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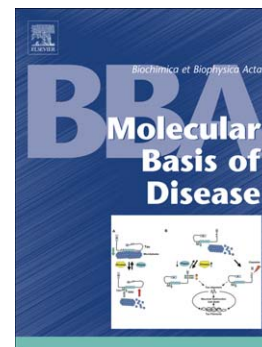
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## The Role of Redox Status on Chemokine Expression in Acute Pancreatitis

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**SUMMARY**

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- $\kappa$ B 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 expression 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- $\kappa$ B and STAT3 activation triggers the chemokine expression in acini but not in non-acinar cells.

## 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<sub>3</sub>C, 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- $\alpha$ /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- $\kappa$ B (NF- $\kappa$ B) 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.

## MATERIALS AND METHODS

### 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,  $\beta$ -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&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).

### Animal groups

Male Wistar rats (250-300 g) were housed individually in cages and maintained at  $22 \pm 1^\circ\text{C}$  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).

### 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 hour before inducing AP followed by a new injection 1 hour 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).

### **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 [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.

### **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<sub>2</sub> 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).

### **Analysis of Phospho-p38MAPK and IκBα**

#### *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.

#### *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 hour 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.

#### **Determination of NF-κB and STAT3 activation:**

##### *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 seconds 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  $-80^{\circ}\text{C}$  until use.

NF- $\kappa$ B- and STAT3-DNA binding was measured in nuclear extracts with the respective ELISA-based commercial kits (NF- $\kappa$ B p65 TransAM and STAT3 activation assays, Active Motif). Nuclear proteins (5  $\mu\text{g}$ ), were added to each well coated with an oligonucleotide containing the consensus binding site for either NF- $\kappa$ B or STAT3 and incubated for 1 h. Activation was detected by incubation for 1 h with the respective primary antibody: anti-NF- $\kappa$ B, 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- $\kappa$ B and STAT 3 by a colorimetric reaction.

#### **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  $\mu$ g) 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'- GACTCACTTGGTTCTGGTCCA-3', product size: 294 bp), CINC (sense: 5'- CTCCAGCCACACTCCAACAGA-3, antisense: 5'- CACCCTAACACAAAACACGAT-3', product size: 600 bp). Oligonucleotide primers for  $\beta$ -actin (sense: 5'-CACGGCATTGTAACCAACTG-3', antisense: 5'- TCTCAGCTGTGGTGGTGAAG-3', product size: 400 bp) were used as internal control. PCR was performed following procedures: 30 cycles of denaturation at 94°C for 30 s, annealing 56°C for 45 s and elongation at 72°C for 45 s. The amplified PCR products were separated on a 2% agarose gel stained with ethidium bromide. The MCP-1, CINC and  $\beta$ -actin PCR products were run together on the same gel in order to normalize the band densities to the  $\beta$ -actin band which were densitometrically quantified with a Gel Doc 1000/2000 image analysis system (BioRad) using the QuantityOne software programme.

### Statistical analysis

Results are expressed as mean  $\pm$  SEM. Statistical analysis was carried out using the analysis of variance (ANOVA) followed by the Dunnett test to evaluate at each time point differences between controls, untreated AP and NAC-treated AP. *P* values lower than 0.05 were considered to be significant.

## RESULTS

A significant GSH depletion was observed in pancreas of rats with AP, which was prevented by NAC at early stages in BPDO but not in NaTc model (Figure 1).

A significant and progressive increase in Il-6 plasma levels was found from early stages in BPDO- and NaTc-induced AP, which did not vary by NAC treatment (Figure 2).

MPO activity is shown in Figure 3. A significant ( $p < 0.01$ ) increase was found 12 h after inducing AP by BPDO. Although neutrophil infiltration was not completely prevented, a significant ( $p < 0.01$ ) reduction was found in NAC-treated BPDO rats. MPO activity significantly ( $p < 0.01$ ) increased from 3 h after inducing AP by NaTc, both in NAC-treated and non-treated rats.

Overexpression of MCP-1 and CINC mRNA was found in acinar cells of rats with AP induced by either BPDO or NaTc (Figure 4). NAC treatment significantly ( $p < 0.05$ ) reduced the acinar chemokine expression in BPDO but not in NaTc-induced AP.

Analysis of chemokine expression in pancreatic tissue (Figure 5) revealed a significant increase in MCP-1 and CINC from 3 h onwards in both AP models. NAC treatment did not vary the upregulation of chemokines in pancreas of rats with AP either induced by BPDO or by NaTc.

Plasma levels of both chemokines, MCP-1 and CINC, (Figure 6) significantly increased from early stages in rats with BPDO- and NaTc-induced AP, either treated or non-treated with NAC.

Figure 7 shows a progressive increase in phospho-p38MAPK in acinar cells of rats with BPDO-induced AP which reached statistical significance ( $p < 0.01$ ) at 12 h. This effect was significantly ( $p < 0.05$ ) inhibited by NAC treatment. In NaTc-AP model, significant phosphorylation of p38MAPK was found in acinar cells from early stages both in rats treated and not treated with NAC.

NF- $\kappa$ B activation was evaluated in by analysis of I $\kappa$ B $\alpha$  (Figure 8A) and p65 nuclear levels (Figure 8B). Intense degradation of I $\kappa$ B $\alpha$  and significant ( $p < 0.01$ ) increase in p65

were found in acinar cells 12 h after inducing AP by BPDO and from 3 h in NaTc-AP model. NAC treatment significantly ( $p < 0.01$ ) reduced NF- $\kappa$ B activation in BPDO- but not in NaTc-induced AP.

STAT3 activity (Figure 9) significantly ( $p < 0.01$ ) increased in acinar cells 12 h after inducing AP by BPDO. This effect, although not completely prevented, was significantly ( $p < 0.01$ ) reduced by NAC treatment. Activation of STAT3 was found in acinar cells from 3 h after inducing AP by NaTc onwards, both in NAC-treated and non-treated rats.

## DISCUSSION

Blocking treatments of chemokine action are shown to reduce the severity of AP [24-26]. The profile of chemokine expression at a site of injury is a main factor that determines the nature of the subsequent infiltration of leukocytes [27, 28]. A complete understanding of the mechanisms involved in the chemokine expression in individual cell sources would be of great interest in the control of both local and systemic inflammatory response in AP.

Our results showed overexpression of MCP-1 and CINC in acinar cells during BPDO- and NaTc-induced AP, thus indicating that acinar cell trigger early inflammatory signals to attract monocytes and neutrophils to the damaged pancreas. Slightly higher chemokine expression was found in BPDO than in NaTc model of AP. Given that this apparent paradox does not occur either in whole pancreatic tissue or in plasma, we suggest that the intense oxidative stress triggered in the severe model of AP may induce an early chemokine expression but could also damage the protein synthesis machinery, so that the chemokine expression was upregulated at lower levels than in milder AP.

Chemokine expression by pancreatic acinar cells has been previously demonstrated in “in vitro” experiments [12, 13, 29] and in other “in vivo” experimental settings. MCP-1 upregulation was shown in rat acinar cells upon mild AP induced by caerulein [6, 30] and

severe AP induced by NaTc [30]. CINC mRNA upregulation was reported in acinar cells of rats with NaTc-induced AP [31], however, no expression was found in AP induced by caerulein [6, 30], thereby suggesting specific patterns of chemokine expression depending on the etiology and severity of the disease. Inflammatory factors are transcriptionally regulated in pancreas by NF- $\kappa$ B, but as has been reported NF- $\kappa$ B requires the support of other transcription factors, such as AP-1, to upregulate chemokine production in acinar cells [11, 13]. Nevertheless, the underlying molecular mechanisms mediating the chemokine production in each pancreatic cell compartment are not well-known. Our results indicated that p38MAPK and the transcription factors NF- $\kappa$ B and STAT3, activated by oxidative stress, act as downstream signalling pathways in the expression of MCP-1 and CINC in acinar cells during AP. To our knowledge this is the first report to demonstrate the STAT3-mediated chemokine expression in AP. To our knowledge this is the first report to demonstrate the STAT3-mediated chemokine expression in AP; however it is supported by a recent report in which Ramudo et al [32] demonstrated STAT3-mediated MCP-1 upregulation in acinar cells cultured in the presence of ascitic fluid collected from rats with NaTc-induced AP. NF- $\kappa$ B is a pleiotropic transcription factor which may be activated by reactive oxygen species (ROS) [33] via MAPK activation [13]. On the other hand ROS activate JAK/STAT pathway [34] by inhibiting phosphatases thus allowing JAK-induced STAT phosphorylation which precedes the STAT activation. NAC treatment prevented the pancreatic depletion of GSH at early stages of BPDO-induced AP and significantly reduced the phosphorylation of p38MAPK as well as the redox activation of NF- $\kappa$ B and STAT3 transcription factors in acinar cells. Therefore, inhibition of the transcriptional MCP-1 and CINC upregulation in acinar cells by NAC treatment occurs in AP induced by BPDO, which leads to a reduction of the infiltration of leukocytes in the pancreas, as MPO activity indicated. In line with these results, ultrastructural morphological alterations in

acinar cells [35] as well as the histological lesions in pancreas [36] of rats with BPDO-induced AP were found to be reduced by NAC treatment.

In contrast, NAC was unable to reduce the oxidative stress developed within the pancreas of rats with severe AP induced by NaTc. The failure of NAC treatment in this AP model can be explained by the fact that, related to the severity of the AP, acinar cells develop intense oxidative stress with the subsequent ROS overproduction in acinar cells. ROS, in addition to acting as second messengers to enhance the production of inflammatory factors, are powerful chemoattractants [37]. As a result, an intense leukocyte recruitment occurs in the pancreas of rats with NaTc-induced AP, as MPO indicated, which in turns leads to an increase in the production of ROS molecules by infiltrated leukocytes. The overproduction of ROS from different pancreatic cell sources overwhelms the antioxidant capability of NAC treatment in severe AP; as a result, NAC failed to hinder the redox-sensitive chemokine expression and its downstream signal pathways. These findings suggest that damaged acinar cells contribute to chemokine production from early stages of AP by activating oxidant dependent signalling mechanisms, which only in mild AP, with lower oxidative stress, were downregulated by NAC treatment.

In contrast with results previously reported in caerulein-induced AP [38], NAC treatment showed no inhibitory effect on the overexpression of chemokines found in pancreatic tissue from 3 h after inducing AP, either in BPDO or NaTc model. This finding suggests that in addition to acinar cells, other cell types resident in the pancreas with the capability of producing chemokines, such as endothelial vascular cells [17, 18], stellate cells [19, 20] and infiltrated leukocytes [7, 39] are also producing MCP-1 and CINC by mechanisms resistant to the antioxidant treatment. Given that high Il-6 levels were found in plasma of rats with BPDO- and NaTc-induced AP, either treated or not treated with NAC, we suggest that in addition to local cytokines produced from early stages by pancreatic

cells, circulating cytokines may act as a stimulus to upregulate chemokine expression during AP in non-acinar cells within the pancreas. This idea is supported by the fact that in addition to ROS, cytokines have demonstrated to be key activating agents of NF- $\kappa$ B and STAT-3 transcription factors in different cell types [40, 41]. In fact, IL-6 is the cytokine most closely associated with STAT transcription factor activity [42].

Neither NAC treatment reduced the high MCP-1 and CINC concentrations found in plasma of rats with BPDO- and NaTc-induced AP. These increased systemic chemokine levels could be the result of the outpouring of chemokines from the pancreas and those produced by inflammatory cells. Given that the profile of chemokines in response to the antioxidant treatment was similar in plasma and in pancreatic tissue, our data suggest that cell sources other than acini would mainly contribute from the damaged tissue to the increase of circulating levels of MCP-1 and CINC.

In summary, our findings indicate that oxidative stress may trigger the overexpression of MCP-1 and CINC in acinar cells of rats with AP by activating MAPK, NF- $\kappa$ B and STAT3, as oxidant-sensitive downstream signalling pathways. The antioxidant capability of NAC only avoided the acinar chemokine expression in BPDO-induced AP but it was ineffective in severe NaTc-induced AP. NAC failed to prevent the chemokine increase in pancreatic tissue and plasma, even in BPDO-induced AP, suggesting that additional stimuli, such as cytokines, activate molecular pathways leading to upregulation of chemokines in non-acinar cell sources.

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ACCEPTED MANUSCRIPT

## LEGENDS

**Figure 1.** Pancreatic glutathione (GSH) content in controls, rats with acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) and 3.5% sodium taurocholate (NaTc) either untreated or treated with N-Acetylcysteine (NAC). Number of animals in each group and AP period: 6. Results are mean  $\pm$  SEM. ANOVA followed by Dunett test showed significant differences *vs* controls (\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ) and BPDO rats ( $p < 0.01$ ).

**Figure 2.** Plasma Interleukin-6 (Il-6) levels in controls, rats with acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) and 3.5% sodium taurocholate (NaTc) either untreated or treated with N-Acetylcysteine (NAC). Number of animals in each group and AP period: 6. Results are mean  $\pm$  SEM. ANOVA followed by Dunett test showed significant differences *vs* controls (\*\*\*  $p < 0.001$ ).

**Figure 3.** Myeloperoxidase (MPO) activity in pancreatic tissue from controls, rats with acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) and 3.5% sodium taurocholate (NaTc) either untreated or treated with N-Acetylcysteine (NAC). Number of animals in each group and AP period: 6. Results are mean  $\pm$  SEM. ANOVA followed by Dunett test showed significant differences *vs* controls (\*  $p < 0.05$ , \*\*  $p < 0.01$ ) and BPDO rats ( $p < 0.01$ ).

**Figure 4.** mRNA expression of MCP-1 and CINC in acinar cells of controls, rats with acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) and 3.5% sodium taurocholate (NaTc) either untreated or treated with N-Acetylcysteine (NAC). A representative RT-PCR and the mean values  $\pm$  SEM (measured as MCP-1/ $\beta$ -actin and CINC// $\beta$ -actin ratios). Number of animals in each group and AP period: 5. ANOVA followed by Dunett test showed significant differences *vs* controls (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ) and BPDO rats ( $p < 0.05$ ).

**Figure 5.** mRNA expression of MCP-1 and CINC in pancreatic tissue of controls, rats with acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) and 3.5% sodium taurocholate (NaTc) either untreated or treated with N-Acetylcysteine (NAC). A representative RT-PCR and the mean values  $\pm$  SEM (measured as MCP-1/ $\beta$ -actin and CINC/ $\beta$ -actin ratios) are shown. Number of animals in each group and AP period: 5. ANOVA followed by Dunett test showed significant differences vs controls (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

**Figure 6.** Plasma monocyte chemoattractant protein-1 (MCP-1) and cytokine-induced neutrophil chemoattractant (CINC) levels in controls, rats with acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) and 3.5% sodium taurocholate (NaTc) either untreated or treated with N-Acetylcysteine (NAC). Number of animals in each group and AP period: 6. Results are mean  $\pm$  SEM. ANOVA followed by Dunett test showed significant differences vs controls (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

**Figure 7.** Phosphorylation of p38 mitogen activated protein kinase (p38MAPK) in acinar cells of controls, rats with acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) and 3.5% sodium taurocholate (NaTc) either untreated or treated with N-Acetylcysteine (NAC). Quantification and representative photograph of Western blot analysis.  $\beta$ -actin was used as a loading control. Relative intensity of the bands in each group vs controls was considered. Results are expressed as means  $\pm$  SEM. Number of animals in each group and AP period: 5. ANOVA followed by Dunett test showed significant differences vs controls (\*  $p < 0.05$ , \*\*  $p < 0.01$ ) and BPDO ( $p < 0.05$ ).

**Figure 8.** NF- $\kappa$ B activation in acinar cells of controls, rats with acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) and 3.5% sodium taurocholate (NaTc) either untreated or treated with N-Acetylcysteine (NAC). A) Representative Western blot of five experiments of I $\kappa$ B $\alpha$  and  $\beta$ -actin, as loading control. B) Mean values  $\pm$  SEM of

p65 nuclear levels of five experiments. ANOVA followed by Dunett test showed significant differences vs controls (\*  $p < 0.05$ , \*\*  $p < 0.01$ ) and BPDO ( $p < 0.01$ ).

**Figure 9.** STAT3 activity in acinar cells of controls, rats with acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) and 3.5% sodium taurocholate (NaTc) either untreated or treated with N-Acetylcysteine (NAC). Results are means  $\pm$  SEM of the nuclear active STAT3 of five experiments. ANOVA followed by Dunett test showed significant differences vs controls (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ) and BPDO ( $p < 0.01$ ).

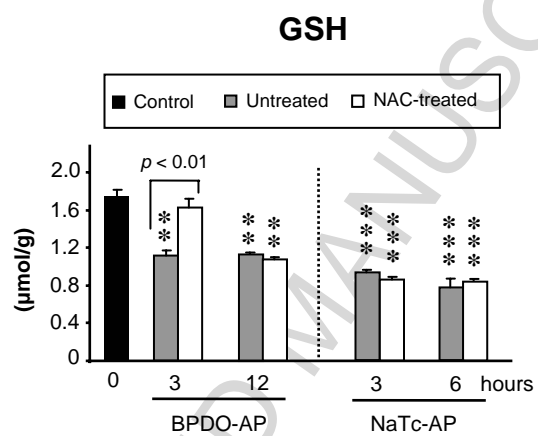


Figure 1

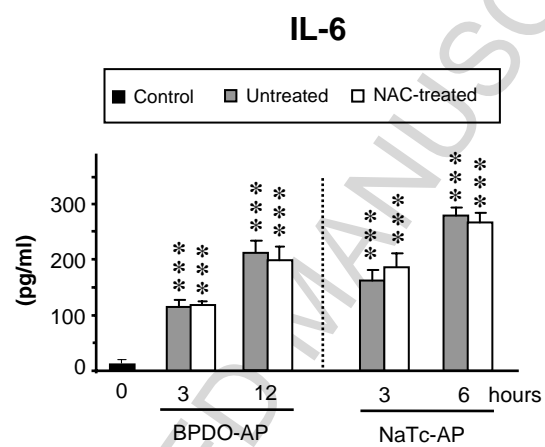


Figure 2

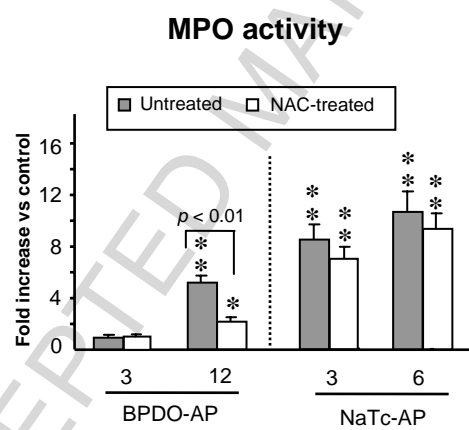


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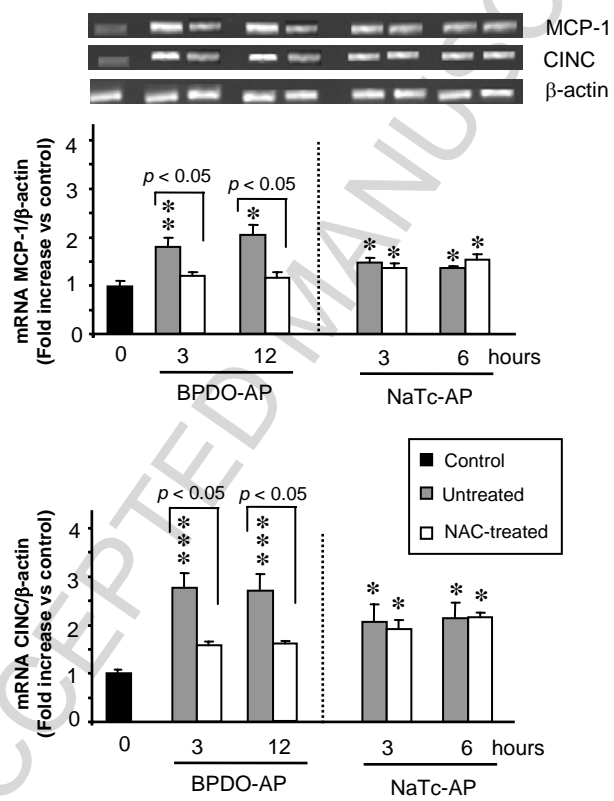


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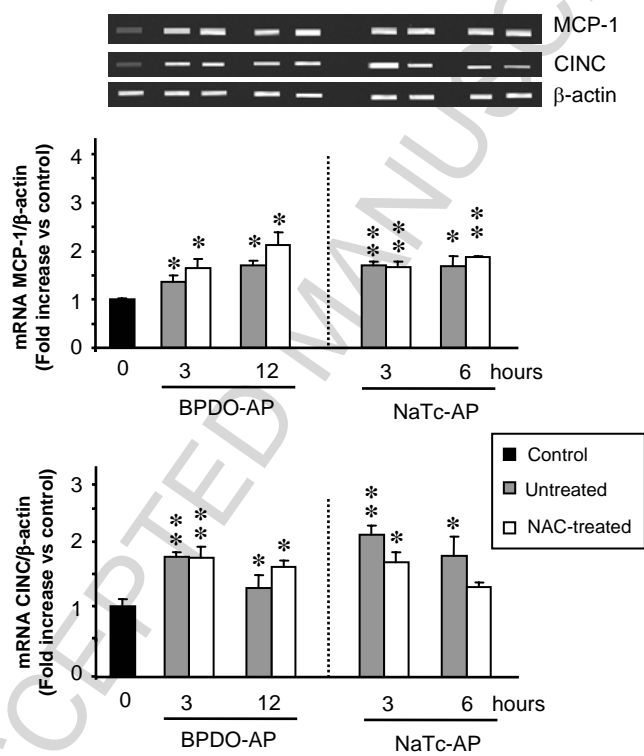


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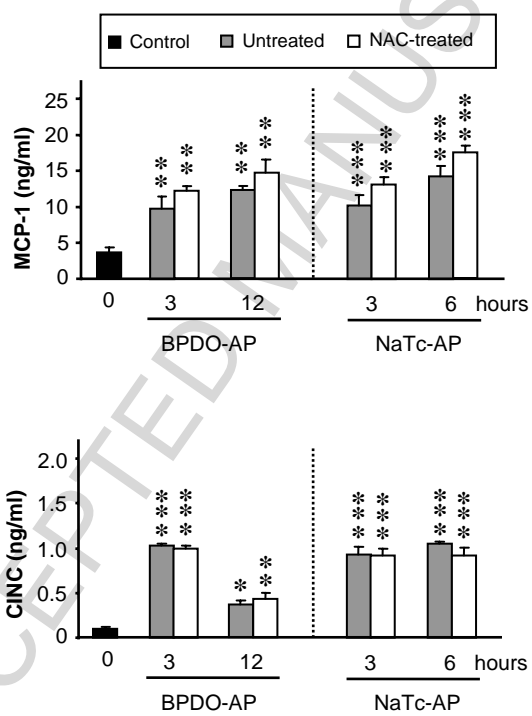


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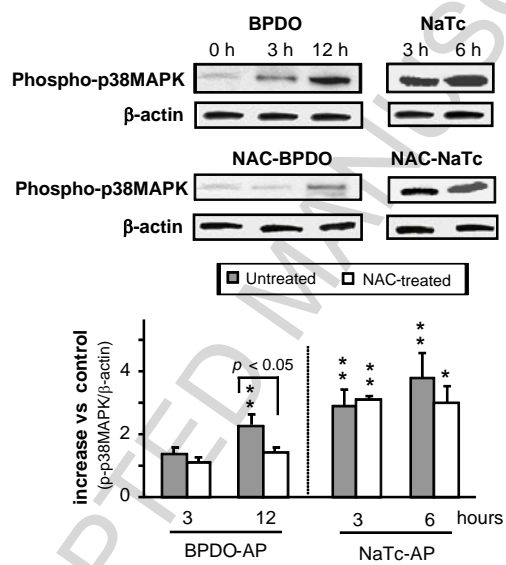


Figure 7

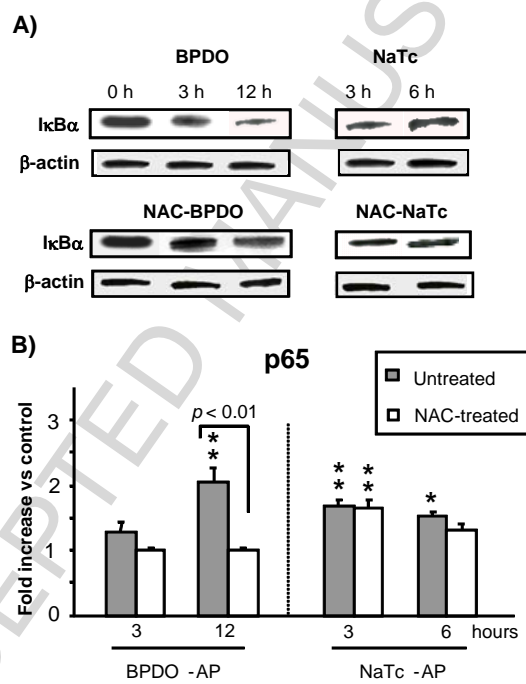


Figure 8

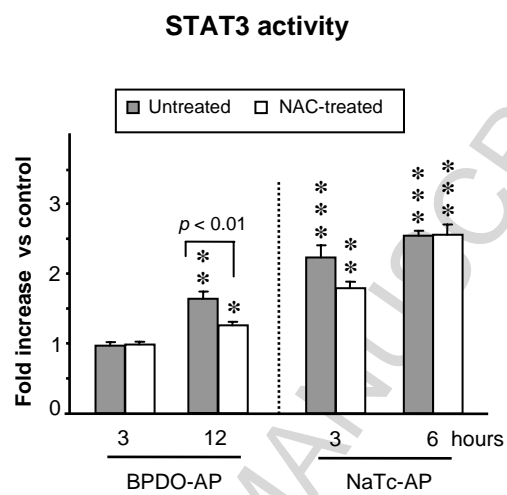


Figure 9