Substance P treatment stimulates chemokine synthesis in pancreatic acinar cells via the activation of NF-κB

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Ramnath, Raina Devi, and Madhav Bhatia. Substance P treatment stimulates chemokine synthesis in pancreatic acinar cells via the activation of NF-κB. Am J Physiol Gastrointest Liver Physiol 291: G1113–G1119, 2006. First published July 27, 2006; doi:10.1152/ajpgi.00177.2006.—Acinar cell injury early in acute pancreatitis leads to a local inflammatory reaction and to the subsequent systemic inflammatory response, which may result in multiple organ dysfunction and death. Inflammatory mediators, including chemokines and substance P (SP), are known to play a crucial role in the pathogenesis of acute pancreatitis. It has been shown that pancreatic acinar cells produce the chemokine monocyte chemoattractant protein-1 (MCP-1) in response to caerulein hyperstimulation, demonstrating that acinar-derived MCP-1 is an early mediator of inflammation in acute pancreatitis. Similarly, SP levels in the pancreas and pancreatic acinar cell expression of neurokinin-1 receptor, the primary receptor for SP, are both increased during secretagogue-induced experimental pancreatitis. This study aims to examine the functional consequences of exposing mouse pancreatic acinar cells to SP and to determine whether it leads to proinflammatory signaling, such as production of chemokines. Exposure of mouse pancreatic acini to SP significantly increased synthesis of MCP-1, macrophage inflammatory protein-1α (MIP-1α), as well as MIP-2. Furthermore, SP also increased NF-κB activation. The stimulatory effect of SP was specific to chemokine synthesis through the NF-κB pathway, since the increase in chemokine production was completely attenuated when pancreatic acini were pretreated with the selective NF-κB inhibitor NF-κB essential modulator-binding domain peptide. This study shows that SP-induced chemokine synthesis in mouse pancreatic acinar cells is NF-κB dependent.

pancreatitis; monocyte chemoattractant protein-1; macrophage inflammatory protein-1α; macrophage inflammatory protein-2; neurokinin-1 receptor

ACUTE PANCREATITIS (AP) is an inflammatory disorder. The activation of digestive enzymes within pancreatic acinar cells plays a critical initiating event in AP, resulting in acinar cell damage and a localized inflammatory response (13, 29, 41). Secreted bioactive molecules from infiltrating leukocytes contribute to local damage and, subsequently, to the systemic inflammatory response, which may result in multiple organ dysfunction and ultimately to death (4). The initial signals that recruit leukocytes into the pancreas are not completely defined, although several inflammatory mediators have been implicated (4, 12). Inflammatory mediators such as chemokines and substance P (SP) play a key role in the pathogenesis of AP (5–7). The chemokines are a family of small (8–10 kDa) inducible cytokines with activating and chemotactic effects on leukocyte subsets. They can be broadly subdivided on a structural basis into the CC subfamily, in which the first two cysteine residues are adjacent, and the CXC subfamily, in which the first two of the four conserved cysteine residues are separated by another amino acid. CC chemokines, such as monocyte chemoattractant protein-1 (MCP-1), are believed to principally affect monocytes, whereas CXC chemokines that possess the Glu-Leu-Arg (ELR motif) at the amino terminal are believed to act on neutrophils (8). We have shown that pancreatic acinar cells produce MCP-1 in response to caerulein hyperstimulation, indicating that acinar-derived MCP-1 is an early mediator of inflammation in AP (5). We have also shown that treatment with bindarit, a blocker of MCP-1 synthesis, protects mice against AP (6).

SP is an 11 amino acid neuropeptide that is released from nerve endings in many tissues. It has been shown to play an important role in asthma, inflammatory bowel disease, arthritis, and other inflammatory processes (8, 40). Subsequent to its release from nerve endings, SP binds to neurokinin-1 receptor (NK1R) on effector cells, increases microvascular permeability, and promotes plasma extravasation from the intravascular to the extravascular space. Pancreatic acinar cells are known to express NK1R, and SP has been detected within the pancreas (18, 27, 35). It has been suggested that this neuropeptide might play a role in the evolution of a pancreatic inflammatory disease such as AP (7). Studies (7) have found that pancreatic levels of SP and the expression of NK1R on pancreatic acinar cells are increased during experimental AP. It has also been shown that genetic deletion of NK1R reduces the severity of pancreatitis and pancreatitis-associated lung injury (7). These observations indicate that SP, acting through NK1R, plays an important proinflammatory role in regulating the severity of AP and associated lung injury.

Recently, NF-κB activation has come out as a key mediator of the inflammatory response in pancreatitis (9, 17, 32, 38). The importance of NF-κB response stems from its ability to upregulate the expression of chemokines/cytokines and other inflammatory molecules that are induced in human and experimental pancreatitis (4, 24). The mechanism and regulation of NF-κB response in the pancreatic acinar cell, however, are not well understood.

In the present study, we have investigated the effect of SP on the synthesis of CC chemokine monocyte chemoattractant protein-1 (MCP-1) and MIP-1α, as well as CXC chemokine MIP-2 in mouse pancreatic acinar cells. Moreover, we have investigated the mechanism by which SP induces the synthesis of chemokines through NF-κB activation.

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MATERIALS AND METHODS

Preparation of mouse pancreatic acini and in vitro treatment with SP or caerulein. All animal experiments were approved by the Animal Ethics Committee of the National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research.

Pancreatic acini were obtained from mouse pancreas by collagenase treatment as described previously (5). Briefly, pancreas from three Swiss mice (20–25 g) were removed, infused with buffer A, containing (in mM) 140 NaCl, 4.7 KCl, 1.13 MgCl2, 1 CaCl2, 10 glucose, and 10 HEPES (pH 7.2) and 200 IU/ml collagenase and 0.5 mg/ml soybean trypsin inhibitor, and incubated in a shaking water bath for 10 min at 37°C. The digested tissue was passed through 50 mg/ml BSA and washed twice with buffer A before further experiments. SP was purchased from Sigma. Caerulein was obtained from Bachem (Bubendorf, Switzerland).

Experiments were performed to examine the effects of SP treatment on chemokine production and NF-κB activation on mouse pancreatic acini. Briefly, acini were incubated with SP at a dose of 10⁻⁶ M (1 μM) for 45 min at 37°C, after which the supernatant was used for chemokine detection by ELISA, whereas the pellet was used for NF-κB isolation and detection.

Chemokine detection. Pancreatic acinar cell supernatants 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 example, MCP-1, briefly, anti-MCP-1 primary antibody, was aliquoted onto ELISA plates and incubated at 4°C overnight. Samples and standards were incubated for 2 h, the plates were washed, and a biotinylated anti-MCP-1 antibody was added for 2 h. Plates were washed again, and streptavidin bound to horseradish peroxidase (HRP) was added for 20 min. After a further wash, tetramethylbenzidine was added for color development, and the reaction was terminated with 2 M H2SO4. Absorbance was measured at 450 nm. The same procedure was followed for the detection of the remaining chemokines MIP-1α and MIP-2.

Nuclear cell extract preparation and NF-κB DNA-binding activity. Nuclear cell extracts were prepared by employing a kit from Active Motif. In brief, cells were washed, collected in ice-cold PBS in the presence of phosphatase inhibitors to limit further protein modifications, and then centrifuged at 24 g for 5 min. The pellets were resuspended in a hypotonic buffer, treated with detergent, and centrifuged at 14,000 g for 30 s. After collection of the cytoplasmic fraction, the nuclei were lysed and nuclear proteins solubilized in lysis buffer containing proteasome inhibitors. The binding of NF-κB to DNA was measured in nuclear extracts with a fast, user-friendly ELISA-based TransAM NF-κB p65 assay kit (Active Motif). This assay uses multilwell plates coated with an unlabeled oligonucleotide containing the consensus binding site for NF-κB (5'-GGGACTTTCG-3') (26).

Nuclear proteins (20 μg) were added to each well and incubated for 1 h to allow NF-κB DNA binding. Subsequently, with the use of an antibody that is directed against the NF-κB p65 subunit, the NF-κB complex, bound to the oligonucleotide, is detected. Addition of the secondary antibody, conjugated to HRP, provides a sensitive colorimetric readout that is easily quantified by spectrophotometry.

NF-κB inhibition. Pancreatic acini were incubated for 2 h with the 50 μM of the NF-κB essential modulator (NEMO)-binding domain peptide (NBD) (39), purchased from Calbiochem, or placebo (DMSO) before stimulation with SP. Subsequently, the supernatant was used for chemokine detection.

Preparation of cell lysates for Western blot analysis. After treatment, pancreatic acinar cells were homogenized on ice in radioimmuno-precipitation assay buffer, supplemented with 1 mM PMSF and the protease inhibitor cocktail containing pepstatin, leupeptin, chymostatin, antipain, and aprotinin (5 μg/ml of each), and centrifuged at 4°C for 15 min at 13,000 rpm. The supernatants were collected and stored at ~80°C. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Western blot analysis. Cell lysates (50 μg) were separated on 12% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. Nonspecific binding was blocked by 1-h incubation of the membranes in 5% nonfat dry milk in 0.05% Tween 20 in PBS (PBST). The blots were then incubated overnight with the primary antibody IκB-α (purchased from Cell Signaling Technology) at 1:1,000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST, after which they were washed four times with PBST and finally incubated for 1 h with goat anti-rabbit HRP-conjugated secondary antibody (urchased from Santa Cruz Biotechnology) at 1:2,000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST. The blots were developed for visualization using enhanced chemiluminescence detection kit (Pierce, Rockford, IL). Hypoxanthine-guanine phosphoribosyl transferase (HPRT), purchased from Santa Cruz Biotechnology, was used as the housekeeping protein.

Amylase estimation. Pancreatic acini were incubated with SP or caerulein (10⁻¹¹ to 10⁻⁶ M) for 45 min, and amylase assays were performed. Amylase activity was measured by using a kinetic spectrophotometric assay. Acinar cell supernatants were incubated with the substrate 4,6-ethylidene (G7) p-nitrophenol (G1) 1-d-maltoheptoside (Sigma, St. Louis, MO) for 2 min at 37°C, and absorbance was measured every minute for the subsequent 2 min at 405 nm (3, 7, 28). The change in absorbance was used to calculate the amylase activity.

Statistical analysis. Results are presented as means ± SE with six replicates for each condition. Each experiment was repeated at least three times. The significance of changes was evaluated by using ANOVA, and Tukey’s method was used as a post hoc test for the difference between groups. A P value < 0.05 was taken as the level of significance.

RESULTS

Viability of mouse pancreatic acinar cells. Viability of the pancreatic acinar cells was determined by dye exclusion assay. One drop of 0.4% trypan blue dye was added to one drop of the isolated acinar cells, and the viability was checked under a light microscope. Viability of the cells was >95%.

SP induces chemokine production in a dose-dependent manner. To investigate the effect of different doses of SP on chemokine synthesis in mouse pancreatic acini, isolated pancreatic acinar cells were challenged for 45 min at 37°C with different concentrations of SP, ranging from 0.01 to 1 μM. Subsequently, the supernatant was used to assess the levels of chemokines, MCP-1, MIP-1α, and MIP-2, by ELISA. As shown in Fig. 1, SP increases MCP-1, MIP-1α, and MIP-2 production in a dose-dependent manner. Maximal activation of MCP-1, MIP-1α, and MIP-2 were observed with 1 μM SP. The increase in MCP-1, MIP-1α, and MIP-2 synthesis was significantly higher compared with that in the placebo-treated control. A dose of 1 μM (10⁻⁶ M) SP was used to carry out subsequent experiments.

SP or caerulein induces NF-κB activation in mouse pancreatic acinar cells. Pancreatic acinar cells were stimulated with 1 μM SP or 0.1 μM caerulein for 45 min at 37°C, after which the pelts were used for nuclear extraction and NF-κB detection. As shown in Fig. 2A, SP at a concentration of 1 μM significantly upregulated NF-κB activation compared with that in the placebo-treated control. This finding was further confirmed by Western blot analysis. As shown in Fig. 2B, when mouse pancreatic acini were stimulated with 1 μM SP for 45 min at
37°C, there was an increased degradation of total IκB compared with that in the placebo-treated control. As shown in Fig. 2C, 0.1 μM caerulein significantly increased NF-κB activation compared with that in the placebo-treated control. Further-

Fig. 1. Substance P (SP) induces chemokine production in a dose-dependent manner in mouse pancreatic acinar cells. Pancreatic acini, obtained from 3 mice, were incubated for 45 min at 37°C with different concentrations of SP ranging from 0.01 to 1 μM, after which the suspension was centrifuged at low speed (240 g for 5 min). The supernatant obtained was used for monocyte chemoattractant protein (MCP)-1 (A), macrophage inflammatory protein (MIP)-1α (B), and MIP-2 (C) detection. Results shown are means ± SE. *P ≤ 0.05 when 1 μM SP-treated acini were compared with placebo-treated acini. #P ≤ 0.05 when NF-κB essential modulator-binding domain peptide (NBD) and caerulein-treated acini were compared with caerulein alone-stimulated cells. HPRT, hypoxanthine-guanine phosphoribosyl transferase.

Fig. 2. SP or caerulein (Cae) induces NF-κB activation in mouse pancreatic acinar cells. Pancreatic acini, obtained from 3 mice, were incubated for 45 min at 37°C with 1 μM SP or 0.1 μM Cae. Acini were separated from incubation medium by centrifugation. A: the pellet (acini) was used for NF-κB extraction and detection. Results shown are means ± SE. *P ≤ 0.05 when 1 μM SP-treated acini were compared with placebo-treated acini. B: Western blot analysis was performed as described in MATERIALS AND METHODS. C: *P ≤ 0.05 when 0.1 μM Cae-treated acini were compared with placebo-treated acini. #P ≤ 0.05 when NF-κB essential modulator-binding domain peptide (NBD) and Cae-treated acini were compared with Cae alone-stimulated cells. HPRT, hypoxanthine-guanine phosphoribosyl transferase.

more, pretreatment of acini with 50 μM NBD significantly attenuated the caerulein-induced NF-κB activation.

SP or caerulein-induced chemokine synthesis is prevented by NBD, an NF-κB inhibitor. We further investigated the specificity of SP or caerulein effects on chemokine production in mouse pancreatic acinar cells by pretreating the cells with NBD, an NF-κB inhibitor. Pancreatic acini were pretreated with NBD peptide for 2 h, followed by stimulation with 1 μM SP or 0.1 μM caerulein, after which the levels of MCP-1, MIP-1α, and MIP-2 were determined by ELISA. As shown in Fig. 3, treatment of pancreatic acini with 1 μM SP or 0.1 μM
caerulein caused a significant production in MCP-1, MIP-1α, and MIP-2. Pretreatment of acini with 50 μM NBD peptide, followed by stimulation with 1 μM SP or 0.1 μM caerulein, significantly attenuated the production in MCP-1 (Fig. 3A), MIP-1α (Fig. 3B), and MIP-2 (Fig. 3C) compared with that in SP- or caerulein-treated pancreatic acini.

**SP and caerulein may act via distinct pathways in inducing chemokine synthesis.** Studies (3, 5, 7) have shown that treatment of pancreatic acinar cells with supramaximal dose of caerulein (0.1 μM) leads to activation of NF-κB as well as production of chemokine, both in vitro and in vivo. Our aim was to investigate the effect of both SP and caerulein on mouse pancreatic acini. We, therefore, incubated the cells with both 1 μM SP and 0.1 μM caerulein for 45 min at 37°C, after which the supernatant obtained was used for chemokines MCP-1, MIP-1α, and MIP-2 detection by ELISA. As shown in Fig. 4, stimulation of acini with SP or caerulein caused a significant increase in chemokine synthesis compared with that in placebo-treated acini. Furthermore, when mouse pancreatic acinar cells were treated with both SP and caerulein, the increase in chemokines MCP-1 (Fig. 4A), MIP-1α (Fig. 4B), and MIP-2 (Fig. 4C) production was significantly higher compared with that in either SP- or caerulein-treated cells. These data show that SP and caerulein act via overlapping, yet distinct, pathways in activating chemokine synthesis.

**Effect of SP treatment on amylase secretion in mouse pancreatic acinar cells.** Treatment of mouse pancreatic acini with different doses ranged from 10−12 to 10−6 M SP. After incubation for 45 min at 37°C, the supernatant was used for the amylase assay. As shown in Fig. 5, SP did not have any effect on amylase secretion. As a positive control, mouse pancreatic acini were also treated with different concentration of caerulein, ranging from 10−12 to 10−7 M. In accord with previously reported findings (7), a biphasic stimulation/inhibition of amylase secretion by increasing concentration of caerulein was observed. Moreover pretreatment of acini with 50 μM NBD peptide, followed by stimulation with caerulein ranging from 10−12 to 10−7 M, had no effect on amylase secretion (data not shown).

**DISCUSSION**

It is generally believed that the earliest events in the evolution of AP lead to intraacinar cell activation of digestive zymogens and that those enzymes, once activated, cause acinar cell injury (30, 31). Recent studies (10, 15, 33) have suggested that the ultimate severity of the resulting pancreatitis may be determined by events that occur subsequent to acinar cell injury, such as inflammatory cell recruitment, activation, generation, and release of cytokines and other chemical mediators of inflammation, including SP and chemokines. SP has been detected within the pancreas, and it has been suggested that it may act as a neurotransmitter for sensory afferent nerves in the pancreas. Receptors for SP have also been detected on guinea pig pancreatic acinar cells (34, 36, 37), and now it is known that mouse pancreatic acini also express NK1R (7). SP has been shown to activate the transcription factor NF-κB in macrophages (22). Intrapulmonary administration of SP results in rapid activation of NF-κB in lung tissues. Its administration also results in the appearance of the neutrophil-attracting CXC chemokine MIP-2 in bronchoalveolar lavage fluid (25). Al-

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*Fig. 3. SP or Cae-induced chemokine synthesis is abolished with NBD, an NF-κB inhibitor. The effect of NF-κB inhibitor NBD on MCP-1 (A), MIP-1α (B), and MIP-2 (C) production after being stimulated by SP or Cae. Freshly isolated mouse acini, obtained from 3 mice, were preincubated with or without NBD (50 μM) for 2 h, followed by stimulation with 1 μM SP or 0.1 μM Cae for 45 min at 37°C. MCP-1, MIP-1α, and MIP-2 levels in conditioned media were measured by ELISA. Results shown are means ± SE. *P ≤ 0.05 when SP or Cae-treated acini were compared with placebo-treated acini. †P ≤ 0.05 when NBD and SP-treated acini were compared with SP alone-stimulated cells. †P ≤ 0.05 when NBD and Cae-treated acini were compared with Cae alone-stimulated cells.*
though pancreatic acinar cells have earlier been shown to express NK1R (7, 20, 21), the mechanism by which SP induces synthesis of chemokines in AP is not yet known. To that end, we went on to investigate the effect of SP on chemokine synthesis, such as CC chemokines MCP-1, MIP-1α, and CXC chemokine MIP-2 on mouse pancreatic acini. Furthermore, we showed that the increase in chemokine synthesis was mediated by the activation of NF-κB. We demonstrated that SP induced chemokine synthesis in a dose-dependent manner. SP at a dose of 1 μM significantly increased NF-κB activation, resulting in significant chemokine synthesis compared with that in the placebo-treated control.

NF-κB is a ubiquitous transcription factor that is implicated in the regulation of many genes which code for mediators of the immune, acute phase and inflammatory responses (2). The activation mechanism of NF-κB by proinflammatory cytokines IL-1 and TNF-α is well known. Briefly, NF-κB is usually composed of the two subunits p65 (also called RelA) and p50, although these polypeptides belong to a family of proteins that can form homo- or heterodimers with each other (11). NF-κB is sequestered in the cytoplasm of most resting cells through its association with an inhibitory protein called IκB. During stimulation by IL-1 or TNF-α, a whole cascade of adaptor proteins and protein kinases is activated, leading to the phosphorylation of IκB by the IKK-α/IKK-β (19). This depends on the regulatory protein NEMO (NF-κB essential modifier), associated with the complex containing two kinases, IKK-α and IKK-β (1, 16). Once phosphorylated, IκB is ubiquitinated and subsequently degraded through 26S proteasome. Consequently, NF-κB is freed to migrate into the nucleus and binds to its consensus decameric sequence located in the promoter region of several genes involved in the proinflammatory response, encoding various immunoreceptors, cell adhesion molecules, cytokines, and chemokines (2).

To our knowledge, this is the first time that a neuropeptide SP is shown to induce activation of this transcriptional activator NF-κB, as well as chemokine synthesis in mouse pancreatic acinar cells. To prove that the effect of SP was specific on NF-κB activation and chemokine synthesis, we pretreated the acini with NBD. NBD is a short cell-permeable peptide spanning IKK-β. This peptide was already shown to disrupt the association of NEMO with IKK-β in vitro, to block TNF-α-induced NF-κB activation, and to effectively ameliorate responses to various inflammatory stimuli in vivo (23). Pretreat-
ment of mouse pancreatic acini with NBD completely attenuated the chemokine synthesis induced by SP. This shows that the increase in chemokine synthesis induced by SP was specifically due to NF-κB activation.

It is known that treatment with a supramaximal dose of caerulein, a CCK analog, induces NF-κB activation as well as chemokine synthesis in pancreatic acini both in vitro as well as in vivo (5, 6). A similar observation was made when mouse pancreatic acini were treated with a supramaximal dose of caerulein: there was a significant upregulation in NF-κB activation. Furthermore, we showed that pretreatment with NBD significantly attenuated caerulein-induced NF-κB activation. To investigate the combined effect of SP and caerulein on mouse pancreatic acini, the cells were incubated simultaneously with SP and caerulein. SP and caerulein independently activated the synthesis of chemokine MCP-1, MIP-1α, and MIP-2 in mouse pancreatic acini. However, when cells were treated with both SP and caerulein, the increase in chemokine synthesis was significantly higher compared with that in cells treated with either SP or caerulein alone. Furthermore, SP has no effect on amylase secretion. This is in contrast to caerulein, which is known to produce a dose-dependent response in amylase secretion (7). Studies characterizing acinar cell secretion in the presence of increasing caerulein concentrations typically reveal a biphasic dose-response relationship, with stimulation at low CCK concentrations and inhibition at supramaximally stimulating concentrations (10−7 M). Treatment of mouse pancreatic acini with different concentration of SP does not have any effect on amylase secretion, unlike that observed with caerulein treatment. These results suggest that SP and caerulein act via overlapping, yet distinct, pathways to stimulate chemokine synthesis in pancreatic acinar cells.

In conclusion, SP induced synthesis of CC chemokines MCP-1, MIP-1α, and CXC chemokine MIP-2 via NF-κB dependent pathway. This is the first direct evidence of the role of SP acting via NK1R present on mouse pancreatic acini in inflammation and points to the mechanism by which SP contributes to inflammation in acute pancreatitis.

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GRANTS

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