Toxicology and Applied Pharmacology 230 (2008) 276–282

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Salmon trypsin stimulates the expression of interleukin-8 via protease-activated receptor-2

Anett K. Larsen [a,b], Ole-Morten Seternes [b], Merethe Larsen [a], Lisbeth Aasmoe [a,b], Berit Bang [a,b,*]

[a] Department of Occupational and Environmental Medicine, University Hospital of North Norway, Tromsø, Norway
[b] Department of Pharmacology, Institute of Medical Biology, University of Tromsø, Tromsø, Norway

ARTICLE INFO

Article history:
Received 28 November 2007
Revised 22 February 2008
Accepted 22 February 2008
Available online 4 March 2008

Keywords:
Protease-activated receptor-2
Interleukin-8
Purified salmon trypsin

ABSTRACT

In this study, we focus on salmon trypsin as an activator of inflammatory responses in airway cells in vitro. The rationale behind the investigation is that salmon industry workers are exposed to aerosols containing enzymes, which are generated during industrial processing of the fish. Knowing that serine proteases such as trypsin are highly active mediators with diverse biological activities, the stimulation of nuclear factor-kappa B (NF-κB) and interleukin (IL)-8 and the role of protease-activated receptors (PAR) in inflammatory signal mediation were investigated. Protease-activated receptors are considered important under pathological situations in the human airways, and a thorough understanding of PAR-induced cellular events and their consequences in airway inflammation is necessary. Human airway epithelial cells (A549) were exposed to trypsin isolated from fish (*Salmo salar*), and we observed that purified salmon trypsin could generate secretion of IL-8 in a concentration-dependent manner. Furthermore, we demonstrate that PAR-2 activation by salmon trypsin is coupled to an induction of NF-κB-mediated transcription using a PAR-2 transfected HeLa cell model. Finally, we show that the release of IL-8 from A549 following stimulation with purified salmon trypsin is mediated through activation of PAR-2 using specific small interfering RNAs (siRNAs). The results presented suggest that salmon trypsin, via activation of PAR-2, might influence inflammation processes in the airways if inhaled in sufficient amounts.

Introduction

Inhalation of particulate matter may affect the airway epithelial cells, and surface epithelium in the upper and lower parts of the respiratory tract can contribute to an inflammation response by the production and secretion of cytokines. Previous research has shown that production workers in the seafood industry inhale aerosols containing biological material (Bang et al., 2005; Jeebhay et al., 2005). The biological material may originate from the raw material itself or from microbiological organisms in the environment.

Processing of clam, shrimp, and crab is associated with increased risk of occupational asthma (Bang et al., 2005; Bønløkke et al., 2004; Jeebhay et al., 2001; Douglas et al., 1995; Desjardins et al., 1995; Sherson et al., 1989; Gaddie et al., 1980). Allergic diseases associated with occupational exposure to crab is well characterized, whereas for other seafood agents the evidence is somewhat limited (Jeebhay et al., 2001). Workers in the salmon industry show enhanced occurrence of symptoms from the upper parts of the airways that cannot be explained by asthma or allergy (Bang et al., 2005). None of the examined workers in the study conducted by Bang et al. had specific IgE toward salmon antigen, and earlier researches contain few clear correlations between airway symptoms and enhanced levels of specific immunoglobulins against fish. These findings raised the question of whether there are biological components in the work environment that trigger airway symptoms via non-IgE-mediated pathways.

Proteases are present in biological materials, although good methods for quantification in bioaerosol samples are not yet available. Several recent reports indicate that serine proteases are important for the induction of cytokine production following exposure to mites (King et al., 1998; Tomee et al., 1998), molds (Kauffman et al., 2000; Borger et al., 1999), and cockroach extracts (Bhat et al., 2003). This protease-induced cytokine production is shown to be mediated, at least partly, through the activation of the protease-activated receptor (PAR)-2 (Hong et al., 2004; Page et al., 2003; Asokananthan et al., 2002b; Sun et al., 2001).

PAR-2 is a member of the protease-activated receptor family of G protein-coupled receptors consisting of four known subtypes, namely PAR-1, PAR-2, PAR-3, and PAR-4 (Ossovskaya and Bunnett, 2004). Receptor activation by proteases is achieved by proteolytic cleavage of the N-terminal sequence, and thereby unmasking a new amino terminus that serves as a tethered ligand that binds to conserved regions in the body of the receptor, resulting in the initiation of signal transduction. Exogenously applied synthetic peptides based on the sequence of the tethered ligand are also capable of activating PARs by directly binding to the body of the receptors.

* Corresponding author. Department of Occupational and Environmental Medicine, University Hospital of North Norway, P.O. Box 16 N-9038, Tromsø, Norway. Fax: +47 77 62 74 71.
E-mail address: berit.bang@unn.no (B. Bang).

0041-008X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2008.02.019

The PARs are widely distributed in various tissues and cells throughout the mammalian body, including the respiratory system where particularly PAR-2 and PAR-1 are expressed in the epithelial and smooth muscle cells (Ossovskaya and Bunnett, 2004; Cocks and Moffatt, 2001). PAR-2 is mainly considered pro-inflammatory in the respiratory system, and Knight et al. (2001) have found an upregulation of PAR-2 in the respiratory epithelium from patients with asthma.

In human alveolar epithelial cells (A549), activation of PAR-2 induces enhancement of expression and release of important mediators of inflammation such as matrix-metalloproteinase-9 (MMP-9) (Vliagoftis et al., 2000) and granulocyte macrophage-colony stimulating factor (GM-CSF) (Vliagoftis et al., 2001), as well as IL-6, IL-8, and prostaglandin E_2 (Asokananthan et al., 2002a).

The transcriptional regulation of IL-8 expression in airway epithelial cells is complex and involves the transcription factors nuclear-factor kappa-B (NF-κB), NF-IL6 (C/EBP), and activating protein (AP)-1 (Mastronarde et al., 1998; Garofalo et al., 1996; Mastronarde et al., 1996). Binding of the NF-κB element is required for activation in all cell types studied, while the AP-1 and NF-IL6 sites are not essential for induction but are required for maximal gene expression (Hoffmann et al., 2002; Kunsch et al., 1994; Mukaida et al., 1994; Matsusaka et al., 1993).

A wide range of proteases cleave and activate PARs, including proteases from the digestive tract, the coagulation cascade, and inflammatory cells (Ossovskaya and Bunnett, 2004). Trypsin (1, 2, and IV) (Cottrell et al., 2004; Alm et al., 2000; Böhm et al., 1996; Nystedt et al., 1995), tryptase (Molino et al., 1997), tissue factor (TF)-factor VIIa/factor Xa complex (Camerer et al., 2000), neutrophil proteinase 3 (Uehara et al., 2002), membrane-type serine protease-1 (MT-SP1) (Takeuchi et al., 2000), dust mite allergens Der P3 and Der P9 (Sun et al., 2001), and the bacterial protease RgpB produced by Porphyromonas gingivalis (Lourbakos et al., 2001) have all been shown to activate PAR-2 in an in vitro setting. It is unclear, however, how many of these proteases can activate PAR-2 in vivo. Mast cell tryptase has been considered a possible endogen activator of PAR-2 in the airways, but it is considerably less potent than trypsin (Ossovskaya and Bunnett, 2004). During inflammation and trauma, proteases from the coagulation cascade (TF-FVIIa/FXa) and from inflammatory cells (neutrophil proteinase 3) could regulate pulmonary cells by activating PAR-2.

Unpublished data from our laboratory showed protein-cleaving enzymatic activity in storage water and fish juice sampled from work benches, with the highest activity of trypsin. Outzen et al. (1996) have established that salmon trypsin has a higher catalytic efficiency than trypsin from mammals (bovine, porcine). Knowing that mammalian trypsin is a PAR-2 agonist and PAR-2 being considered important for inflammation in the airways, we wished to explore if purified salmon trypsin could have similar properties as reported for mammalian trypsin.

In this study, we show that salmon trypsin induces IL-8 production in airway epithelial cells. Furthermore, we demonstrate that purified salmon trypsin activates NF-κB mediated gene expression in HeLa cells over-expressing human PAR-2. Finally, we provide evidence that the production of IL-8 by salmon trypsin is mediated by PAR-2 using specific siRNA-mediated knockdown.

Materials and methods

Materials. Selective PAR-2 peptide agonist, Ser-Leu-Ile-Gly-Arg-Leu-amide (SLIGRL-NH_2), the inactive control peptide, Leu-Arg-Gly-Ile-Leu-Ser-amide (LRGILS-NH_2), Na-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride (DL-BAPNA), Protease Inhibitor Cocktail (PIC), and aprotinin were purchased from Sigma-Aldrich, MO, USA. Purified salmon trypsin was kindly provided by Dr. Nils Peder Willassen (UoT). Recombinant human TNFα was purchased from Invitrogen, CA, USA.

Cells cultures. A549 cells, a human pulmonary epithelial cell line, were purchased from American Type Culture Collection (ATCC; no CCL-185) and were cultured in Dulbecco's minimum essential medium/Ham's F12 medium (1:1) supplemented with 10% fetal calf serum (Gibco, NY, USA), 50 IU/ml penicillin, and 0.05 mg/ml streptomycin. HeLa cells were obtained from ATCC (no CCL-2) and were cultured in Eagle's minimum essential medium (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine, 1× non-essential amino acids (BioWhittaker), 1000 IU/ml penicillin, and 1 mg/ml streptomycin. The cells were passed without the use of trypsin by using a non-enzymatic cell dissociation solution (Sigma-Aldrich).

Protease activity determination. The enzymatic activity of the purified salmon trypsin was determined by a serine protease assay where the hydrolyzation of a chromogenic substrate (DL-BAPNA) was measured spectrophotochemically by the increase in absorbance at 405 nm at room temperature and was expressed as units (U)/ml (Outzen et al., 1996). One unit of activity was defined as 1 μmol substrate hydrolyzed per minute using an extinction coefficient of 8800 M^{-1} cm^{-1} (Erlanger et al., 1961).

Cell stimulation assays. A549 cells (2×10^5 cells/well) were seeded in 6-well plates (9.6 cm^2) and were cultured until 80–90% confluence was reached, before the cells were starved for serum overnight. Cells were exposed to different concentrations of purified salmon trypsin, PAR-2 peptide agonist, or inactive control peptide for the appropriate time. In the inhibition assay 1 mU of purified salmon trypsin was pre-incubated with PIC (1040 nmol AEBSF, 0.8 nmol aprotinin, 20 nmol leupeptin, 40 nmol bestatin, 15 nmol pepstatin A, and 14 nmol E-64) or aprotinin (0.8 nmol) for 15 min at 37 °C. Heat-inactivation was conducted by incubating the purified salmon trypsin at 65 °C for 15 min. Culture supernatants were harvested and kept at −20 °C until the level of IL-8 was measured.

Measurement of IL-8 secretion. The amount of secreted IL-8 in the supernatant was determined by using an ELISA (enzyme-linked immunosorbent assay)-kit from BD Biosciences, NJ, USA, according to the manufacturer's protocol. The absorbance was read using an iEMS Multiscan EX (Thermo Labsystems). IL-8 production was expressed as pg/ml supernatant.

Small interfering RNA. A mix of three pre-designed siRNAs (Ambion, USA, catalogue number 16704, ID nr 1960, 1876, and 1783) for the PAR-2 gene was used. The sequences of the siRNA primers were as follows: nr 1960; forward primer 5′-GGAGUUACAGUU-GAAACAGTT-3′, and reverse primer 5′-CUGUUUCAACUGUAACUCCTT-3′, nr 1876; forward primer 5′-GGAAGAAGCCUUAUUGGUATT-3′, and reverse primer 5′-UAC-CAAUAAGGCUUCUUCCTT-3′, and nr 1783; forward primer 5′-GGAACCAAUAGAUCCU-CUATT-3′, and reverse primer 5′-UAGAGGAUCUAUUGGUUCCTT-3′. The Silencer negative control nr 1 (Ambion, catalogue number 4611) was used as a control siRNA. The primer sequences of the negative control were as follows: forward primer 5′-AGUACUGCUUACGAUACGGTT, and reverse primer 5′-CCGUAUCGUAAGCAGUACUTT-3′. A549 cells (2×10^5 cells/well) were seeded in 6-well plates (9.6 cm^2) and were transfected at 70–80% confluency using Lipofectamine 2000 (Invitrogen)-assisted transfection according to the manufacturer's protocol.

Real-time PCR. Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Synthesis of cDNA from total RNA was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems) with the choice of random hexamers as primers. For the real-time PCR reaction, primers and probe for the PAR-2 gene were from the TaqMan Gene Expression Assays (Applied Biosystems, assay ID: Hs00608346_m1). The TaqMan MGB probe used has a FAM reporter dye at the 5′ end and a non-fluorescent quencher at the 3′ end. The primers and probe in the gene expression assay were premixed to a concentration of 18 μM for each primer and 5 μM for the probe. The human house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems, part nr 4333764F) was used as an endogenous control. The TaqMan Fast Universal PCR Master Mix (Applied Biosystems) was used to run the real-time PCR reaction on a 7900HT Fast real-time PCR System from Applied Biosystems according to the manufacturer's protocol. The data obtained were analyzed using the relative standard curve method for quantification (Applied Biosystems; real-time PCR Systems Chemistry Guide, PN 04348358).

DNA constructs. The primer sequences for human PAR-2 were as follows: forward primer, 5′ CACCATGCGGAGCCCCAGCGCGGCGTG-3′, and reverse primer, 5′-ATAG-GAGGTCTTA ACAGTGG-3′. The PCR was performed using Pfx platinum polymerase (Invitrogen). The PCR-product was cloned into a pENTR-D-TOPO vector (Invitrogen) before the open-reading frame (ORF) was transferred to the appropriate C-terminal fusion expression vector pdEYFP (Simpson et al., 2000) by an LR-recombination using the Gateway LR Clonase II Enzyme Mix (Invitrogen) according to the manufacturer's instructions. The integrity of the cloned PCR product was verified by sequencing.

The luciferase reporter vector κB-ConA-luc containing the binding site for NF-κB and the ConA-luc control vector were kindly provided by Dr. Estelle Sontag (University of Texas South Western Medical Center) (Sontag et al., 1997). The pEGFP (Clontech, CA, USA) was used as a non-functional vector to accomplish equal amounts of DNA transfected to the cells when necessary. The reporter vector pCH110 encoding β-galactosidase was from Pharmacia (now GE Healthcare). All vectors used in the reporter gene assays were purified for endotoxins by using an EndoFree Plasmid Maxi kit (Qiagen, Hilden, Deutchland).

Western blot analysis. Cells were harvested and lysed using 2x NUPAGE LDS Sample Buffer (Invitrogen), were separated by SDS-PAGE (4–12% NUPAGE; Invitrogen), were transferred to a nitrocellulose membrane (Amersham Biosciences), and were probed