Local disruption of the celiac ganglion inhibits substance P release and ameliorates caerulein-induced pancreatitis in rats

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Submitted 19 September 2005; accepted in final form 14 February 2006

Noble, Marc D., Joelle Romac, Yu Wang, Jay Hsu, John E. Humphrey, and Rodger A. Liddle. Local disruption of the celiac ganglion inhibits substance P release and ameliorates caerulein-induced pancreatitis in rats. Am J Physiol Gastrointest Liver Physiol 291: G128--G134, 2006; doi:10.1152/ajpgi.00442.2005.—Primary sensory neurons of the C and Aδ subtypes express the vanilloid capsacin receptor TRPV1 and contain proinflammatory peptides such as substance P (SP) that mediate neurogenic inflammation. Pancreatic injury stimulates these neurons causing the release of SP in the pancreas resulting in pancreatic edema and neutrophil infiltration that contributes to pancreatitis. Axons of primary sensory neurons innervating the pancreas course through the celiac ganglion. We hypothesized that disruption of the celiac ganglion by surgical excision or inhibition of C and Aδ fibers through blockade of TRPV1 would reduce the severity of experimental pancreatitis by inhibiting neurogenic inflammation. Resiniferatoxin (RTX) is a specific TRPV1 agonist that, in high doses, selectively destroys C and Aδ fibers. Sprague-Dawley rats underwent surgical ganglionectomy or application of 10 μg RTX (vs. vehicle alone) to the celiac ganglion. One week later, pancreatitis was induced by six hourly intraperitoneal injections of caerulein (50 μg/kg). The severity of pancreatitis was assessed by serum amylase, pancreatic edema, and pancreatic myeloperoxidase (MPO) activity. SP receptor (neurokinin-1 receptor, NK-1R) internalization in acinar cells, used as an index of endogenous SP release, was assessed by immunocytochemical quantification of NK-1R endocytosis. Caerulein administration caused significant increases in pancreatic edema, serum amylase, MPO activity, and NK-1R internalization. RTX treatment and ganglionectomy significantly reduced pancreatitis edema by 46% (P < 0.001) and NK-1R internalization by 80% and 51% (P < 0.001 and P < 0.05, respectively). RTX administration also significantly reduced MPO activity by 47% (P < 0.05). Neither treatment affected serum amylase, consistent with a direct effect of caerulein. These results demonstrate that disruption of or local application of RTX to the celiac ganglion inhibits SP release in the pancreas and reduces the severity of acute secretagogue-induced pancreatitis. It is possible that selectively disrupting TRPV1-bearing neurons could be used to reduce pancreatitis severity.

TRPV1; endocytosis; resiniferatoxin; neurogenic inflammation; neurokinin-1 receptor

NEUROGENIC INFLAMMATION RESULTS from activation of specific afferent sensory nerves leading to the release of neurotransmitters from nerve terminals that ultimately cause vasodilation, plasma extravasation, and cellular infiltration (11, 14). These effects can lead to mast cell degranulation, neutrophil adhesion, and the production of reactive oxygen species (1, 32, 34). Neurogenic inflammation is a major factor in the inflammatory response to injury in a number of organs including the pancreas. Recent data suggest that activation of primary sensory neurons and release of inflammatory mediators are important in determining the severity of pancreatitis (25).

Substance P (SP) is a neuropeptide located within primary afferent sensory neurons and has a substantial role in mediating neurogenic inflammation. SP release causes extravasation of plasma from postcapillary venules in the pancreas as well as other gastrointestinal organs (5). SP is the specific ligand for the neurokinin-1 receptor (NK-1R).

NK-1Rs are located on a variety of cell types including inflammatory cells, smooth muscle cells, vascular endothelial cells, surface epithelia, and pancreatic acinar cells (3, 24, 26, 31). Activation of this receptor has been shown to mediate the release of various proinflammatory agents (24). Further experiments using NK-1R knockout mice have shown that pancreatic inflammation can be significantly attenuated in caerulein-induced pancreatitis (7). Alternatively, local infusion of the NK-1R antagonist CP-96345 into the pancreatic duct has been shown to significantly reduce the severity of pressure-induced pancreatitis (8). These data demonstrate that activation of the NK-1R is an important step in initiating the inflammatory cascade in the pancreas leading to more severe forms of pancreatitis and that blocking this receptor can reduce the severity of pancreatitis. Activation of the vanilloid capsacin receptor TRPV1 located on C and Aδ primary sensory neurons has been shown to mediate the release of SP (24). Pancreatic injury stimulates these neurons through the activation of TRPV1. Moreover, blocking TRPV1 significantly reduced SP release and pancreatitis severity (24).

Resiniferatoxin (RTX) is a specific TRPV1 agonist that, in high doses, selectively destroys C and Aδ fibers by dramatically increasing intracellular calcium concentrations and disrupting vital organelles (28). In the current study, we hypothesized that local denervation of the C and Aδ fibers by surgical ganglionectomy or administration of RTX to the ganglion would reduce the severity of acute secretagogue-induced pancreatitis. The celiac ganglion has nerves coursing it that directly innervate the pancreas, thus making it an attractive target for surgical or pharmacological denervation. We demonstrate that surgical excision or application of RTX to the celiac ganglion inhibits SP release and reduces the severity of secretagogue-induced pancreatitis.

MATERIALS AND METHODS

Animal protocol and experimental design. All animal experiments were performed with approval of the Duke University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats, weigh-

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ing 250–300 g were purchased from Charles River Laboratories (Wilmington, MA) and housed in climate-controlled rooms with a 12:12-h light-dark cycle. All rats were fed standard laboratory diet until an overnight fast before the experiment. Rats were allocated to the following groups (n = 12): control, ganglionectomy, or RTX administration. On the day of the experiment, the rats were anesthetized with xylazine and ketamine in a 1:10 ratio at a dose of 0.1 ml/100 g body wt administered intramuscularly. At laparotomy, the intraperitoneal organs were moved superiorly in the abdominal cavity allowing for exposure of the aorta, superior mesenteric artery, and celiac artery. The technique of celiac ganglionectomy has been described previously (23), but briefly, the celiac ganglion was located within the area between the superior mesenteric artery and celiac artery. For those rats undergoing celiac ganglionectomy, the ganglion was removed. For those rats in the RTX group, 100 μl of RTX at a concentration of 100 μg/ml were applied to the ganglion over 30 min to apply a total dose of 10 μg. For those rats in the sham surgical group, 100 μl of vehicle were applied over 30 min. Rats were observed for 7 days after surgery during which time they were allowed food and water ad libitum. Seven days postoperatively, six rats from each group were designated to receive either caerulein or vehicle.

**Caerulein-induced pancreatitis.** The CCK analog caerulein was purchased from Bachem California (Torrance, CA) and dissolved in 0.1 M NaHCO₃ followed by dilution in isotonic saline. Caerulein was prepared the morning of the experiment and stored on ice. After an overnight fast, caerulein was administered as six hourly intraperitoneal injections at a supramaximal-stimulating dose of 50 μg/kg per injection (15). Control rats received six hourly intraperitoneal injections of isotonic saline. One hour after the last caerulein injection, rats were euthanized in a carbon dioxide precharged chamber. Mixed arteriovenous blood was collected by decapitation for measurement of serum amylase activity. The pancreas was then removed, weighed, and compared with body weight as a measure of edema and divided for histological grading, measurement of tissue myeloperoxidase (MPO), and measurement of NK-1R endocytosis.

**Serum amylase concentration.** Mixed arteriovenous blood was centrifuged for 10 min at 1,500 g. The serum amylase concentration was measured by using the Procin yellow starch assay as previously described (13). A standard curve was prepared by using crude type VI-B α-amylase (Sigma, St. Louis, MO), and serum amylase was expressed as milligrams per milliliter.

**MPO activity.** Portions of pancreas were immediately frozen. MPO, an enzyme produced by neutrophils, was used as a marker of inflammation associated with neutrophil infiltration, as previously described (4). The assay was performed in microtiter plates using a Safire plate reader from Tecan, with measurement wavelength at 450 nm and reference wavelength at 650 nm. Human MPO (Sigma) was used as standard. Protein concentration of the pancreatic extract was determined using the micro BCA protein assay (Pierce). Results were expressed in MPO units per milligram protein.

**Histology.** Portions of the pancreas were fixed overnight at room temperature in a pH-neutral, phosphate-buffered, 10% formalin solution. The tissue was then embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Histological severity of pancreatitis was rated by a histologist blinded to the study design using a scale of 0–4 and included parameters of edema, necrosis, and neutrophil infiltration.

**Immunocytochemical analysis of SP release.** SP release was assessed by measurement of NK-1R endocytosis as described previously (19, 24).

**Quantification of NK-1R endocytosis.** Immunostained sections were analyzed using a Zeiss LSM-410 inverted krypton-argon confocal laser-scanning coupled to a Zeiss Axiovert 100 microscope as described (19, 24). Optical sections (0.5 μm) of 512 × 512 pixels were obtained and processed using Adobe PhotoDeluxe. Quantification of NK-1R endocytosis was performed by analyzing 10 NK-1R immunoreactive acinar cells per mouse (n = 5) and determining the number of these cells containing >15 NK-1R-immunoreactive endosomes. Cytoplasmic endosomes were distinguished from plasma membrane-associated NK-1R immunoreactivity by ensuring that the nucleus of the acinar cells was in the same optical section as the NK-1R-immunoreactive endosomes.

**Statistical analysis.** Results are means ± SE. Statistical comparisons among groups were examined by one-way ANOVA with the Tukey posttest, using GraphPad Prism version 4.02. Statistical significance was set at P < 0.05.

**RESULTS**

Repeated administration of supramaximal-stimulating doses of caerulein caused pancreatitis as evidenced by elevations in pancreatic edema, MPO activity, and serum amylase. Caerulein-treated, sham-operated rats had a 3.25-fold increase in pancreas-to-body weight ratio (0.004 vs. 0.013, P < 0.001, n = 6) (Fig. 1). As shown in Fig. 2, caerulein substantially increased the size of the gross pancreas. Caerulein-treated, sham-operated rats also demonstrated a 2.8-fold increase in MPO levels (27.5 vs. 76.1 U/mg pancreatic protein, P < 0.01) (Fig. 3) and a 3.5-fold increase in amylase levels (1.2 vs. 6.6 mg/ml, P < 0.001) (Fig. 4) compared with vehicle-treated, sham-operated rats.

We hypothesized that primary sensory neuronal function, particularly SP release, was important in the pancreatic response to caerulein-induced injury. To investigate whether SP release occurred in the pancreas following caerulein administration, NK-1R internalization on pancreatic acinar cells was quantified. Caerulein treatment significantly increased NK-1R internalization on pancreatic acinar cells that was quantified. Caerulein treatment significantly increased NK-1R internalization with only 6% of cells demonstrating ≥15 vesicles per cell under basal conditions compared with 92% of cells following caerulein treatment (P < 0.0001) as shown in Figs. 5 and 6.

The axons of primary sensory nerves innervating the pancreas course through the celiac ganglion. To determine whether interruption of these nerves might affect SP release and the inflammatory response following caerulein administration, su-
gical ganglionectomy was performed. Celiac ganglionectomy caused a 46% reduction in pancreas-to-body weight ratio (0.013 vs. 0.007, \( P < 0.001 \)) (Fig. 1), indicating a significant reduction in caerulein-induced pancreatic edema. Similarly, NK-1R internalization was reduced by 51% in caerulein-treated rats postceliac ganglionectomy. Although MPO activity was lower in rats that underwent ganglionectomy, this result was not statistically significant. Amylase activity was not statistically different between any of the observed groups, which is consistent with caerulein having a direct effect on the pancreas (24, 25).

To determine whether selective destruction of primary sensory neurons in the celiac ganglion may alter caerulein-induced pancreatitis, RTX was applied to the celiac ganglion. RTX is a selective agonist of TRPV1 that, in high concentrations, selectively destroys C and A\(\delta\) fibers. Importantly, RTX administration to the celiac ganglion reduced caerulein-induced pancreatic edema by 46% (0.013 vs. 0.007 g, \( P < 0.001 \)) (Fig. 1),

Fig. 2. Effects of caerulein, GN, and RTX treatment on pancreatic size. Photographs are representative of pancreata from rats treated with vehicle alone (A), caerulein alone (B), caerulein + GN (C), and caerulein + RTX (D).

Fig. 3. Effect of GN and RTX on caerulein-induced MPO activity. Caerulein administration significantly increased pancreatic MPO activity that was inhibited by RTX pretreatment. (*\( P < 0.05 \), **\( P < 0.01 \), \( n = 5 \) or 6).

Fig. 4. Effect of GN and RTX on serum amylase. Caerulein treatment significantly increased serum amylase levels (**\( P < 0.001 \), \( n = 6 \)). These elevated values were unaltered by GN or RTX pretreatment.
MPO activity by 47% (76.1 vs. 40.2 U/mg protein, P < 0.05) (Fig. 3), and NK-1R internalization by 80% (Figs. 5 and 6). To confirm that RTX was acting locally at the ganglion and did not have a systemic effect, an eye-wipe test was performed. The eye-wipe responses to corneal application of 0.1% capsaicin were identical in both RTX- and sham-treated rats, indicating that RTX did not cause systemic destruction of primary sensory neurons (results not shown).

Ganglionectomy and RTX treatments alone had no effect on any pancreas measurements and were not different from rats that had sham surgery (results not shown). The gross appearances of the pancreas from vehicle- and caerulein-treated rats with and without ganglionectomy or RTX pretreatment are shown in Fig. 2. Both celiac ganglionectomy and RTX substantially reduced the marked edematous enlargement of the pancreas stimulated by caerulein. Figure 7 shows histological sections of pancreas from the same groups. Caerulein treatment caused edema, cellular disruption, acinar cell vacuolization, and necrosis. These parameters were significantly reduced in pancreata from animals treated with celiac ganglionectomy or RTX.

DISCUSSION

It has previously been shown that SP mediates many of the changes that are characteristic of neurogenic inflammation (5). Through interaction with the NK-1R, SP induces plasma extravasation from postcapillary venules and neutrophil infiltration (22). Mice with genetic deletion of NK-1R are protected against secretagogue-induced pancreatitis (2) and choline-deficient, ethionine-supplemented diet-induced hemorrhagic pancreatitis (18), suggesting that blocking the effects of SP mitigates neurogenic inflammation, which appears to be important in the pathogenesis of acute pancreatitis. Conversely, it has been shown that preventing the degradation of SP by inhibition of the membrane protease, neutral endopeptidase, causes a relative increase in SP and can increase plasma extravasation (17). These studies suggest that SP through its interaction with...
the NK-1R is instrumental in the pathogenesis of neurogenic inflammation.

Primary sensory neurons of the C and Aδ subtype synthesize SP, which is released from nerve endings upon stimulation (9, 10, 29). It has been demonstrated in secretagogue and obstructive models of pancreatitis that primary sensory denervation reduces the severity of pancreatic inflammation (25). This finding supported a role for these neurons being a common pathway for the development of neurogenic inflammation.

TRPV1 is a nonselective cation channel that resides on C and Aδ fibers, which when activated causes the release of SP. Systemic administration of the TRPV1 inhibitor capsazepine has been shown to reduce the severity of secretagogue-induced pancreatitis (24). However, local denervation of primary sensory neurons of the pancreas as a means to reduce neurogenic inflammation has not been reported previously. We hypothesized that selective disruption of the nerves that directly innervate the pancreas could decrease the severity of secretagogue-

Fig. 7. Effects of caerulein, GN, and RTX treatment on pancreatic histology. A: representative histology sections of pancreas from rats treated with vehicle alone (A), caerulein alone (B), caerulein + GN (C), and caerulein + RTX (D). B: histological severity of pancreatitis was evaluated using a scale of 0–4 (see MATERIALS AND METHODS). ***P < 0.001.

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induced pancreatitis. Innervation of the pancreas by primary sensory neurons originates from dorsal root ganglion cells (35). The axons of these nerves fibers course through the celiac ganglion making it a possible target for pharmacological or surgical manipulation.

In this study, we sought to compare surgical vs. pharmacological denervation of pancreatic primary sensory neurons. RTX is a member of the vanilloid family but is more potent than capsaicin for activating the TRPV1 receptor (30). When RTX is applied to TRPV1-expressing cells, a significant and prolonged increase in free intracellular calcium occurs (33). The resultant calcium toxicity destroys only the TRPV1-expressing cells (28). In the present study, we applied RTX to the celiac ganglion to selectively eliminate TRPV1-expressing primary sensory neurons. To confirm that the effect of RTX administration to the celiac ganglion was local rather than systemic, we evaluated chemogenic pain perception by forepaw eye-wiping movements in response to application of 0.1% capsaicin (16). Previous studies have shown that systemic denervation of primary sensory nerves attenuates the eye-wipe response (12). Our finding that the eye-wipe response was similar in RTX and sham groups indicates that animals did not have systemic denervation.

Both in vitro and in vivo studies have demonstrated that after binding SP, the NK-1R is rapidly internalized by endocytosis and eventually recycled to the plasma membrane after degradation of bound SP (3, 20, 21). In the current study, we used quantification of NK-1R-immunoreactive endosomes in pancreatic acinar cells to provide an estimate of endogenous SP release. This is a measure of dynamic SP release (rather than total SP content in the pancreas, which may not reflect SP release) and has been used in a number of other studies for evaluating SP release (3, 6, 19, 20, 21). In the current study, we demonstrated that caerulein-induced pancreatitis was associated with an increase in NK-1R internalization, which was reduced by both surgical celiac ganglionectomy and RTX treatment to the celiac ganglion. We believed that both of these maneuvers interrupt primary sensory nerve signaling, thereby reducing SP release in the pancreas. Both RTX administration and ganglionectomy reduced NK-1R internalization. The magnitude of this reduction was similar to the decline in other parameters of inflammation such as decreases in pancreatic MPO levels and edema. It is not surprising that RTX application to the celiac ganglion was not identical to celiac ganglionectomy. Treatment with RTX selectively destroys TRPV1-bearing neurons, which, in turn, prevents the subsequent release of SP and other transmitters of C and Aδ fibers (e.g., CGRP). Therefore, RTX treatment would be expected to have some actions similar to administration of an SP antagonist. Ganglionectomy, on the other hand, destroys the nerves and axons of the celiac ganglion. Because of this, ganglionectomy affects multiple types of neurons, some of which may even play a protective role against pancreatitis.

Although the application of RTX to the celiac ganglion and celiac ganglionectomy both appear to decrease the severity of pancreatitis as demonstrated by reductions in MPO activity, edema, and histological scoring, they do not completely prevent pancreatitis. These findings suggest that these interventions are affecting only one pathway that contributes to the development of pancreatitis. Extensive cellular injury can produce inflammation that involves other mediators that may not have a neurogenic component. Although it is unknown whether inhibition of other neural pathways could further reduce the severity of pancreatitis, we expect that neurogenic influences represent only one of several important processes in the pathogenesis of the disease.

Similar to other studies, reduction of pancreatitis severity was not accompanied by a decrease in serum amylase levels, which is consistent with direct hyperamylasemia of the pancreas by caerulein (25). Even in experimental pancreatitis, hyperamylasemia is not synonymous with pancreatitis, and we believe that the hyperamylasemia observed in the current study is consistent with caerulein-induced pancreatic injury that does not necessarily lead to pancreatitis. Previous studies have also found that the levels of amylase in the disease course of pancreatitis can be variable and do not correlate accurately with the severity of pancreatitis (25, 27).

In conclusion, caerulein-induced pancreatitis is associated with an increase in NK-1R internalization, consistent with local release of SP; marked pancreatic edema and neutrophil infiltration; and changes in pancreatic histology, indicative of acute pancreatitis. Surgical disruption or local application of RTX to the celiac ganglion reduced NK-1R internalization and reduced the severity of acute secretagogue-induced pancreatitis. These findings indicate that activation of primary sensory nerves is involved in the pancreatic response to injury. It is possible that selectively disrupting TRPV1-bearing neurons could be used to reduce pancreatitis severity.

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