Preprotachykinin-A gene deletion protects mice against acute pancreatitis and associated lung injury

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Submitted 11 April 2002; accepted in final form 14 January 2003

Preprotachykinin-A gene deletion protects mice against acute pancreatitis and associated lung injury. Am J Physiol Gastrointest Liver Physiol 284: G830–G836, 2003; 10.1152/ajpgi.00140.2002.—Impaired lung function in severe acute pancreatitis is the primary cause of morbidity and mortality in this condition. Preprotachykinin-A (PPT-A) gene products substance P and neurokinin (NK)-A have been shown to play important roles in neurogenic inflammation. Substance P acts primarily (but not exclusively) via the NK1 receptor. NKA acts primarily via the NK2 receptor. Earlier work has shown that knockout mice deficient in NK1 receptors are protected against acute pancreatitis and associated lung injury. NK1 receptors, however, bind other peptides in addition to substance P, not all of which are derived from the PPT-A gene. To examine the role of PPT-A gene products in acute pancreatitis, the effect of PPT-A gene deletion on the severity of acute pancreatitis and the associated lung injury was investigated. Deletion of PPT-A almost completely protected against acute pancreatitis-associated lung injury, with a partial protection against local pancreatic damage. These results show that PPT-A gene products are critical proinflammatory mediators in acute pancreatitis and the associated lung injury.

ACUTE PANCREATITIS IS A COMMON clinical condition, whose incidence has been increasing over recent years (1, 3, 22, 26). In the majority of patients, the condition is mild, but up to 25% of patients suffer a severe attack, and between 30 and 50% of these will die (1, 3). Most cases are secondary to biliary disease or excess alcohol consumption. The exact mechanisms by which diverse etiological factors induce an attack are still unclear, but once the disease process is initiated, common inflammatory and repair pathways are invoked. If this inflammatory reaction is very strong, it leads to a systemic inflammatory response syndrome (SIRS), and it is this systemic response that is ultimately responsible for the majority of the morbidity and mortality (1, 3). Systemic leukocyte activation is a direct consequence of SIRS, and if excessive, it can lead to distant organ damage and multiple organ dysfunction syndrome (MODS) (1, 3). Lung injury that manifests itself clinically as adult respiratory distress syndrome is a major component of MODS associated with acute pancreatitis.

Substance P and neurokinin (NK)-A are neuropeptide products of the preprotachykinin-A (PPT-A) gene. The primary RNA transcript of the PPT-A gene is spliced to yield three different forms of mRNA termed α-, β-, and γ-forms. The α-, β-, and γ-PPT-A mRNAs code for the synthesis of substance P, whereas β- and γ-PPT-A mRNAs code for the synthesis of both substance P and NKA. Substance P is released from nerve endings in many tissues. Subsequent to its release, substance P binds primarily, but not exclusively, to NK1 receptors on the surface of effector cells and, in addition to being a mediator of pain, acts as a proinflammatory mediator in many inflammatory states (20) including asthma (15, 27), immune-complex-mediated lung injury (7), experimental arthritis (40), and inflammatory bowel disease (41). NKA binds primarily to NK2 receptors. Substance P and NKA bind to NK3 receptors as well, although with a lower affinity. Substance P has been detected within the pancreas, and it has been suggested that substance P may act as a neurotransmitter for sensory afferent nerves in the pancreas. Receptors for substance P have also been detected on guinea pig pancreatic acinar cells and the neuropeptide acts as a secretagogue, stimulating amylase secretion from acinar cells via a G protein, phospholipase-α, inositol phosphate-, and calcium-mediated mechanisms in that species (37–39). Rat pancreatic acinar cells apparently do not express receptors for substance P, and the neuropeptide does not stimulate enzyme secretion from rat acinar cells. By contrast, recent work (4) has shown the presence of substance P in the mouse pancreas and of NK1 receptors on mouse pancreatic acinar cells. On induction of pancreatitis in mice, there is a severalfold upregulation of pancreatic substance P levels and of NK1 receptors on pancreatic acinar cells (4). Moreover, knockout mice deficient in

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NK1 receptors are protected against pancreatitis and associated lung injury (4, 18). These results suggest an important proinflammatory role for neurogenic inflammation and NK1 receptors in acute pancreatitis and associated lung injury. These results are further substantiated by the observation that knockout mice deficient in neutral endopeptidase, the enzyme that hydrolyzes substance P, thereby terminating its action, are more susceptible to acute pancreatitis and associated lung injury (5, 24).

NK1 receptors, however, bind other peptides in addition to substance P, and not all of these are products of the PPT-A gene. Also, the PPT-A gene products substance P and NKA bind to other receptors, albeit with a lower affinity, in addition to NK1 and NK2 receptors (8, 19, 20, 33). The present study, therefore, aims to investigate the contribution of the PPT-A gene products on the pathogenesis of acute pancreatitis and associated lung injury by using mice deficient in PPT-A gene.

MATERIALS AND METHODS

Induction of acute pancreatitis. All experiments were performed under a current Home Office Project License within the regulations of the Animals (Scientific Procedures) Act 1986. PPT-A knockout mice were generated and bred as described previously (10). Caerulein was obtained from Research Plus (Bayonne, NJ). PPT-A−/− mice with BALB/c background and PPT-A+−/− BALB/c mice (16–18 g) were randomly assigned to control or experimental groups by using eight animals for each group. Animals were given hourly intraperitoneal injections of normal saline or saline containing caerulein (50 μg/kg) for 3, 6, or 10 h. One hour after the last caerulein injection, animals were killed by an intraperitoneal injection of a lethal dose of pentobarbital, and samples were prepared for storage.

Harvested heparinized blood was centrifuged (3,000 g, 15 min, 4°C), and the plasma was removed and stored at −70°C until analysis. Random cross sections of the head, body, and tail of the pancreas and samples of the right lung were fixed in 4% neutral phosphate-buffered formalin for 48 h and then embedded in paraffin wax. A sample of pancreas was weighed and then dried for 72 h at 60°C and reweighed to determine pancreatic water content (4). Samples of pancreas and lung were removed, weighed, and then stored at −70°C for subsequent measurement of tissue MPO activity as described below. A separate set of mice (8 animals in each group) was used to determine pulmonary microvascular permeability.

Preparation of pancreatic acini. Pancreatic acini were obtained from mouse pancreas by collagenase treatment as described previously (4). Briefly, pancreata from PPT-A−/− and PPT-A−/− mice were removed under aseptic conditions, infused with collagenase buffer A (in mM: 140 NaCl, 4.7 KCl, 1.13 MgCl2, 1 CaCl2, 10 glucose, and 10 HEPES, pH 7.2) containing 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. Digested tissue was passed through 50 μm nylon mesh, washed twice with buffer A (10% CO2, 90% air) before further experiments. Cell viability was assayed by trypan blue exclusion. Acini were incubated with varying concentrations of caerulein (Research Plus) for 30 min, and amylase secretion was determined as described in Amylase estimation.

Amylase estimation. Amylase activity was measured by using a kinetic spectrophotometric assay. Plasma samples and acinar cell supernatants were incubated with the substrate, 4,6-ethylenediamine (G7)-p-nitrophenyl (G1)-1-b-maltoside (Sigma, Dorset, UK) for 2 min at 37°C, and absorbance was measured every minute for the subsequent 2 min at 405 nm (4, 31). The change in absorbance was used to calculate the amylase activity.

MPO estimation. Neutrophil sequestration in pancreas and lung was quantified by measuring tissue MPO activity (2). Tissue samples were thawed, homogenized in 20 mM phosphate buffer (pH 7.4), centrifuged (10,000 rpm, 4°C), and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonicating (40 s). The sample was then centrifuged (10,000 rpm, 5 min, 4°C), and the supernatant was used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mM tetramethylbenzidine (Sigma), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide. This mixture was incubated at 37°C for 10 min, the reaction was terminated with 0.18 M H2SO4, and the absorbance was measured at 450 nm. This absorbance was then corrected for the calculated dry weight of the tissue sample, and results were expressed as activity per unit of dry weight (fold increase over control).

Measurement of pulmonary microvascular permeability. Two hours before death, each animal received an intravenous bolus injection containing FITC-albumin (5 mg/kg, Sigma). Immediately after death, the trachea was exposed, and the lungs were lavaged three times with 1 ml of normal saline. The lavage fluid was combined, and FITC fluorescence was measured in the lavage fluid and plasma (excitation = 494 nm, emission = 520 nm). The ratio of fluorescence emission in lavage fluid to plasma was calculated and used as a measure of pulmonary microvascular permeability (4).

Morphological examination. Paraffin-embedded pancreas and lung samples were sectioned (4 μm), stained with hematoxylin and eosin, and examined with light microscopy.

Statistics. Data are expressed as the means ± SE. In all figures, vertical bars denote the SE, and the absence of such bars indicates that the SE is too small to illustrate. The significance of changes was evaluated by using Student’s t-test when the data consisted of only two groups or by ANOVA when three or more groups were compared. 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

Effect of PPT-A gene deletion on caerulein-induced acute pancreatitis. Wild-type mice given intraperitoneal injections of supramaximally stimulating doses of the secretagogue caerulein developed acute necrotizing pancreatitis. As shown in Figs. 1–4, this was manifested by a dose and time-dependent rise in plasma amylase activity, pancreatic water content (a measure of pancreatic edema), pancreatic MPO activity (an indicator of neutrophil sequestration in the pancreas), and morphological evidence of extensive acinar cell necrosis. PPT-A−/− mice also showed evidence of pancreatic injury in acute pancreatitis. Genetic deletion of PPT-A, however, markedly reduced the severity of cae-
rulein-induced pancreatitis. At induction of acute pancreatitis, the rise in plasma amylase activity (Fig. 1), pancreatic water content (Fig. 2), and pancreatic MPO activity (Fig. 3) were significantly subdued in PPT-A−/− mice compared with the wild-type controls. Histological examination of pancreas sections showed significant protective effect of PPT-A gene deletion on acinar cell injury/necrosis in acute pancreatitis (Fig. 4).

Effect of PPT-A gene deletion on caerulein-induced acute pancreatitis-associated lung injury. Acute pancreatitis in wild-type mice induced by 6 and 10 (but not 3) hourly injections of caerulein 50 μg/kg was associated with lung injury. As shown in Fig. 5, caerulein-induced pancreatitis was associated with a rise in lung MPO activity, indicating the presence of sequestered neutrophils. Furthermore, leakage of intravenously administered FITC-labeled albumin into the alveolar space, a measure of pulmonary microvascular permeability, was increased during secretagogue-induced pancreatitis (Fig. 6). Histological examination of lung sections confirmed evidence of lung injury in acute pancreatitis (Fig. 7). Genetic deletion of PPT-A resulted in a marked reduction in the severity of pancreatitis-associated lung injury. Lung MPO activity, lung microvascular permeability, and microscopic evidence of lung injury are reduced in PPT-A−/− mice when compared with the PPT-A+/+ controls (Figs. 5–7). Indeed there was an almost complete protection against acute pancreatitis-associated lung injury in PPT-A−/− mice.

Effect of PPT-A gene deletion on caerulein-stimulated enzyme secretion from pancreatic acini. In accordance with previously reported findings (4), biphasic stimulation/inhibition of amylase secretion by increasing concentrations of caerulein was observed when freshly prepared pancreatic acini from wild-type PPT-A+/+ mice was evaluated (Fig. 8). Similar changes were observed when acini obtained from PPT-A−/− mice were evaluated. These findings indicate that the deletion of PPT-A does not alter pancreatic cell responsiveness to the secretagogue caerulein.

DISCUSSION

Substance P is a major mediator of neurogenic inflammation in several tissues including skin (20, 21), cardiovascular tissue (6, 9, 25), cephalic structures (13, 17, 26), respiratory tract (7, 15, 27), genitourinary tract (23, 30), and gastrointestinal tract (12, 16, 25, 32). NKA has also been shown to play an important role in neurogenic inflammation in several conditions. Indeed neurogenic inflammation likely underlies several disease conditions (20), such as asthma (15, 27), immune-complex-mediated lung injury (7), experimental arthritis (40), and inflammatory bowel disease (41). Based on our studies with NK1 receptor knockout mice, we pre-
Previously proposed a proinflammatory contribution of substance P in the pathogenesis of acute pancreatitis and associated lung injury (8). In that study, mice genetically deficient in the NK1 receptor were protected against acute pancreatitis and associated lung injury. However, NK1 receptors bind other peptides in addition to substance P (and NKA), and not all of these are products of PPT-A gene. Also, substance P and NKA bind to other receptors, albeit with a lower affinity (8, 19, 20, 33).

This paper aims to investigate the contribution of PPT-A gene products to the pathogenesis of acute pancreatitis and associated lung injury, by using knockout mice deficient in the PPT-A gene. It has previously been shown that although the behavioral response to mildly painful stimuli is intact in these mice, the response to moderate-to-intense pain is significantly reduced. Neurogenic inflammation, evidenced by plasma extravasation and hindpaw edema induced by capsaicin treatment, which results from peripheral release of substance P, is almost absent in the mutant mice (10). By contrast, inflammation produced by complete Freund’s adjuvant, which is nonneurogenic, is intact in these mice (10).

In PPT-A knockout mice, we induced acute pancreatitis by giving them supramaximally stimulating doses of the secretagogue caerulein. This secretagogue-induced model of acute pancreatitis is characterized by hyperamylasemia, pancreatic edema (increased pancreatic water content), sequestration of neutrophils within the pancreas (i.e., increased pancreatic MPO activity), and morphological evidence of acinar cell injury/necrosis. Severe, but not mild, acute pancreatitis is also associated with lung injury characterized by sequestration of neutrophils within the lung (increased lung MPO activity) and increased pulmonary microvascular permeability (increased leakage of the intravenously administered FITC-labeled albumin into the bronchoalveolar lavage fluid). Our results show that deletion of PPT-A results in a marked decrease in each
of the parameters that characterize the severity of secretagogue-induced acute pancreatitis. The earliest events in acute pancreatitis are mediated by the action of caerulein on the CCK receptors on pancreatic acinar cells, which may account for the residual pancreatic injury in the mutant mice. A lack of effect of PPT-A gene deletion on the biphasic caerulein dose-response curve for amylase secretion indicates that the early acinar cell responses to caerulein are unaltered in the PPT-A null mice and that the deletion of PPT-A does not alter pancreatic cell responsiveness to caerulein. Subsequently, however, PPT-A gene products, acting via the NK1 receptors on pancreatic acinar cells, may increase the severity of acinar cell injury in wild-type but not PPT-A<sup>−/−</sup> mice. Indeed, PPT-A gene deletion does not appear to have an effect on mild, acute pancreatitis induced by three hourly injections of caerulein (50 μg/kg), whereas the severity of more severe acute pancreatitis induced by 6 and 10 hourly injections of caerulein is significantly reduced.

Results presented in this paper show that mild acute pancreatitis is not associated with lung injury, whereas severe pancreatitis is. Lung injury in this model is unlikely to be due to a direct effect of caerulein. In one of the earliest studies on this subject (14), it was shown that perfusion of isolated lungs with caerulein did not lead to any evidence of lung injury or functional alterations. A similar picture is observed in clinical acute pancreatitis (1, 3). Significantly, there is an almost complete protection against acute pancreatitis-associated lung injury in PPT-A knockout mice. It has previously been shown (4) that pancreatic levels of the PPT-A gene product substance P are elevated within 3–4 h of starting caerulein administration before any evidence of lung injury can be observed and remain elevated for 12 h, pointing to the pancreas being the primary site of action of substance P. Results also indicate that leukocytes, particularly neutrophils, are most likely to reflect the downstream lung injury, because protection in the pancreas by plasma amylase and pancreatic water content is minimal, whereas that in terms of pancreatic MPO activity is more impressive. On the basis of these data, however, we cannot conclusively say whether the protection against lung injury in PPT-A<sup>−/−</sup> mice is because acute pancreatitis is not severe enough in PPT-A<sup>−/−</sup> mice or because of a direct protective effect on lung injury (or both). Nevertheless, the reduction in pancreatitis and lung injury severity that is brought about by PPT-A deletion leads us to conclude that the PPT-A gene product(s) substance P and/or NKA are important proinflammatory mediators in the pathogenesis of acute pancreatitis and associated lung injury.

A similar partial protection against local pancreatic injury in, and an almost complete protection against, lung injury in acute pancreatitis was observed in mice genetically deficient in the NK1 receptor (4, 18). The protective effect of the gene deletion is not limited to the caerulein model, because it has been shown (24) that NK1 receptor gene deletion protects mice against acute pancreatitis induced by the administration of a
choline-deficient diet supplemented with ethionine. The question of the contribution of other NK1-receptor agonists and of individual contribution of substance P and NKA (possibly via the NK2 receptor) remains to be answered and will be the subject of future investigations.

Mechanisms by which PPT-A gene products act as proinflammatory mediators in acute pancreatitis are not very clear, although there are several possibilities. Of the PPT-A gene products, substance P (and possibly NKA) may act via NK1 receptors present on pancreatic acinar cells (4), contributing to acinar cell injury in acute pancreatitis. Other NK receptors, such as NK2 and NK3 receptors, have not yet been found on pancreatic acinar cells.

Alternatively, PPT-A gene products may act on endothelial cells to increase vascular permeability and promote edema formation (16, 18). Interactions between different NK receptors are mediated by β-arrestin (35). It was previously shown (17a, 32a) that some cells, such as enteric neurons and microvascular endothelial cells, coexpress NK1 and NK3 receptors. Schmidlin et al. (35) reported that in cells coexpressing NK1 and NK3 receptors, prior activation of the NK1 receptors inhibited homologous desensitization of the NK3 receptors, but activation of NK3 receptors had no effect on NK1 receptor desensitization. PPT-A gene products have been shown to act on both NK1 and NK3 receptors, and it is likely that on activation of the NK1 receptor, the NK3 receptor would become resistant to desensitization and be the major receptor responding to the tachykinins.

Yet another possibility is that PPT-A gene products may act via the inflammatory cells, such as neutrophils (by inducing chemotaxis) or mast cells (11, 29, 34, 36). Further studies shall be directed at defining the mechanism of action of PPT-A gene products as proinflammatory mediators in the pathogenesis of acute pancreatitis and associated lung injury.

This work was supported by Academic Research Fund Start-Up Grant R-184–000–048–112, Academic Research Fund Grant R-184–000–054–112, Biomedical Research Council Grant 02/12/19/110, and Wellcome Trust Project Grant 003393.

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