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Suppression of IL-1β expression by the Jak 2 inhibitor AG490 in cerulein-stimulated pancreatic acinar cells

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ABSTRACT

Cerulein pancreatitis is similar to human edematous pancreatitis with dysregulation of the digestive enzyme production and cytoplasmic vacuolization, the death of acinar cells, edema formation, and an infiltration of inflammatory cells into the pancreas. Cytokines are up-regulated in pancreatic acinar cells stimulated with cerulein. In various cells and tissues, Janus kinase (Jak)/signal transducer and activator of transcription (Stat) pathway mediates inflammatory process. In the present study, we investigated whether the activation of Jak/Stat signaling mediates IL-1β expression in pancreatic acinar AR42J cells stimulated with cerulein in vitro as well as the rats with cerulein pancreatitis in vivo using AG490, the Jak2 inhibitor. Activation of Jak2 and Stat3 were monitored by Western blot analysis for phosphorylated Jak2 and phosphorylated Stat3. mRNA expression and protein level of IL-1β were determined by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbant assay (ELISA). Histological examination of pancreatic tissues were performed and serum IL-1β levels of the rats were determined by ELISA. As a result, cerulein induced the activation of Jak2 and Stat3 as well as IL-1β expression, which was inhibited by the treatment of AG490 in AR42J cells. In cerulein pancreatitis of the rats, edematous and inflammatory changes of the pancreas and increased serum levels of IL-1β were suppressed by AG490 treatment. In conclusion, Jak2/Stat3 pathway may be the underlying mechanism in the pathogenesis of pancreatitis by inducing cytokines such as IL-1β.

1. Introduction

Acute pancreatitis is a multifactorial disease associated with the release of digestive enzymes to the pancreatic interstitium and to the systemic circulation as well as with the increased cytokine production and release, which can ultimately lead to deleterious local and systemic effects [1–3]. Evidence suggests that pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 act as mediators of the local and systemic manifestations of acute pancreatitis [4–6]. Activated pancreatic macrophages release IL-1β and TNF-α in response to local tissue damage. These cytokines act locally to aggravate acute pancreatitis and systemically with IL-6 to increase capillary permeability and promote leukocyte adherence and extravasation to cause multiple organ failure [4–11].

Among a number of animal models of experimental pancreatitis that exhibit the biochemical, morphological, and pathophysiological similarities to various aspects of human pancreatitis, cerulein pancreatitis was shown to be one of the best-characterized and widely used experimental

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models [12,13]. Doses of a cholecystokinin (CCK) analogue cerulein beyond those that cause the maximum pancreatic secretion of amylase and lipase [14,15] result in pancreatitis, which is characterized by a dysregulation of the production and secretion of digestive enzymes, particularly, the inhibition of pancreatic secretion and an elevation in their serum levels, cytoplasmic vacuolization and the death of acinar cells, edema formation, and an infiltration of inflammatory cells into the pancreas [12,13,16]. However, we showed that the expression of cytokines (IL-1β, IL-6) were up-regulated in cerulein-stimulated pancreatic acinar cells in the absence of inflammatory cells [17]. The levels of IL-1β and IL-6 of the pancreatic acinar cells stimulated with cerulein or the activated neutrophils were similar at the late stage of stimulation. However, IL-1β was earlier detected and more expressed in the cells treated with cerulein at early stage of stimulation [17,18]. Therefore, to determine the involvement of Janus kinase/signal transducers and activators of transcription (Jak/Stat) signaling pathways as an early event on cytokine expression, IL-1β was selected as the marker cytokine and alteration in IL-1β expression was observed in the present study.

Janus kinase/signal transducers and activators of transcription (Jak/Stat) signaling pathways have been reported to be involved not only in the immune response of numerous cytokines but also in the actions of primarily non-immune mediators such as growth factors and hormones. Specific subtypes of Jak and Stat molecules are activated by different signals, resulting in specificity of response [11,19]. The binding of ligand to its receptor induces assembly of an active receptor complex and consequent phosphorylation of the receptor-associated Jaks (Jak1, Jak2, Jak3, and Tyk2). Phosphorylated Jaks lead to the activation of neighboring Jaks, receptor subunits, and several other substrates. Phosphorylation of Jaks provides the docking sites for Stats, which in turn become phosphorylated on tyrosine and serine residues; the phosphorylation of both amino acid species is required for full Stat activity. Phosphorylated Stats are released from the receptor complex and form dimers. These dimers translocate to the nucleus where they directly bind to the promoter region of specific target genes, thus regulating transcription of these genes, many of which are involved in immune responses [19–22].

AG490 is a recently developed Jak2 inhibitor and involved in the synthetically derived tyrphostin family of tyrosine kinase inhibitors [23–25]. At relatively low concentrations, AG490 has been shown to block hyperactive forms of Jak2 found in B cell precursors of acute lymphoblastic leukemia (ALL) patients [25]. AG490 has been shown to inhibit cytokine-induced activation of Jak2 in eosinophils stimulated with granulocyte-macrophage colony stimulating factor (CSF) [26] and in vascular smooth muscle cells and cardiac myocytes activated by angiotensin II [27,28]. AG490 effectively blocks Stat3 activation and suppressed the proliferation of different cancer cell lines [29–33]. However, the patho-physiological mechanism of pancreatitis in relation to Jak/Stat pathways has not been established yet. Our preliminary study showed that Jak2 activation in pancreatic acinar cells stimulated with cerulein (unpublished data). In the present study, we investigated whether the activation of Jak2/Stat3 signaling mediates IL-1β expression in pancreatic acinar AR42J cells stimulated with cerulein in vitro and the rats receiving cerulein in vivo, using the Jak2 inhibitor AG490.

2. Materials and methods

2.1. Cell culture and in vitro experimental protocol

The rat pancreatic acinar AR42J cells (pancreatoma, ATCC CRL 1492) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were incubated at 37 °C in a humidified atmosphere of 90% air–10% CO_2. AR42J cells were stimulated with cerulein (10^{-8} M) at indicated time points for 60 min (for the activation of Jak2 and Stat3) and 24 h (for mRNA expression and protein levels of IL-1β). AG490 was dissolved in dimethylsulphoxide (DMSO) and treated 2 h prior to cerulein treatment at final concentration of 50 μM. Concentration (50 μM) and pretreatment time (2 h) of AG490 were adapted from previous studies showing to inhibit Stat activation in human large intestinal epithelial cells [34] and mouse proximal tubular cells [35]. mRNA expression and protein level of IL-1β were determined by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbant assay (ELISA), respectively. Western blotting for nonphosphorylated and phosphorylated forms of Jak2, and Stat3 were performed in the cells stimulated with cerulein for 60 min-culture.

2.2. Animals and in vivo experimental protocol

Male Sprague–Dawley rats (200–250 g) were housed in standard cages in a climate-controlled room with an ambient temperature of 23 ± 2 °C and 12 h light/dark cycle. They were fed standard laboratory chow, given water ad libitum, and randomly assigned to control or experimental groups. Care and use of rats were followed by the Animal Care Guideline of Yonsei University College of Medicine (Seoul, South Korea). Pancreatitis was induced by seven intraperitoneal injections of cerulein (50 μg/kg body weight) at hourly interval. At the end of this period (3 days after final cerulein treatment), venous blood was collected and the rats were killed by administration of a lethal dose of pentobarbital. All rats were fasted for 12 h before the experiment and permitted water ad libitum. AG490 (20 mg/kg body weight) were injected once 2 h prior to the first injection of cerulein to the rats. AG490 was dissolved in DMSO and intraperitoneal injected to the animals at final volume of 200 μl. The rats treated without AG490 received 200 μl of DMSO instead of AG490. The histology of pancreas was compared between the rats received DMSO alone and the rats without DMSO. Dose of AG490 (20 mg/kg body weight) was reported to efficiently block Jak/Stat signaling in animals in the previous studies [36,37] and used in the present study. Pancreas was harvested, fixed, embedded with paraffin, and stained with hematoxylin–eosin (H & E) for histological examination. Serum IL-1β levels were determined by enzyme-linked immunosorbant assay.

2.3. Reverse transcription-polymerase chain reaction analysis

Gene expression of IL-1β mRNA was assessed using RT-PCR standardized by coamplifying with the housekeeping gene GAPDH, which served as an internal control. Total RNA was isolated from the cells stimulated without (none) or with cerulein (cerulein) treated with or without AG490 by the guanidine thiocyanate extraction method [38]. Total RNA was reverse transcribed into DNA and used for PCR with rat specific primers for IL-1β and GAPDH. Sequences of IL-1β primers were 5′-TCCTAGGAAACAGCAATGGTCG-3′ (forward primer) and 5′-TTCATCCCATACACACGGACAAC-3′ (reverse primer), giving 363 bp PCR product. For GAPDH, the forward primer was 5′-ACCACAGTCCATGCCATCAC-3′ and the reverse primer was 5′-TCCACCACCCTGTTGCTGTA-3′, giving a 460 bp PCR product. Briefly, the PCR was amplified by 32 repeat denaturation cycles at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. During the first cycle, the 95 °C step was extended to 5 min. PCR products were separated on 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide and visualized by UV transillumination.

2.4. Enzyme-linked immunosorbent assay

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

2.5. Western blot analysis

The whole cell extracts (50 μg 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 (1:1000; sc-7229, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Stat3 (1:1000, Cat. No. 06–596, Upstate Biotechnology, Lake Placid, NY, USA), phospho-Jak2 (1:1000, Cat. No. 3771, Cell Signaling, Beverly, MA, USA) and phospho-Stat3 (1:1000, Cat. No. 9131, Cell Signaling, Beverly, MA, USA) 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 conjugated to horseradish peroxidase, which was followed by enhanced chemiluminescence (Amersham). Exactly equal amount of protein, determined by Bradford method [39], was loaded in each lane.

2.6. Histological examination of pancreatic tissues

Pancreatic tissues were harvested, fixed in 4% paraformaldehyde buffered with 0.01 M sodium phosphate, pH 7.4 (PBS) overnight at 4 °C, 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 ×40 magnification with Olympus CKX 41 microscope (Olympus, Tokyo, Japan).

2.7. Statistical analysis

The statistical differences were determined using one-way ANOVA and Newman–Keul's test. All values are expressed as mean ± S.E. for five different experiments.

3. Results

3.1. Cerulein induced mRNA expression and protein level of IL-1β in AR42J cells

mRNA expression (Fig. 1A) and protein level (Fig. 1B) of IL-1β were determined in cerulein-treated AR42J cells. RT-PCR analysis shows that IL-1β mRNA expression was observed at 2 h-culture and increased at 4 h-culture (Fig. 1A). As shown in Fig. 1B, IL-1β protein increased in a time-dependent manner up to 24 h. IL-1β levels in the medium (pg/ml) released from the cells at the start of experiment was 14.4 ± 1.5, which increased by cerulein to 24.7 ± 1.8, 35.7 ± 2.0, 40.8 ± 1.5, 72.1 ± 1.3, and 110.5 ± 5.2 at culture time of 4, 8, 12, 16, and 24 h, respectively. Thus, for the following experiments using treatment with AG490, the time point of 3 h (mRNA expression) or 24 h (protein level), at which IL-1β mRNA expression and protein level were evident, were used. GAPDH was constitutively expressed in AR42J cells and not changed with incubation time.

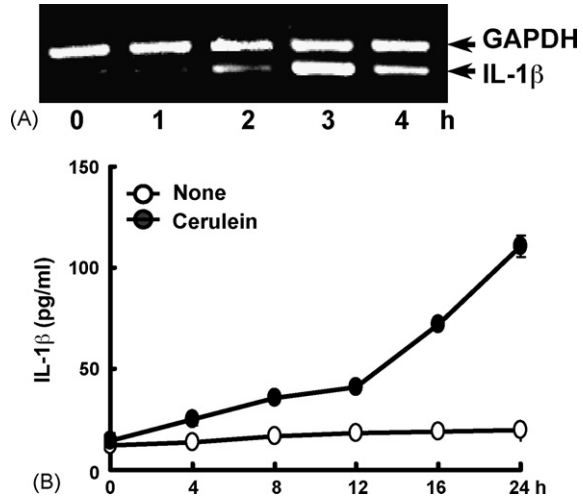

Fig. 1 – Time-response of pancreatic acinar cells to cerulein for mRNA expression of IL-1β, as determined by a reverse transcription-polymerase chain reaction (RT-PCR) (A) and protein levels of IL-1β in the medium determined by enzyme-linked immunosorbant assay (ELISA) (B). Cerulein (10^{-8} M) was treated to AR42J cells and mRNA expression and protein levels of IL-1β were assessed at an indicated time point. The internal standard (GAPDH) was co-amplified with IL-1β (A). Each point indicates mean ± S.E. of five different experiments (B). None, the acinar cells without cerulein; cerulein, the acinar cells with cerulein.

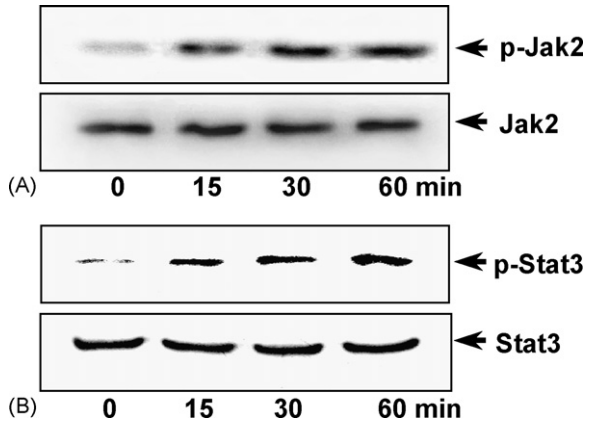

Fig. 2 – Time-dependent activation of Jak2 and Stat3 in pancreatic acinar cells stimulated with cerulein. AR42J cells were stimulated with cerulein (10^{-8} M) at indicated time points for 60 min. Phosphorylated and nonphosphorylated forms of each Jak2 (A) and Stat3 (B) were determined in the cells by Western blot analysis.

3.2. *Cerulein induced activation of Jak2 and Stat3, which was inhibited by AG490 in AR42J cells*

We examined the activation of Jak2 and Stat3 in AR42J cells stimulated with cerulein at the indicated time points for 60 min. Phosphorylated Jak2 and phosphorylated Stat3 were observed at 15 min stimulation with cerulein, which was increased up to 60 min (Fig. 2). These results demonstrate that cerulein activates Jak2/Stat3 signaling in pancreatic acinar cells. As shown in Fig. 3, 2 h pretreatment of the Jak2 inhibitor AG490 suppressed the activation of Jak2 and Stat3 in AR42J cells stimulated with cerulein at 60 min-culture. These results show that AG490 may be beneficial in pancreatitis by inhibiting the activation of Jak2/Stat3 inflammatory signaling in pancreatic acinar cells.

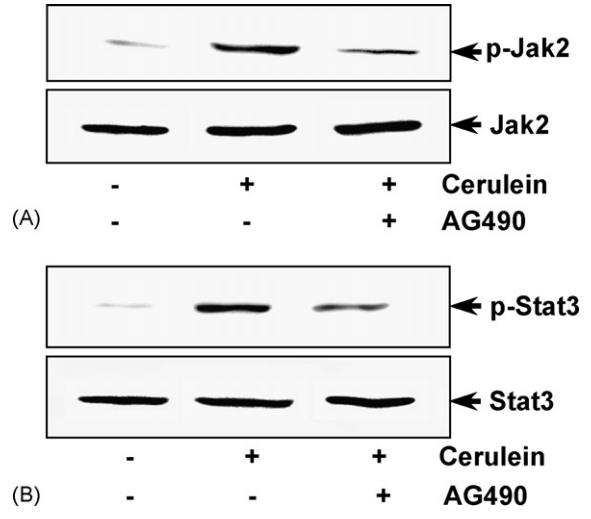

Fig. 3 – Inhibitory effect of AG490 on cerulein-induced activation of Jak2 and Stat3 in pancreatic acinar cells. AR42J cells were treated with (+) or without (−) AG490 for 2 h and stimulated with (+) or without (−) cerulein (10^{-8} M) for 60 min. Phosphorylated and nonphosphorylated forms of each Jak2 (A) and Stat3 (B) were determined in the cells by Western blot analysis.

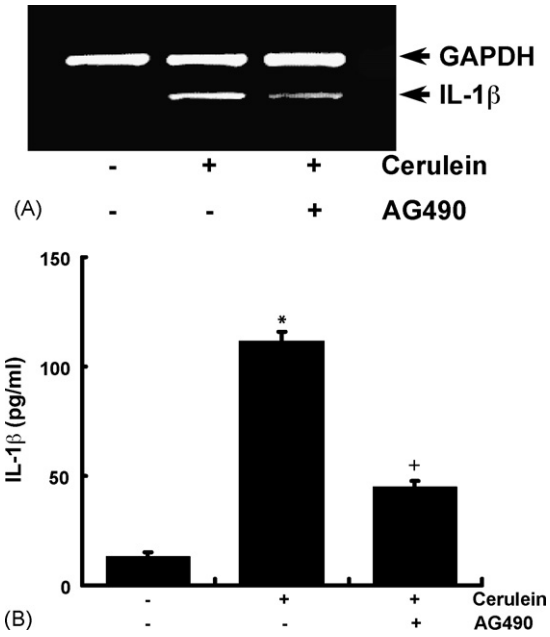

Fig. 4 – Inhibitory effect of AG490 on cerulein-induced mRNA expression of IL-1β in pancreatic acinar cells, as determined by a reverse transcription-polymerase chain reaction (RT-PCR) (A) and protein levels of IL-1β in the medium determined by enzyme-linked immunosorbant assay (ELISA) (B). AR42J cells were treated with (+) or without (−) AG490 for 2 h and stimulated with (+) or without (−) cerulein (10^{-8} M) for 3 h (mRNA expression, A) and 24 h (protein level, B). Each bar indicates mean ± S.E. of five different experiments (B). $^{*}P < 0.05$ vs. the cells treated without cerulein (−) and without AG490 (−); $^{+}P < 0.05$ vs. the cells treated with cerulein (+) and without AG490 (−).

3.3. *AG490 inhibits cerulein-induced mRNA expression and protein level of IL-1β in AR42J cells*

To determine whether cerulein-induced Jak2/Stat3 activation mediates IL-1β expression in pancreatic acinar cells, the cells were pretreated with AG490 for 2 h prior to cerulein stimulation. mRNA expression and protein level of IL-1β were analyzed (Fig. 4). IL-1β mRNA expression was determined by RT-PCR at 3 h-culture while protein levels of 1β in the medium were analyzed by ELISA at 24 h-culture. mRNA expression of IL-1β was inhibited by treatment of AG490 (Fig. 4A). We examined the effects of AG490 on production of IL-1β in AR42J cells stimulated with cerulein at 24 h-culture. As shown in Fig. 4B, pretreatment of AG490 markedly suppressed the cerulein-induced production of IL-1β in AR42J cells. IL-1β levels in the medium (pg/ml) released from the cells without cerulein without AG490 was 13.1 ± 1.7 while those of the cells

received cerulien without AG490 and with AG490 were 111.2 ± 4.3 and 44.6 ± 2.7, respectively. These results suggest that AG490 has inhibitory effect on IL-1β expression by suppressing Jak2/Stat3 inflammatory signaling and thus may regulate pancreatic inflammation.

3.4. *Cerulein induced pancreatic damage and increase in serum levels of IL-1β, which was inhibited by AG490 in vivo*

Chronic pancreatitis was induced by one hourly intraperitoneal injections of cerulein. As shown in Fig. 5A, all rats treated

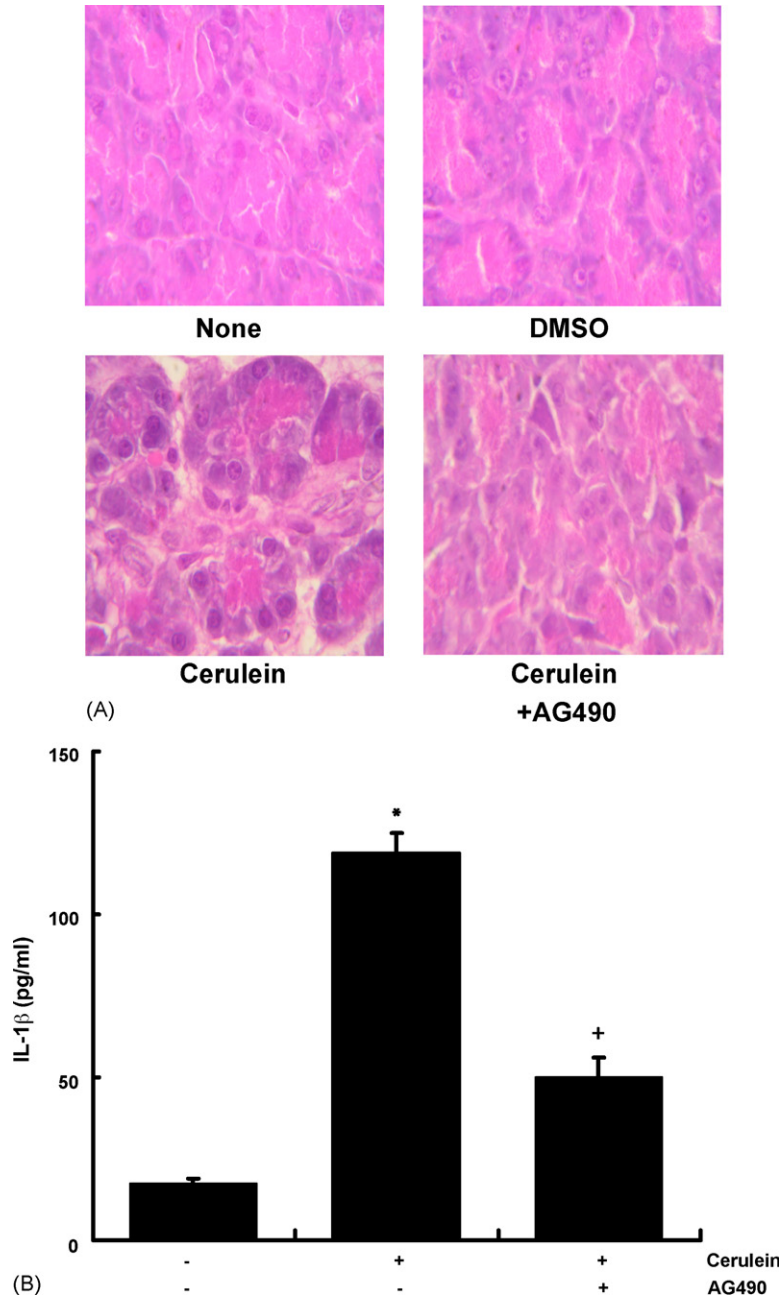

Fig. 5 – Inhibitory effect of AG490 on cerulein-induced pancreatic changes (A) and increase in serum levels of IL-1β of the rats (B). Cerulein injections displayed histopathological signs of chronic pancreatitis at the time of sacrifice at day 3, as reflected by edema, vacuole formation, and infiltration 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 200 μl (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 (A). Serum levels of IL-1β were determined by enzyme-linked immunosorbant assay (ELISA) (B). The rats were repeatedly injected with (+) or without (−) cerulein (10^{-8} M) and pretreated with (+) or without (−) AG490. Each bar indicates mean ± S.E. of five different experiments (B). $^{*}P < 0.05$ vs. the rats received without cerulein (−) and without AG490 (−); $^{+}P < 0.05$ vs. the rats received with cerulein (+) and without AG490 (−).

with repeated cerulein injections displayed histopathological signs of chronic pancreatitis at the time of sacrifice at day 3, as reflected by edema, vacuole formation and inflammatory cell infiltrate (cerulein). Administration of AG490 at 20 mg/kg significantly reduced the edema, vacuole formation, and inflammatory cell infiltrate induced by cerulein (cerulein + AG490). The rats treated without AG490 received 200 μl of DMSO instead of AG490 (DMSO). The histology of pancreas was compared between the rats received DMSO alone and the rats without DMSO (none). DMSO did not affect the histology of the pancreas.

Serum levels of IL-1β were highly increased at the end of experiment period (at day 3) by repeated injections of cerulein (Fig. 5B). We examined the effects of AG490 on production of IL-1β reflected in rat serum. Pretreatment of AG490 markedly suppressed the cerulein-induced increase in IL-1β in the serum. Serum IL-1β levels (pg/ml) of the rats without cerulein without AG490 was 17.0 ± 1.6 while those of the rats received cerulien without AG490 and with AG490 were 119.0 ± 5.6 and 50.2 ± 5.7, respectively.

4. Discussion

The present study aims to investigate whether the activation of Jak2/Stat3 signaling mediates IL-1β expression in pancreatic acinar AR42J cells in vitro as well as the rats in vivo using cerulein stimulation with or without AG490, the Jak2 inhibitor. Jak-Stat signaling has been reported to be closely involved in inflammation. Although Stat proteins were discovered during the course of analysis of interferon signaling, recent studies have revealed that Stat signaling can account for various cellular responses to a number of cytokines, growth factors, and hormones [40,41].

Tha Jak/Stat pathway is well known to be activated by the family of cytokine receptors and to mediate a wide variety of biological effects, such as immune response, differentiation, cell survival, proliferation, or oncogenesis [42]. The mechanism of Jak activation is well known for the cytokine receptors. Ligand binding induces oigomerization of the receptors subunits, constitutively associated to Jaks, and a tranphosphorylation of the tyrosine kinases. Activated Jaks in turn phosphorylate the receptor that recruits the Stat protein [42].

Cerulein, a CCK analogue (pGlu-Gln-[Met^3]-CCK8, sulphated), acts on two distinct CCK receptor subtypes, namely CCK_1 (previously named CCK_A) and CCK_2 (previously named CCK_B) receptors [43]. While CCK_1 receptors are highly discriminating with respect to the presence of a sulphate moiety on the tyrosine residue in the sequence of CCK and peptide analogues including cerulein, CCK_2 receptors (CCK2R) bind sulphated and non-sulphated analogues with much less difference in affinity [44,45]. CCK1R mediates for example the secretion of pancreatic digestive enzymes and may also be involved in the regulation of satiety and feeding behavior, while CCK2R stimulate gastric acid production [46]. Increase in blood flow induced by sulphated CCK-8 analogue and nonsulphated gastrin-17 in the gastric mucosa of rats have been demonstrated to be due to the activation of CCK2R [47]. CCK1R has a role in the exocrine effects of cerulein such as amylase secretion. The CCK receptors (CCKR) are seven-helix membrane-spanning receptor principally coupled to G proteins [48]. Initially, this type of receptor was implicated in the secretory effects of digestive peptide hormones. However, CCK2R is now recognized to mediate the mitogenic and anti-apoptotic effects of gastrin on gastrointestinal and pancreatic cells. In a pancreatic tumor cell line expressing the endogenous CCK2R, the proliferative effects of the CCK2R have been induced by the activation of the Jak2/Stat3 pathway by this receptor [49].

In the present study, cerulein induces the activation of Jak2/Stat3 signaling, a key pathway in inflammation, which leads to the expression of inflammatory cytokine IL-1β. Cerulein mediates the secretory and inflammatory events through CCK receptors. Therefore, it is possible that cerulein-induced Jak2/Stat3 activation may be mediated by CCK receptor(s) even though we could not conclude which type of receptor is involved in this signaling. In cerulein pancreatitis model in vitro and in vivo, AG490 effectively inhibited Jak2 phosphorylation. Likewise, Stat3 phosphorylation was blocked by AG490. Presumably it may be caused by inactivation of Jak2, which would be required to catalyze the event of Stat phosphorylation. These data provide a strong evidence to indicate that cerulein activate Stat3 through the activation of Jak2. AG490 prevented antigen-induced eosinophil recruitment into the airways of the sensitized animals. The results showed the important clinical relevance of Jak2 inhibition to the treatment of airway inflammation in asthma [50].

In the present study, the increase in serum levels of IL-1β was accompanied with the edematous and inflammatory pancreatic damages. The severity of inflammatory response by cerulein, determined by serum levels of IL-1β and pancreatic changes in vivo, were significantly inhibited by treatment of AG490. AG490 significantly reduced ceruelin-induced IL-1β expression, indicating that IL-1β expression is dependent on Jak2/Stat3 signaling. The present study demonstrates that cerulein induced phosphorylation of Jak2 and Stat3 in pancreatic acinar AR42J cells. Interestingly, we found that AG490 inhibited phosphorylation of Jak2 and Stat3 and rapidly reduced the expression of IL-1β, regulators of immune responses for acute pancreatitis. Therefore, AG490 may negatively regulate inflammation in the episodes of acute pancreatits.

In conclusion, cerulein triggers Jak2/Stat3 inflammatory signaling and may result in several important inflammatory events in pancreas, including production of inflammatory cytokines such as IL-1β. We suggest Jak2/Stat3 activation as the molecular mechanism underlying pathogenesis of acute pancreatitis.

Even though AG490 has been known to inhibit the activation of Jak2 [23–28] and Stat3 [29–33] in various cells and tissues, further study should be performed using the cells transfected with dominant negative gene of Stat3 or examining at the promoter level by using IL-1β promoter reporter construct to determine the transcriptional regulation of IL-1β by Stat3. In addition, the possible involvement of Jak3 in signaling for IL-1β expression in pancreatic acinar cells should be determined since recent studies showed that AG490 achieved inhibition of Jak3 and its downstream substrates Stat5a/b in vivo [36,37].

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