# Inhibition of NADPH Oxidase Suppresses Cerulein-Induced Inflammatory Signaling in Pancreatic Acinar AR42J Cells

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# Inhibition of NADPH Oxidase Suppresses Cerulein-Induced Inflammatory Signaling in Pancreatic Acinar AR42J cells

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This certifies that the Doctoral Dissertation of Kyung Don JU is approved.

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# ABSTRACT

## Inhibition of NADPH Oxidase Suppresses Cerulein-Induced Inflammatory Signaling in Pancreatic Acinar AR42J Cells

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(Directed by Professor Kyung Hwan KIM)

Reactive oxygen species (ROS) are important pathogenic factors in the development of pancreatitis and activate NF-κB, a regulator of inflammatory cytokine gene expression, in pancreatic acinar cells. Previous results demonstrated that cerulein induces activation of NADPH oxidase in pancreatic acinar AR42J cells and produces ROS. Present study was designed to elucidate the role of NADPH-oxidase on cerulein-induced signaling cascade leading to inflammatory cytokine expression such as IL-1β and TGF-β1 in pancreatic acinar AR42J cells.

As a result, JAK2/STAT3, ERK/JNK and NF-κB were activated by cerulein in AR 42J cells. IL-1β and TGF-β1 expressions increased by cerulein. Cerulein-activated JAK2/STAT3, ERK/JNK and NF-κB were inhibited by NADPH oxidase inhibitor DPI and by transfection with AS ODNs for NADPH oxdase subunits in AR42J cells. Cerulein-induced expression of IL-1β and TGF-β1 were suppressed by DPI and by transfection with AS ODNs for NADPH oxidase subunits in AR 42J cells. Further, ceruelin-induced expression of IL-1β and TGF-β1, and NF-κB activation were inhibited by JAK2 inhibitor AG490 in AR 42J cells, which indicates that the activation of ERK/JNK, NF-κB and the expression of IL-1β, and TGF-β1 are mediated by activated JAK2. These results suggest that cerulein-

stimulated NADPH-oxidase mediates the expression of inflammatory cytokine such as IL-1β and TGF-β1 by signaling pathways that involve JAK2/STAT3 and ERK/JNK as signaling molecules.

In conclusion, NADPH oxidase mediates the inflammatory signaling involving the activation of JAK/STAT, ERK/JNK and NF-κB, and results in the induction of IL-1β and TGF-β1 expression in pancreatic acinar AR42J cells. NADPH oxidase may be the target molecule for new drug to prevent the progression of acute pancreatitis.

Key words: NADPH oxidase, ROS, Cerulein, Janus kinase 2/signal transducer and activator of transcription 3, ERK/JNK, AR42J Cells, Acute pancreatitis

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## I. INTRODUCTION

Acute pancreatitis is a multifactorial disease leading to deleterious local and systemic effects. This is associated with the release of digestive enzymes into the pancreatic interstitium and to the systemic circulation as well as with the increase of cytokine production and release[1–3]. Supramaximal cerulein stimulation, a cholecystokinin (CCK) analogue[4], results in experimental pancreatitis. Cerulein-induced pancreatitis is similar to edematous pancreatitis in human. It is characterized by dysregulation of the production, elevation of pancreatic enzymes in serum, cytoplasmic vacuolization, death of acinar cells, and edema formation[5-7]. Cytokines play a role in the pathogenesis of pancreatitis by driving the subsequent inflammatory response. Previous reports demonstrated that the expression of cytokine (IL-1β and IL-6) were up-regulated in cerulein-stimulated pancreatic acinar cell in the absence of inflammatory cells[8]. In pancreatic acinar cells stimulated with either cerulein or the activated neutrophils, the level of IL-1β and IL-6 expression were similar at the late stage of stimulation. However, IL-1β was

detected earlier and expressed more in the cells treated with cerulein at early stage of stimulation[9]. TGF-β1 is the required for the regulation of extracellular matrix remodeling in the pancreas and to be involved in the pathogenesis of pancreatic fibrosis[10-14]. TGF-β1 cerulein promotes acinar apoptosis and eliminates damaged acinar cells in the cerulein induced pancreatitis model [14]. Inhibition of TGF-β1 not only alleviate pancreatic fibrosis but also protect from chronic injury due to excessive pancreatic cell apoptosis[15].

Oxidative stress is regarded as a major pathogenic factor in acute pancreatitis[1]. Reactive oxygene species (ROS) act as molecular triggers of various inflammatory responses. Once produced, ROS attack the biological membranes directly and trigger the accumulation of neutrophils and their adherence to the capillary wall[16]. Therefore, it is plausible that ROS may play a critical role in perpetuating pancreatic inflammation and the development of extrapancreatic complication[17]. ROS are important regulators in the pathogenesis of pancreatitis, and an activator of the transcription factor-κB (NF-κB)[18-20].

During inflammation, NADPH oxidase produces large amount of ROS in phagocytic and non-phagocytic cells[23,24]. It was found that NADPH oxidase subunits Nox1, $p27^{phox}$, $p47^{phox}$ and $p67^{phox}$ are present, which activation is involved in the ROS production in cerulein-stimulated pancreatic acinar cells[25]. However, the exact inflammatory mechanism produced by the activation of NADPH oxidase in pancreatic acinar cells are not clear.

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway represents the main effector for many cytokines[26]. JAK/STAT signaling pathways are involved primarily in the actions of non-immune mediators such as growth factors and hormones, as well as in the immune response of several cytokines. It is activated not only by several cytokines but also by stress stimuli[27-29]. However, little is known about functional roles of JAK/STAT pathways in the pancreatitis.

Knockdown of STAT1 or JAK1 suppresses the reduction of both of ERK and mitogene –activated protein (MAP) EKR kinase (MEK)[30]. JAK2 is required for

activation of extracellular signal-regulated kinase (ERK) by growth hormone[31]. Treatment with JAK/STAT inhibitor AG490 significantly inhibits ERK activation. The inhibition of JAK activation with chemical inhibitors suppresses phosphorylation of ERK, indicating JAK signaling mediates ERK1/2 activation[32].

MAP kinases are serine-threonine-directed kinases which are activated by various stimuli including cytokine, growth factor, hormones and cell stress[33,34]. MAP kinases regulate many cellular processes including gene transcription, protein translation and metabolism. In addition, MAP kinases are involved in the control of cell growth, differentiation, survival and apoptosis. Cerulein induces AP-1 DNA binding and selectively activates the MAPK cascade in pancreatic AR42J cells[35]. Three major subfamilies of MAPKs have been identified in mammalian cell: the extracellular ERKs, the c-Jun NH2-terminal protein kinases (JNKs) or stress-activated protein kinase (SAPKs), and the p38 MAPK. The signaling cascades of all three MAPK have been evaluated in pancreatic acinar cells. CCK activates MEK, and Ras in rat pancreas acini[36-37]. JNK are rapidly activated by CCK in rat pancreatic acini[38]. Cerulein activates AP-1 through ERK signaling and JNK signaling in pancreatic AR 42J cells[39].

The cellular mechanism of these inflammatory molecules involves activation of transcription factors such as NF-κB and AP-1[40]. NF-κB is a family of transcription factors expressed ubiquitously and controls the expression of numerous genes involved in inflammatory and immune responses, and cell proliferation. Activation of NF-κB in the pancreas has been demonstrated in cerulein induced pancreatitis of rat [41]. Inhibition of NF-κB activation results in a decrease in the cytokine expression[41,42]. AP-1 as well as NF-κB represent early responsive transcriptional complexes essential for the gene expression of inflammatory molecules.

In the present study, it was aimed to investigate NADPH oxidase-mediated inflammatory signaling whether JAK/STAT and MAPK signaling are activated by suprmaximal cerulein stimulation in pancreatic acinar AR42J cells. Further, the role of JAK/STAT signaling on NF-κB-mediated IL-1β and TGF-β1 expression was investigated.

# II. MATERIALS AND METHODS

## 1. Cell lines and culture condition

The rat pancreatic acinar cells (AR42J cells) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) and 3.7g/L sodium bicarbonate, supplemented with 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY, USA) and 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were used for experiments after incubation for 20–24 h at 37°C in a humidified atmosphere of 90% air–10% $CO_2$.

## 2. Preparation of extracts

Total cell extracts and nuclear extracts were prepared using the method previously described [29]. The cells were trypsinized, washed with PBS buffer and then centrifuged at 500 rpm for 5 min. The cells were resuspended with lysis buffer containing 10 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 50 μM leupeptin, 25 μM pepstatin, 25 μM aprotin, and 1 M PMSF, lysed by drawing the cells through a 1 ml syringe with several rapid strokes, and centrifuged at 10,000 *g* for 10 min. The supernatant was collected and used as a whole cell extract. To prepare nuclear extracts, the cells were resuspended in 500 μl of hypotonic buffer and centrifuged. The supernatant was removed. The pellets were washed once with a hypotonic buffer, resuspended in the extraction buffer and placed on ice for 20 min, and then centrifuged. The supernatants were used as nuclear extracts. The protein concentration was determined using the Bradford method.

## 3. Western blotting

The whole cell extracts (50μg of protein/lane) was loaded, separated by 8% SDS polyacrylamide gel electrophoresis under reducing conditions, and transferred onto nitrocellulose membranes (Amersham Inc., Arlington Heights, IL, USA) by

electroblotting. The transfer of the protein and the equality of the loading in the lanes were verified using reversible staining with Ponceau S. The membranes were blocked with 5% nonfat dry milk in TBS-T (Tris-buffered saline and 0.15% Tween 20) for 3 h at room temperature. The proteins were detected with polyclonal antibodies for 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, Beverly, MA, USA), ERK (1: 1000, Cat. No 9102 , cell signaling), phospho-ERK (1: 1000, Cat. No 9101 , cell signaling), JNK (1: 1000, Cat. No 9152, cell signaling), phospho-JNK (1: 1000, Cat. No 9251, cell signaling) diluted in TBS-T containing 5% dry milk, and incubated at 4°C overnight. After washing in TBS-T, the immunoreactive proteins were visualized using goat anti-rabbit secondary antibodies (1:2000, Cat. No. sc-2004, Santa Cruz Biotechnology, Santa Cruz, CA, USA), conjugated to horseradish peroxidase, which was followed by enhanced chemiluminescence (Amersham).

## 4. Reverse transcription-polymerase chain reaction

Total RNA is isolated from cells by guanidine thiocyanate extraction method. cDNA was synthesized with 2 μg total RNA. Polymerase chain reaction (PCR) was carried out with 160 ng cDNA and cytokine (IL-1β, TGF-β1) primer. Total volume of PCR was adjusted to 20 μl with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.1 mM dNTP, and 0.6U Tag polymerase. The internal control (GAPDH) is coamplified with a cytokine. Expression level of cytokine mRNA is normalized to that of housekeeping gene (GAPDH) in the same sample. The sequence for IL-1β primer is 5'-TCCTAGGAA ACAGCAATGGTCG-3'(forward primer) and 5'-TTCATCCCATACACACGGACAAC-3'(reverse primer). The sequence for TGF-β1 primer is 5'-GCGGACTACTACGCCAAAGA -3'(forward primer) and 5'-TGGTTGTAGAGGGCAAGGAC-3'(reverse primer). GAPDH sequence is 5'-ACCACAGTCCATGCCATCAC-3'(forward primer) and 5'-TCCACCACCCTGT

TGCTGTA-3'(reverse primer). PCR amplification was performed employing the following conditions: denaturizing at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec for a total of 32 cycles. Five-micro liter samples from each 25 μl PCR product were removed and analyzed by electrophoresis and UV transillumination on 1.5 % agarose gel involving 0.5 μg/ml ethidium bromide.

## 5. Enzyme-linked immunosorbent assay

The medium was immediately removed from cultures, and centrifuged at 15,000 x g to remove cell pellets and cellular debris. Levels of IL-1β and TGF-β1 in the medium were determined by enzyme-linked immunosorbent assay (ELISA) kit (Bio-source Inc., Canton, MA, USA) according to the manufacturer's instructions.

## 6. Electrophoretic mobility shift assay

EMSA was carried out by slightly modified method of Dignam *et al*[44]. Enzyme-linked immunosorbent assay (EMSA) was performed with nuclear extracts prepared from non-stimulated cells or cerulein-stimulated cells. Briefly, 30 μg of nuclear extract was incubated with $^{32}$P-labeled probes in binding buffer (20 mM HEPES (pH 7.4), and 5 mM EDTA, 300 mM KCl, 25% glycerol) for 40 min at room temperature. γ-$^{32}$P-labeled probes contained the following double-stranded oligonucleotides, which were generated by a kinase reaction with polynucleotide kinase and [γ-$^{32}$P] ATP (Amersham, Piscataway, NJ, USA): NF-κB (5'–AGTTGAGGGGACTTTCCCAGGC-3'),AP-1(5'-CGCTTGATGACTCAGCCGG AA-3'). Reaction mixtures were analyzed on 6% polyacrylamide gels containing 0.25M TBE buffer (22.5 mM tris, 22.5 mM borate and 500 μM EDTA, pH 8.0). The gel was then dried for 1 hr at -80 °C and exposed to Kodak film (Eastman Kodak Co., Rochester, NY, USA) at -80 °C.

## 7. Transfection with antisense or sense oligonucleotides

Phosphothioate-modified oligonucleotides (ODNs) were produced commercially (GIBCO-BRL). The sequence of p22$^{phox}$ antisense (AS) and sense (S) ODNs was

GATCTGCCCCATGGTGAGGACC and GGTCCTCACCATGGGGCAGATC, respectively. The sequence of the $p47^{phox}$ AS and S ODN was CTGTTGAAGTACTCGGTGAG and CTCACCGAGTACTTCAACAG, respectively. The cells were treated with ODNs using a transfectionreagent DOTAP (Boehringer-Mannheim, Mannheim, Germany). When DOTAP was employed, the appropriate amounts of ODNs were incubated with DOTAP (15μg/ml) to achieve the final concentration of the ODNs to 0.5μM for 15 min. Then the mixture was added directly to the cells. The cells were incubated for 24 h and then used.

## 8. Histological examination of pancreatic acinar cells

Pancreatic tissues were harvested, fixed in 4% paraformaldehyde buffered with 0.01M sodium phosphate, pH 7.4 (PBS) overnight at 4 days, and embedded in paraffin, Paraffin sections were stained with hematoxylin-eosin (H&E). Five rats were analyzed for each group. Ten random fields were observed per section at x40 magnification with Olympus CKX 41microscpe (Olympus, Tokyo, Japan).

## 9. Statistical analysis

Each set of experiment was performed at least three times. Results were expressed as means ± standard errors obtained from four separate experiments. Analysis of variance (ANOVA) followed by Newman-Keul's test was used for statistical analysis[45]. $P<0.05$ was considered statistically significant.

# III. RESULTS

## 1. Cerulein activates JAK2 and STAT3

It has been well known that interferon gamma activates JAK and STAT. Therefore, it has been investigated whether cerulein induces JAK/STAT activation. As shown in Figure 1, levels of phosphorylated JAK2 and STAT3 were increased by cerulein, whereas those of total JAK2 and STAT3 were unchanged. Pretreatment of JAK2 inhibitor AG490 suppressed cerulein-induced phosphorylation of STAT3 and JAK2 (Fig. 1B). This indicated that phosphorylation of STAT3 is required for JAK2 activation in pancreatic acinar AR42J cells.

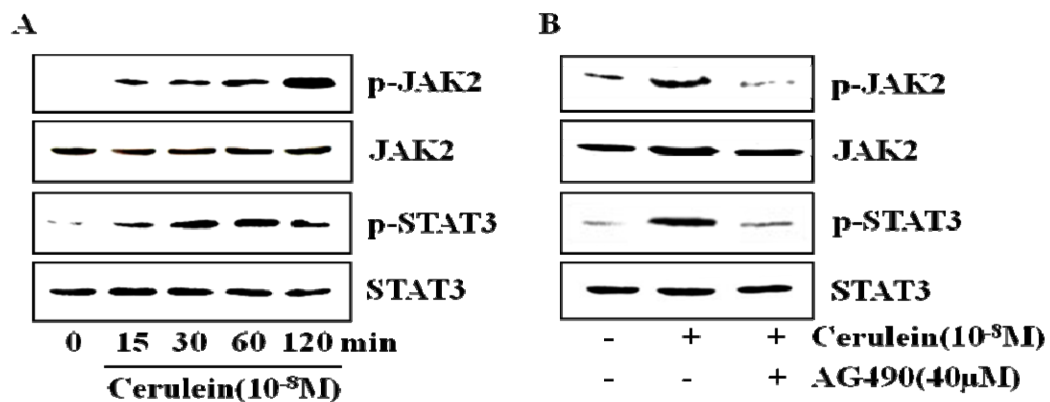


Figure 1. **Effects of JAK2 inhibitor AG490 on activation of JAK2 and STAT3 in cerulein-stimulated AR42J cells.** (A) The cells were stimulated with cerulein ($10^{-8}$M) for the indicated times. (B) The cells were pretreated with (+) or without (-) AG490 for 2h, and then stimulated with cerulein ($10^{-8}$ M) for 60 min. Phosphorylated and nonphosphorylated forms of JAK2 and STAT3 in the total cell lysates were determined by Western blot analysis.

## 2. JAK2 inhibitor AG490 suppresses IL-1β and TGF-β1 expression

As shown in Figure 2, levels of mRNA and protein of IL-1β and TGF-β1 in medium were increased by ceruelin up to 24 h. To investigate whether cerulein-

induced JAK2/STAT3 signaling mediates IL-1β and TGF-β1 expression, the cells were pretreated with JAK2 inhibitor AG490 for 2 hr prior to cerulein stimulation (Fig. 2C).

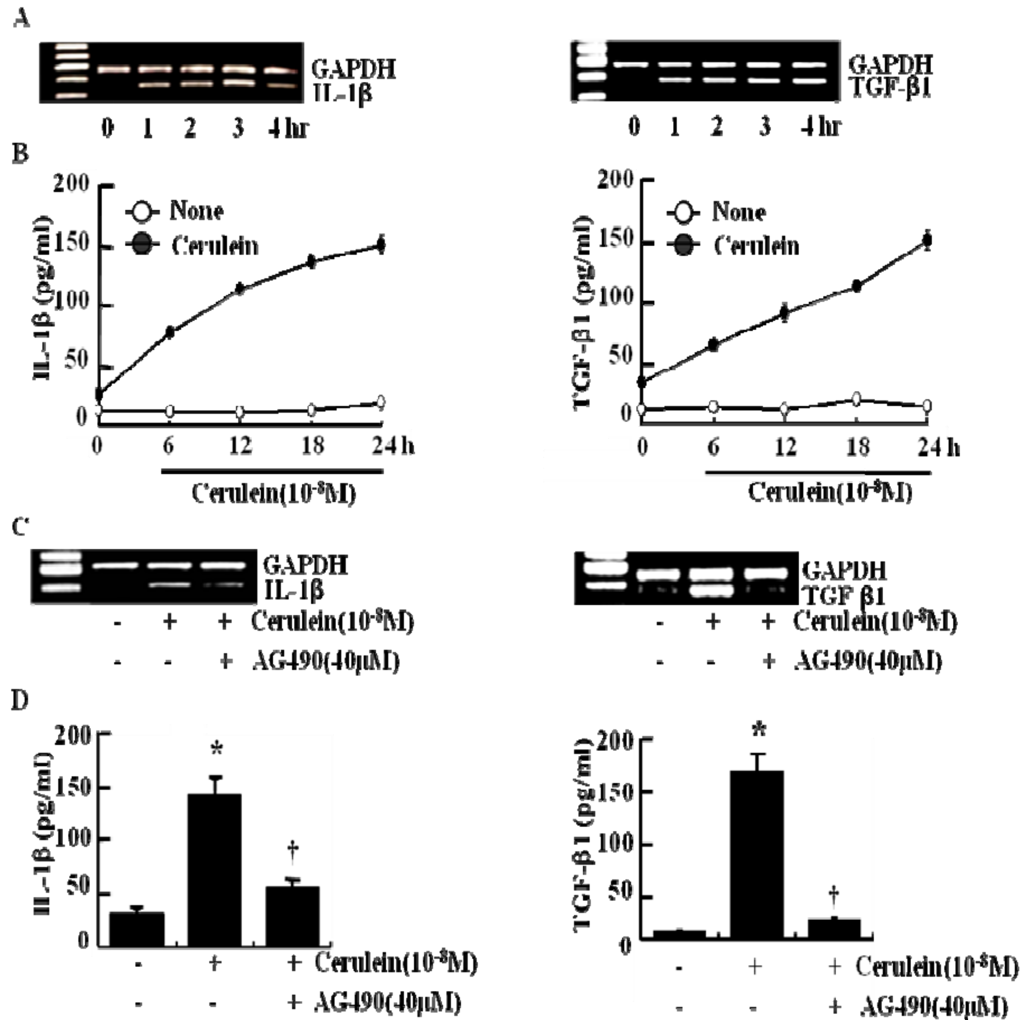


Figure 2. **Effect of JAK2 inhibitor AG490 on the mRNA and protein levels of IL-1β and TGF-β1 in cerulein-stimulated AR42J cells.** (A, B) The cells were stimulated with cerulein ($10^{-8}$M) for the indicated time periods. (C, D) The cells were pretreated with (+) or without (-) AG490 for 2 h, and then stimulated with cerulein ($10^{-8}$ M) for 24 h. After treatment of cerulein, protein levels of IL-1β and TGF-β1 in the medium were determined by enzyme-linked immunosorbant assay (ELISA). Each bar indicates mean±S.E. of five different experiments. $^{*}P < 0.05$ vs. the cells cells non-treated (None); $^{\dagger}P < 0.05$ vs. the cells treated with cerulein (+) and without AG490 (-).

The increase of IL-1β and TGF-β1 levels by cerulein was significantly suppressed by pretreatment of AG490 (Fig. 2D). These results clearly suggested that expression of IL-1β and TGF-β1 was mediated by JAK2/STAT3 signaling in ceruelin-stimulated pancreatic acinar cells.

## 3. NADPH oxidase mediates expression of IL-1β and TGF-β1 and activation of JAK/STAT signaling

Previous results demonstrated that NADPH oxidase, mediated production of reactive oxygen species (ROS), and activated by cerulein in AR42J cells[23]. It was found that cerulein increased IL-1β and TGF-β1 expression through activation of JAK2/STAT3. To investigate the involvement of NADPH oxidase in ceruelin-induced JAK/STAT signaling, and IL-1β and TGF-β1 expression, the cells were pretreated with NADPH oxidase inhibitor diphenylene iodonium (DPI) or transfected with antisense oligonucleatides (AS ODNs) for NADPH oxidase subunits $p22^{phox}$ and $p47^{phox}$ prior to stimulation of cerulein. As shown in Figure 3, pretreatment of DPI suppressed cerulein-induced phosphorylation of JAK2 and STAT3 in AR42J cells. Cerulein-induced mRNA and protein expression levels of IL-1β and TGF-β1 expression were suppressed by pretreatment of DPI (Fig. 3B, C). AS ODNs for NADPH oxidase subunits $p22^{phox}$ and $p47^{phox}$ suppressed cerulein-induced phosphorylation of JAK2 and STAT3 (Fig. 4A, B). Cerulein-induced IL-1β and TGF-β1 expression was also inhibited by transfection of AS ODNs for NADPH oxidase subunits $p22^{phox}$ and $p47^{phox}$ (Fig. 5A, B). It was also found that the inhibition of JAK2/STAT3 signaling by AG490 suppressed cerulein-induced IL-1β and TGF-β1 expression (Fig. 2C). These results suggested that NADPH oxidase regulated cerulein-induced IL-1β and TGF-β1 expression via JAK/STAT signaling in pancreatic acinar AR42J cells.

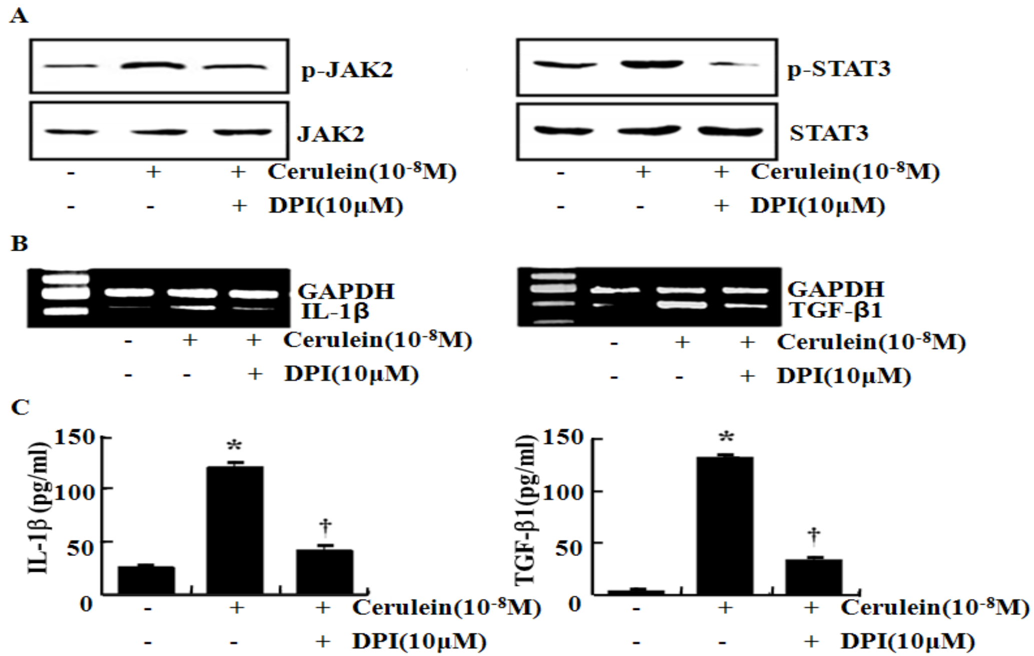


Figure 3. **Effects of DPI on activation of JAK2 and STAT3, and the expression of IL-1β and TGF-β1 in cerulein-stimulated AR42J cells.** The cells were pretreated with (+) or without (-) DPI for 2 h, and then stimulated with cerulein ($10^{-8}$ M). (A) After the treatment of cerulein for 2h, the cell isolation of total cell lysates, the phosphorylated and nonphosphorylated forms of JAK2 and STAT3 in the total cell lysates were determined by Western blot analysis. (B, C) mRNA and protein levels of IL-1β and TGF-β1 in the medium were determined by enzyme-linked immunosorbant assay (ELISA). Each bar indicates mean±S.E. of five different experiments. $^{*}P < 0.05$ vs. the cells cells non-treated (None); $^{\dagger}P < 0.05$ vs. the cells treated with cerulein (+) and without DPI(-).

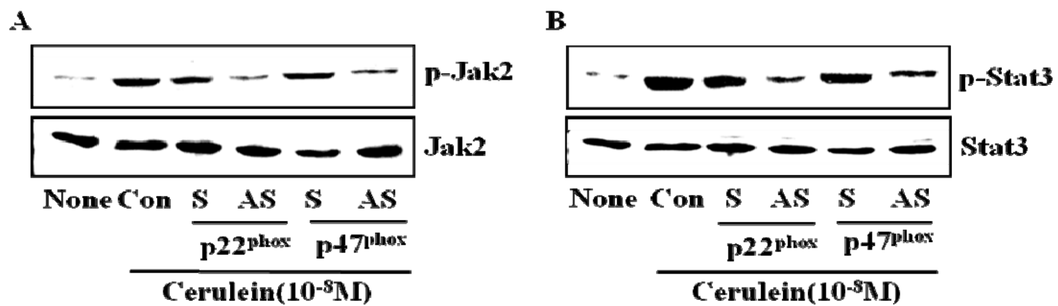


Figure 4. **Effects of AS ODN for NADPH oxidase p22$^{phox}$ and p47$^{phox}$ on activation of JAK2 and STAT3 in cerulein-stimulated AR42J cells.** The cells were transfected with antisense oligonulceatides (AS ODNs) or sense ODNs (S ODNs) for NADPH oxidase

subunits p22$^{phox}$ and p47$^{phox}$ for 24 h and then stimulated with cerulein ($10^{-8}$ M) for 4 h. After isolation of total cell lysates from the cells, phosphorylated and nonphosphorylated forms of JAK2 and STAT3 in the total cell lysates were determined by Western blot analysis. Con: the cells cultured in the presence of ceruelin, S:the cells transfected with S ODN and cultured in the presence of ceruelin, AS: the cells transfected with AS ODN and cultured in the presence of ceruelin.

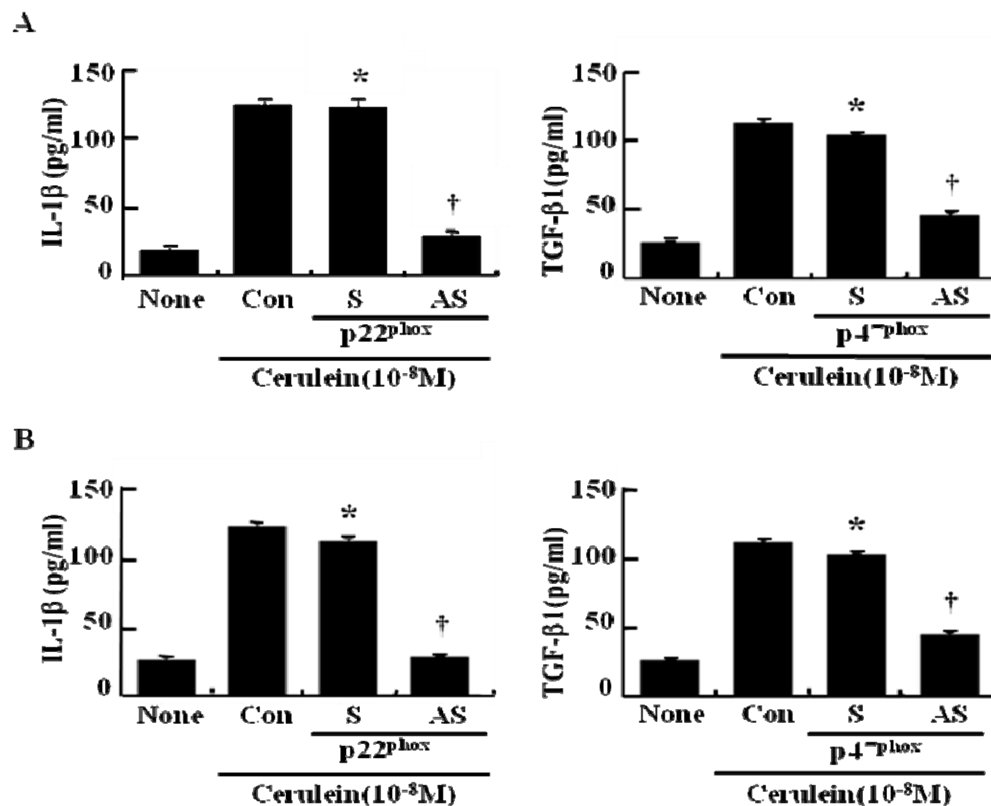


Figure 5. **Effects of AS ODN for NADPH oxidase p22$^{phox}$ and p47$^{phox}$ on the expression of IL-1 β and TGF-β1 in cerulein-stimulated AR42J cells.** The cells were transfected with antisense oligonucleatides (AS ODNs) or sense ODNs (S ODNs) for NADPH oxidase subunits p22$^{phox}$ and p47$^{phox}$, and then stimulated with cerulein ($10^{-8}$ M) for 24 h. Protein levels of IL-1β and TGF-β1 in the medium were determined by enzyme-linked immunosorbant assay (ELISA). Each bar indicates mean±S.E. of five different experiments. $^{*}P < 0.05$ vs. cells non-treated (None); $^{\dagger}P < 0.05$ vs. the cells treated with cerulein (+) and without DPI(-). Con: the cells cultured in the presence of ceruelin, S: the cells transfected with S ODN and cultured in the presence of ceruelin, AS: the cells transfected with AS ODN and cultured in the presence of ceruelin.

## 4. NADPH oxidase Mediates NF-κB Activation

Previously results demonstrated that inhibition of NF-κB activation suppressed the expressions of inflammatory cytokines such as IL-1β, IL-6 and IL-8 in cerulein-stimulated AR42J cells[45,46]. In order to investigated whether NADPH oxidase is involved in cerulein-induced NF-κB activation, NF-κB was activated by cerulein (Fig. 6A). As shown in Figure 6, pretreatment of DPI suppressed cerulein-induced NF-κB activation in AR42J cells. AS ODNs for NADPH oxidase subunits $p22^{phox}$ and $p47^{phox}$ suppressed the cerulein-induced NF-κB activation (Fig. 7A, B). These results demonsterated that NADPH-oxidase regulated the cerulein-activated NF-κB in AR42J cells.

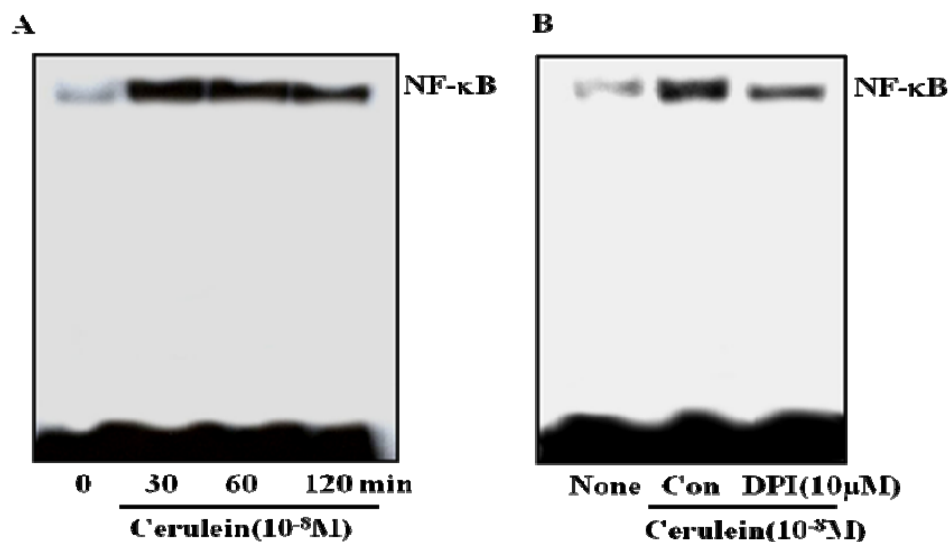


Figure 6. **Effects of DPI on NF-κB activation in cerulein-stimulated AR42J cells.** (A) The cells were cultured for various time points with cerulein ($10^{-8}$ M). (B) The cells were pre-treated with (+) or without (-) DPI for 2 h, and then stimulated with cerulein ($10^{-8}$ M) for 1 h. The nuclear extracts isolated from the cells were incubated with buffer containing $^{32}$P-labeled NF-κB consensus oligonucleotide for 30 min, and subjected to electrophoretic separation on a nondenaturing acrylamide gel. The gels were dried and exposed to the radiography film at -70℃. None: the cells cultured in the absence of cerulein, Con: the cells cultured in the presence of ceruelin, DPI: the cells pre-treated with DPI and cultured in the presence of cerulein.

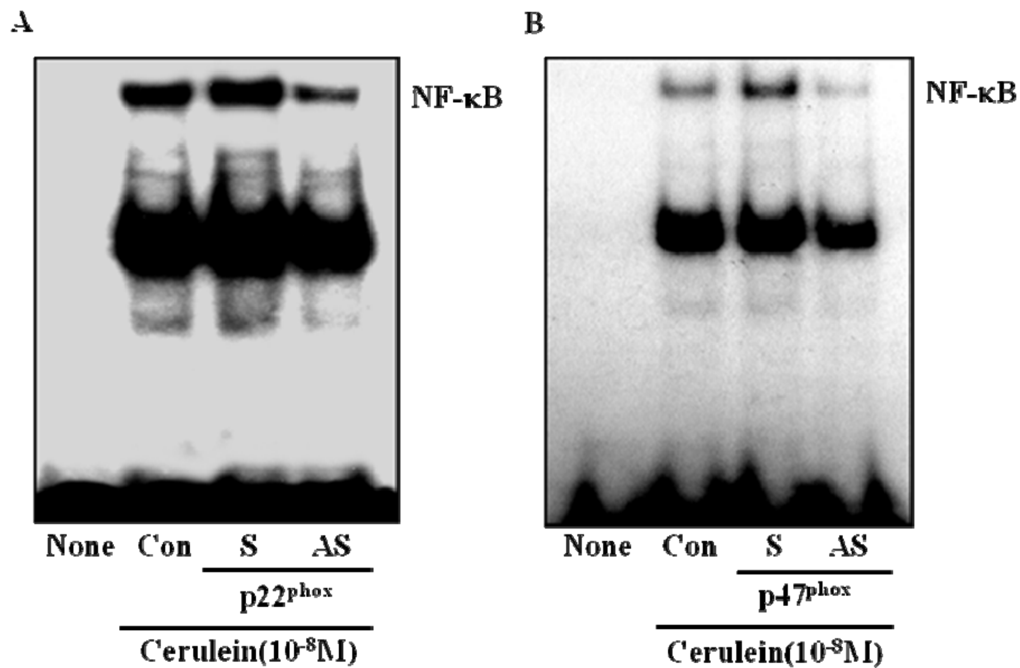


Figure.7. **Effects of AS ODN for NADPH oxidase p22$^{phox}$ and p47$^{phox}$ on NF-κB activation in cerulein-stimulated AR42J cells.** The cells were transfected with antisense oligonucleatides (AS ODNs) or sense ODNs (S ODNs) for NADPH oxidase subunits p22$^{phox}$ and p47$^{phox}$,and then stimulated with cerulein ($10^{-8}$ M) for 1 h. The nuclear extracts isolated from the cells were incubated with buffer containing $^{32}$P-labeled NF-κB consensus oligonucleotide for 30 min, and subjected to electrophoretic separation on a nondenaturing acrylamide gel. The gels were dried and exposed to the radiography film at -70℃. Con: the cells cultured in the presence of ceruelin, S: the cells transfected with S ODN and cultured in the presence of ceruelin, AS: the cells transfected with AS ODN and cultured in the presence of ceruelin.

## 5. JAK/STAT signaling mediates NF-κB activation

To investigate the association of JAK/STAT signaling with NF-κB activation, the cells were pretreated with JAK2 inhibitor AG490 prior to cerulein stimulation. As shown in Figure 8, pretreatment of AG490 suppressed cerulein-induced NF-κB activation. This result strongly indicated that NF-κB pathway was a down-stream signaling of JAK2 in pancreatic acinar cells.

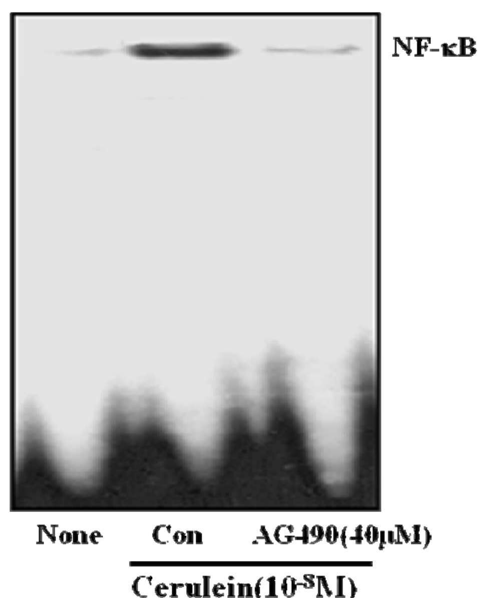


Figure 8. **Effect of JAK2 inhibitor AG490 on NF-κB activation in cerulein-stimulated AR42J cells.** The cells were pre-treated with (+) or without (-) AG490 for 2 h, and then stimulated with cerulein ($10^{-8}$ M) for 1 h. The nuclear extracts isolated from the cells were incubated with buffer containing $^{32}$P-labeled NF-κB consensus oligonucleotide for 30 min, and subjected to electrophoretic separation on a nondenaturing acrylamide gel. The gels were dried and exposed to the radiography film at -70℃. None: the cells cultured in the absence of cerulein, Con: the cells cultured in the presence of ceruelin, DPI: the cells pre-treated with DPI and cultured in the presence of ceruelin.

## 6. Cerulein induces pancreatic damage

Pancreatitis was induced by intraperitoneal injections of 40μg/kg cerulein every hour for 7hours. As shown in Figure 9, all rats treated with repeated cerulein injections displayed histological signs of pancreatitis, such as edema, vacuole formation and inflammatory cell infiltrate. Adminstration of AG490 (20mg/kg) significantly ameliorated these changes. The rat treated with 200μl of DMSO, the solvent AG490, did not show any histologic changes.

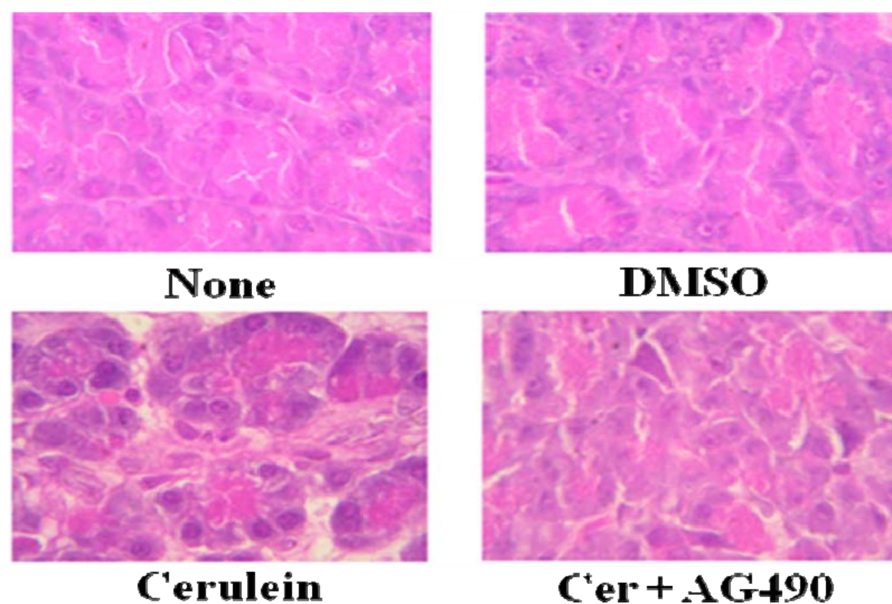


Figure 9. **Inhibitory effect of AG490 on cerulein-induced histologic changes**. Cerulein injections showed histopathological signs of pancreatitis at day, as reflected by edema, vacuole formation, and infiltration of inflammatory cells (cerulein). AG490 at 20 mg/kg was treated 2 h prior to the first injection of cerulein. AG490 was dissolved in DMSO and intraperitoneal injected to the rats at final volume of 200μl (cerulein + AG490). The rats without AG490 received DMSO instead of AG490 (DMSO). Pancreas was harvested, fixed, embedded with paraffin, and stained with hematoxylin and eosin (H & E). None: the rats without cerulein, DMSO: the rats received DMSO alone, cerulein: the rats with cerulein, cerulein + AG490: the rats received cerulein and AG490.

## 7. Cerulein induces MAP kinase activation via JAK 2 signaling

It was reported that activation of MAP kinase (ERK, JNK and p38) are associated with cerulein-induced inflammation in pancreatic AR42J cells. In this experiment, it was confirmed that cerulein induces activation of ERK and JNK (Fig10. A). More specifically, it was carried out that the activation of ERK/JNK by NADPH are mediated NADPH oxidase and JAK2 activation stimulated with cerulein. Pretreatment with DPI (NADPH oxidase inhibitor) and AG490 (JAK2 inhibitor) suppressed the levels of cerulein induced ERK and JNK phosphorylation. This indicated that ERK/JNK activation was regulated by NADPH oxidase and

JAK/STAT signaling (Fig10. B). These results imply that cerulein activated ERK/JNK via JAK2 activation.

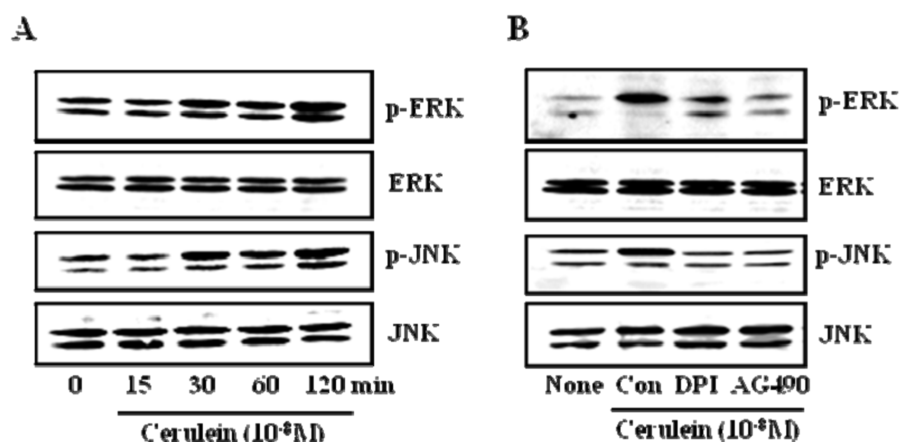


Figure.10. **Effects of DPI and AG490 on activation of MAP kinase in cerulein-stimulated AR42J cells.** (A) The cells were stimulated with cerulein ($10^{-8}$M) for incubation time. (B) The cells were pre-treated with (+) or without (-) AG490 for 2 h, and then stimulated with cerulein ($10^{-8}$ M) for 60 min. Phosphorylated and nonphosphorylated forms of ERK and JNK in the total cell lysates were determined by Western blotting. Levels of phosphorylated or nonphosphorylated forms of ERK1/2, JNK1/2 were determined by western blotting.

# IV. DISCUSSION

JAK/STAT pathway is well known to mediate a wide variety of biological effects such as immune response, differentiation, cell survival, proliferation and oncogenesis[48]. This study showed that cerulein-induced activation of JAK2/STAT3 leads to the expression of inflammatory cytokine IL-1 β and TGF-β1. It was found that NADPH oxidase was required for cerulein-activated JAK/STAT signaling. Therefore, these observations suggested that cerulein-stimulated ROS production mediated by NADPH oxidase participated in the induction of IL-1β and TGF-β expression via JAK/STAT and MAPK signaling. The phosphorylation of STAT was suppressed in the presence of JAK2 inhibitor AG490, indicating a dependence of STAT3 activation on JAK2 activation.

Cytokine binding to the receptor induces oligomerization of the receptor subunits, constitutively associated to the JAK, and a transphorylation of the tyrosine kinases. Activated JAKs in turn phosphorylate the receptor that recruits the STAT protein[48]. The observations suggested that JAK activation is required for traditional receptor-ligand interaction. However, several reports demonstrated that JAK2 is activated by ROS[49]. These results also showed that the inhibition of NADPH oxidase suppressed the cerulein-induced JAK2 signaling. In other words, these results indicated that the certain signaling steps in JAK activation became activated by ROS. Although it is unclear wheter NADPH oxidase-derived ROS interacts with receptors, which initiates the JAK/STAT signaling cascade, several reports have suggested that ROS can function by inhibiting protein Tyr phosphatase including SHP-1, thereby increasing the Tyr phosphorylation of JAK[50,51].

MAP kinase are involved in the control of cell growth, differentiation, survival, and apoptosis. All three MAPK signaling cascades have been recently evaluated in pancreatic acinar cells[37-39]. It was revealed that cerulein activates JAK/STAT and ERK/JNK signaling. Leptin-induced activation of JAK/STAT pathway activated via ERK signaling[32]. Treatment with DPI and AG490 suppressed the levels of ERK and JNK activation. Taken together, these results indicate that ERK/JNK activation

is regulated by NADPH oxidase and JAK/STAT signaling. Therefore it seems evident that cerulein activates the ERK/JNK pathway through JAK2 activation.

Additionally, it was found that the inhibition of NADPH oxidase suppressed the ceruelin-induced NF-κB activation, indicating ROS produced by cerulein-activated NADPH oxidase activates NF-κB activation. The oxidant-sensitive nuclear transcription factor NF-κB is known to be activated by ROS. Every step of the NF-κB signaling cascade comprises redox-sensitive proteins[52]. In particular, the IKK that phosphorylates IκBs is known to be the prime target of redox regulation[52]. However, it have not yet been clarified which NF-κB signaling step is directly and specifically affected by ROS. Several investigators could not detect NF-κB activation in HeLa, 293, fibroblast, or Jurkat T cells[53,54]. It is becoming clear from the available data that ROS-induced NF-κB activation is highly cell type-dependent and therefore ROS is unlikely to be a general mediator of NF-κB activation. It was here found that the JAK2 inhibitor AG490 suppressed activation of MAP kinase, NF-κB, as well as cytokine expression, indicating that NF-κB signaling is directly regulated by JAK2 activation but not by ROS in AR42J cells. This result is consistent with previous reports that demonstrated the regulation of MAP kinase and NF-κB by JAK activation[55,56]. These reports also indicated that STAT may not be involved in NF-κB activation. These results suggested that MAP kinase and NF-κB signaling act as a downstream of JAK2 activation in cerulein-stimulated AR42J cells. JAK2 activation was also inhibited by the inhibition of NADPH oxidase-mediated ROS production. Therefore, these observations indicated that ROS produced by NADPH oxidase may induce MAP kinase and NF-κB activation through the regulation of JAK2 activation. However, the precise mechanisms of the involvement of JAK2 in MAP kinase and NF-κB activation are not yet established. The inhibition of JAK activation with chemical inhibitors suppresses phosphorylation of extracellular signal-regulated kinase (ERK)[33], indicating JAK signaling mediates ERK1/2 activation. Additionally, we found the inhibition of ERK1/2 with the ERK inhibitor U0126 suppressed ceruelin-induced NF-κB

activation, as well as the expression of cytokine such as IL-8 in AR42J cells[36]. The active ERK1/2 but not ROS directly phosphorylate and degrade IκB, and then activate NF-κB in rat vascular smooth muscle cells (VSMCs)[57,58]. These results suggested that ceruelin-induced JAK2 activation could induce the activation of NF-κB through extracellular signal-regulated kinase (ERK) signaling pathways.

In conclusion, cerulein induces ROS through NADPH oxidase and triggers JAK2/STAT3 inflammatory signaling, and results in several important inflammatory events in pancreatic acini including the activation of ERK/JNK, NF-κB and the production of inflammatory cytokines such as IL-1β and TGF-β1.

# V. CONCLUSION

Present study was designed to elucidate the role of NADPH-oxidase on cerulein-induced signaling cascade leading to inflammatory cytokine expression such as IL-1β and TGF-β1 in pancreatic acinar AR42J cells.

The results are as follows.

1. Cerulein activates JAK/STAT, ERK/JNK, NF-κB and induces the expression of IL-1β and TGF-β1 in pancreatic AR42J cells.
2. Cerulein-activated JAK/STAT, ERK/JNK, and NF-κB were inhibited by NADPH oxidase inhibitor DPI and AS ODNs for NADPH oxidase subunits in AR42J cells.
3. Cerulein-induced protein levels of IL-1β and TGF-β1 were suppressed by DPI and transfection with AS ODN for NADPH oxidase subunits.
4. Cerulein-induced ERK/JNK, NF-κB, and the expression of IL-1β and TGF-β1were inhibited by JAK2 inhibitot AG90.

In conclusion, NADPH oxidase mediates inflammatory signaling involving JAK/STAT, ERK/JNK, and NF-κB and thus induces the expression of cytokines (IL-1β and TGF-β1) in pancreatic acinar AR42J cells. NADPH oxidase may be the target molecule for new drug to prevent the progression of acute pancreatitis.

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## Abstract (in Korean)


췌장선세포에서 Cerulein에 의한 염증 신호전달에 대한
NAPDPH Oxidase의 억제 효과 연구



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주 경 돈



활성산소종은 췌장 선세포에서 췌장염의 발달단계, 핵전사인자인 NF-κB의 활성화와 염증성 cytokine의 발현에 중요한 조절인자로 고려되어지고 있다. 이전 연구에서 췌장 선세포에서 cerulein에 의해 NADPH oxidase가 활성화됨을 확인하였고, 이로 인해 활성산소가 생성됨을 확인하였다. 본 연구에서는 췌장 선세포에서 cerulein에 의해서 유도되는 염증성 cytokine발현을 유도하는 신호전달 기전에서 NADPH oxidase의 역할에 대해서 알아보고자 하였다.

연구결과 (1) Cerulein에 의해서 염증매개 단백인 JAK2/STAT3과 핵전사인자인 NF-κB가 활성화 되었으며, 대표적인 염증성 cytokine인 IL-1β and TGF-β1 또한 발현되었다. (2) NADPH oxidase 억제제인 DPI와 NADPH oxidase subnits에 대한 AS ODNs을 처치하여 cerulein으로 활성화되는 JAK2/STAT3, ERK/JNK, NF-κB의 활성화, IL-1β 와 TGF-β1의 발현을 억제하였다. (3) 부가적으로 JAK2 억제제인 AG490을 처치하였을 때 ERK/JNK, NF-κB 활성화, IL-1β 와 TGF-β1의 발현이 억제됨을 확인 하였다. 이는 cerulien으로 유도되는 NADPH oxidase가 JAK2/STAT3, ERK/JNK 신호전달기전을 통해 염증성 cytokine이 발현됨을 시사한다.

본 연구를 통하여 NADPH oxidase는 급성 췌장염 진전을 막기 위한 신약 개발에 표적물질일 것으로 생각된다.


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핵심되는 말: NADPH oxidase, 활성산소, cerulein, JAK2/STAT3, ERK/JNK, 췌장 선세포, 급성 췌장염