Adrenergic signaling mediates mechanical hyperalgesia through activation of P2X3 receptors in primary sensory neurons of rats with chronic pancreatitis

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Chronic pancreatitis (CP) is a progressive inflammatory disorder characterized by repeated attacks of abdominal pain, fibrosis, and destruction of the glandular pancreas (15, 29, 39). Pain is a cardinal feature of CP, featured as burning, intermittent, and shooting pain (13). It is, not only the most frequent and dominant symptom of patients with CP, but also the most difficult to treat (1, 3, 22, 35). Our lack of knowledge about what causes pain in pancreatitis has been a serious obstacle to treatment on these patients, with pain frequently relapsing or persisting (1, 31). Basic research of pancreatic nerves and experimental human pain studies have provided evidence that pain processing is abnormal in these patients and in many conditions resembles that seen in neuropathic pain disorders (34). Despite much speculation, little is known about the detailed pathophysiology of pain in CP.

Recent studies demonstrated that ATP is increasingly being recognized as a participant in diverse biological events including neurotransmission and pain (4, 16), which is mediated by a variety of P2 receptors. Sensory neurons in dorsal root ganglion (DRG) express P2X receptors (P2XRs), ligand-gated cation channels that are assembled from at least seven different subunits (5, 6, 10, 30). Spinal afferents predominantly display homomeric P2X3 and heteromeric P2X2/3 subtypes, and this system has been shown to be important in several animal models of visceral injury, including the urinary bladder (12, 24) and the colon (42). In inflammatory pancreas, ATP may release from macrophages and acinar cells that then act on P2X3 homomeric and P2X2/3 heteromeric receptors on intramural spinal nerves, triggering pain signaling in pancreas (26). Furthermore, this pathway appears to be amplified in inflammatory conditions. Although these data imply a role for purinergic receptors in pancreatic inflammation, it is unknown whether they also contribute to pain signaling in pancreatic hyperalgesia.

Increased sympathetic activity may also play a role in patients with CP with chronic pain syndromes (7). It is reported that increased supine plasma norepinephrine (NE) levels and pancreatic hyperalgesia appear associated in patients with severe CP (7). However, there are minimal data detailing the interaction of adrenergic signaling and P2XR plasticity in CP. We addressed this question using a previously validated rat model of CP, which is induced by an intraductal injection of trinitrobenzene sulfonic acid (TNBS) in the rat (43, 44). In the present study, we investigated activities of purinergic receptors in DRG neurons innervating the pancreas in response to chronic inflammation. In particular, we examined modulation of NE signaling on P2XR-mediated responses in these neurons under CP conditions. Our findings indicate that CP results in sensitization of P2X3Rs and that this is associated with activation of β2-adrenergic receptor (β2-AR) signaling in pancreatic DRG neurons. Our study therefore provides mechanistic insight into painful CP and identifies a potential molecular target for the treatment of pancreatic pain hypersensitivity.
MATERIALS AND METHODS

Animals. Experiments were performed on adult male Sprague-Dawley rats (200–220 g). Animals were housed under controlled conditions (07:00, 19:00 lighting, 24 ± 2°C) with free access to a standard laboratory diet and fresh water. The protocol was approved by the Institutional Animal Care and Use Committee of Soochow University. All experiments were performed in strict accordance with the guidelines of the International Association for the Study of Pain. Abdominal surgery was carried out under chloral hydrate (360 mg/kg body wt) anesthesia, and all efforts were taken to minimize suffering. Following tissue harvest, rats were killed by decapitation. Following tissue harvest, rats were killed by decapitation.

Induction of CP and cell labeling. CP was induced by an intraduodenal injection of TNBS, and 1,1'-dioleoyl-3,3',3-tetramethylindocarbocyanine methanesulfonate (DiI) was injected into the pancreas areas designated for stimulation were marked in relation to the pancreas before testing. Rats were placed in a plastic cage with a mesh floor and were given 30 min to adapt before testing. Various filaments (Stoelting) were applied in ascending order to the designated abdominal area 10 times each for 1–2 s, with a 10-s interval between applications. A response was considered positive when the rat raised its belly (withdrawal response). The data were expressed as percentages of the numbers of positive responses in 10 times of stimulation with each filament for each rat. All tests were performed in a blinded manner.

Figure 1. Increase in expression and function of P2X3 receptors (P2X3Rs) in chronic pancreatitis (CP) rats. A: trinitrobenzene sulfonic acid (TNBS) injection significantly enhanced response frequency of CP rats to von Frey filament (VFF) stimuli (*P < 0.05, compared with control, n = 8 for each group). Dunn’s post hoc test following Friedman ANOVA). Normal saline (NS)-injected rats were used as controls (CON). B: TNBS injection remarkably enhanced expression of P2X3Rs in pancreatic dorsal root ganglion (DRGs) compared with controls (CON, n = 5; TNBS, n = 3; *P < 0.05, 2-sample t-test). C: enhanced expression of P2X3 mRNA after TNBS injection (CON, n = 6; TNBS, n = 4; *P < 0.05, 2-sample t-test). D: TNBS treatment did not significantly alter the expression of P2X3Rs in spinal dorsal horn when compared with control group. E: TNBS injection enhanced the ATP-evoked calcium signal of pancreatic DRG neurons (*P < 0.05, 2-sample t-test), which were labeled by 1,1'-dioleoyl-3,3',3-tetramethylindocarbocyanine methanesulfonate (DiI) in red (arrow). F: TNBS injection did not enhance the ATP-evoked calcium signal of non-DiI-labeled DRG neurons. G: percentage of pancreatic DRG neurons with action potentials (APs) in response to ATP application was greatly increased in TNBS-injected rats when compared with controls (*P < 0.05 vs. CON, χ² test). H: example of ATP-evoked inward current of DiI-labeled DRG neurons from control (left) and TNBS-injected (right) rat was shown on the top. TNBS injection significantly enhanced the ATP-evoked current density in DiI-labeled DRG neurons of CP rats (CON, n = 12; TNBS, n = 11, **P < 0.05, 2-sample t-test).
are as follows: P2X3 forward: CCAGTGCATCCTAATATTCC, P2X3 reverse: CCAGCGTTCATCATAACCAG; actin forward: GCAGGTCATCCTATCGGCA, actin reverse: GCAGAGAGTCCTTACCGGT.

Immunofluorescence study. Triple-labeling techniques were performed to examine the colocalization of P2X3R and β2-AR in DRG neurons innervating the pancreas, as described previously (48). Primary antibodies for P2X3R (1:200) and β2-AR (1:200) were used in the present study.

Intracellular calcium measurements. Fura 2 Ca$^{2+}$ imaging of acutely dissociated DRG neurons was performed as previously described (25, 28). The ratio of fluorescence signal measured at 340 nm, divided by the fluorescence signal measured at 380 nm, was used as an indicator for intracellular calcium mobilization. Amplitudes of peak [Ca$^{2+}$i], responses were computed as the difference between the peak value and the baseline value. To be considered a drug-induced response, changes in [Ca$^{2+}$i] had to occur within ~2–3 min after drug application, and the amplitudes had to exceed baseline by two times standard deviation.

Western blotting. Protein extracts from acute dissected DRGs (T9-T13) from control and CP rats were used for protein measurement by Western blotting as described previously (42). Primary antibodies used in this experiment included P2X1R (1:200), P2X2R (1:1,000), or P2X3R (1:1,000). These primary antibodies were purchased from Neuronics. AR antibodies included β1-AR (1:200, Santa Cruz Biotechnology), β2-AR (1:1,000, Abcam), and β3-AR (1:200, Santa Cruz Biotechnology). GAPDH or β-actin was used as a loading control.

Measurement of NE levels. NE levels of plasma, DRG, and pancreas were measured using the respective radioimmunoassay kits from Abnova as described previously (47). Blood samples were collected from the rat abdominal aorta in tubes containing 2.5% sodium citrate and 0.45% citric acid at the time of animal euthanasia under 2% isoflurane general anesthesia. After centrifuge, plasma was quickly aliquoted and stored at ~80°C for assays. DRG (T9-T13 DRGs) and pancreas from TNBS-treated (4 wk) or age-matched control rats were dissected out and lysed in 120 μl of radioimmunoprecipitation assay buffer containing 1% NP-40, 0.5% SDS, PMSF (10 μl/ml), and aprotinin (30 μl/ml; Sigma). The cell lysates were then microfuged at 15,000 revolution/min for 30 min at 4°C. The concentration of protein in homogenate was determined using a BCA reagent (Beyotime). The results were expressed as nanograms per liter.

**Fig. 2.** Antagonism of P2XRs on pancreatic pain behavior. A: administration of P2 receptor antagonist suramin (intrathecal, it) significantly attenuated response frequency of CP rats to VFF stimulation [*P < 0.05, compared with pretreatment (Pre), respectively, n = 8, Dunn’s post hoc test following Friedman ANOVA]. B: time course of effect of P2 receptor antagonist suramin (500 μg) on mechanical hyperalgesia. The antinociceptive effect lasted for more than 4 h (*P < 0.05, compared with Pre; n = 8, Dunn’s post hoc test following Friedman ANOVA). C: administration of suramin did not produce any effect on response frequency in age-matched healthy control rats. D: administration of a selective P2X3R/P2X2R antagonist A314791 (100 μM) significantly reduced response frequency of CP rats to VFF stimulation (*P < 0.05, compared with Pre, respectively; n = 8, Dunn’s post hoc test following Friedman ANOVA). E: time course of effect of A314791 (30 nmol) on pancreatic hyperalgesia. The antinociceptive effect lasted for more than 4 h (*P < 0.05, compared with Pre; n = 8, Dunn’s post hoc test following Friedman ANOVA). F: administration of A314791 did not produce any effect on response frequency in age-matched healthy rats. G: NS treatment did not change the response frequency in rats 4 wk after TNBS infusion. H: administration of suramin did not produce any effect on time of Rota-rod test in healthy control rats. I: administration of A314791 did not produce any effect on time of Rota-rod test in healthy control rats.
for blood plasma and as nanomoles per gram of proteins for DRG and the pancreas.

Evaluation of motor performance. The effects of purinergic or adrenergic receptor antagonists on motor performance of the rat were tested using a Rota-rod test. Motor performance was evaluated at 30 min following treatments, and the amount of time to stay on the revolving bar (30 revolution/min) was recorded.

Drug application and intrathecal injection. Suramin or A317491, propranolol (Prop), phenotiamine (Phen), Butoxamine (Buto), NE, ATP, H-89, and forskolin were purchased from Sigma-Aldrich and were freshly prepared in 0.9% normal saline. Suramin or A317491 was intrathecally (it) injected once. Intrathecal injection was made with a 27 G needle between the L5 and L6 intervertebral space to deliver the reagents to the cerebral spinal fluid as described previously (21, 45). Buto, Phen, or Prop was intraperitoneally injected once daily for 7 consecutive days for molecular expression experiments and once for behavioral test. The choice of doses for Phen and Prop (47) and H-89 and forskolin (20, 36) in this study was mainly based on our pilot studies and previous observations because these doses of drugs showed maximal analgesic effects without producing inhibitory effects on motor activities in rats.

Data analysis. All data in the present study were expressed as means ± SE or as percentages. Statistical analyses were conducted using OriginPro 8 (OriginLab) and Matlab (Mathworks). Normality was checked for all analyses. Student's t or $\chi^2$ test was used to determine significance of changes between two groups. Two-way repeated-measures ANOVA followed by Tukey's post hoc test and Friedman ANOVA followed by Dunn's post hoc test or Mann-Whitney test were performed where appropriate. A P value <0.05 was considered statistically significant.

RESULTS

TNBS injection sensitizes P2X3Rs. We first determined whether TNBS injection enhanced response frequency in rats. Four weeks after TNBS injection, CP rats displayed an enhanced response frequency compared with control rats (Fig. 1A, *P < 0.05, n = 8 for each group). TNBS injection significantly enhanced P2X3R expression in T9-T13 DRGs both at protein (Fig. 1B, *P < 0.05, n = 3 for each group) and at mRNA levels (Fig. 1C, *P < 0.05, n = 6 and 4 for control and TNBS group, respectively) compared with age-matched controls. To confirm the specificity of P2X3R expression in pancreas DRGs, expression of P2X1R and P2X2R in T9-T13 DRGs was also studied. TNBS injection did not alter expression of P2X1Rs and P2X2Rs in T9-T13 DRGs (data not shown). In addition, expression of P2X3Rs in spinal dorsal horn levels was not significantly altered (Fig. 1D).

We then examined the effect of TNBS injection on ATP-evoked intracellular calcium mobilization in DRG neurons innervating the pancreas. As described above, pancreas-projecting DRG neurons were labeled by DiI (Fig. 1E, top, arrow). Application of 20 μM ATP elicited a significantly larger calcium signal of DiI-labeled DRG neurons in TNBS-injected animals than in control rats (Fig. 1E, bottom, *P < 0.05, n = 16 cells for each group). However, application of the same concentration of ATP did not elicit a significant change in calcium signal of non-DiI-labeled DRG neurons isolated from control and TNBS-injected rats (Fig. 1F, n = 30 and 25 cells for control and TNBS group, respectively). Under current-clamp conditions, ATP at 20 μM induced more cells to generate action potentials in CP rats than in control rats (Fig. 1G, *P < 0.05, $\chi^2$ test). The percentage of cells with action potentials in responding to ATP application was 28.6% in control rats and was 90.9% in TNBS-injected rats. Under voltage-clamp conditions, ATP at 20 μM was used to record ATP-evoked inward currents in DiI-labeled pancreas DRG neurons. Compared with control rats, TNBS injection dramatically enhanced ATP-evoked peak current density (Fig. 1H, **P < 0.01). The mean ATP current densities were 31.34 ± 10.7 pA/pF (n = 12) for control rats and 91.45 ± 27.57 pA/pF (n = 11) for TNBS rats. The ATP-induced current was blocked by A317491, a selective P2X3 and P2X2R/P2X3R antagonist (data not shown). These data suggest that TNBS injection enhanced nociceptive responses, which was correlated with sensitization of P2X3Rs in pancreatic DRG neurons.

P2X3R antagonist suppresses pancreatic hyperalgesia. We next determined whether nociceptive responses observed in TNBS-treated rats were mediated by purinergic receptors. Suramin was first used in the present study. Suramin is a nonselective P2 receptor antagonist. It blocks both P2XRs and P2YRs. Administration of suramin (500 μg in 30 μl iT) significantly reduced the response frequencies to stimulation forces of 6, 8, and 10 g when compared with pretreatment baseline (Fig. 2A, *P < 0.05, n = 8). In contrast, normal saline
treatment did not alter the response frequency in TNBS-injected rats (Fig. 2G). Time course of suramin effects was also determined. The stimulation force of 10 g was used to examine the time course. The effect of suramin at a dose of 500 μg lasted for 4 h (Fig. 2B, n = 8, *P < 0.05 vs. pretreatment, Dunn’s post hoc test following Friedman ANOVA). Maximal inhibition effect was at 30 min after treatment. However, suramin at dose of 500 μg has no significant effects on response frequency in control rats (Fig. 2F, n = 8), indicating that those P2 receptors do not normally participate in the responses to von Frey filament stimulation. To further determine which subunit of P2 receptors mediated TNBS-induced hyperalgesia, A317491 was tested. A317491 was a selective P2X3 and P2X2R/P2X3R antagonist (23). Following an intrathecal application of A317491 (30 nmol) to CP rats, response frequency significantly decreased (Fig. 2D, n = 8), and the effect persisted for ~4 h (Fig. 2E, n = 8, *P < 0.05). However, A317491 at a dose of 30 nmol has no significant effects on response frequency in control rats (Fig. 2F, n = 8).

To exclude the possible effect of suramin or A317491 on motor performance, Rota-rod test was performed. The time for rats to stay on Rota rod was not significantly altered after administration of suramin (Fig. 2H) or A317491 (Fig. 2I).

Together, these data suggest that P2X3 and/or P2X2R/P2X3R are sensitized and involved in the development of TNBS-induced pancreatic hyperalgesia.

Adrenergic signaling sensitizes P2X3R activities. We then determined whether adrenergic signaling is involved in regulation of P2X3R expression and function in rats with CP. Treatment with β-AR antagonist Prop (2 mg/kg ip) once daily for 7 consecutive days markedly suppressed expression of P2X3R in DRGs from CP rats (Fig. 3A, *P < 0.05, n = 3 for each group). In contrast, treatment with α-AR antagonist Phen (5 mg/kg ip) once daily for 7 consecutive days did not alter expression of P2X3R in DRGs from CP rats (Fig. 3B, n = 3 for each group). In addition, administration of Phen significantly suppressed ATP-evoked inward current density (Fig. 3C, normal saline: 91.5 ± 27.6 pA/pF, n = 11; Prop: 37.6 ± 10.9 pA/pF, n = 16 cells; TNBS, n = 19 cells; #P < 0.05, compared with Phen, paired t-test; EXTRA, extracellular solution).

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Fig. 4. Potentiation of P2XR-mediated calcium signals by norepinephrine (NE). A: application of A317491 greatly reduced the ATP-evoked intracellular calcium mobilization (n = 29 cells). ATP concentration was 20 μM (**P < 0.05, 2-sample t-test; EXTRA, extracellular solution). B: potentiation effect of NE at different concentrations (2, 20, and 200 μM) on ATP induced Ca2+ transients in pancreatic DRG neurons (n = 21) (**P < 0.05, 2-sample t-test). C: potentiation of ATP-induced transients by NE (20 μM) was not blocked by CdCl2 (50 μM) (**P < 0.05, 2-sample t-test). D: in CP rats, NE (20 μM) significantly enhanced the ATP-induced Ca2+ transients when compared with control rats (CON, n = 16 cells; TNBS, n = 16 cells, *P < 0.05, 2-sample t-test). E: Phen, an antagonist of α-ARs, had little effect on potentiation of ATP-induced Ca2+ transients by NE (20 μM) (**P < 0.05, 2-sample t-test). F: potentiation of ATP-induced Ca2+ transients by NE (20 μM) was blocked by Prop, a selective blocker of β-ARs (*P < 0.05, compared with EXTRA, paired t-test, n = 19 cells; #P < 0.05, compared with NE, paired t-test, n = 19 cells).
pA/pF, n = 12, **P < 0.01) and reduced the percentage of numbers of neurons demonstrating action potentials after ATP application (Fig. 3D, *P < 0.05, χ² test). These data suggest that adrenergic signaling participates in sensitization of P2X3Rs following pancreatic inflammation.

NE treatment potentiates P2XR-mediated calcium signals. We next examined whether NE treatment potentiates P2XR-mediated responses in acutely dissociated DRG neurons from healthy control rats. Application of ATP at 20 μM for 2 s evoked a large intracellular calcium signal in more than 80% of the neurons tested (Fig. 4A). In the presence of A317491 (1 μM), ~90% of ATP-induced responses were blocked and returned to baseline level after wash. The vehicle solutions did not alter intracellular Ca²⁺ concentration (data not shown). We next examined the effect of NE on ATP-induced calcium mobilization. Incubation of NE (up to 200 μM), by itself, did not evoke any calcium signal but enhanced ATP-induced calcium signal (Fig. 4B). In the presence of CdCl₂ (50 μM), the potentiation effect by NE was not greatly altered (Fig. 4C), indicating that voltage-gated calcium channels may not be involved in potentiation by NE of ATP-evoked calcium signals. We also compared the ATP-evoked intracellular calcium mobilization of DRG neurons from control and TNBS-treated rats, in the presence of NE at 20 μM. Surprisingly, the ATP-evoked intracellular calcium mobilization in the presence of NE at 20 μM was more significantly enhanced in CP rats than in age-matched control rats (Fig. 4D, *P < 0.05). The percentage of increase in Δratio was 23.5 ± 1.7 (n = 12) for control rats and 34.2 ± 4.3 (n = 13) for TNBS-treated rats. We next determined which subtype of adreceptors (α- or β-ARs) mediated the potentiation of ATP responses by NE. Application of Phen at 10 μM did not alter the potentiation of ATP effect by NE [Fig. 4E, *P < 0.05, compared with extracellular solution (extra)]. In contrast, application of Prop at 10 μM greatly reduced the potentiation of ATP effect of NE (Fig. 4F, *P < 0.05, compared with NE). When Prop perfusion was suspended, the potentiation effect of NE appeared again. These results suggest that β-ARs mediated the potentiation effect of NE on ATP responses.

PKA is involved in NE-induced potentiation of ATP responses. Because PKA is known to participate in the actions of NE (46), its role in the potentiation of ATP responses was then studied. We first determined the effect of H-89, a membrane-permeable PKA inhibitor, on the enhancement of ATP responses by NE. In the presence of H-89 (1 μM), the potentiation effect of NE was completely blocked (Fig. 5, A and B, *P < 0.05, n = 19, compared with extra; #P < 0.05, compared with NE). When H-89 was washed out, the potentiation effect of NE appeared again. In addition, we investigated the action of the adenylyl cyclase activator, forskolin, on ATP-evoked calcium signals and demonstrated that forskolin at 1 μM significantly increased ATP-evoked calcium signal, indicating that forskolin mimicked the action of NE (Fig. 5, C and D, *P < 0.05, n = 16).

TNBS injection sensitizes adrenergic signaling. We next determined whether TNBS injection enhanced NE concentration in DRGs, pancreas, and blood plasma. NE concentration was significantly enhanced in DRGs (Fig. 6A, control, n = 4; TNBS, n = 7, **P < 0.01) and pancreas (Fig. 6B, control, n = 4; TNBS, n = 5, *P < 0.05) in CP rats compared with age-matched control rats. However, NE concentration in blood plasma was not significantly altered after TNBS injection (Fig. 6C, control, n = 3; TNBS, n = 4). Because β-ARs were involved in upregulation of P2X3R expression, three subtypes of β-AR expression were then determined. Interestingly, TNBS injection significantly enhanced β₂-AR expression in DRGs when compared with aged-matched controls (Fig. 6D, n = 4 for each group, *P < 0.05). However, expression of β₁ (Fig. 6D, n = 4 for each group) and β₃ (Fig. 6F, n = 4 for each group) adrenergic receptors was not significantly altered. We next examined whether P2X3Rs were coexpressed with β₂-AR in DRG neurons innervating the pancreas. Triple-labeling tech-

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![Image](http://ajpgi.physiology.org/)
Fig. 6. Activation of adrenergic signaling following TNBS injection. A: TNBS injection significantly increased NE concentration in DRGs when compared with CON (CON, n = 4 rats; TNBS, n = 7 rats; **P < 0.01, 2-sample t-test). B: TNBS injection remarkably elevated NE level in the pancreas when compared with CON (CON, n = 4 rats; TNBS, n = 5 rats; *P < 0.05, 2-sample t-test). C: TNBS injection had little effect on NE concentration in blood plasma (CON, n = 3 rats; TNBS, n = 4 rats). D: β1-AR expression was not significantly altered in DRGs of CP rats (n = 4 for each group). E: β2-AR expression was significantly increased in DRGs of CP rats (*P < 0.05, n = 3 for each group). F: β2-AR expression was not significantly altered in DRGs of CP rats (n = 4 for each group). G: pancreatic T9 DRG cells are labeled with DiI (red). H: P2X3R-positive cells are shown in green. I: β2-AR-positive cells are shown in blue. J: merge of double labeling of DiI and P2X3R. K: merge of β2-AR-positive staining and DiI labeling. L: merge of β2-AR staining and P2X3R labeling. Bar = 50 μm.

Figures 6A-L: In the figures, the significance levels are indicated as follows: * for P < 0.05, ** for P < 0.01, and *** for P < 0.001. The data are presented as mean ± SEM. The statistical analyses were performed using IBM SPSS version 22.0 (IBM) and GraphPad Prism version 5.0 (GraphPad Software). The comparisons between the experimental and control groups were made using a two-tailed Student's t-test or one-way ANOVA followed by a Bonferroni multiple comparison test. The level of statistical significance was set at P < 0.05.
P2X3Rs at the DRG levels play a role in this in the spinal cord dorsal horn, we concluded that, in terms of hypersensitivity in this model. Because suramin is administered in the sensitized state. Thus the present study adds P2X3Rs to the list of key nociceptive molecules that participate in visceral pain hypersensitivity under CP conditions. Administration of a nonselective P2 receptor antagonist suramin or a selective P2X3 and P2X2R/P2X3R antagonist A317491 significantly attenuated pancreatic pain behaviors time dependently. Of note is that these two antagonists did not produce any effect on healthy control rats. They did not alter the duration time for rats to stay on the bar (n = 8 rats for each group).

Mechanisms of pain generation in CP have not been completely understood. The initial hypothesis on the development of pancreatic pain was attributed to ductal strictures, increased intraductal pressure, interstitial hypertension, and pancreatic pseudocysts (19). Nowadays, a variety of ligands and their respective receptors have been identified to play roles in initiation and maintenance of pancreatic pain (2, 44). In the present study, we demonstrated that TNBS infusion sensitizes P2X3Rs of DRG neurons innervating the pancreas. This conclusion was based on several key steps. First, expression of P2X3Rs was greatly enhanced both at protein and mRNA levels, suggesting a plasticity of P2X3Rs on primary sensory neurons after pancreatic inflammation. Although DRG neurons also express P2X1Rs and P2X2Rs, expression of these two subtypes of P2XRs was not significantly altered 4 wk after TNBS infusion into the pancreas. Second, ATP-evoked inward currents were greatly potentiated in pancreatic DRG neurons in rats following TNBS infusion. Third, ATP-induced intracellular calcium mobilization was significantly enhanced after TNBS treatment. These results are consistent with those of previous studies of this signaling system in inflammatory models of bladder (12), hindpaw (41), and the colon (42). Together, our results support the concept that pain in CP is neuropathic attributable to the prominent neuroplastic alterations (18). This specific pancreatic neuropathy is featured by enlarged intrapancreatic nerves that are increased in number
and frequently infiltrated by inflammatory cells, leading to pancreatic neuritis (9, 14). In addition, pancreatic neuropathy is characterized by numerous molecular and morphological alterations at both the peripheral (17, 43, 44) and the central nervous system level (18). Increased peripheral nociceptive signals mediated by neurotransmitters and inflammatory factors are paralleled by hypersensitive DRG (43) and spinal cord neurons (17). These phenomena are closely associated with increased abdominal pain sensation in the respective patients (8). Of note is that the central nervous system might also play a role in pain sensation in CP (27). The detailed mechanism needs to be further investigated.

An interesting finding of the present study is that adrenergic signaling is involved in sensitization of purinergic receptors. This is based on the following observations. First, upregulation of P2X3R expression was reduced by administration of β-AR antagonist propranolol but not by α-AR antagonist phentolamine. Second, the ATP-evoked current density and the number of cells with action potentials evoked by ATP application were significantly reduced after incubation of β-AR antagonist propranolol. Third, incubation of NE enhanced the ATP-induced intracellular calcium mobilization of DRG neurons in vitro. Fourth, triple-labeling experiments showed that P2X3Rs were coexpressed with β2-ARs in pancreas innervating DRG neurons. Finally, administration of β-AR antagonist Prop significantly attenuated the pancreatic pain behaviors, whereas administration of α-AR antagonist Phen did not significantly alter pancreatic pain behaviors of CP rats, which is consistent with gene expression and patch-clamp experiments. Collectively, these data strongly suggest that adrenergic signaling is involved in sensitization of purinergic receptors and thus contributed to pancreatic hyperalgesia. In particular, our data demonstrated for the first time that β2-ARs might play a crucial role in the regulation of P2X3R sensitization under CP conditions because expression of β2-ARs was markedly enhanced, whereas expression of β1-ARs and β3-ARs was not significantly altered. In addition, β2-AR antagonist Buto greatly suppressed pancreatic hyperalgesia in CP rats. We conclude that β2-ARs might play a crucial role in the regulation of P2X3R sensitization and the development of hyperalgesia under CP conditions. Of note is that β2-ARs actually have the highest affinity for epinephrine and that epinephrine is known to sensitize DRG neurons (37). Thus, epinephrine released from the adrenals might potentially play a role on regulation of P2X3R function and expression. Further investigations of epinephrine level and the origins of NE in pancreas and DRGs are definitely warranted in this setting.

The mechanism by which adrenergic signaling sensitizes P2X3Rs remains largely unknown. In this study, we provided evidence to support an idea that enhanced adrenergic signaling sensitizes P2XR through a PKA-dependent mechanism. Recent studies have shown that P2XR expression and function are modulated by various forms of intracellular signaling molecules such as calcium/calmodulin protein kinase II (11, 40), PKC (38), and PKA (36, 38). Because PKA is involved in inflammatory inflammation (33), we therefore investigated whether PKA is also involved in NE-induced P2X3R sensitization. In the present study, we showed that acute application of NE enhanced ATP-evoked intracellular calcium mobilization and that H-89, an inhibitor of PKA, blocked the potentiation of NE effect on P2X3Rs, indicating a role for PKA in the potentiation of NE action. This is further confirmed by the experiment that acute incubation of forskolin, an activator of adenylyl cyclase, mimics the effect of NE application. Although further experiments are needed to investigate the detailed mechanisms underlying how adrenergic signaling regulates sensitization of purinergic receptors under chronic inflammation conditions, the present data suggest that PKA might be one of the mechanisms involved in the potentiation of P2X3R function induced by adrenergic signaling pathways.

In summary, the present study demonstrated that sensitization of purinergic receptors (i.e., P2X3Rs) in primary sensory neurons contributes to pancreatic hyperalgesia in a rat model of CP induced by TNBS infusion. The enhanced adrenergic signaling (i.e., upregulation of β2-ARs) sensitizes the P2X3Rs through a PKA-dependent signaling pathway. This and future studies on the mechanism of purinergic receptor activation might provide potential targets for the treatment for pancreatic pain for patients with CP.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.W., H.-Y.Z., Y.J., and Y.Z. performed experiments; G.-Y.X. interpreted results of experiments; G.-Y.X. and H.-Y.Z. designed research; G.-Y.X. and S.H. contributed new reagents or analytic tools; S.W., H.-Y.Z., Y.J., and X.J. drafted manuscript; T.L. and G.-Y.X. edited and revised manuscript; G.-Y.X. conceived and designed of research; G.-Y.X. interpreted results of experiments; G.-Y.X. approved final version of manuscript.

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