Signal transduction of MCP-1 expression induced by pancreatitis-associated ascitic fluid in pancreatic acinar cells

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Abstract

Pancreatitis-associated ascitic fluid (PAAF) is known to contribute to the progression of acute pancreatitis (AP). We have investigated the capability of PAAF to activate the expression of MCP-1 in pancreatic acinar cells and the involvement of MAPK, NF-κB and STAT3 as downstream signalling transduction pathways. The actions of dexamethasone (Dx) and N-acetylcysteine (NAC) on the PAAF’s acinar effects have also been evaluated. Acinar cells were incubated for 1 hr with PAAF collected from rats with severe AP induced by sodium taurocholate in the absence or presence of Dx (10^{-7}M) or NAC (30 mM). MCP-1 mRNA expression, phospho-p38-MAPK, IκBα, nuclear p65 levels and nuclear translocation of STAT3 were analysed. In response to PAAF, overexpression of MCP-1, phosphorylation of p38-MAPK, degradation of IκBα and increases in p65 nuclear levels and STAT3 activity were found in acinar cells. PAAF-mediated MCP-1 up-regulation was completely suppressed by Dx and NAC. MAPK activation was only inhibited by NAC, NF-κB activation was repressed by Dx and NAC, and STAT3 pathway was strongly blocked by Dx and significantly reduced by NAC. In conclusion, acinar cells were activated by PAAF to produce MCP-1, mainly via NF-κB and STAT3 pathways. Both downstream pathways were targeted by Dx and NAC to repress the PAAF-mediated acinar MCP-1 up-regulation.

Keywords: acinar cells • dexamethasone • MCP-1 • N-acetylcysteine • pancreatitis-associated ascitic fluid • signal transduction

Introduction

Premature activation of pancreatic enzymes and secondary oxidative stress are associated with impairment in the pancreatic microcirculation, which leads to the accumulation of ascitic fluid in the abdominal cavity [1, 2]. Pancreatitis-associated ascitic fluid (PAAF) is known to be important in the progression of acute pancreatitis (AP) [3, 4], because it contains mediators involved in the multiple organ failure, the main cause of death of the disease [5]. PAAF has been shown to up-regulate the expression of adhesion molecules in human endothelial cells [6], as well as cytokines in lung [7], leukocytes [8] and pancreatic acinar cells [9, 10]. Downstream signal transduction pathways have been demonstrated to mediate the expression of inflammatory mediators in acinar cells during AP, including nuclear factor-κB (NF-κB) [11–13] and mitogen-activated protein kinases (MAPK) [13–15]. Signal transducers and activators of transcription (STAT) family of transcription factors have similar profiles of target genes as NF-κB [16]. The STAT proteins are in a latent form in the cytoplasm and, on receptor activation by cytokines, become phosphorylated by members of the Janus kinase (JAK) family, which are physically associated with the receptor [17]. Upon phosphorylation, the STAT proteins dimerize and migrate to the nucleus where they bind to DNA and regulate specific gene expression [18]. Studies carried out in STAT knockout mice demonstrated that STATs are involved in cytokine signalling and immune responses [19]. Expression of JAK and STAT proteins has been demonstrated in pancreas of rats, acinar cells being the main source of these proteins rather than ductal, endocrine, vascular or blood cells [20]. In vitro experiments [20, 21] showed STAT1 and STAT3 activation in acinar cells induced by IFN-γ and TNF-α, respectively; however, their role in mediating the inflammatory response in AP is not well established.

Dexamethasone (Dx), a potent anti-inflammatory corticosteroid, and N-acetylcysteine (NAC), a powerful antioxidant, have shown to be beneficial in the treatment of AP by affecting the
cytokine network [13, 22, 23]; however, the mechanisms of how this occurs remain unclear.

The aim of this study was to investigate the effect of PAAF on the expression of chemokine MCP-1 in pancreatic acinar cells as well as the involvement of MAPK, NF-xB and STAT3 as its signalling transduction pathways. The actions of Dx and NAC on PAAF-stimulated acinar cells were also investigated in order to find out whether the mechanisms mediating the acinar chemokine expression are sensitive to glucocorticoid and/or antioxidant treatments.

**Methods**

**Reagents**

N-Acetyl-l-cysteine (NAC), dexamethasone 21 phosphate disodium salt (Dx), sodium taurocholate (NaTc), buprenorphine, aminoacid mixture, bovine serum albumin (BSA), collagenase type XI, soybean trypsin inhibitor (STI), N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES), streptomycin and penicillin solution, Nonidet P-40, aprotinin, leupeptin, pepstatin, antipapain, chymostatin, phenylmethanesulfonyl fluoride (PMSF) and dithiothreitol (DTT) were supplied by Sigma Chemical Co (Madrid, Spain). Medium 199 (Gibco, Paisley, Scotland) and calf foetal serum (BioWhittaker, Walkersville, MD, USA) were also used. Other standard analytical grade laboratory reagents were obtained from Merck (Darmstadt, Germany).

**Animals**

Male Wistar rats (250–300 g) were housed individually in cages and maintained at 22 ± 1°C under a 12-hr 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).

**Production and preparation of PAAF**

After 12 hrs of fasting, under strict sterile conditions and anaesthesia with 2–3% isofluorane, Forane® (Abbott, Madrid, Spain) laparotomy was performed in order to induce AP in rats by the intraductal administration of a previously sterilized solution of 3.5% sodium taurocholate (NaTc). Following clamping of the proximal common bile duct 0.1 ml of NaTc solution was infused at 0.03 ml/min. After infusion, the bile duct clamp was maintained on ice for 20 min., after which Nonidet P-40 (0.4% v/v) was added for 2 min. and then centrifuged at 4°C for 5 min. at 14,000 g. The supernatants were collected and immediately stored at −80°C.

**Analysis of MCP-1 mRNA expression by RT-PCR**

Total RNA was extracted from acinar cells 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 and its integrity by the presence of well-defined 28S and 18S rRNA bands.

**Analysis of phospho p-38 MAPK and IκBα**

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

Nuclear protein extracts were obtained using a commercial nuclear extract kit following the recommendations of the manufacturer (Active Motif, Reixenart, 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. Nonidet P-40 (0.4% v/v) was added after 15 min. and incubated for 1 min. Nuclear fraction was collected in the pellet after centrifuging 30 sec. at 14,000 g and resuspended in lysis buffer containing 0.1% Tween 20 and 5% (w/v) BSA overnight at 4°C. After washing for 1 hr with TBS containing 0.1% Tween 20, the blots were incubated for 1 hr 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) non-fat dry milk and finally they were developed for visualization. The bands were detected with Phototope-HRP Detection kit (Cell Signalling Technology). Image J 1.32 software from http://rsbweb.nih.gov/ij/download.html was used to quantify the intensity of the bands.

Determination of NF-κB and STAT3 activation

NF-κB and STAT3 DNA binding was measured in nuclear extracts with the respective ELISA-based commercial kits (Trans AM NF-κB p65 and TransAM STAT3 activation assay, Active Motif, Reixenart, Belgium). 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 hr. Activation was detected by incubation for 1 hr with the respective primary antibody: anti-NF-κB, which specifically recognizes an epitope (p65) accessible only when the factor is activated and bound to its target DNA or anti-STAT3, which recognizes epitopes 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. Absorbance was read within 5 min. at 450 nm with a reference wavelength of 655 nm.

Statistical analysis

Data are expressed as mean ± S.E.M. Paired Student’s t-test was used in the comparison of data between non-PAAF-stimulated and PAAF-stimulated cells. Analysis of variance (ANOVA) followed by the Dunnett test was applied to evaluate the differences between more than two experimental groups. P-values lower than 0.05 were considered to be significant.

Results

Time-course of PAAF activation

As Fig. 1 shows, overexpression of MCP-1 was found in acinar cells incubated with PAAF for 1 hr and 3 hrs. However, phosphorylation of p38-MAPK and activation of NF-κB (measured by p65 nuclear levels) and STAT3 were only significantly (P < 0.01) increased in cells cultured with PAAF for 1 hr (time of PAAF activation in all subsequent experiments).

Viability of pancreatic acinar cells

Viability of the pancreatic acinar cells, determined 1 hr after cell culture by trypan blue dye exclusion assay, was >95%.

MCP-1 mRNA expression

Prominent MCP-1 RT-PCR products were found in acinar cells incubated with PAAF (Fig. 2). MCP-1/β-actin mRNA ratio indicated a four-fold increase in MCP-1 expression when compared with acinar cells cultured in the absence (basal conditions) and the presence of PAAF. Dx and NAC abrogated the PAAF-induced MCP-1 gene expression in pancreatic acinar cells, but they did not show any effect on non-stimulated acinar cells.

Downstream signalling pathways

Phosphorylation of p38-MAPK was analysed by Western blot (Fig. 3) to evaluate the activation of MAPK. Basal p38-MAPK phosphorylation did not vary by adding Dx or NAC. A significant (P < 0.01) increase in phospho-p38-MAPK was found in acinar cells cultured in the presence of PAAF. Pre-treatment with Dx did not prevent the PAAF-induced MAPK activation. However, a significant (P < 0.01) reduction in phospho-p38 was found in acinar cells cultured with PAAF in the presence of NAC.
The activation of NF-κB was evaluated by analysis of IκBα (Fig. 4A) and p65 (Fig. 4B). Dx and NAC did not show any effect in acinar cells cultured under basal conditions. PAAF significantly activated NF-κB in acinar cells as indicated by the significant (P < 0.01) degradation of IκBα and the significant (P < 0.01) increase in p65 levels. Both Dx and NAC significantly inhibited NF-κB activation induced by PAAF, by preventing the degradation of IκBα and maintaining p65 values at similar levels to those that were found in acinar cells cultured in the absence of PAAF (basal conditions).

Figure 5 shows the STAT3 activity. A non-significant decrease was induced by Dx and NAC in the basal STAT3 activity. PAAF significantly (P < 0.01) up-regulated the activation of STAT3 in acinar cells. Dx significantly (P < 0.01) reduced the PAAF-induced STAT3 activation to values significantly (P < 0.05) lower than those found in acinar cells cultured in the absence of PAAF. NAC significantly (P < 0.05) diminished the STAT3 activation in PAAF-stimulated acinar cells, although the STAT3-DNA binding

Fig. 4 Activation of NF-κB in pancreatic acinar cells under basal conditions and under stimulation with pancreatitis-associated ascitic fluid (PAAF, 20% v/v) in the absence or presence of dexamethasone (Dx, 10^−7 M) and N-acetylcysteine (NAC, 30 mM). (A) Representative Western blot of IκBα and the mean values of five experiments are shown. Band intensity was determined by densitometry. (B) p65 nuclear levels (NF-κB-DNA binding). Results are mean ± S.E.M. ANOVA followed by Dunnett’s test showed significant differences versus non-PAAF-stimulated cells (basal conditions) (*** P < 0.001) and versus PAAF-stimulated cells (●●● P < 0.001).

Fig. 3 Phosphorylation of p38-MAPK expression in acinar cells under basal conditions and under stimulation with pancreatitis-associated ascitic fluid (PAAF, 20% v/v) in the absence or presence of dexamethasone (Dx, 10^−7 M) and N-acetylcysteine (NAC, 30 mM). A representative Western blot and the mean values of five experiments are shown. Band intensity was determined by densitometry. Results are mean ± S.E.M. ANOVA followed by Dunnett’s test showed significant differences versus non-PAAF-stimulated cells (basal conditions) (** P < 0.01) and versus PAAF-stimulated cells (●● P < 0.01).

Fig. 2 MCP-1 mRNA expression in acinar cells under basal conditions and under stimulation with pancreatitis-associated ascitic fluid (PAAF, 20% v/v) in the absence or presence of dexamethasone (Dx, 10^−7 M) and N-acetylcysteine (NAC, 30 mM). A representative RT-PCR and the mean values of five experiments are shown. Results are mean ± S.E.M. ANOVA followed by Dunnett’s test showed significant differences versus non-PAAF-stimulated cells (basal conditions) (*** P < 0.001) and versus PAAF-stimulated cells (●●● P < 0.001).

The activation of NF-κB was evaluated by analysis of IκBα (Fig. 4A) and p65 (Fig. 4B). Dx and NAC did not show any effect in acinar cells cultured under basal conditions. PAAF significantly
Discussion

In response to PAAF, up-regulation of cytokines and adhesion molecules in endothelial cells [6] and leukocytes [8] has been reported. In addition, PAAF has shown to be able to modulate the phenotype of pancreatic acinar cells to produce TNF-α [20, 21]. The role of the STAT pathway in the transcriptional regulation of inflammatory factors is increasingly being appreciated. It provides a direct link between the cytokine receptors and cytokine-induced gene transcription [32]. Up-regulation of STAT3 in response to TNF-α has been reported in acinar cells [21], an interesting data due to the fact that acinar cells have been demonstrated to have TNF-α receptors, which are up-regulated by cerulein [33]. In addition, STAT has also been demonstrated to be a redox sensitive transcription factor [34]. Given that PAAF is a cytokine-rich biological fluid and under its presence acinar cells generate a high amount of reactive oxygen species (ROS), MAPKs as well as both transcription factors, NF-κB and STAT3, may be involved in the acinar MCP-1 up-regulation induced by PAAF. Data concerning this are of great interest in the consideration of the downstream signalling pathways as potential targets of therapeutic strategies designed to reduce both local and systemic inflammation during AP.

Glucocorticoids have a long history of use as anti-inflammatory agents by down-regulating inflammatory factors at transcriptional level. NF-κB and Activator protein-1 (AP-1) have been shown to be candidates as targets for the genomic action of glucocorticoids [35, 36]. On the other hand, interaction of glucocorticoids with the JAK/STAT pathway has been reported with positive [37] and negative [22, 23, 38] effects on cytokine signalling. Our results showed a negative cross-talk in acinar cells between Dx and either NF-κB or STAT3 pathway in parallel with a total repression of the PAAF-induced MCP-1 expression. This suggests that NF-κB and STAT3 may synergically act as downstream signalling transduction pathways of the PAAF-mediated inflammatory response in acinar cells. Dx prevented the phosphorylation and subsequent degradation of IκBα induced by PAAF in acinar cells.
Because IL-1bX acts as an inhibitory subunit [31], NF-κB remained in a latent form in the cytoplasm instead of being translocated to the nucleus. In fact, basal levels of p65 were found in acinar cells cultured with PAAF in the presence of Dx, thus indicating that NF-κB-DNA binding was inhibited. STAT3 pathway has shown to be especially sensitive to the Dx action. It was able to block the STAT3 activation to levels even lower than those detected in non-stimulated acinar cells (basal conditions), which may be the result of the oxidative stress triggered by the pancreas removal and during acini isolation, as Gallmeier et al. reported [20]. Inhibitory effects of Dx on the activation of STAT family members have also been reported in macrophages [22] and keratinocytes [23] stimulated by cytokines. Our results strongly suggest that STAT3 may be selected as a potential target for therapeutic intervention of AP and give new insight into the anti-inflammatory actions of glucocorticoids in AP. In contrast, Dx did not affect the acinar p38-MAPK phosphorylation induced by PAAF. As a possible non-genomic rapid action, glucocorticoids have been reported to cause inhibition [39] and activation [40, 41] of p38-MAPK. This indicates that the glucocorticoid action is cell type-dependent. Our results suggest that p38-MAPK activation does not seem to play a pivotal role in the glucocorticoid-mediated MCP-1 up-regulation, because Dx completely abrogated the acinar MCP-1 expression with no change in the PAAF-induced p38-MAPK phosphorylation.

NAC has demonstrated in in vitro [9] and in vivo [10, 13] studies to reduce the production of TNF-α in acinar cells. In line with this, we show that NAC down-regulated the PAAF-induced MCP-1 expression in acinar cells. In response to PAAF, acinar cells develop oxidative stress, which has been demonstrated to activate MAPK [42] and NF-κB [29, 43]. On the other hand, the inhibition of phosphatases induced by ROS allows the JAK-induced STAT phosphorylation required for STAT activation [18, 31]. Accordingly, the anti-inflammatory action of NAC may be explained by preventing the redox-activation of p38-MAPK and NF-κB and significantly reducing the STAT3 activity. The different effects of NAC versus Dx on the PAAF-activated signalling pathways may be explained by their specific actions as antioxidant and anti-inflammatory agents, respectively. Because MAPK cascade is especially sensitive to the cellular redox state, NAC totally repressed p38-MAPK phosphorylation. However, NAC was not as effective as Dx to inhibit STAT3 activation, probably due to the fact that JAK may be activated not only by ROS but also by cytokines, whose presence, in turns, are especially sensitive to Dx action.

In summary, PAAF may be involved in the pathophysiology of AP by activating acinar cells to produce MCP-1, particularly via NF-κB and STAT3 signalling pathways. Dx and NAC suppressed the PAAF-mediated acinar MCP-1 cell up-regulation by inhibiting both downstream mechanisms. STAT3 activity was especially sensitive to Dx, thus becoming a key target in therapeutic interventions in AP.

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