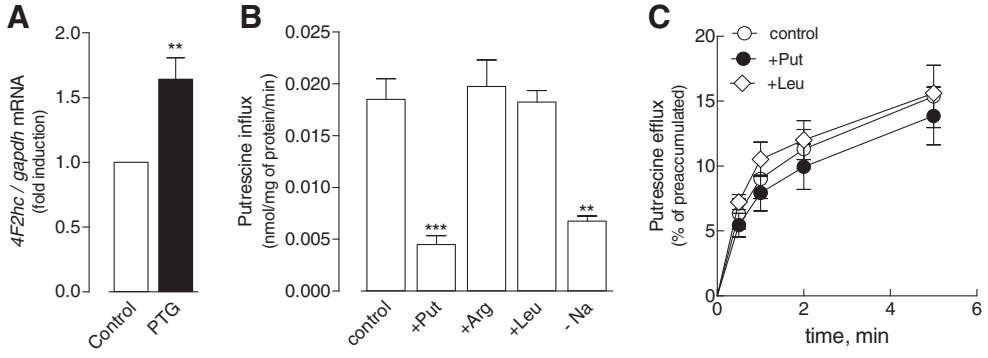

Fig. 5. 4F2hc expression and putrescine transport in RAW264.7 macrophages. Panel A: RAW264.7 cells were either untreated (control) or treated for 24 h with 1 mg/ml PTG. At the end, mRNA was extracted and the expression of *4F2hc* measured as described in Methods. Data are means ± SEM of 3 experiments, each performed in duplicate. Panel B: Putrescine uptake was assessed as described in Methods with 30-min incubation in EBSS supplemented with [1,4-^{3}H(N)]-putrescine (19 μM, 2 μCi/ml) in the absence (control) or in the presence of an excess (2 mM) of putrescine (+Put), arginine (+Arg) or leucine (+Leu), or in a sodium-free EBSS (−Na^{+}), as indicated. Data are means ± SEM of 3 experiments, each performed in quadruplicate. Panel C: For the measurement of putrescine efflux, cells were maintained for 15 min in EBSS containing 50 μM [1,4-^{3}H(N)]-putrescine, rapidly washed, then incubated in 100 μl EBSS, in the absence (control) or in the presence of 1 mM putrescine (+Put) or leucine (+Leu). At the indicated times, putrescine efflux was determined as described in Methods. Data are means ± SEM of 3 independent experiments, each performed in triplicate. **$p < 0.01$, ***$p < 0.001$ *vs.* control, untreated cells.

seems to be played by alterations of the cell-to-cell junctional complexes in the intestinal epithelial layer [32].

Actually, the maintenance of gastrointestinal epithelial homeostasis as well as of its selective barrier function depend on a complex interplay of processes involving intestinal epithelial cell proliferation, differentiation, migration, and apoptosis, but also protein–protein networks that mechanically link adjacent cells and seal the intercellular space, including tight junctions (TJs), adherens junctions, desmosomes, and gap junctions [33]. Multiple factors are known to modulate epithelial permeability in *in vitro* and *in vivo* models of intestinal barrier; among the others, polyamines are gaining peculiar relevance in the field of intestinal disorders, due to the multiple roles they hold in the maintenance of epithelial functions [9,34]. Accordingly, in the present paper we provide evidence for the involvement of these molecules also in the modification of intestinal epithelial integrity driven by gliadin-activated macrophages *in vitro*. In a previous contribution, we reported, indeed, that gliadin peptides (PTG) induce the stimulation of arginase pathway in both murine macrophages and human monocytes [16]. Here, we further address this issue, demonstrating that this event ultimately leads to a massive production of polyamines by RAW264.7 macrophages (see Fig. 1), that are released in the extracellular medium. The use of PTG as an *in vitro* model of gluten digests is still a matter of debate, since some of the effects described in literature are likely due to peptic tryptic digests, rather than to gluten itself [35]. In our hands, however, the lack of stimulation of arginine metabolism by albumin peptides (PTA), obtained with the same enzymatic digestion as PTG (see Fig. 1), confirms the specific role of gluten peptides, and rules out the possibility that pepsin-tryptic aspecific digests are responsible for the effects observed in PTG treated macrophages.

Gliadin-driven release of putrescine, in turn, affects epithelial integrity: the incubation of intestinal epithelial monolayers with conditioned medium obtained from PTG-, but not IFNγ-treated macrophages causes, indeed, a significant impairment of epithelial permeability, as indicated by the decrease in TEER in Caco-2 epithelial monolayers, which is not further modified by the presence of the cytokine (see Fig. 3). Since TEER decrease is efficiently prevented by the inhibition of polyamines biosynthetic pathway in PTG-treated macrophages (see Fig. 4), we can definitely refer the alteration of epithelial monolayer permeability to the production of putrescine consequent to the interaction of macrophages with gluten peptides and to the release of this polyamine in the extracellular milieu. The incubation of polarized Caco-2 cultures in fresh growth medium containing PTG, both alone or combined with IFNγ, lead to an almost negligible decrease of TEER (see Fig. 3, panel C). Although these results are apparently in contrast with other contributions addressing the same issue (see [36–39] for examples), it must be underlined that our results have been obtained by incubating intestinal monolayers at the basolateral, rather than at the apical side, likely accounting for the discrepancies observed. Although the mechanisms responsible for polyamines transport have been thus far only roughly defined, evidences indicate that both endocytosis and solute carrier transporters are involved in their uptake in the gastrointestinal tract [40]. In particular, NO production is known to be required for the release of polyamines from intracellular vesicles [41], while a DiAmine eXporter (DAX) containing SLC3A2 has been identified and proposed to mediate putrescine efflux in colon epithelial cells [29]. Interestingly, we show here that both the pathway of NO (see Fig. 1) and the expression of SLC3A2/4F2hc (see Fig. 5) are induced in RAW264.7 macrophages by the incubation with gliadin peptides, hence suggesting that both processes could be involved in polyamines release under our experimental conditions.

As previously reported, polyamines contribution to gut homeostasis is well documented [9]. However, their role in the intestinal compartment is thus far not definitely understood, due to their ambivalent effects: their depletion is, indeed, known to increase epithelial paracellular permeability partially by repressing the expression of TJ proteins Zona Occludens-1 (ZO-1), occludin and E-cadherin [42–44]; on the other hand, it has been also demonstrated that the accumulation of polyamines within intestinal cell lines promotes their apoptosis through multiple signaling pathways, hence affecting intestinal integrity [9,45]. In our contribution, the molecular mechanisms responsible for the observed reduction of epithelial integrity by PTG-treated macrophages were not addressed; hence, nor an alteration of tight junctions nor the eventual induction of epithelial cell death by polyamines can be thus far excluded and will be object of further analyses.

The massive production of polyamines observed in murine macrophages upon incubation with gliadin peptides associates with the induction of C/EBPβ (see Fig. 2), the transcription factor specifically responsible for toll-like receptor (TLR) mediated induction of arginase in macrophages [46]. Similarly, a comparable PTG-driven induction of the transcription factor has been obtained in human monocytes isolated from peripheral blood samples of healthy donors, along with a concomitant induction of IL-1β mRNA expression (Barilli et al., unpublished results). These results are consistent with literature findings stating that C/EBPβ is involved in myeloid development and macrophage activation [25,47], and is a crucial mediator of inflammation in activated macrophages [48], particularly responsible for IL-1β transcription [49]. Our hypothesis is, hence, that this protein could be involved both in the gliadin-dependent activation of arginase pathway in macrophages [16] and, at least in part, in the onset of the inflammatory dysfunction described upon interaction of immune cells with gliadin [50]. Whether this transcription factor can be actually activated in macrophages *in vivo* by the presence of gluten deserves to be elucidated.

Altogether our results add to the plethora of cellular pathways activated by gliadin and its undigested peptides not only in cells and the intestinal mucosa of patients with CD, but also in normal subjects or in different diseases [6]. We can speculate that these pathways, constitutively altered in celiac cells, render CD patients more susceptible than healthy subjects to the effects of gluten, ultimately producing, once in the celiac background, the well-known clinical feature of the disease; however, further investigations addressing this issue are required to shade light on the role of these pathways in gluten driven cellular and intestinal defects typical of CD patients.

Conflicts of interest

The authors declare that they have no competing interests.

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