Biochimica et Biophysica Acta 1792 (2009) 148–154

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

The role of redox status on chemokine expression in acute pancreatitis

S. Yubero, L. Ramudo, M.A. Manso, I. De Dios *

Department of Physiology and Pharmacology, University of Salamanca, 37007, Salamanca, Spain

ARTICLE INFO

Article history:
Received 12 September 2008
Received in revised form 12 November 2008
Accepted 2 December 2008
Available online 9 December 2008

Keywords:
Acinar cell
Acute pancreatitis
Chemokine
Oxidative stress
Signalling pathway

ABSTRACT

This study focused on the involvement of oxidative stress in the mechanisms mediating chemokine production in different cell sources during mild and severe acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) and 3.5% NaTc, respectively. N-Acetylcysteine (NAC) was used as antioxidant treatment. Pancreatic glutathione depletion, acinar overexpression of monocyte chemoattractant protein-1 (MCP-1) and cytokine-induced neutrophil chemoattractant (CINC), and activation of p38MAPK, NF-κB and STAT3 were found in both AP models. NAC reduced the depletion of glutathione in BPDO- but not in NaTc-induced AP, in which oxidative stress overwhelmed the antioxidant capability of NAC. As a result, inhibition of the acinar chemokine expression and signalling pathways occurs in mild, but not in severe AP. However, MCP-1 and CINC expressions in whole pancreas and plasma chemokine levels were not reduced by NAC, even in BPDO-induced AP, suggesting that in addition to acini, other pancreatic cells produced chemokines by antioxidant resistant mechanisms. The high Il-6 plasma levels found during AP, both in NAC-treated and non-treated rats, pointed out cytokines as activating factors of chemokine expression in non-acinar cells. In conclusion, from early AP oxidant-mediated MAPK, NF-κB and STAT3 activation triggers the chemokine expression in acini but not in non-acinar cells.

1. Introduction

Acute pancreatitis (AP) is initiated by premature activation of trypsinogen within acinar cells [1,2]. It leads to a local pancreatic inflammation followed by a systemic inflammatory response which could result in multiple organ dysfunction (MODS) [3]. Different inflammatory mediators, including chemokines, play a key role in the pathogenesis of AP [4]. Chemokine circulating levels are elevated in human and experimental acute pancreatitis [5–7]. Chemokines are a family of small cytokines with chemotactic and activating effects on inflammatory cells which direct the migration of leukocytes into the injured tissues [8]. On a structural basis, chemokines have been divided into four subfamilies: C, CC, CXC and CX_3C, according to the position of the first two cysteine residues [9]. CC chemokines, such as monocyte chemoattractant protein-1 (MCP-1), mainly affect monocytes and CXC chemokines, such as cytokine-induced neutrophil chemoattractant (GRO-α/CINC), predominantly act on neutrophils [4,9,10]. In response to inflammatory stimuli, pancreatic acinar cells [11–15], leukocytes [16], endothelial cells [17,18] and stellate cells [19,20] are shown to produce cytokines by mechanisms sensitive to oxidative stress, among which mitogen activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) play a key role. However, little is known about the involvement of signal transducers and activators of transcription (STAT) proteins, latent transcription factors initially identified as cytokine-inducible DNA-binding proteins [21,22]. STAT proteins could mediate the transcriptional control mechanisms that regulate both local and systemic inflammatory response. The aim of this study was to examine the pattern of expression of MCP-1 and CINC in two models of AP of different severity and to assess the involvement of the oxidative stress on the underlying mechanisms mediating the chemokine production in different cell sources.

2. Materials and methods

2.1. Chemicals

N-Acetyl-L-cysteine (NAC), taurocholic acid sodium salt hydrate, aminoacid mixture, bovine serum albumin (BSA), collagenase type XI, soybean trypsin inhibitor (STI), N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), glutathione (GSH), β-nicotinamide adenine nucleotide phosphate reduced form (NADPH), glutathione reductase type III, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), hexadecyltrimethylammonium bromide (HDTAB), 3,3′,5′5-tetramethylbenzidine (TMB) liquid substrate system and buprenorphine, were supplied by Sigma Chemical Co. (Madrid, Spain). MCP-1 and Il-6 enzyme-linked immunosorbent assay (ELISA) kits were supplied by Bender MedSystems (Vienna, Austria) and CINC ELISA kit by R and D Systems (Minneapolis, MN, USA). Agarose was supplied by Iberlabo (Madrid, Spain). All other standard analytical grade laboratory reagents were obtained from Merck (Madrid, Spain).

* Corresponding author. Departamento Fisiología y Farmacología, Edificio Departamental, Campus Miguel de Unamuno, 37007 Salamanca, Spain. Fax: +34 923 294673.
E-mail address: bel@usal.es (I. De Dios).

0925-4439/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2008.12.002

2.2. Animal groups

Male Wistar rats (250–300 g) were housed individually in cages and maintained at 22±1 °C using a 12-h light/dark cycle. The animals were fasted overnight before the experiment but they were allowed free access to water. All experiments were performed in accordance with European Community guidelines on ethical animal research, established by the European Community (86/609/EEC). The study was approved by the Institutional Animal Care and Use Committee of the University of Salamanca (Spain).

2.3. Animal models of pancreatitis and treatment

Under anaesthesia with 2%–3% isofluorane, Forane® (Abott, Madrid, Spain) mild and severe AP were induced in two different experimental models: bile-pancreatic duct obstruction (BPDO) and retrograde infusion of 3.5% sodium taurocholate (NaTc) into bile-pancreatic duct, respectively. N-Acetylcysteine (NAC) was administered at dose of 50 mg/kg by intraperitoneal injection 1 h before inducing AP followed by a new injection 1 h afterwards. Studies were carried out at the following time periods: 3 h and 12 h in BPDO-induced AP and 3 h and 6 h in NaTc-induced AP. Postoperative analgesia was maintained by intramuscular injections of buprenorphine (0.2 mg/kg).

2.4. Biochemical assays

Pancreatic GSH content was measured using the technique described by Tietze [23]. Neutrophil infiltration was estimated in pancreas by measuring tissue myeloperoxidase (MPO) activity following the method of Bhatia et al. [24]. Il-6, MCP-1 and CINC plasma levels were determined by ELISA kits strictly following the recommendations of manufacturers. All samples were run in duplicate and averaged.

2.5. Isolation of acinar cells

Acinar cells were isolated by collagenase digestion as previously described [15]. Briefly, pancreata were minced in a previously oxygenated solution composed of (in mM) 25 HEPES (pH 7.4), 110 NaCl, 5 KCl, 1 $CaCl_2$ 14 D-glucose, 2 L-glutamine as well as 2% (w/v) BSA, 0.01% (w/v) STI and 2% (v/v) aminoacids mixture. Collagenase (25 U/ml) was then added and the suspension was vigorously shaken in a water bath at 20 °C for 10 min under continuous oxygenation. Afterwards, the medium was replaced by a fresh oxygenated solution without collagenase and then gently pipetted on ice through tips of decreasing diameter (3–1 mm). After filtering through a double layer of muslin gauze acinar cells were washed twice by centrifugation (540 g, 3 min, 4 °C).

2.6. Analysis of phospho-p38MAPK and IκBα

2.6.1. Preparation of total cell lysates

Acinar cells were homogenized on ice in HEPES buffer, 10 mM, pH 7.9, containing 2 mM EDTA, 25 mM KCl and supplemented with 1 mM PMSF and a protease inhibitor cocktail containing aprotinin, leupeptin, pepstatin, antipain and chymostatin (5 μg/ml each). The mixture was maintained on ice for 20 min, after which Nonidet P-40 (0.4%) was added for 2 min and then centrifuged at 4 °C for 3 min at 14,000 g. The supernatants were collected and immediately stored at −80 °C until use.

2.6.2. Western blot analysis

Cytoplasmic extracts from acinar cells (40 μg) were separated by 12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Non-specific binding was blocked by incubating the blot in Tris-buffered saline (TBS) pH 7.6, containing 0.1% (v/v) Tween 20 and 5% (w/v) nonfat dry milk for 1 h. Afterwards, blots were incubated with the primary antibody against either phospho-p38MAPK, IκBα, or β-actin (Cell Signalling Technology, Beverly, MA) at 1:1,000 dilution in TBS buffer pH 7.6, containing 0.1% (v/v) Tween 20 and 5% (w/v) BSA overnight at 4 °C. After washing for 1 h with TBS containing 0.1% Tween 20, the blots were incubated for 1 h at room temperature with the respective horseradish peroxidase-conjugated secondary antibody at 1:2000 dilution in TBS buffer pH 7.6, containing 0.1% Tween 20 and 5% (w/v) nonfat dry milk and finally they were developed for visualization. The bands were detected with Phototope-HRP Detection kit (Cell Signalling Technology, Beverly, MA). Image J 1.32 software from http://rsbweb.nih.gov/ij/download.html was used to quantify the intensity of the bands. Relative protein levels were calculated compared to the β-actin standard. Results are expressed as changes *vs* controls.

2.7. Determination of NF-κB and STAT3 activation

2.7.1. Nuclear cell extract preparation

Nuclear protein extracts were obtained using a commercial nuclear extract kit following the recommendations of the manufacturer (Active Motif, Rixensart, Belgium). Basically, isolated acinar cells were washed with ice-cold phosphate-buffered saline (PBS) containing phosphatase inhibitors and then lysed on ice in hypotonic buffer containing a cocktail of protease inhibitors (aprotinin, leupeptin, pepstatin, antipain and chymostatin) by pipetting up and down several times. The homogenate was maintained on ice for 15 min. Nonidet P-40 (0.4%) was then added and after vortexing and incubation on ice for 1 min, nuclear fraction was collected in the pellet after centrifuging 30 s at 14,000 g. The nuclear pellet was resuspended in a complete lysis buffer containing dithiothreitol (DTT) and protease inhibitor cocktail. After vortexing and incubating for 30 min on ice with shaking, nuclear membranes were pelleted by centrifugation at 14,000 g for 10 min and nuclear extract was collected from the supernatant and aliquoted and stored at −80 °C until use.

NF-κB- and STAT3-DNA binding was measured in nuclear extracts with the respective ELISA-based commercial kits (NF-κB p65 TransAM and STAT3 activation assays, Active Motif). Nuclear proteins (5 μg), were added to each well coated with an oligonucleotide containing the consensus binding site for either NF-κB or STAT3 and incubated for 1 h. Activation was detected by incubation for 1 h with the respective primary antibody: anti-NF-kB, which specifically recognizes an epitope (p65) accessible only when the factor is activated and bound to its target DNA and anti-STAT3, which recognizes epitope only accessible when STAT3 is activated. A secondary anti-IgG horseradish peroxidase conjugate allows detection of the activated NF-κB and STAT3 by a colorimetric reaction.

2.8. Analysis of mRNA expression for MCP-1 and CINC

Total RNA was extracted from isolated acinar cells and pancreas using RNAeasy kit treated with amplification grade DNase 1 (Quiagen, Valencia, Spain) according to the manufacturer's instructions. Purity of RNA was verified by ethidium bromide staining on 1% agarose gels. The purity of RNA was assessed by a 260/280 ratio and the integrity of RNA was verified by the presence of well-defined 28S and 18S rRNA bands.

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to analyze mRNA expression of MCP-1 and CINC in acinar cells and pancreas. Total RNA (1 μg) was reversed transcribed by using 1st Strand cDNA synthesis kit (Roche, Mannheim, Germany). The cDNA synthesized was used as template for PCR amplification by using Taq DNA polymerase, dNTPack (Roche). The following primer pairs (Roche) were used: MCP-1 (sense: 5′-CACTATGCAGGTCTCTGTCACG-3′, antisense; 5′-GACTCACTTGGTTC-TGGTCCA-3′, product size: 294 bp), CINC (sense: 5′-CTCCAGCCAC-ACTCCAACAGA-3, antisense: 5′-CACCCTAACACAAAACACGAT-3′, product size: 600 bp). Oligonucleotide primers for β-actin (sense: