INVOLVEMENT OF SRC FAMILY KINASES IN SUBSTANCE P-INDUCED CHEMOKINE PRODUCTION IN MOUSE PANCREATIC ACINAR CELLS, AND ITS SIGNIFICANCE IN ACUTE PANCREATITIS

Raina Devi Ramnath, Jia Sun and Madhav Bhatia

Department of Pharmacology, National University of Singapore, Singapore 117456 (R.D.R., J.S., M.B.)
RUNNING TITLE PAGE

a) Running title: Role of SFKs in acute pancreatitis

b) Address correspondence to: Madhav Bhatia, Ph.D.
Department of Pharmacology, National University of Singapore,
Yong Loo Lin School of Medicine,
Centre for life Sciences, 28 Medical Drive, Singapore 117456
Tel. (65)-6516-8256;
Fax. (65)-6775-7674;
email. mbhatia@nus.edu.sg

c) Number of text pages: 27
Number of tables: 0
Number of figures: 9
Number of references: 40
Number of words in the Abstract: 243
Number of words in the Introduction: 295
Number of words in the Discussion: 701

d) List of abbreviations
AP-1, Activator protein-1; BSA, Bovine serum albumin; DMSO, Dimethyl sulfoxide; ECL, Enhanced chemiluminescence; GPCRs, G protein coupled receptors; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; HPRT, Hypoxanthine-guanine phosphoribosyl transferase; HRP, Horse radish peroxidase; MCP, Monocyte chemoattractant protein; MIP-1α, Macrophage inflammatory protein-1 alpha; MIP-2, Macrophage inflammatory protein-2; MPO, Myeloperoxidase; NFKB, Nuclear factor kappa B; NK1R, Neurokinin1 receptor; PBST, 0.05% Tween-20 in PBS; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal Kinase; PMSF; Phenylmethylsulfonyl Fluoride; RIPA, Radio-immunoprecipitation assay; SDS, Sodium dodecyl sulfate; SFK, Src family kinase; STAT, Signal transducers and activators of transcription

e) Recommended section: Inflammation, Immunopharmacology, and Asthma
ABSTRACT

Substance P is known to play a key role in the pathogenesis of acute pancreatitis. Src family kinases (SFKs) are known to be involved in cytokine signaling. However, the involvement of SFKs in substance P-induced chemokine production and its role in acute pancreatitis have not been investigated yet. To that end, we have used primary preparations of mouse pancreatic acinar cells as our model to show that substance P/NK1R induced activation of SFKs. SFKs mediated the activation of mitogen activated protein kinases (ERK, JNK), transcription factors (STAT3, NFκB, AP-1) and production of chemokines in pancreatic acinar cells. We further tested the significance of SFKs signaling pathway in acute pancreatitis. Our results show for the first time that treatment of mice with the potent and selective SFKs inhibitor PP2, but not its negative inhibitor PP3, reduced the severity of pancreatitis. This was evidenced by a significant attenuation of hyperamylasemia, pancreatic myeloperoxidase activity, chemokines and water content. Histological evidence of diminished pancreatic injury also confirmed the protective effect of the inhibition of SFKs. Moreover, treatment with substance P receptor antagonist CP96345 attenuated acute pancreatitis-induced activation of SFKs, ERK, JNK, STAT3, NFκB and AP-1. The proposed signaling pathway through which substance P mediates acute pancreatitis is through substance P/NK1R - SFKs - (ERK, JNK) - (STAT3, NFκB, AP-1) - chemokines. In light of our study, we propose that drugs targeting the substance P mediated signaling pathways could prove beneficial in improving treatment efficacy in acute pancreatitis.
INTRODUCTION

Acute pancreatitis is increasing in incidence and is often a fatal human disease, in which the pancreas digests itself and its surroundings (Bhatia and Moochhala, 2004; Bhatia et al., 2000; Bhatia et al., 2000; Bhatia et al., 2001; Bhatia, 2002). Accumulating experimental evidence has suggested that substance P/NK1R as well as chemokines play critical roles in the pathogenesis of acute pancreatitis. We have previously shown that substance P by itself caused an increased synthesis in CC and CXC chemokines in pancreatic acinar cells (Ramnath and Bhatia, 2006). Moreover, blockade of substance P receptor, NK1R, attenuated chemokine production in pancreatic acinar cells as well as protected mice from acute pancreatitis (Ramnath et al., 2007; Lau et al., 2005; Sun and Bhatia, 2007).

SFKs specifically Src has been widely studied in tumorigenesis. However, recent evidence has revealed that SFKs are among the most important families for the intracellular signal transduction related to acute inflammatory responses (Armstrong, 2004; Lowell, 2004; Yuan, 2002). Several animal studies have shown that inhibition of SFKs with small chemical inhibitors prevented ischemia-reperfusion-induced injury in the brain and heart. Moreover, blockade of SFKs attenuated sepsis, acute lung injury, and other organ damage (Khadaroo et al., 2004; Severgnini et al., 2005; Kusaka et al., 2004; Lennmyr et al., 2004; Akiyama et al., 2004; Paul et al., 2001; Weis et al., 2004). SFKs are activated in response to the stimulation of a variety of cell surface receptors (Thomas and Brugge, 1997). One such receptor type is GPCR. Once activated, Src is then capable of interacting with and activating the transcription factor STAT3 (Brown, 1996). The STAT family of proteins has been implicated in the functions of a wide range of cells (Akira, 2000; Bromberg and Darnell, 2000) and it is known to activate various key inflammatory mediators, for example the cytokine signaling pathway (Severgnini et al., 2004).
Limited knowledge is available on the role of SFKs in chemokine production in acute pancreatitis. Therefore, the aim was to investigate the role of SFKs in mediating substance P-induced chemokine production in pancreatic acinar cells and also the underlying signal transduction mechanisms involved. Moreover, we sought to test the significance of our in vitro findings in an in vivo model of acute pancreatitis.
METHODS

Animal Welfare

All animal experiments were approved by the Animal Ethics Committee of National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research. Male Swiss albino mouse (25-30 g) were maintained in the Animal Housing Unit of this University in an environment with controlled temperature (21-24°C) and lighting (12:12 h light-darkness cycle). Standard laboratory chow and drinking water were provided ad libitum. A period of 2 days was allowed for animals to acclimatize before any experimental manipulations were undertaken.

Test system used

Pancreatic acinar cells were obtained from mouse pancreas by collagenase treatment as described previously (Ramnath et al., 2008; Bhatia et al., 2002). Briefly, pancreas from three Swiss mice (20-25 g) were removed, infused with buffer A (in mM: 140 NaCl, 4.7 KCl, 1.13 MgCl2, 1 CaCl2, 10 glucose, 10 HEPES, pH 7.2) containing 200 IU/ml collagenase (Worthington) and 0.5 mg/ml soybean trypsin inhibitor (Sigma-Aldrich) and incubated in a shaking water bath for 10 min at 37°C. The digested tissue was centrifuged through 50 mg/ml bovine serum albumin (BSA) and washed twice with buffer A for further experiments. A cell suspension (in buffer A) consisting of only small clumps, around 3 to 5 acinar cells, was used to carry out the following experiments.

Viability of mouse pancreatic acinar cells

Viability of the pancreatic acinar cells was determined by trypan blue dye exclusion assay. One drop of 0.4% trypan blue dye was added to one drop of the isolated acinar cells. The viability was determined under the light microscope (Carl Zeiss, Oberkochen, Germany). The number of
unstained cells/clumps was expressed as a percentage of the total number of cells/clumps. This process was repeated for different fields and the average was then calculated. In all experiments, cell viability was greater than 95%.

**In vitro experimental design**

Pancreatic acinar cells (500 μl of cell suspension) were treated with substance P (Sigma-Aldrich) at a concentration of 1 μM for 0, 3, 5, 10, 15, 30 and 45 min at 37°C. Following treatment with substance P, the cells were lysed to detect for SFK activation by Western blot analysis. In some experiments, cells were either pre-treated with a potent and selective inhibitor of the Src family of protein tyrosine kinases, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine, PP2, (Calbiochem) at 1 and 10 μM or a negative control for the Src family protein tyrosine kinase inhibitor, 4-Amino-7-phenylpyrazol [3,4-d] pyrimidine, PP3, (Calbiochem) at 1 μM for 30 min followed by stimulation with 1 μM substance P or vehicle for 10 or 45 min at 37°C. Subsequently, the supernatant was used for chemokine detection and the pellet was used for either nuclear extract, to detect STAT3, NFκB (p65) and AP-1(c-Jun) activation, or cell lysis for Western blot analysis to detect SFKs, ERK and JNK. In another experiment, isolated pancreatic acinar cells were pre-incubated with the selective NK1R antagonist, CP96345, at 1 µM (Pfizer Diagnostics) for 30 min followed by treatment with 1 μM substance P or vehicle for 10 min at 37°C. Subsequently, the cells were lysed and used for Western blot analysis to detect SFKs.

**Preparation of total cell lysates for Western blot analysis**

After treatment, pancreatic acinar cells or pancreatic tissue were homogenized on ice in RIPA assay buffer supplemented with 1 mM PMSF and the protease inhibitor cocktail (Sigma-Aldrich) containing pepstatin, leupeptin, chymostatin, antipain and aprotinin (5 μg/ml of each), and centrifuged at 4 °C for 15 min at 14,000 g. The supernatants were collected and stored at -80°C.
until use. Protein concentrations were determined by using Bio-Rad protein assay. 5 μl of sample was added to 250 μl of Bradford reagent (Bio-Rad Laboratories, Hercules, CA) and read at 595 nm after a short incubation (5 min) at room temperature.

**Western blot analysis**

Cell lysates (50 μg protein) were separated on 12% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Non-specific binding was blocked by 1 h incubation of the membranes, at room temperature, in 5% nonfat dry milk in phosphate buffered saline Tween 20 (PBST) (0.05 % Tween 20 in phosphate buffered saline, pH 7.4). The blots were then incubated overnight at 4°C with the primary antibodies (at a 1:1000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST) phospho-Src family, phospho-ERK1/2, phospho-SAPK/JNK, (Cell Signaling Technology) and HPRT, purchased from Santa Cruz Biotechnology. HPRT was used as the housekeeping protein. Phospho-Src Family (Tyr416) antibody detects endogenous levels of Src only when phosphorylated at tyrosine 416. The antibody may cross-react with other Src family members (Lyn, Fyn, Lck, Yes and Hck) when phosphorylated at equivalent sites. It does not cross-react with Src phosphorylated at tyrosine 527. After which the membranes were washed four times with PBST, and finally incubated for 1 h at room temperature with goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2000 dilutions in the buffer containing 2.5 % nonfat dry milk in PBST. The blots were developed for visualization using enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL). The densities of the bands were quantified using a UVP GelDoc-It Imaging Systems.

**Nuclear extract preparation**
Nuclear extracts were prepared by employing a kit (Active Motif, SciMed, Asia), as described previously (Ramnath et al., 2008). Nuclear extract was prepared from both isolated pancreatic acinar cells and pancreatic tissue following caerulein-induced acute pancreatitis.

**STAT3/NFκB/AP-1 DNA-binding activity**

The binding of STAT3/NFκB/AP-1 to DNA was measured in nuclear extracts with ELISA-based TransAM STAT3/NFκB p65/AP-1 c-jun assay kits (Active Motif, SciMed, Asia). This assay uses multi-well plates coated with an unlabeled oligonucleotide containing the consensus binding site for STAT (5’-TTCCCGGAA-3’)/NFκB (5’ -GGGACTTTCC-3’)/AP-1 (5’ -TGAGTCA-3’). Nuclear proteins (5 µg) were added to each well and incubated for 1 h at room temperature to allow STAT3/NFκB/AP-1 DNA binding. Subsequently, by using an antibody that is directed against STAT3/NFκB p65/AP-1 c-jun subunit, the STAT3/NFκB/AP-1 complex bound to the oligonucleotide is detected. Addition of the secondary antibody conjugated to horseradish peroxidase (HRP) provides sensitive colorimetric readout that is easily quantified by spectrophotometry. Absorbance was read at 450 nm within 5 min by using a 96-well microplate reader (Tecan Systems, San Jose, CA). The wild-type consensus oligonucleotide is provided as a competitor for STAT3/NFκB/AP-1 binding to monitor the specificity of the assay. Results were expressed as fold increase over the control group.

**Chemokine detection**

Pancreatic acinar cell supernatant and pancreatic homogenate were assayed for MCP-1, MIP-1α and MIP-2 using a sandwich ELISA, according to the manufacturer’s instructions (Duoset kit; R&D Systems, Minneapolis, MN). For MCP-1 assay, anti-MCP-1 primary antibody was aliquoted onto ELISA plates and incubated at room temperature overnight. Samples (100 µl of cell supernatant) and standards were incubated at room temperature for 2 h, the plates were...
washed, and a biotinylated anti-MCP-1 antibody was added and left at room temperature for 2 h. Plates were washed again, and streptavidin bound to horseradish peroxidase was added for 20 min, at room temperature. After a further wash, tetramethylbenzidine was added for colour development, and the reaction was terminated with 2 M H_2SO_4. Absorbance was read at 450 nm within 5 min by using a 96-well microplate reader (Tecan Systems, San Jose, CA). The same procedure was followed for the detection of the remaining chemokines MIP-1α and MIP-2.

**Induction of Acute Pancreatitis**

Swiss mice (20-25 g) were randomly assigned to control or experimental groups using 10 or more animals for each group. Animals were given hourly intra-peritoneal (i.p.) injections of normal saline or saline containing caerulein (50 µg/kg) for 10 h. PP2 at doses of 0.5, 1.0 and 1.5 mg/kg, PP3 at a dose of 1.0 mg/kg and CP96345 at a dose of 2.5 mg/kg were administered to mice i.p. either 1 h before or 1 h after the first caerulein injection. 1 h after the last caerulein injection animals were sacrificed by an i.p. injection of a lethal dose of pentabarbitone. Harvested heparinized blood was centrifuged, the plasma removed and stored at -80°C. Random cross-sections of the head, body, and tail of the pancreas were fixed in 4% neutral phosphate-buffered formalin then embedded in paraffin wax. Freshly harvested samples of pancreas were weighed and then dried and reweighed to determine pancreatic water content (Bhatia et al., 1998). Samples of pancreas were stored at -80°C for subsequent measurement of tissue myeloperoxidase (MPO) activity and for ELISA and Western blot analysis.

**Amylase estimation**

Amylase activity was measured using a kinetic spectrophotometric assay. Plasma samples were incubated with the substrate, 4, 6-ethylidene (G_7)-p-nitrophenyl (G_1)-1-D-maltoheptoside (Sigma, St. Louis, MO, USA) for 2 minutes at 37°C and absorbance measured every minute for
the subsequent 2 minutes at 405 nm (Bhatia et al., 2000; Bhatia et al., 1998; Pierre et al., 1976). The change in absorbance was used to calculate the amylase activity.

Myeloperoxidase estimation

Neutrophil sequestration in pancreas was quantified by measuring tissue MPO activity (Bhatia et al., 2000; Bhatia et al., 1998). Tissue samples were thawed, homogenised in 20 mM phosphate buffer (pH 7.4), centrifuged (10,000 × g, 10 min, 4°C) and the resulting pellet resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO, USA). The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication (40 sec). The sample was then centrifuged (10,000 × g, 5 min, 4°C) and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mM tetramethylbenzidine (Sigma, St. Louis, MO, USA), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide. This mixture was incubated for 120 sec, the reaction terminated with 2M H₂SO₄ and the absorbance measured at 450 nm. This absorbance was then corrected for the protein content of the tissue sample.

Morphological Examination

Paraffin-embedded pancreas samples were sectioned (5 µm), stained with hematoxylin/eosin, and examined under light microscopy at ×400 magnifications. For these studies, 8 randomly chosen microscopic fields (×125) were examined for each tissue sample. Pancreatic damage was assessed based on the presence of acinar-cell ghosts, vacuolization, swelling of acinar cells, edema and infiltration of neutrophils.

Statistics

Data are expressed as the mean ± standard error of the mean (SEM). The significance of changes was evaluated by using analysis of variance (ANOVA). If ANOVA indicated a significant
difference, the data were analyzed by using Tukey's method as a post hoc test for the difference between groups. A $P$ value of $< 0.05$ was considered to indicate a significant difference.
RESULTS

Substance P/NK1R induces a rapid and transient increase in phosphorylation of Src family (Tyr 416) in mouse pancreatic acinar cells

Pancreatic acinar cells were stimulated with 1 μM substance P or vehicle (saline) for 0, 3, 5, 10, 15, 30 and 45 min at 37°C. Cells were then lysed, and cell proteins were subjected to Western blot analysis. As shown in Figure 1 (a, b), substance P induced phosphorylation of Src family (Tyr 416) in a time dependent manner up to 10 min, followed by a time-dependent decrease. Densitometric analysis of Western blot experiments revealed a significant increase in phosphorylation Src family (Tyr 416) at 10 and 15 min when compared to 0 min control. No significant change was observed when pancreatic acinar cells were treated with the vehicle at different time points. Pancreatic acini were also pre-treated with 1 μM of CP96345, a selective NK1R antagonist, for 30 min followed by stimulation with 1 μM of substance P for 10 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis. As demonstrated in Figure 1 (c, d), CP96345 significantly reduced substance P-induced phosphorylation of Src family (Tyr 416) in pancreatic acinar cells. No significant change was detected in the housekeeping proteins HPRT. This shows that substance P-induced phosphorylation of Src family is mediated through substance P/NK1R in pancreatic acinar cells.

Involvement of SFKs in substance P-induced MAP Kinases in pancreatic acinar cells

Stimulation of pancreatic acinar cells with 1 μM substance P significantly upregulated phosphorylation of Src family (Tyr 416), ERK and JNK. Pancreatic acinar cells were pre-treated for 30 min with the selective inhibitor of the Src family of protein tyrosine kinases, PP2, at 1 and 10 μM, followed by stimulation with 1 μM of substance P for 10 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis. As shown in Figure 2, PP2 significantly
decreased substance P-induced phosphorylation of Src family (Tyr 416), hence confirming its inhibitory effect. Moreover, PP2 dose dependently attenuated substance P-induced phosphorylation of ERK and JNK in pancreatic acinar cells (Figure 2 c, d). No significant change was detected in the housekeeping proteins HPRT.

**Substance P-induced SFKs is involved in activation of STAT3, NFκB and AP-1 in pancreatic acinar cells.**

Pancreatic acinar cells were pre-incubated for 30 min with PP2, at 1 and 10 μM followed by stimulation with 1 μM of substance P for 45 min. This time point was established from earlier data (Ramnath and Bhatia, 2006). STAT3, NFκB and AP-1 DNA-binding assay revealed that treatment with substance P led to a notable increase in the activity of STAT3, NFκB and AP-1 in pancreatic acinar cell. As shown in Figure 3 (a, b, c), pre-treatment with PP2 attenuated substance P-induced DNA-binding activity of STAT3, NFκB and AP-1.

**SFKs mediate substance P-induced production of CC and CXC chemokines in pancreatic acinar cells.**

Treatment of pancreatic acini with 1 μM substance P resulted in increased production of CC chemokine MCP-1, MIP-1α and CXC chemokine MIP-2 (Figure 4). Pancreatic acinar cells were pre-treated with either PP2 at 1 and 10 μM or its negative control PP3 at 1 μM for 30 min followed by stimulation with 1 μM of substance P for 45 min. The protein levels of these chemokines were determined by ELISA. Figure 4 (a, b, c) shows that pre-treatment with PP2 markedly decreased MCP-1, MIP-1α, and MIP-2 production. However, treatment with the negative control PP3 had no significant effect on chemokine production when compared to substance P only treated cells.
Effect of prophylactic and therapeutic treatment with PP2 on the severity of caerulein-induced acute pancreatitis.

Acute pancreatitis was induced by intraperitoneal administration of caerulein at a dose of 50 μg/kg hourly for 10 h. Evidence of pancreatic injury in acute pancreatitis mice was confirmed by a rise in plasma amylase, as shown in Figure 5 (a, b). There was also an increase in pancreatic edema as evidenced by elevated pancreatic water content, as shown in Figure 5 (c, d). Animals were administered either PP2 or PP3 1h before or after starting caerulein injection. Both prophylactic and therapeutic treatment with PP2 significantly attenuated plasma amylase levels, pancreatic water content when compared with animals treated with caerulein alone Figure 5 (a, c). Qualitative histological examination of pancreas sections confirmed a protection by PP2 treatment on acute pancreatitis in terms of acinar cell injury/necrosis, neutrophil infiltration as well as edema (Figure 6). However, prophylactic and therapeutic treatment with PP3 had no protective effect on pancreatic injury in acute pancreatitis when compared to mice treated with caerulein alone.

Involvement of SFKs in the mobilization of neutrophils and chemokines in acute pancreatitis.

Induction of acute pancreatitis by caerulein hyperstimulation resulted in a heightened pancreatic MPO, a measure of neutrophil infiltration (Figure 7). Acute pancreatitis also resulted in increased levels of pancreatic CC chemokine MCP-1, MIP-1α and CXC chemokine MIP-2 when compared to placebo-treated animals. Prophylactic treatment with PP2 resulted in a decrease in MPO activity, MCP-1, MIP-1α and MIP-2 levels in the pancreas when compared to caerulein-treated animals. Moreover, mice administered with PP2 therapeutically had a significant
reduction in pancreatic MPO activity, chemokines MCP-1, MIP-1α and MIP-2 levels when compared to caerulein treated mice (Figure 7).

**Inhibition of SFKs attenuated the activation of pancreatic STAT3, NFκB, AP-1 and MAP Kinases in acute pancreatitis**

Caerulein-induced acute pancreatitis resulted in significant activation of transcription factors STAT3, NFκB and AP-1 in pancreas when compared to placebo-treated animals (Figure 8). Treatment with PP2 both prophylactically and therapeutically significantly attenuated the activation of pancreatic STAT3, NFκB and AP-1 when compared to caerulein treated animals, as shown in Figure 8 (a, b, c). To further explore the signaling pathways mediated by SRC tyrosine kinases in acute pancreatitis, we examined the role of MAP kinases. Acute pancreatitis increased phosphorylation of MAP kinases ERK and JNK in pancreas when compared to placebo-treated animals. Both prophylactic and therapeutic treatment with PP2 significantly attenuated activation of ERK and JNK when compared to caerulein treated mice Figure 8 (d, e, f). No significant change was observed in the housekeeping protein HPRT in the pancreas.

**Substance P/NKIR mediated activation of pancreatic SFKs and its downstream signaling pathway in acute pancreatitis.**

Treatment with CP96345 both prophylactically and therapeutically attenuated the activation of pancreatic SFKs, MAP kinases ERK and JNK when compared to caerulein treated mice as shown in Figure 9. No significant change was observed in the housekeeping protein HPRT in the pancreas. Moreover, as shown in Figure 8 (a, b, c), CP96345 blocked the upregulation of pancreatic STAT3, NFκB and AP-1 in acute pancreatitis.
DISCUSSION

SFKs have been shown to play a key role in cytokine signaling and inflammatory responses (Song et al., 2003; Chaturvedi et al., 1998). However, the involvement of SFKs in substance P-induced chemokine production and also its role in acute pancreatitis have not been investigated yet.

To that end, we have demonstrated that substance P/NK1R induced activation of Src family in pancreatic acinar cells. SFKs also mediated substance P-induced activation of MAP Kinases ERK and JNK. We also illustrated that substance P-induced phosphorylation of Src tyrosine kinases was involved in the activation of STAT3, NFκB, AP-1 and production of chemokines MCP-1, MIP-1α and MIP-2 in pancreatic acinar cells. Our results are in agreement with several other studies, whereby substance P was found to induce Src activation, which was also implicated in the activation of MAP Kinases (Della Rocca et al., 1997; Yamaguchi et al., 2005; Yamaguchi et al., 2005; DeFea et al., 2000). Moreover, MAP Kinases were found to modulate the transcriptional activity of STAT3 (Chung et al., 1997; Turkson et al., 1999). We have previously demonstrated the involvement of ERK, JNK, NFκB and AP-1 in substance P induced chemokine synthesis (Ramnath et al., 2007). Substance P-induced activation of SFKs most likely is mediating chemokine production through the ERK and JNK induced transcription factors NFκB and AP-1. However, whether substance P-induced STAT3 activation is directly involved in the production of chemokines remained to be investigated. On the one hand, studies have suggested that STAT3 plays a pivotal role in orchestrating inflammatory responses by increasing the expression of inflammatory mediators, cytokines and chemokines (Severgnini et al., 2004; Schumann et al., 1996). However, on the other hand, studies have revealed that STAT3 modulates anti-inflammatory responses (Takeda et al., 1999; Matsukawa et al., 2003). Hence,
more work needs to be carried out to elucidate the complexity of STAT3 signaling mechanisms in acute pancreatitis.

We further sought to investigate the in vivo relevance of the in vitro findings. Our results show that treatment of animals with the potent and selective inhibitor of the Src family of protein tyrosine kinases PP2 but not its negative inhibitor PP3 (either prophylactic or therapeutic) reduces the severity of pancreatitis as evidenced by a significant attenuation of hyperamylasemia, pancreatic MPO activity, chemokine production and water content which is a measure of edema. Moreover, histological evidence of diminished pancreatic injury such as acinar cell injury/necrosis, neutrophil infiltration as well as edema also confirmed the protective effect of the inhibition of SFKs on acute pancreatitis. In line with our study, Src tyrosine kinase inhibitors were found to suppress inflammatory responses in vivo by blocking the function of inflammatory cells including neutrophils, monocytes and macrophages (Okutani et al., 2006).

Using the in vivo model of acute pancreatitis, we further explored the molecular mechanisms through which SFKs protected against acute pancreatitis, we found that SFKs mediate their protective effects through the same signaling pathway that we have shown in our in vitro model of isolated acinar cells. It has been shown that treatment with the NK1R antagonist CP96345 protected mice against acute pancreatitis by attenuating chemokine production (Sun and Bhatia, 2007; Lau et al., 2005). In the present study we have further shown that CP96345 attenuated activation of SFKs, ERK, JNK, STAT3, NFκB and AP-1 in acute pancreatitis. Based on our results we proposed that elevated level of substance P, which is produced as a result of acute pancreatitis, binds to NK1R to activate several signaling molecules to mediate chemokine production. One such signaling complex is SFKs and its blockade attenuated MAP Kinases, STAT3, NFκB, AP-1 and chemokines, thus resulting in protection against acute pancreatitis. The
The proposed signaling pathway through which substance P mediates acute pancreatitis is through substance P/NK1R - SFKs - (ERK, JNK) - (STAT3, NFκB, AP-1) - (MCP-1, MIP-1α, MIP-2).

The mechanism of activation of SFKs in acute pancreatitis is most likely to be multifactorial, however one of the factors involved is the neuropeptide substance P. Using isolated pancreatic acinar cell preparations we were able to show that substance P/NK1R indeed induces activation of SRC tyrosine kinases. Blockade of SFKs attenuated chemokine production both in vitro and in vivo as well as protected the mice against acute pancreatitis. This study gives us a deeper insight into the mechanisms by which substance P contributes to acute pancreatitis. Increased understanding of the signal transduction mechanisms involved in acute pancreatitis would facilitate the discovery of novel therapeutic approaches that can target selective pathways to prevent disease progression in acute pancreatitis and/or improve treatment efficacy.
ACKNOWLEDGMENTS

We would like to give our thanks to Mei Leng Shoon for technical assistance.
REFERENCES


**FOOTNOTES**

National Medical Research Council Grant No. [R-184-000-156-213] and Biomedical Research Council Grant [R-184-000-069-305].

Address for reprint requests and other correspondence: M. Bhatia, Cardiovascular Biology Group, Department of Pharmacology c/o Centre for Life Sciences, #03-02 National Univ. of Singapore, 28 Medical Dr., Singapore 117456 (E-mail. mbhatia@nus.edu.sg).
LEGENDS FOR FIGURES

Figure 1 Substance P (SP)/NK1R induces a time dependent increase and decrease in phosphorylation of Src family (Tyr 416) in mouse pancreatic acinar cells. Freshly isolated pancreatic acini were incubated with either 1 μM SP/vehicle (saline) for 0, 3, 5, 10, 15, 30 and 45 min at 37°C or pre-incubated with 1 μM CP96345 for 30 min followed by stimulation with 1 μM SP for 10 min. The cells were lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a, c) phospho-Src family (Tyr 416) and HPRT. Densitometric analysis of Western blot experiments were performed and the group data from 3 independent preparations (n=3) are presented in (b, d). The results are representative of three independent (n=3) experiments. Results shown are the means ± SE. *P ≤ 0.05 when compared to 0 min control, +P ≤ 0.05 when compared to SP.

Figure 2 Substance P (SP)-induced activation of ERK and JNK is mediated by SFKs in pancreatic acinar cells. Freshly isolated pancreatic acini were pre-incubated with either PP2 at 1 μM, 10 μM or vehicle (DMSO) for 30 min at 37°C followed by stimulation with 1 μM SP for 10 min at 37°C. Cells were subsequently lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-Src family (Tyr 416), phospho-ERK, phospho-JNK and HPRT. The phosphorylated sub-units such as p-Src family, p-44 ERK, p-42 ERK, p-54 JNK and p-46 JNK have been quantified. Densitometric analysis of Western blot experiments were performed and the group data from 3 independent preparations (n=3) are presented in (b, c, d). Results shown are the means ± SE. *P ≤ 0.05 when compared to control, +P ≤ 0.05 when compared to SP.

Figure 3 SFKs are involved in substance P (SP)-induced STAT3, NFκB and AP-1 activation in pancreatic acinar cells. Freshly isolated pancreatic acini were pre-incubated with
PP2 at 1 and 10 µM or vehicle (DMSO) followed by stimulation with 1 µM of SP for 45 min. The cells were separated from incubation medium by centrifugation. The pellet (cells) was used for (a) STAT3, (b) NFκB and (c) AP-1 nuclear extraction. STAT3, NFκB and AP-1 DNA-binding assays were then carried out as described in MATERIALS AND METHODS. The results are representative of three independent (n=3) experiments. Results shown are the means + SE. *P≤ 0.05 when compared to control, +P≤ 0.05 when compared to SP.

Figure 4 SFKs are involved in the secretion of CC and CXC chemokines in pancreatic acinar cells. Freshly isolated pancreatic acini were pre-incubated with either PP2 at 1 and 10 µM, or PP3 at 1 µM for 30 min followed by stimulation with 1 µM of substance P (SP) for 45 min. The supernatant was used to measure (a) MCP-1, (b) MIP-1α and (c) MIP-2 levels by ELISA as described in MATERIALS AND METHODS. The results are representative of three independent (n=3) experiments. Results shown are the means + SE. *P≤ 0.05 when compared to control, +P≤ 0.05 when compared to SP.

Figure 5 Effects of prophylactic and therapeutic PP2 administration on the severity of acute pancreatitis. Mice (n =10 in each group) were given 10 hourly injections of caerulein (50 µg/kg i.p.). PP2 or PP3 was administered to mice i.p. either prophylactically (1h before) or therapeutically (1h after) the first caerulein injection. 1h after the last caerulein injection, mice were sacrificed by an i.p. injection of a lethal dose of pentobarbitone. Plasma amylase activity (a, b) and pancreatic edema (water content c, d) were determined as described in MATERIALS AND METHODS. Results shown are the means + SE. *P ≤ 0.05 when caerulein or PP3-treated animals were compared with placebo-treated animals. +P ≤ 0.05 when PP2-treated animals were compared to caerulein-treated animals.
Figure 6 Morphological changes in mouse pancreas on induction of acute pancreatitis with/without prophylactic and therapeutic treatment with PP2 or PP3. Representative micrographs from each group were shown (× 400 magnifications). A: control; no pancreatitis. B: caerulein-induced acute pancreatitis. C: caerulein-induced acute pancreatitis in mice administered PP3 1mg/kg (prophylactic). D: caerulein-induced acute pancreatitis in mice administered PP3 1mg/kg (therapeutic). E: caerulein-induced acute pancreatitis in mice administered PP2 1mg/kg (prophylactic). F: caerulein-induced acute pancreatitis in mice administered PP2 1mg/kg (therapeutic).

Figure 7 Involvement of SFKs in the mobilization of pancreatic neutrophils and chemokine in acute pancreatitis. Mice (n=10 in each group) were given 10 hourly injections of caerulein (50 μg/kg i.p). PP2 was administered in mice at doses of 0.5, 1, 1.5 mg/kg i.p. 1h before or at a dose of 1 mg/kg 1 h after the first caerulein injection. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentobarbitone, and pancreatic MPO (a), MCP-1 (b), MIP-1α (c) and MIP-2 (d) levels were measured as described in MATERIALS AND METHODS. Results shown are the means + SE. *P ≤ 0.05 when caerulein-treated animals were compared with placebo-treated animals. +P ≤ 0.05 when PP2-treated animals were compared to caerulein-treated animals.

Figure 8 Inhibition of SFKs attenuated the activation of pancreatic STAT3, NFκB, AP-1 and MAP Kinases in acute pancreatitis. Mice (n=10 in each group) were given 10 hourly injections of caerulein (50 μg/kg i.p). PP2 was administered to mice at a dose of 1 mg/kg i.p. 1h before or 1 h after the first caerulein injection. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentabarbitone. The activation of pancreatic STAT3 (a), NFκB (b), AP-1 (c) and MAP Kinases (d, e, f) was quantified as
described in MATERIALS AND METHODS. Results shown are the means + SE. *$P \leq 0.05$ when caerulein-treated animals were compared with placebo-treated animals. +$P \leq 0.05$ when PP2-treated animals were compared to caerulein-treated animals.

Figure 9 Blockade of NK1R reduced acute pancreatitis-induced activation of MAP Kinases and transcription factors STAT3, NFκB, and AP-1 in the pancreas.

Mice (n=6 in each group) were given 10 hourly injections of caerulein (50 μg/kg i.p). CP96345 was administered to mice at a dose of 2.5 mg/kg i.p. 1h before or 1 h after the first caerulein injection. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentobarbitone. The activation of pancreatic STAT3 and MAP Kinases (a) and the densitometry analysis (c, d) were described in MATERIALS AND METHODS. Results shown are the means + SE. *$P \leq 0.05$ when caerulein-treated animals were compared with placebo-treated animals. +$P \leq 0.05$ when CP96345-treated animals were compared to caerulein-treated animals.
Figure 1

(a) Time

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>3</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>30</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-Src Family (Tyr 416)</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
</tr>
<tr>
<td>HPRT</td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
</tr>
</tbody>
</table>

(b) Densitometry

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>3</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>30</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-Src Family</td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
<td><img src="image21.png" alt="Image" /></td>
</tr>
</tbody>
</table>

(c) Control SP CP96345

<table>
<thead>
<tr>
<th>Control</th>
<th>SP</th>
<th>CP96345</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-Src Family (Tyr 416)</td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
</tr>
<tr>
<td>HPRT</td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
</tr>
</tbody>
</table>

(d) Densitometry

<table>
<thead>
<tr>
<th>Control</th>
<th>SP</th>
<th>CP96345</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-Src Family</td>
<td><img src="image28.png" alt="Image" /></td>
<td><img src="image29.png" alt="Image" /></td>
</tr>
</tbody>
</table>

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 2

(a) PP2 + SP (μM)

Phospho-Src Family (Tyr 416)

p-ERK

p-JNK

HPRT

(b)

(c)

Phospho-Src Family

p-44 ERK

p-54 JNK

P-46 JNK

PP2 + SP

Densitometry

Control SP 1 µM 10 µM

Densitometry

Control SP 1 µM 10 µM

Densitometry

Control SP 1 µM 10 µM

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 3

(a) STAT3

(b) NFkB

(c) AP-1

Fold increase over control
Figure 4

(a) MCP-1

(b) MIP-1α

(c) MIP-2

Fold increase over control

Control SP 1 μM 10 μM 1 μM

PP2 + SP PP3 + SP

PP2 + SP PP3 + SP

Fold increase over control

Control SP 1 μM 10 μM 1 μM

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 5

(a) Prophylactic PP2 (mg/kg)

(b) Therapeutic PP2 (mg/kg)

(c) Prophylactic PP3 (1 mg/kg)

(d) Therapeutic PP3 (1 mg/kg)

Pancreas Water Content (%)

Control Caerulein 0.5 1 1.5 1

Prophylactic PP2 (mg/kg)

Therapeutic PP2 (mg/kg)

Amylase U/L

Control Caerulein 0.5 1 1.5 1

Prophylactic PP3 (1 mg/kg)

Therapeutic PP3 (1 mg/kg)

Pancreas Water Content (%)

Control Caerulein 0.5 1 1.5 1

Prophylactic PP2 (mg/kg)

Therapeutic PP2 (mg/kg)

Amylase U/L

Control Caerulein 0.5 1 1.5 1

Prophylactic PP3 (1 mg/kg)

Therapeutic PP3 (1 mg/kg)
Figure 7

Pancreas MPO

Pancreas MPO

Pancreas MIP-1α

Pancreas MIP-2

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 8

Pancreas STAT3

(a) Pancreas STAT3

(b) Pancreas NFκB

(c) Pancreas AP-1

(d) Pancreas p-JNK

(e) Pancreas p-44 ERK

(f) Pancreas p-42 ERK

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 9

(a) Phospho-Src Family (Tyr 416)
- Control
- Cae
- Pro
- The

(b) p-ERK

(c) p-JNK

(d) HPRT

Pro
CP96345 (2.5 mg/kg)
Control
The