

# Potential role of NADPH oxidase-mediated activation of Jak2/Stat3 and mitogen-activated protein kinases and expression of TGF- $\beta$ 1 in the pathophysiology of acute pancreatitis

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## Abstract

**Objective** NADPH oxidase is potentially associated with acute pancreatitis by producing reactive oxygen species (ROS). We investigated whether NADPH oxidase mediates the activation of Janus kinase (Jak)2/signal transducers and activators of transcription (Stat)3 and mitogen-activated protein kinases (MAPKs) to induce the expression of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in cerulein-stimulated pancreatic acinar cells.

**Treatment** AR42J cells were treated with an NADPH oxidase inhibitor diphenyleneiodonium (DPI) or a Jak2 inhibitor AG490. Other cells were transfected with antisense or sense oligonucleotides (AS or S ODNs) for NADPH oxidase subunit p22<sup>phox</sup> or p47<sup>phox</sup>.

**Methods** TGF- $\beta$ 1 was determined by enzyme-linked immunosorbent assay. STAT3-DNA binding activity was measured by electrophoretic mobility shift assay. Levels of MAPKs as well as total and phospho-specific forms of Jak1/Stat3 were assessed by Western blot analysis.

**Results** Cerulein induced increases in TGF- $\beta$ 1, Stat3-DNA binding activity and the activation of MAPKs in AR42J cells. AG490 suppressed these cerulein-induced

changes, similar to inhibition by DPI. Cerulein-induced activation of Jak2/Stat3 and increases in MAPKs and TGF- $\beta$ 1 levels were inhibited in the cells transfected with AS ODN for p22<sup>phox</sup> and p47<sup>phox</sup> compared to S ODN controls. **Conclusion** Inhibition of NADPH oxidase may be beneficial for prevention and treatment of pancreatitis by suppressing Jak2/Stat3 and MAPKs and expression of TGF- $\beta$ 1 in pancreatic acinar cells.

**Keywords** Cerulein · NADPH oxidase · Jak2/Stat3 · TGF- $\beta$ 1 · Pancreatic acinar cells

## Introduction

Acute pancreatitis is a multifactorial disease that involves both local and systemic effects caused by the release of digestive enzymes into the pancreatic interstitium and systemic circulation, and by the production and release of cytokines [1–3]. High doses of cerulein, a cholecystokinin (CCK) analogue [4], result in experimental pancreatitis. Similar to human pancreatitis, experimental pancreatitis involves dysregulation of the production and secretion of digestive enzymes leading to an elevation in their serum levels, as well as cytoplasmic vacuolization, death of acinar cells and edema formation [5–7]. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is known to be one of the cytokines that regulate extracellular matrix remodeling in the pancreas and is involved in the pathogenesis of pancreatic inflammation and fibrosis [8–11]. TGF- $\beta$ 1 eliminates the damaged acinar cells by promoting acinar apoptosis during acute pancreatitis induced by cerulein [12]. Inhibition of TGF- $\beta$ 1 not only alleviates pancreatic fibrosis but also protects the pancreas from chronic injury due to excessive apoptosis [13].

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Reactive oxygen species (ROS) are regarded as the molecular triggers of various inflammatory responses including pancreatitis [14]. ROS attack biological membranes directly, triggering the accumulation of neutrophils and their adherence to the capillary wall [14]. ROS may thus play a critical role in perpetuating pancreatic inflammation and the development of extrapancreatic complications [15]. ROS activate nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinases (MAPKs) which regulate the expression of inflammatory cytokines including interleukin (IL)-1 $\beta$ , IL-6 [16], IL-8 [17] and TGF- $\beta$ 1 [18] as well as substance P [19] in cerulein-stimulated pancreatic acinar cells. NADPH oxidase is activated by inflammatory stimuli and produces large amounts of ROS in phagocytic and non-phagocytic cells [20, 21]. In phagocytic cells, NADPH oxidase is composed of membrane-bound subunits gp91<sup>phox</sup> and p22<sup>phox</sup>, and separate cytosolic subunits p67<sup>phox</sup> and p47<sup>phox</sup>. Upon activation, a complex of the cytosolic subunits translocates to the membrane and facilitates NADPH-dependent formation of superoxide (O<sub>2</sub><sup>-</sup>) and other secondary ROS (H<sub>2</sub>O<sub>2</sub>). Several non-phagocytic cells, such as vascular endothelial cells and pulmonary and systemic smooth muscle cells, contain NADPH oxidase to produce toxic ROS [22]. Recently, we found that NADPH oxidase subunits Nox1, p27<sup>phox</sup>, p47<sup>phox</sup> and p67<sup>phox</sup> are present in pancreatic acinar AR42J cells and produce ROS upon cerulein stimulation [23]. The activation of MAPKs and NF- $\kappa$ B to induce the expression of cytokines [16–18] may therefore be mediated by ROS in cerulein-stimulated pancreatic acinar cells. However, the precise mechanism whereby activated NADPH oxidase induces inflammation in pancreatic acinar cells during pancreatitis remains unclear.

Several lines of evidence support the activation of MAPKs in cerulein-stimulated pancreatic acinar cells. Extracellular signal-regulated kinase (ERK), c-Jun NH<sub>2</sub>-terminal protein kinase (JNK) and intracellular cAMP levels all modulate the expression of protein tyrosine phosphatases SHP-1 and SHP-2 in pancreas, and play an essential role in cerulein-induced acute pancreatitis [24]. Inhibition of JNK and ERK suppresses the expression of protein tyrosine phosphatase 1B which is increased in the early phase of acute cerulein-induced pancreatitis [25]. In addition, ERK, p38 and JNK are activated and cytokine (IL-1 $\beta$ , tumour necrosis factor [TNF]- $\alpha$ ) concentrations are increased in pancreatic fragments stimulated with cerulein [26].

The Janus kinase (Jak)/signal transducers and activators of transcription (Stat) mediate the expression of inflammatory cytokines [27]. Jak/Stat signaling pathways function primarily through non-immune mediators such as growth factors, hormones and ROS, but also play a role in the immune response of various cytokines [27–29]. Jak2

and Stat1 proteins are expressed and activated by interferon (IFN)- $\gamma$  in rat pancreatic acinar cells, perhaps contributing to cytokine secretion and stimulation of immune cells [30]. Since cerulein is a CCK analogue, it binds to the CCK receptor to activate signaling in pancreatic acinar cells. The CCK2 receptor is a Gq protein coupled receptor that mediates the activation of Jak2/Stat3 for cell proliferation in pancreatic tumor cells [31]. Previously, we showed that cerulein-activated Jak2/Stat3 induces the expression of IL-1 $\beta$  in pancreatic acinar cells [32]. AG490, a Jak2 inhibitor [33], reduced edema, vacuole formation and inflammatory cell infiltration induced by cerulein in vivo. Jak/Stat activation may therefore affect the development of acute pancreatitis by the induction of inflammatory mediators.

We hypothesized that NADPH oxidase may be involved in signaling pathways such as those of Jak2/Stat3 and MAPKs to induce inflammatory cytokines in cerulein-stimulated pancreatic acinar cells. Here we investigated the role of NADPH oxidase in inflammatory signaling and TGF- $\beta$ 1 induction in pancreatic acinar cells, to determine whether inhibition of the activation of NADPH oxidase is beneficial for the prevention and treatment of pancreatitis.

The present study aims to investigate whether NADPH oxidase mediates the activation of Jak2/Stat3 and MAPKs (ERK, JNK, p38) to induce the expression of TGF- $\beta$ 1 in cerulein-stimulated pancreatic acinar AR42J cells. Cells were treated with an NADPH oxidase inhibitor diphenyleneiodonium (DPI), or transfected with antisense oligonucleotides (AS ODN) or sense oligonucleotides (S ODN) for NADPH oxidase subunit p22<sup>phox</sup> or p47<sup>phox</sup>. The activation of Jak2/Stat3 and MAPKs, Stat3-DNA binding activity and the level of TGF- $\beta$ 1 in the medium were determined. In addition, to investigate the role of Jak2/Stat3 on the expression of TGF- $\beta$ 1, cells were treated with Jak2 inhibitor AG490. Stat3-DNA binding activity, MAPK activation and the level of TGF- $\beta$ 1 released into the medium were determined. Even though AG490 was developed as a Jak2 inhibitor [33], it also blocks Stat3 activation in some cancer cells [34, 35]. We therefore used AG490 to inhibit both Jak2 and Stat3.

## Materials and methods

### Cell culture condition

Rat pancreatic acinar cells (AR42J cells) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 3.7 g/L sodium bicarbonate, 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA), and 1% antibiotics (100 units/mL penicillin and 100  $\mu$ g/mL

streptomycin). Cells were used for experiments after incubation for 20–24 h at 37°C in a humidified atmosphere of 90% air, 10% CO<sub>2</sub>.

#### Experimental protocol

AR42J cells ( $5 \times 10^5$  cells per well in a 12-well plate) were stimulated with cerulein ( $10^{-8}$  M) for 24 h to assess the level of TGF- $\beta$ 1 in the medium, or for 30 min to assess the activation of Jak2/Stat3 and MAPKs, and Stat3-DNA binding activity. Cells were treated with DPI (10  $\mu$ M) or AG490 (50  $\mu$ M) for 2 h before stimulation with cerulein. The concentrations of cerulein, DPI and AG490 and the culture period were adapted from our previous studies [16, 17, 21, 32]. For knockdown experiments, cells were transfected with antisense oligonucleotides (AS ODN) or sense oligonucleotides (S ODN) (0.5  $\mu$ M) for NADPH oxidase subunit p22<sup>phox</sup> or p47<sup>phox</sup> for 24 h before stimulation with cerulein.

#### Western blot analysis of total or phosphorylated Jak2, Stat3, ERK, JNK and p38

Cells were trypsinized, washed, and then homogenized in Tris-HCl (pH 7.4) buffer containing 1% NP-40 and protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN, USA). The protein concentration of each sample was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Total cell extracts (50  $\mu$ g) were separated by 8% SDS polyacrylamide gel electrophoresis under reducing conditions and electroblotted onto nitrocellulose membranes (Amersham Inc., Arlington Heights, IL, USA). Membranes were blocked for 2 h with 5% nonfat dry milk and then incubated with polyclonal antibodies against Jak2 (1:500, sc-7229, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Stat3 (1:500, Cat. No. 06-596, Upstate Biotechnology, Lake Placid, NY, USA), phospho-Jak2 (1:500, Cat. No. 3771, Cell Signaling, Beverly, MA, USA), and phospho-Stat3 (1:500, Cat. No. 9131, Cell Signaling) diluted in TBS-T (Tris-buffered saline and 0.15% Tween 20) containing 5% nonfat dry milk at 4°C overnight. For antibodies against MAPKs, polyclonal antibodies for pan-ERK1/2 (1:500, Cat. No. 3771, Cell Signaling), pan-JNK1/2 (1:500; sc-7345, Santa Cruz Biotechnology), pan-p38 (1:500, Cat. No. 9212, Cell Signaling), phospho-ERK1/2 (1:500; sc-7383, Santa Cruz Biotechnology), phospho-JNK (1:500; sc-6254, Santa Cruz Biotechnology) and phospho-p38 (1:500; sc-7975, Santa Cruz Biotechnology) were used. After washing with TBS-T, the immunoreactive proteins were visualized with enhanced chemiluminescence (Santa Cruz Biotechnology) using goat anti-rabbit secondary antibodies (1:2,000, Cat. No. sc-2004, Santa Cruz Biotechnology) conjugated to horseradish peroxidase.

#### Electrophoretic mobility shift assay (EMSA) for Stat3-DNA binding activity

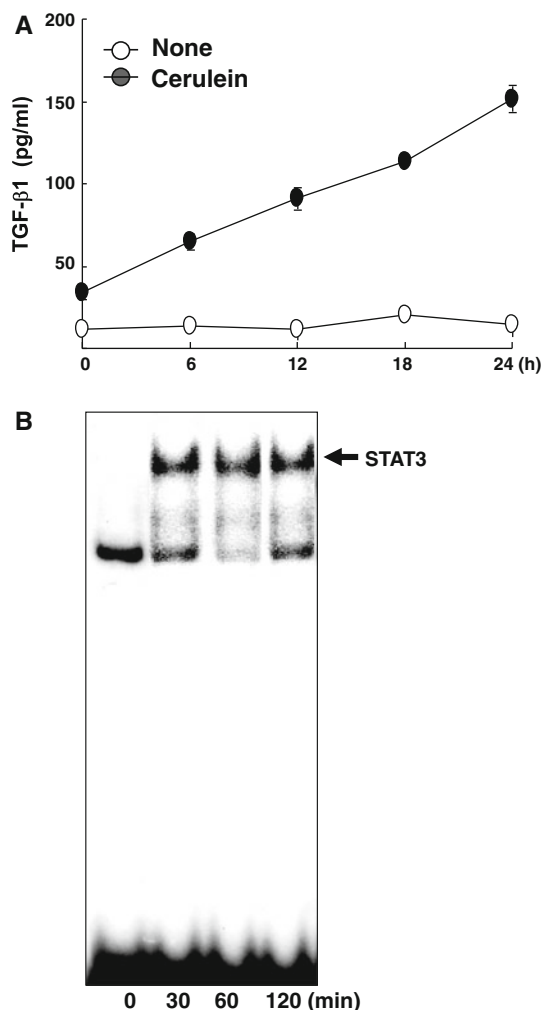
EMSAs were carried out essentially as described by Dignam et al. [36]. Cells were rinsed with ice-cold phosphate buffered saline (PBS), harvested by scraping into PBS, and pelleted by centrifugation at 1,500 rpm for 5 min. The cells were extracted in buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1.5 mM MgCl<sub>2</sub>, 0.2% Nonidet P-40, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethyl-sulfonyl fluoride (PMSF). After centrifugation at 13,000 rpm for 10 min, the nuclear pellet was resuspended on ice in a nuclear extraction buffer containing 20 mM HEPES, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 25% glycerol, 1 mM DTT and 0.5 mM PMSF. The protein concentrations of nuclear extracts were determined by Bradford assay (Bio-Rad Laboratories). A Stat3 EMSA oligonucleotide (5'-GATCCTTCTGGGAATTCCTAGAT C-3', Santa Cruz Biotechnology) was labeled with [<sup>32</sup>P]-dATP (Amersham) using T4 polynucleotide kinase (Gibco). The end-labeled probe was separated from unincorporated [<sup>32</sup>P]-dATP on a Bio-Rad purification column (Bio-Rad Laboratories) and recovered in Tris-EDTA buffer (TE). Nuclear extracts (3  $\mu$ g) were incubated with the buffer containing <sup>32</sup>P-labeled NF- $\kappa$ B consensus oligonucleotide for 30 min, and subjected to electrophoretic separation on a nondenaturing acrylamide gel. Gels were dried at 80°C for 2 h and exposed to radiography film for 6–18 h at -70°C with intensifying screens.

#### Enzyme-linked immunosorbent assay (ELISA)

Levels of TGF- $\beta$ 1 in the medium were determined by enzyme-linked immunosorbent assay kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

#### Transfection with antisense or sense oligonucleotides

Phosphothioate-modified oligonucleotides (ODNs) were produced commercially (Gibco-BRL). The sequences of p22<sup>phox</sup> antisense (AS) and sense (S) ODNs were 5'-GATC TGCCCCATGGTGAGGACC-3' and 5'-GGTCCTCA CCATGGGGCAGATC-3', respectively. The sequences of the p47<sup>phox</sup> AS and S ODNs were 5'-CTGTTGAAGTAC TCGGTGAG-3' and 5'-CTCACCGAGTACTTCAACAG-3', respectively. ODNs were incubated with DOTAP transfection reagent (15  $\mu$ g/ml, Boehringer-Mannheim) at final concentrations of 0.5  $\mu$ M ODN, for 15 min. Cells were transfected with ODNs for 24 h before cerulein treatment ( $10^{-8}$  M).



**Fig. 1** Time-response of TGF- $\beta$ 1 levels in the medium and Stat3-DNA binding activity upon cerulein ( $10^{-8}$  M) treatment of AR42J cells. **a** The protein levels of TGF- $\beta$ 1 in the medium were determined at the indicated time points by enzyme-linked immunosorbant assay (ELISA). Each point indicates mean  $\pm$  SE of four different experiments. *None* the cells cultured in the absence of cerulein; *Control* the cells cultured in the presence of cerulein. **b** Cells were harvested and nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) for Stat3-DNA binding activity at the indicated time points

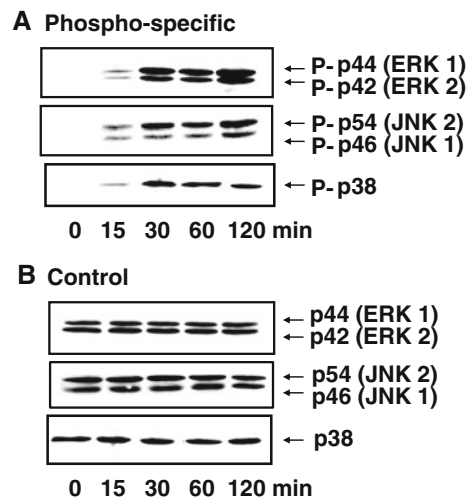
#### Statistical analysis

Statistical differences were determined using one-way ANOVA and Newman-Keul's test [37]. All values are expressed as mean  $\pm$  SE of four different experiments.

## Results

Cerulein-dependent increases in TGF- $\beta$ 1 levels, Stat3-DNA binding activity and MAPK activation are inhibited by AG490 in AR42J cells

We investigated the effects of cerulein on AR42J cells. Cerulein induced a time-dependent increase in TGF- $\beta$ 1 levels

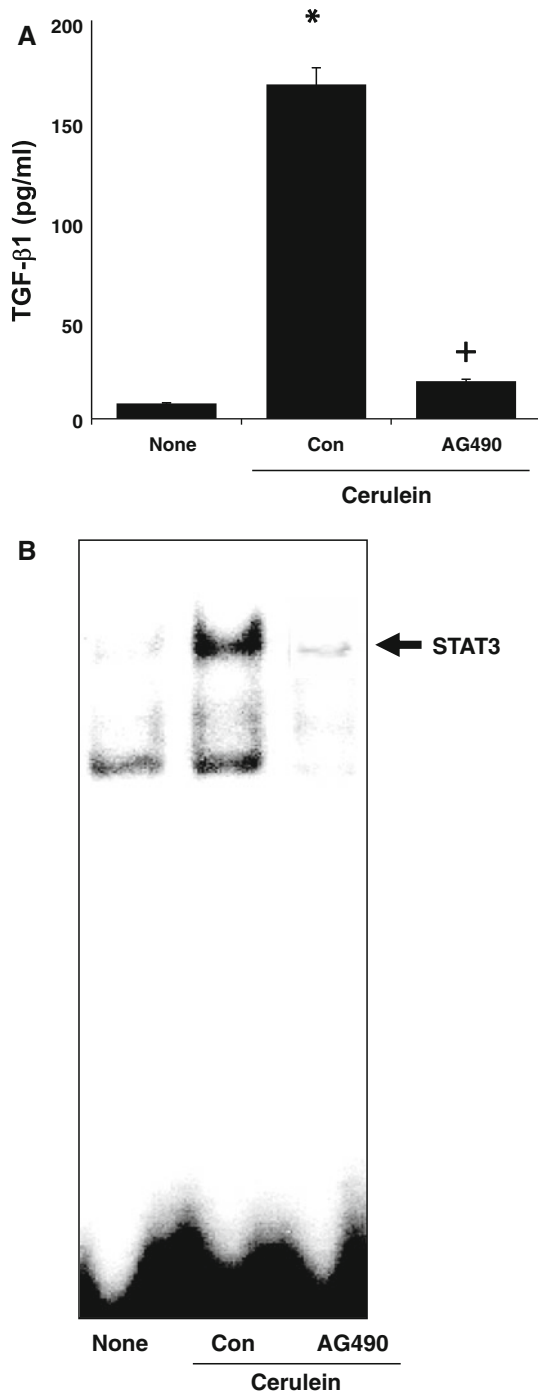


**Fig. 2** Time-dependent activation of MAPKs by cerulein in AR42J cells. Cell lysates were analyzed for total (**b**) and phospho-specific (**a**) ERK1/2, JNK2/1 and p38 levels by Western blot analysis at the indicated time points. For the detection of total MAPKs, antibodies to pan-ERK1/2, pan-JNK2/1 and pan-p38 were used. Antibodies to p-ERK1/2, p-JNK2/1 and p-p38 were used for the determination of phospho-specific proteins. *ERK* extracellular signal-regulated kinase; *JNK* c-Jun NH2-terminal protein kinase

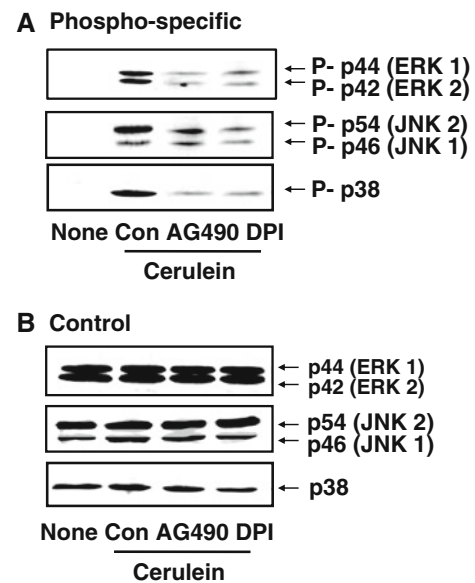
in the medium up to 24 h (Fig. 1a). Stat3-DNA binding activity and MAPK activation were also increased in cerulein-stimulated AR42J cells, evident at 30 min of culture (Figs. 1b, 2). To determine the inhibitory effect of AG490 on cerulein-induced changes, cells were therefore pretreated with AG490 for 2 h, and then cultured in the presence of cerulein for either 24 h to measure TGF- $\beta$ 1 levels, or for 30 min to assay Stat3-DNA binding activity and the activation of MAPKs (ERK, JNK, p38). The cerulein-induced increases in TGF- $\beta$ 1 levels and Stat3-DNA binding activity were inhibited by pretreatment of AR42J cells with AG490 (Fig. 3). Since AG490 inhibits Jak2 and Stat3 in cerulein-stimulated pancreatic acinar cells [32], the results suggested that TGF- $\beta$ 1 expression was mediated by the activation of Jak2 and Stat3. AG490 also inhibited the activation of MAPKs in cerulein-stimulated AR42J cells, demonstrating that Jak2/Stat3 signaling was upstream of MAPK activation in cerulein-stimulated pancreatic acinar cells (Fig. 4). Total levels of ERK, JNK and p38 were not changed by cerulein treatment, with or without AG490 (Fig. 4).

Cerulein-induced activation of Jak2/Stat3 and MAPKs and increase in Stat3-DNA binding activity and expression of TGF- $\beta$ 1 are inhibited by DPI and by transfection with AS ODNs for p22<sup>phox</sup> and p47<sup>phox</sup> in AR42J cells

DPI treatment was found to inhibit the cerulein-induced activation of MAPKs in AR42J cells at 30 min of culture

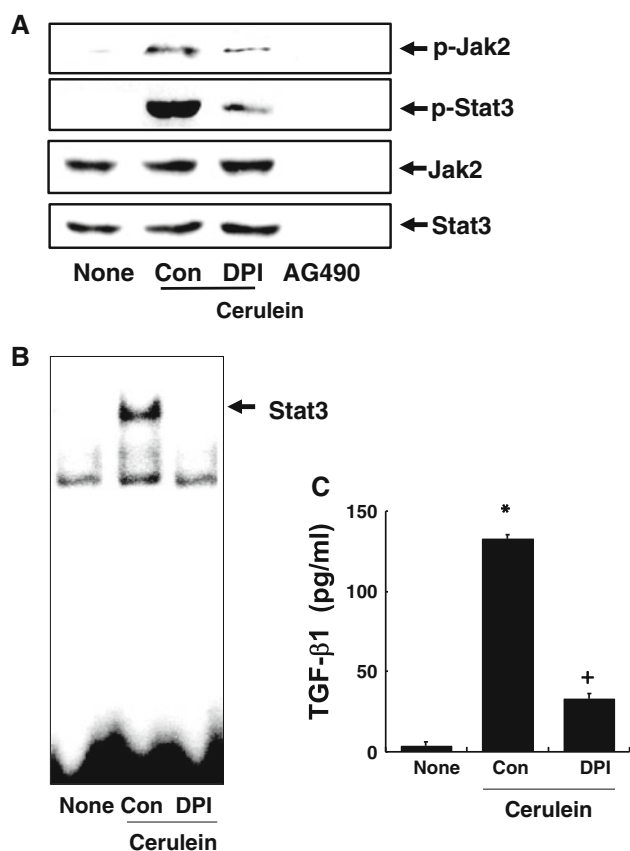


**Fig. 3** Effect of AG490 on the cerulein-induced increase in TGF-β1 levels and Stat3-DNA binding activity in AR42J cells. The cells were pre-treated with or without AG490 (50 μM) for 2 h, and then stimulated with cerulein (10<sup>-8</sup> M) for 24 h for TGF-β1 levels (a) and for 30 min for Stat3-DNA binding activity (b). a The levels of TGF-β1 in the medium were determined by enzyme-linked immunosorbant assay (ELISA). Each bar indicates mean ± SE of four different experiments. *None* cells cultured in the absence of cerulein, *Con* (Control) cells cultured in the presence of cerulein, *AG490* cells treated with AG490 and cultured in the presence of cerulein. \**P* < 0.05 versus *None*; +*P* < 0.05 versus *Con*. b The cells were harvested and nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) for Stat3-DNA binding activity.



**Fig. 4** Effect of AG490 and DPI on cerulein-induced activation of MAPKs in AR42J cells. Cells were pre-treated with or without AG490 (50 μM) or DPI (10 μM) for 2 h, and then stimulated with cerulein (10<sup>-8</sup> M) for 30 min. The cell lysates were analyzed for total (b) and phospho-specific (a) ERK1/2, JNK2/1 and p38 levels by Western blot analysis at the indicated time points. For the detection of total MAPKs, antibodies to pan-ERK1/2, pan-JNK2/1 and pan-p38 were used. Antibodies to p-ERK1/2, p-JNK2/1 and p-p38 were used for the determination of phospho-specific proteins. *None* cells cultured in the absence of cerulein, *Con* (Control) cells cultured in the presence of cerulein, *AG490* cells treated with AG490 and cultured in the presence of cerulein, *DPI* cells treated with DPI and cultured in the presence of cerulein. *ERK* extracellular signal-regulated kinase; *JNK* c-Jun NH2-terminal protein kinase

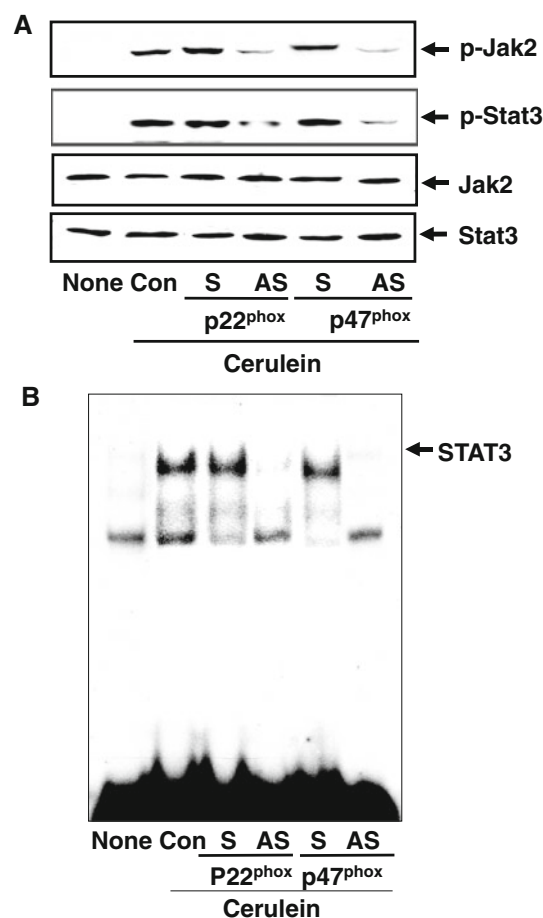
(Fig. 4). In addition, DPI suppressed the phosphorylation of Jak2 and Stat3 as well as Stat3-DNA binding activity without altering the total levels of Jak2 and Stat3 proteins (Fig. 5a, b). Cerulein-induced TGF-β1 expression was inhibited by DPI at 24 h of culture (Fig. 5c). AS ODNs for NADPH oxidase subunits p22<sup>phox</sup> and p47<sup>phox</sup> were also found to inhibit the cerulein-induced activation of Jak2 and Stat3, the increase in Stat3-DNA binding activity, and the activation of MAPKs, compared to the corresponding S ODNs (Figs. 6, 7). In addition, transfection with AS ODNs for p22<sup>phox</sup> and p47<sup>phox</sup> inhibited cerulein-induced TGF-β1 expression (Fig. 8). Total levels of MAPKs (ERK, JNK and p38) (Fig. 4a), Jak2 and Stat3 (Fig. 5a) were not changed by cerulein treatment with or without DPI. Transfection with AS ODNs for NADPH oxidase subunits did not affect the levels of total Jak2 and Stat3 (Fig. 6a) as well as MAPKs (Fig. 7b) in AR42J cells stimulated with cerulein. These results demonstrate that inhibition of NADPH oxidase by DPI and transfection with AS ODNs for NADPH oxidase subunits suppressed both cerulein-induced activation of Jak2/Stat3 and MAPKs, and cerulein-induced TGF-β1 expression.



**Fig. 5** Effect of DPI on cerulein-induced activation of Jak2/Stat3 and increase in Stat3-DNA binding activity and TGF- $\beta$ 1 levels in AR42J cells. The cells were pre-treated with or without DPI (10  $\mu$ M) for 2 h, and then stimulated with cerulein ( $10^{-8}$  M) for 30 min for the activation of Jak2/Stat3 and Stat3-DNA binding activity (**a, b**) and for 24 h for TGF- $\beta$ 1 levels (**c**). **a** Phospho-specific and total forms of Jak2 and Stat3 in total cell extracts were determined by Western blot analysis. **b** Stat3-DNA binding activity was assessed by electrophoretic mobility shift assay (EMSA). **c** Protein levels of TGF- $\beta$ 1 in the medium were determined by enzyme-linked immunosorbant assay (ELISA). Each bar indicates mean  $\pm$  SE of four different experiments. *None* cells cultured in the absence of cerulein, *Con* (Control) cells cultured in the presence of cerulein, *DPI* cells treated with DPI and cultured in the presence of cerulein. \* $P < 0.05$  versus *None*; + $P < 0.05$  versus *Con*

## Discussion

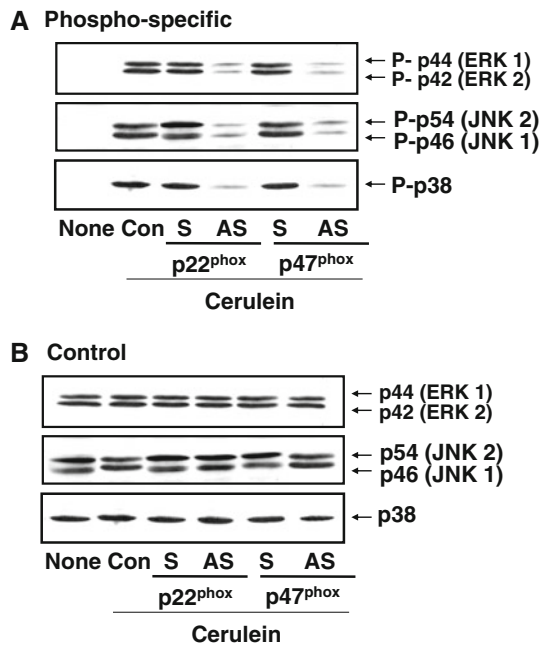
ROS play a key regulatory role in the pathogenesis of pancreatitis [14], suggesting that their production represents an important target for preventing and treating pancreatitis. Previously, we found that NADPH oxidase subunits are expressed in pancreatic acinar cells [22]. Cerulein, which induces experimental pancreatitis, leads to translocation of the p47<sup>phox</sup> and p67<sup>phox</sup> subunits to the membrane and activates NADPH oxidase, which is inhibited by DPI [22]. If NADPH oxidase mediates inflammatory signaling such as pathways involving MAPKs and Jak/Stat to induce inflammatory cytokine expression, inhibition of NADPH oxidase would be the first



**Fig. 6** Effect of AS ODNs for NADPH oxidase p22<sup>phox</sup> and p47<sup>phox</sup> on cerulein-induced activation of Jak2/Stat3 and increase in Stat3-DNA binding activity in AR42J cells. **a** Cells were transfected with AS ODNs or S ODNs for NADPH oxidase subunits p22<sup>phox</sup> and p47<sup>phox</sup> for 24 h and then stimulated with cerulein ( $10^{-8}$  M) for 30 min. Phospho-specific and total forms of Jak2 and Stat3 in whole cell extracts were determined by Western blot analysis. **b** Stat3-DNA binding activity was assessed by electrophoretic mobility shift assay (EMSA). *None* cells cultured in the absence of cerulein, *Con* (Control) cells cultured in the presence of cerulein, *S* cells transfected with S ODN and cultured in the presence of cerulein, *AS* cells transfected with AS ODN and cultured in the presence of cerulein

step to suppressing the propagation of inflammation in pancreas.

The Jak/Stat pathway mediates a wide variety of biological effects including immune responses, differentiation, cell survival, proliferation and oncogenesis [38]. Binding of cytokines to cytokine receptors induces oligomerization of the receptor subunits, constitutive binding to Jak, and trans-phosphorylation of tyrosine kinases. Activated Jaks in turn phosphorylate the receptor that recruits the Stat protein [38]. In addition to the interaction of cytokines with their receptors, Jak2 can also be activated by ROS [39]. Here we showed that inhibition of NADPH oxidase suppressed cerulein-induced activation of Jak2/Stat3. Irrespective of whether ROS inhibit protein tyrosine

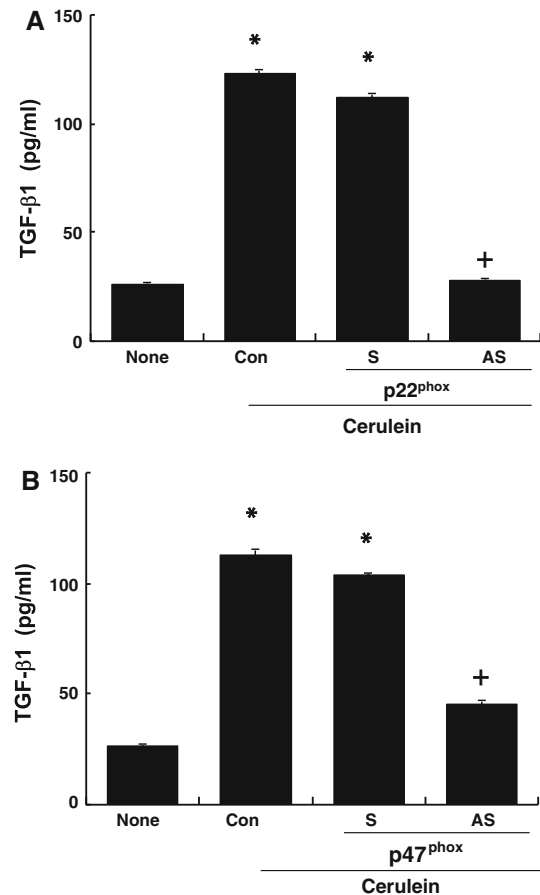


**Fig. 7** Effect of AS ODNs for NADPH oxidase p22<sup>phox</sup> and p47<sup>phox</sup> on cerulein-induced activation of MAPKs in AR42J cells. The cells were transfected with AS ODNs or S ODNs for NADPH oxidase subunits p22<sup>phox</sup> and p47<sup>phox</sup> for 24 h and then stimulated with cerulein (10<sup>-8</sup> M) for 30 min. The cell lysates were analyzed for total (b) and phospho-specific (a) ERK1/2, JNK2/1 and p38 levels by Western blot analysis. *None* cells cultured in the absence of cerulein, *Con* (Control) cells cultured in the presence of cerulein, *S* cells transfected with S ODN and cultured in the presence of cerulein, *AS* cells transfected with AS ODN and cultured in the presence of cerulein. extracellular signal-regulated kinase; c-Jun NH2-terminal protein kinase

phosphatase, thereby inducing tyrosine phosphorylation of Jak as previously reported [40], our findings clearly show that inhibition of NADPH oxidase suppresses the activation of Jak2/Stat3 and Stat3-DNA binding as well as the expression of TGF-β1 in cerulein-stimulated AR42J cells.

In addition to ROS, both JNK and ERK modulate the expression of protein tyrosine phosphatases in pancreas [24]. Inhibition of JNK and ERK suppresses the expression of protein tyrosine phosphatase 1B which is increased in the early phase of cerulein-induced acute pancreatitis [25]. In addition, pancreatic fragments stimulated with cerulein show activation of ERK, p38 and JNK and increased levels of cytokines (IL-1β, TNF-α) [26]. These studies related ROS, the activation of MAPKs, and cytokine expression in pancreatic acinar cells stimulated with cerulein.

In this study, we found that AG490 inhibited cerulein-induced activation of Jak2/Stat3 and MAPKs, and the expression of TGF-β1 in pancreatic acinar cells. We previously demonstrated that AG490 inhibits the activation of both Jak2 and Stat3 in cerulein-stimulated pancreatic acinar cells [32]. AG490 inhibits leukemic cell growth [33] and growth of mycosis fungoides tumor cells [34] by



**Fig. 8** Effect of AS ODNs for NADPH oxidase p22<sup>phox</sup> and p47<sup>phox</sup> on the cerulein-induced increase in TGF-β1 levels in the medium. Cells were transfected with AS ODNs or S ODNs for NADPH oxidase subunits p22<sup>phox</sup> (a) or p47<sup>phox</sup> (b) for 24 h and then stimulated with cerulein (10<sup>-8</sup> M) for 24 h. Protein levels of TGF-β1 in the medium were determined by enzyme-linked immunosorbant assay (ELISA). Each bar indicates mean ± SE of four different experiments. *None* cells cultured in the absence of cerulein, *Con* (Control) cells cultured in the presence of cerulein, *S* cells transfected with S ODN and cultured in the presence of cerulein, *AS* cells transfected with AS ODN and cultured in the presence of cerulein. \**P* < 0.05 versus None; +*P* < 0.05 versus Con

blocking Jak2 and Stat3. Therefore, the activation of both Jak2 and Stat3 appears to be involved in the expression of TGF-β1 in cerulein-stimulated pancreatic acinar cells. In the present study, cerulein-induced activation of MAPKs (ERK, JNK and p38) was suppressed by treatment with AG490 and DPI or by transfection with AS ODN for p22<sup>phox</sup> or p47<sup>phox</sup> in AR42J cells. The results demonstrate that cerulein-induced activation of Jak2/Stat3 may be upstream signaling of MAPK activation in pancreatic acinar cells. It is evident that ROS produced by NADPH oxidase mediate the activation of both MAPKs and Jak2/Stat3 in pancreatic acinar cells stimulated with cerulein, which induces the expression of inflammatory cytokines such as TGF-β1 and thus may contribute to pancreatic inflammation.

DPI is a noncompetitive inhibitor of NADPH oxidase that acts by binding covalently to FAD when the enzyme is activated [41]. Although DPI is a nonspecific inhibitor of flavoenzymes, the decrease of cellular production of ROS in the presence of DPI has increasingly been recognized as a result of its inhibition of NADPH oxidase [42]. DPI not only inhibits flavoenzymes, but also reversibly blocks  $K^+$  and  $Ca^{2+}$  currents [43, 44], thus affecting cell cycle-mediated cell proliferation [45, 46]. Thus, inhibition of NADPH oxidase may not be the sole mechanism of the effect of DPI on TGF- $\beta$ 1 expression. We found that the activation of Jak2/Stat3 was similarly suppressed by either transfection of an AS ODN for NADPH oxidase subunits or treatment with DPI, but TGF- $\beta$ 1 induction was more strongly inhibited by transfection of an AS ODN for NADPH oxidase subunits. While it is clear that inhibition of ROS production by suppression of NADPH oxidase decreases the activation of Jak2/Stat3 in pancreatic acinar cells, DPI may also affect signaling downstream of Jak2/Stat3 to induce TGF- $\beta$ 1. Further study will be required to elucidate the precise inhibitory mechanism of DPI in this system.

In addition to Jak2/Stat3, the oxidant-sensitive nuclear transcription factor NF- $\kappa$ B is also activated by ROS [47]. NF- $\kappa$ B activation by ROS was not observed in HeLa cells, fibroblasts or Jurkat T cells [48, 49], suggesting that ROS-induced NF- $\kappa$ B activation may be highly cell type-specific. We previously found that AG490 inhibited the activation of NF- $\kappa$ B in cerulein-stimulated pancreatic acinar AR42J cells (data not shown). NF- $\kappa$ B signaling thus appears to be directly regulated by Jak2/Stat3 activation in AR42J cells, consistent with previous reports of Jak-mediated NF- $\kappa$ B activation [50]. Jak signaling in turn mediates ERK activation, since chemical inhibition of Jak activation suppresses phosphorylation of ERK [51].

Here we used the pancreatic acinar AR42J cell line instead of freshly isolated and dispersed pancreatic acini to investigate the role of NADPH oxidase on activation of Jak2/Stat3 and TGF- $\beta$ 1 expression. To inhibit NADPH oxidase activation, the cells were transfected with AS ODNs for NADPH oxidase subunits, since there is no specific inhibitor for NADPH oxidase. The commonly used NADPH oxidase inhibitor DPI is a non-specific inhibitor [52]. In addition, AR42J cells were more efficiently transfected with AS ODNs than dispersed pancreatic acini in our preliminary experiments (unpublished data). AR42J is the only currently available cell line that maintains many characteristics of normal pancreatic acinar cells, such as the synthesis and secretion of digestive enzymes [53]. AR42J cell receptor expression and signal transduction mechanisms parallel those of pancreatic acinar cells. This cell line has been widely used as an in vitro model to study cellular secretion, growth, proliferation and apoptosis of the exocrine pancreas [53–56], making it an ideal model

for our study. However, further studies using experimental animals with pancreatitis should be performed to determine the direct involvement and action mechanism of NADPH oxidase on the activation of Jak2/Stat3, MAPKs and TGF- $\beta$ 1 expression in the pathogenesis of pancreatitis.

In conclusion, cerulein-induced activation of NADPH oxidase may trigger the activation of Jak2/Stat3, resulting in several important inflammatory events in pancreas, including Stat3-DNA binding and the production of inflammatory cytokines such as TGF- $\beta$ 1. Jak2/Stat3 signaling acts upstream of the activation of MAPKs (ERK, JNK, p38) in cerulein-stimulated pancreatic acinar cells. Jak2/Stat3 activation may be one of the important molecular mechanisms underlying the pathogenesis of pancreatitis. Therefore, inhibition of the activation of NADPH oxidase may be beneficial for the prevention and treatment of pancreatitis by suppressing inflammatory signaling of Jak2/Stat3 and MAPKs and inhibiting the expression of TGF- $\beta$ 1 in pancreatic acinar cells.

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