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Proteasome inhibitors exacerbate interleukin-8 production induced by protease-activated receptor 2 in intestinal epithelial cells

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ABSTRACT

Protease activated receptors (PARs) and the ubiquitin-proteasome system (UPS) regulate inflammatory response in intestinal cells. We aimed to elucidate putative connections between PARs and UPS pathways in intestinal epithelial cells. Caco-2 cells were treated by agonist peptides of PARs and/or IL-1β and/or proteasome inhibitors, bortezomib or MG132. Inflammatory response was evaluated by measuring IL-8 production. Proteasome activities were also evaluated. We showed that PAR-1 and -2 activation increased release of IL-8 compared with vehicle and independently of IL-1β. In contrast, PAR-4 agonist peptide had no effect. Caspase-like and chymotrypsin-like proteasomal activities were increased by PAR-2 activation only in the presence of IL-1β. Interestingly, in polarized Caco-2 cells, the release of IL-8 was predominantly upregulated in the side where PAR-2 agonist peptide was added, apical or basalolateral. In contrast, proteasome activities were only affected when PAR-2 agonist peptide was added in the apical side. Proteasome inhibitors, bortezomib and MG132, enhanced IL-8 production in both sides, apical and basolateral. In conclusion, PAR-2 activation alone did not affect proteasome but needed inflammatory stimulus IL-1β to synergistically increase chymotrypsin-like activity in intestinal epithelial cells. However, proteasome inhibition led to exacerbate inflammatory response induced by PAR-2 activation.

1. Introduction

Protease-activated receptors (PARs) are highly expressed throughout gastrointestinal tract and are involved in various functions and in the pathophysiology of intestinal diseases such as inflammatory bowel diseases (IBD) or irritable bowel syndrome (IBS) [1]. Four family members have been described, of which three are activated by thrombin (PAR-1, PAR-3 and PAR-4) and one is activated by trypsin and tryptase (PAR-2). Proteases activating receptors PAR may have their origin in the intestinal lumen from digestive proteases or from intestinal microbiota but also in the mucosa secondary to mast cell degranulation. Indeed, alterations of immune cell population in the intestinal mucosa have been described in IBS patients. In particularly, mast cell number is increased in patients with IBS-D compared with controls [2–4] and their localization/proximity from nerves seem to be correlated with abdominal pain [4]. In mice deficient for protease-activated receptor-2 (PAR-2), colonic biopsy supernatant from IBS patients did not induce intestinal hyperpermeability and hypersensitivity as observed in wild-type mice [5].

Previous data underlined the role of the ubiquitin-proteasome system (UPS) in the pathophysiology of IBD [6–8] but also in IBS [2]. UPS is responsible for most of the intracellular proteolysis in eukaryotes and is involved in the regulation of several cell pathways as apoptosis, cell proliferation, inflammatory responses and antigen presentation [9]. Proteasome is a multi-protein complex structure, including a 20S complex for proteolytic activities and two 19S regulatory complexes. Catalytic activities are carried by 3 β subunits of each inner ring β1, β2, β5 [10]. These subunits have a homologous inducible subunit (β1i, β2i, β5i). We [6] and others [7] previously reported that IFNγ increased inducible β subunits in intestinal epithelial cells to form an alternative proteasome called immunoproteasome. In the context of IBD, experimental data underlined that proteasome inhibition has protective effects

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in intestinal damage by limiting inflammatory response [11,12]. However, the effects of proteasome on PAR-induced cytokine production have not yet been evaluated.

In the present study, we thus aimed to investigate whether UPS is involved in the PAR-induced inflammatory response in intestinal epithelial cells.

2. Material and methods

2.1. Cell culture and treatments

Caco-2/TC7 cells were obtained the American Type Cell Collection (ATCC) and were cultured in Dulbecco's modified Eagle's medium (DMEM, Eurobio, Courtaboeuf, France), supplemented with 10% of fetal bovine serum (FBS, Lonza, Verviers, France), 1% antibiotics (ATB, Sigma-Aldrich, Saint Quentin-Fallavier, France), 1% solution of non-essential amino acids (NEAA, Sigma-Aldrich) and 1% glutamine (Sigma-Aldrich).

Cells were seeded and grown in 6-well plates (2.5×10^5 cells/well) or on microporous filters (1×10^6/0.4 μm filter) to obtain a monolayer polarized model as previously described [13]. Cells were pre-treated in FBS-free DMEM 24 h before treatment. FBS-free DMEM was replaced 1 h before treatment with agonist peptides (AP) and/or cytokines and/or proteasome inhibitors. To induce inflammatory conditions, Caco-2 cells were treated for 24 h with IL-1β (1 ng/ml) alone or in combination (called Cytomix) with TNF α (20 ng/ml) and IFN γ (10 ng/ml) as previously [14]. In polarized cells, IL-1β (1 ng/ml) was added at the basolateral side. Cells were then stimulated with PAR1-AP (TLLR-NH_2), PAR2-AP (SLIGRL-NH_2) or PAR4-AP (GYPGKF-NH_2) at a final concentration of 100 μmol/L, as previously described [15], at the apical or basolateral sides as appropriate. Inhibitors of proteasome MG132 (20 μmol/L) or bortezomib (20 μM) were added 1 h before AP at the basolateral side. After 18 h, culture media and cells were removed and stored until analysis.

2.2. Proteoasome activities

Fluorometric assays using fluorogenic substrates specific for chymotrypsin-like, trypsin-like, caspase-like (PGPH) or peptidase activities were performed as previously described [2] by using, respectively, specific fluorogenic substrates: Suc-LLVY-AMC (Calbiochem, Merck Millipore, Darmstadt, Germany); Boc-LSTR-AMC (Sigma-Aldrich); Z-LLE-AMC (Sigma-Aldrich) and Z-LLL-AMC (Sigma-Aldrich). In a black 96-well plate, 25 μg of protein were loaded in a final volume of 90 μL in 30 mM Tris-HCl, 5 mM EDTA, 10% SDS buffer. Each sample is placed in two wells into which were added either proteasome inhibitor, MG132 (20 μmol/L) or vehicle. Finally, specific substrates were added to each well and plates were incubated at 37 °C for 30 min. Then, reading was performed in a fluorimeter Chameleon V (Hidex, Turku, Finland) with the following characteristics: excitation wavelength at 355 nm and emission wavelength at 460 nm. Values of proteasome activities correspond to the difference between fluorescence obtained in the absence of inhibitor and in presence of MG132. The results were expressed in relative fluorescence units (RFU)/20 μg of proteins.

2.3. Determination of IL-8 release

The amount of IL-8 released into the supernatant was quantified using a human IL-8 ELISA Duoset kit (R&D Systems, Abingdon, UK), according to manufacturer's instructions. Concentrations were expressed as pg/ml.

2.4. Measurement of Transepithelial Electrical Resistance (TEER)

An epithelial volt-ohm meter (Millipore Corporation, Molsheim, France) was used for measurements of Caco-2 monolayer transepithelial electrical resistance as previously reported [13]. Values of TEER are expressed as % between values obtained before and after treatment. TEER was calculated after subtraction of the resistance value of the filters alone.

2.5. Western blot analysis

After washes, cells were lysed in Triton X100, DL-Dithiothreitol 500 mM (DTT), Tris-HCl 30 mM pH 7.2. The protein sample (25 μg/lane) were separated by electrophoresis on mini-protean TGX stain-free gels (Bio-Rad Laboratories, Marne la Coquette, France) and transferred into a nitrocellulose blotting membrane, amersham™ hybond ECL (GE healthcare Life Sciences). The primary antibodies were mouse anti-β1, goat anti-β5 (1:1000, Santa Cruz Biotechnology Inc, CA), rabbit anti-β1i, β2i and β5i, (1:1000, Biomol International LP, Palatine House, UK), rabbit anti-c-fos (1:1000, Merck Millipore, Darmstadt, Germany), mouse anti-β-actin (1:5000, Sigma-Aldrich). After washing followed by incubation with IgG conjugated with HRP, immunoreactive proteins were visualized by enhanced chemiluminescence detection system (GE healthcare). Protein bands were quantified by densitometry using ImageScanner III and ImageQuant TL software (GE Healthcare).

2.6. RT-qPCR

Cells were homogenized in Trizol buffer (LifeTechnologies, Saint Aubin, France). Total RNA (1.5 μg) were reverse transcribed into cDNA using SuperScript II Reverse Transriptase (SSII, LifeTechnologies). Then, qPCR was performed with BIO-RAD CFX96 (Bio-Rad Laboratories). cDNA (4 μL) and 6 μL of reaction mixture were added and placed in a 96 well plate by using EpMotion 5070 automate (Eppendorf, Le Pecq, France). Reaction mixture was composed of specific primers and SYBRgreen supermix (Bio-Rad Laboratories). Primers specific for encoding the GAPDH and IL-8 cDNA were: TGGCAGCCTTCCTGATTT 5′-3′, 5′-AACTTCTCCA CAACCCTCTG-3′ for IL-8 and 5′-TGCCATCAATGACCCCTTCA-3′, 5′-TGACCTTGCCCACAGCCTTG-3′ GAPDH. Primers were designed and controlled by the NCBI BLAST search engine and synthesized (LifeTechnologies).

3. Statistical analysis

Results from 4 to 6 independent experiments were expressed as mean ± sem and compared using one-way ANOVA followed by Tukey post-tests. A p value < 0.05 was considered significant.

4. Results

4.1. Effects of PAR-agonist peptides on IL-8 production in Caco-2 cells

In Caco-2 cells, IL-1β (1 ng/ml) alone did not affect IL-8 production whereas Cytomix (mixture of IL-1β, TNF α and IFNγ) increased IL-8 release ($p < 0.05$, Fig. 1A). PAR1-agonist peptide (AP) and PAR2-AP alone significantly increased release of IL-8 compared with vehicle-treated cells (Fig. 1A, both $p < 0.05$) but we did not observe additive or synergic effects between PAR1 or PAR2-AP and cytokines (Fig. 1A). PAR4-AP alone or in combination with cytokines had no effect (Fig. 1A). We then evaluate IL-8 mRNA level in response to PARs-AP. In presence of PAR2-AP, a significant increase of IL-8 mRNA was observed compared with vehicle-treated cells (data not shown). A trend for increased IL-8 mRNA