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

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Abstract

The mechanism of pain in chronic pancreatitis (CP) is poorly understood. The aim of this study was designed to investigate roles of norepinephrine (NE) and P2X receptor signaling pathway in the pathogenesis of hyperalgesia in a rat model of CP. CP was induced in male adult rats by intraductal injection of trinitrobenzene sulfonic acid (TNBS). Mechanical hyperalgesia was assessed by referred somatic behaviors to mechanical stimulation of rat abdomen. P2X receptor-mediated responses of pancreatic DRG neurons were measured utilizing calcium-imaging and whole cell patch clamp recording techniques. Western blot analysis and immunofluorescence were performed to examine protein expression. TNBS injection produced a significant upregulation of P2X3 receptor (P2X3R) expression and an increase in ATP-evoked responses of pancreatic DRG neurons. The sensitization of P2X3Rs was reversed by administration of β-adrenergic receptor antagonist propranolol. Incubation of DRG neurons with NE significantly enhanced ATP-induced intracellular calcium signals, which was abolished by propranolol, and partially blocked by protein kinase A inhibitor H-89. Interestingly, TNBS injection led to a significant elevation of NE concentration in DRGs and the pancreas, an upregulation of β2-adrenergic receptor expression in DRGs, and amplification of the NE-induced potentiation of ATP responses. Importantly, pancreatic hyperalgesia was markedly attenuated by administration of purinergic receptor antagonist suramin or A317491 or β2-adrenergic receptor antagonist butoxamine. Sensitization of P2X3Rs, which was likely mediated by adrenergic signaling in primary sensory neurons, contributes to pancreatic pain, thus identifying a potential target for treating pancreatic pain caused by inflammation.

Key words: Chronic pancreatitis; Dorsal root ganglion; Purinergic receptors; Norepinephrine; Protein kinase A
INTRODUCTION

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 CP patients 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 acts on P2X3 homeric and P2X2/3 heteromeric receptors on intramural spinal nerves triggering pain signaling in pancreas (26). Further, 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 CP patients with chronic pain syndromes (7). It is reported that increased supine plasma norepinephrine
(NE) level and pancreatic hyperalgesia appear associated in severe CP patients (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 chronic pancreatitis, which is induced by an intraductal injection of trinitrobenezene 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 norepinephrine 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 weight) anesthesia, and all efforts were taken to minimize suffering. Following tissue harvest, rats were sacrificed by decapitation.

**Induction of CP and Cell Labeling**

CP was induced by an intraductal injection of TNBS, and Dil was injected into the pancreas before injection of TNBS, as described in details previously (43, 44).

**VFF measurements**
Von Frey Filament (VFF) testing was performed as described previously (44). In brief, the belly was shaved and 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 minutes to adapt before testing. Various filaments (Stoelting, Wood Dale, IL) were applied in ascending order to the designated abdominal area 10 times each for 1-2 seconds, with a 10-second interval between applications. A response was considered positive when the rat raised its belly (withdrawal response). The data were expressed as a percentage of the number of positive responses in 10 times stimulation with each filament for each rat. All tests were performed in a blinded manner.

**Dissociation of DRG Neurons and patch clamp recording techniques**

Isolation and whole cell patch clamp recordings of DRG neurons from adult rats were performed as previously described (43, 44).

**Real-time quantitative polymerase chain reaction (qPCR) for mRNA**

qPCR was performed using SYBR Green fluorescence, as previously described (48). The sequences of the primers for P2X3 receptors, and β-actin (as an internal control) used in qPCR are: P2X3-F: GAACCAGACGGAGCAAACAG, P2X3-R: GGCGAAGGAATCGTCATCA, Actin-F: TCAGGTCATCACTATCGGCA, Actin-R: GGCATAGAGGTCTTTACGGAT.

**Immunofluorescence study**

Triple labeling techniques were performed to examine the co-localization 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 $[\text{Ca}^{2+}]_i$ responses were computed as the difference between the peak value and the baseline value. To be considered a drug-induced response, changes in $[\text{Ca}^{2+}]_i$ had to occur within 2~3 minutes after drug application, and the amplitudes had to exceed baseline by 2 times standard deviation.

**Western blotting**

Protein extract 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 (1:1000) or P2X3R (1:1000). These primary antibodies were purchased from Neuromics, USA. Adrenergic receptor antibodies included $\beta_1$- (1:200, Santa Cruz), $\beta_2$- (1:1000, Abcam) and $\beta_3$- (1:200, Santa Cruz) adrenergic receptor antibodies. GAPDH or $\beta$-actin was used as 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% isofluorane general anesthesia. After centrifuge, plasma was quickly aliquoted and stored at -80°C for assays. Dorsal root ganglion (T9-T13 DRGs) and pancreas from TNBS-treated (4 weeks) or age-matched control rats were dissected out and lysed in 120 μl of radioimmunoprecipitation assay buffer containing 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, PMSF (10 μl/ml), and aprotinin (30 μl/ml; Sigma). The cell lysates were then microfuged at 15,000 rpm for 30 min at 4°C. The concentration of protein in homogenate was determined using a BCA reagent (Beyotime, CHN). The results were expressed as ng/L for blood plasma and as nmol/g proteins for DRG and the
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 rpm) was recorded.

Drug application and intrathecal injection

Suramin or A317491, propranolol (Prop), phentolamine (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 (i.t.) 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 consecutive 7 days for molecular expression experiments and once for behavioral test. The choice of doses for phentolamine and propranolol (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 mean ± SEM or as percentage. Statistical analyses were conducted using OriginPro 8 (OriginLab, US) and Matlab (Mathworks, US). Normality was checked for all analyses. Student’s t or χ² test was used to determine significance of changes between two groups. Two-way repeated measures ANOVA followed by Tukey 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 P2X3 receptors**

We first determined whether TNBS injection enhanced response frequency in rats. Four weeks after TNBS injection, CP rats displayed an enhanced response frequency when compared to 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) when compared to 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 P2X1 and P2X2 receptors 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 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 Dil (Fig. 1E, top, arrow). Application of 20 µM ATP elicited a significant larger calcium signal of Dil 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-Dil 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 (APs) in CP rats than in control rats (Fig. 1G, *p<0.05, χ² 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 Dil 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 P2X2/3
receptor antagonist (data not shown). These data suggest that TNBS injection enhanced nociceptive responses, which was correlated with sensitization of P2X3 receptors in pancreatic DRG neurons.

**P2XR 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 non-selective P2 receptor antagonist. It blocks both P2X receptors and P2Y receptors. Administration of suramin (500 μg in 30 μl, i.t.) significantly reduced the response frequencies to stimulation forces of 6, 8, 10 g when compared with pretreatment baseline (Fig. 2A, *p<0.05, n=8). In contrast, normal saline (NS) treatment did not alter the response frequency in TNBS-injected rats (Fig. 2G). Time course of suramin effects were also determined. The stimulation force of 10 g was used to examine the time course. The effect of suramin at dose of 500 μg lasted for 4 hours (Fig. 2B, n=8, *p<0.05 vs. Pre, 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 2C, 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 P2X2/3 receptor 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 hours (Fig. 2E, n= 8, *p<0.05). However, A317491 at dose of 30 nmol has no significant effects on response frequency in control rats (Fig 2F, n= 8).

To exclude 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 P2X2/3 receptors 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-adrenergic receptor (β-AR) antagonist Prop (2 mg/kg, i.p.) once daily for consecutive 7 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-adrenergic receptor (α-AR) antagonist Phen (5 mg/kg, i.p.) once daily for consecutive 7 days did not alter expression of P2X3R in DRGs from CP rats (Fig. 3B, n=3 for each group). In addition, administration of Prop significantly suppressed ATP-evoked inward current density (Fig. 3C, NS: 91.5±27.6 pA/pF, n=11; Prop: 37.6±10.9 pA/pF, n=12, **p<0.01) and reduced the percentage of numbers of neurons demonstrating action potentials (AP) 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 P2X receptor-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). Next, we examined 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 Δ ratio 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 adrenoceptors (α- or β-ARs) mediated the potentiation of ATP responses by NE. Application of phent at 10 µM did not alter the potentiation of ATP effect by NE (Fig. 4E, *p<0.05, compared with 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 Extra; #p<0.05, compared with NE). When Prop perfusion was suspended, potentiation effect of NE was appeared again. These results suggest that β-ARs mediated the potentiation effect of NE on ATP responses.

PKA involves in NE-induced potentiation of ATP responses

Since protein kinases A (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), potentiation effect of NE was completely blocked (Figs. 5A and B, *p<0.05, n=19, compared with Extra; #p<0.05, compared with NE). When H-89 was washed out, potentiation effect of NE was 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 (Figs. 5C and D, *p<0.05, n=16).

TNBS injection sensitizes adrenergic signaling

We next determined whether TNBS injection enhanced NE concentration DRGs, pancreas and blood plasma. NE concentration was significantly enhanced in DRGs (Fig. 6A, CON, n=4; TNBS, n=7, **p<0.01) and pancreas (Fig. 6B, CON, n=4; TNBS, n=5, *p<0.05) in CP rats when compared to age-matched control rats. However, NE concentration in blood plasma was not significantly altered after TNBS injection (Fig.
Since 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. 6E, 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 co-expressed with β2-AR in DRG neurons innervating the pancreas. Triple-labeling techniques were used in this experiment. Pancreatic projection DRG neurons were retrogradely labeled by Dil (Fig. 6G). P2X3R positive neurons were labeled in green (Fig. 6H). β2-AR positive neurons were labeled in blue (Fig. 6I). DRG sections containing Dil labeled neurons were stained with P2X3R (Fig. 6J) and β2-AR (Fig. 6K) antibodies. About 95% pancreatic projection DRG neurons that were immunoreactive for P2X3R were also positive for β2 adrenoceptor (Fig. 6L). Similarly, all pancreatic projection DRG neurons that were immunoreactive for β2-AR also were positive for P2X3R (Fig. 6L).

**Adrenergic β2-receptor inhibitor attenuates pancreatic hyperalgesia**

We then determined whether adrenergic signaling is involved in TNBS-induced pancreatic hyperalgesia. Adrenergic receptor inhibitor, Prop or Phen, was administrated intraperitoneally. Injection of Phen with two different doses (1 and 5 mg/kg body weight) failed to alter nociceptive responses in CP rats (Fig. 7A). In contrast, injection of Prop at 2 mg/kg body weight significantly reduced nociceptive responses in CP rats (Fig. 7B). The effect of Prop at doses of 2 mg/kg body weight lasted for ~1 hour (Fig. 7C, n=8 for each group; *p<0.05 vs. Pre, Dunn’s post hoc test following Friedman ANOVA). Furthermore, Butoxamine (Buto), an antagonist of β2-ARs, was administrated intraperitoneally. Injection of Buto at 1.5 mg/kg body weight significantly reduced nociceptive responses in CP rats (Fig. 7D). The effect of Buto at doses of 1.5 mg/kg body weight lasted for ~1 hour (Fig. 7E, n=8 for each group; *p < 0.05 vs. Pre, Dunn’s post hoc test following Friedman ANOVA). In addition, either Prop or Buto treatment did not alter the time for rats to stay on the bar.
DISCUSSION

In the present study we demonstrated for the first time that purinergic receptors play an important role in the development of chronic pain in rats with chronic pancreatitis. During the past several decades, much progress has been made in understanding the roles for ATP and purinergic receptors in pain signaling under a variety of pathophysiological conditions (6). Tissue inflammation or injury causes release of ATP from damaged cells and then acts on P2X3 homomeric and P2X2/3 heteromeric receptors on primary afferent nerves, triggering pain signaling. In visceral organs, this was first demonstrated in urological organs (12, 24), but subsequently also shown to apply to the colon (42). These results of the present study are significant because they provide further evidence that ATP-P2XR signaling plays an important role in pancreatic pain hypersensitivity under chronic pancreatitis conditions. Administration of a non-selective P2 receptor antagonist suramin or a selective P2X3 and P2X2/3 receptor 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 Rota rod after administration of either antagonist. These data indicate that this antinociceptive effect was not a non-specific analgesic effect and that the role of the P2X3R pathway in mediating pancreatic pain hypersensitivity may not be as important in health as in the sensitized state. Thus, the present study adds P2X3 receptors to the list of key nociceptive molecules that participate in visceral hypersensitivity in this model. Since suramin is administrated intrathecally, suramin can reduce central sensitization by blocking P2X4 receptors on microglia as well as by pre-synaptic blockade of P2X3Rs on primary afferent fibers. As described by shiokawa et al (32), post-synaptic P2X receptors in spinal dorsal horn neurons might be involved in excitation. Since we showed an upregulation of P2X3R expression in DRGs but not in the spinal cord dorsal horn, we concluded that in...
terms of expression P2X3Rs at the DRG levels play a role in this setting. However, we cannot exclude the involvement of P2X4 receptors in spinal microglia. Further investigation is definitely warranted.

Mechanisms of pain generation in chronic pancreatitis 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. Firstly, 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 are also expressing P2X1Rs and P2X2Rs, expression of these two subtypes of P2XRs was not significantly altered 4 weeks after TNBS infusion into the pancreas. Secondly, ATP-evoked inward currents were greatly potentiated in pancreatic DRG neurons in rats following TNBS infusion. Thirdly, 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 chronic pancreatitis is neuropathic due 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 dorsal root ganglia (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. Firstly, upregulation of P2X3R expression was reduced by administration of β-adrenergic receptor (β-AR) antagonist propranolol but not by α-adrenergic receptor (α-AR) antagonist phentolamine. Secondly, the ATP-evoked current density and the number of cells with action potentials evoked by ATP application was significantly reduced after incubation of β-AR antagonist propranolol. Thirdly, incubation of NE enhanced the ATP-induced intracellular calcium mobilization of DRG neurons in vitro. Fourthly, triple-labeling experiments showed that P2X3Rs were co-expressed with β₂-ARs in pancreas innervating DRG neurons. Finally, administration of β-AR antagonist propranolol significantly attenuated the pancreatic pain behaviors while administration of α-AR antagonist phentolamine 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 since expression of β₂-ARs was markedly enhanced while expression of β₁-ARs and β₃-ARs was not significantly altered. In addition, β₂-ARs antagonist Buto greatly suppressed pancreatic hyperalgesia in CP rats. We conclude that β₂-ARs might play a crucial role in the regulation of P2X3 receptor sensitization and the development of hyperalgesia under CP conditions. Of note is that β₂-ARs actually have 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 norepinephrine 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 P2XRs 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). Since PKA is involved in pancreatic 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 potentiation of NE action. This is further confirmed by the experiment that acute incubation of forskolin, an activator of adenylate 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. P2X3 receptors) in primary sensory neurons contributes to pancreatic hyperalgesia in a rat model of chronic pancreatitis induced by TNBS infusion. The enhanced adrenergic signaling (i.e. upregulation of β2-ARs) sensitizes the P2X3 receptors 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 the patients with
chronic pancreatitis.
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Competing interests: None.

Ethics approval: Animal care and handling of the animals were approved by the Institutional Animal Care and Use Committee at Soochow University and were strictly in accordance with the guidelines of the International Association for the Study of Pain.
Figure Legends

Fig. 1  Increase in expression and function of P2X3Rs in CP rats.
(A) 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 DRGs when compared to controls (CON, n=5, TNBS, n=3, *p<0.05, two sample t-test). (C) Enhanced expression of P2X3R mRNA after TNBS injection (CON, n=6; TNBS, n=4, *p<0.05, two sample t-test). (D) TNBS treatment did not significantly alter the expression of P2X3 receptors 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, two sample t-test, which were labeled by Dil in red (arrow). *p<0.05, two sample t-test. (F) TNBS injection did not enhance the ATP-evoked calcium signal of non-Dil 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) An example of ATP-evoked inward current of Dil 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 Dil labeled DRG neurons of CP rats (CON, n=12; TNBS, n=11, *p<0.05, two sample t-test).

Fig. 2  Antagonism of P2XR on pancreatic pain behavior.
(A) Administration of P2 receptor antagonist suramin (intrathecal, i.t.) significantly attenuated response frequency of CP rats to von Frey filament (VFF) stimulation (*p<0.05, compared with 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 four hours (*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 P2X3/P2X2 receptor antagonist A317491 (i.t.) 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 A317491 (30 nmol) on pancreatic hyperalgesia. The antinociceptive effect lasted for more than four hours (*p<0.05, compared with Pre. n=8, Dunn’s post hoc test following Friedman ANOVA). (F) Administration of A317491 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 weeks after TNBS infusion. (H).

**Fig. 3** Propranolol treatment suppresses P2X3R expression and function. (A) Administration of propranolol (Prop), a selective blocker of β adrenergic receptors, significantly reduced expression of P2X3Rs (CON, n=3; TNBS, n=3, *p<0.05, two sample t-test). (B) Administration of phentolamine (Phen), a selective blocker of α adrenergic receptors, did not alter expression of P2X3 receptors. (C) Administration of Prop significantly reduced ATP-evoked current density of pancreatic projection DRG neurons (NS, n=11, Prop, n=12, *p<0.05, two sample t-test). (D) Administration of Prop significantly reduced the percentage of numbers of cells (NS, n=11, Prop, n=13) with action potentials (AP) in responding to ATP application (*p<0.05, χ² test).

**Fig. 4** Potentiation of P2XR-mediated calcium signals by norepinephrine. (A) Application of A317491 greatly reduced the ATP-evoked intracellular calcium mobilization (n=29 cells). ATP concentration was 20 μM. (B). The potentiation effect of norepinephrine (NE) at different concentrations (2, 20 and 200 μM) on ATP-induced Ca²⁺ transients in pancreatic DRG neurons (n=21). (C). The potentiation of ATP-induced transients by NE (20 μM) was not blocked by CdCl₂ (50 μM). (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, two sample t-test). (E). Phentolamine (Phen), an antagonist of α adrenergic receptors, had little effect on potentiation of ATP induced Ca\(^{2+}\) transients by NE (20 μM). (F). The potentiation of ATP induced Ca\(^{2+}\) transients by NE (20 μM) was blocked by propranolol (Prop), a selective blocker of β adrenergic receptors (*p<0.05, compared with EXTRA, paired t-test, n=19 cells; #p<0.05, compared with NE, paired t-test, n=19 cells). EXTRA: extracellular solution.

Fig. 5  PKA inhibitor H-89 inhibits NE-induced potentiation of ATP-evoked responses.

(A) An example from a cell showing responses to ATP before and after incubation of NE (20 μM) in the presence and absence of H-89 (1 μM). (B) Bar graph showing enhancement of ATP-evoked response by NE and that potentiation of ATP induced Ca\(^{2+}\) transients by NE was blocked by H-89 preincubation (*p<0.05, compared with EXTRA, paired t-test, n=19; #p<0.05 compared with NE, paired t-test, n=19 cells). (C) An example from a cell showing responses to ATP before and after incubation of forskolin (1 μM). (D) Bar graph showing that ATP-induced Ca\(^{2+}\) transients were potentiated by incubation of forskolin (*p<0.05, compared with EXTRA, paired t-test, n=16).

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, two 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, two sample t-test). (C) TNBS injection had little effect on NE concentration in blood plasma (CON, n=3 rats, TNBS, n=4 rats). (D) \(β_1\)-AR expression was not significantly altered in DRGs of CP rats (n=4 for each group). (E) \(β_2\)-AR expression was significantly increased in DRGs of CP rats (*p<0.05, n=3 for each group). (F) \(β_3\)-AR expression was not significantly altered in DRGs of CP rats (n=4 for each group). (G) Pancreatic T9 DRG cells were labeled
with Dil (red). (H) P2X3R positive cells were shown in green. (I) $\beta_2$-AR positive cells were shown in blue. (J) Merge of double labeling of Dil and P2X3Rs. (K) Merge of $\beta_2$-AR-positive staining and Dil labeling. (L) Merge of $\beta_2$-AR staining and P2X3Rs labeling. Bar=50 μm.

Fig. 7  Suppression of $\beta_2$-AR inhibitor on pancreatic hyperalgesia

(A) Systemic administration of $\alpha$-adrenergic receptor antagonist Phen (ip., 1 and 5 mg/kg) did not produce any effect on pancreatic hyperalgesia in CP rats. (B) Administration of $\beta$-adrenergic receptor antagonist Prop (ip., 2 mg/kg) greatly attenuated CP-induced pancreatic hyperalgesia (*p<0.05, compared with Pre. n=8 rats, Kruskal-Wallis ANOVA followed by Tukey post hoc test). (C) The effect of $\beta$-adrenergic receptor antagonist Prop at 2 mg/kg lasted for one hour (*p<0.05, compared with Pre. n=8 rats, Dunn’s post hoc test following Friedman ANOVA). (D) Administration of $\beta_2$-adrenergic receptor antagonist butoxamine (Buto, ip., 1.5 mg/kg) markedly attenuated CP-induced pancreatic hyperalgesia (*p<0.05, compared with Pre. n=8 rats, Kruskal-Wallis ANOVA followed by Tukey post hoc test). (E) The effect of Buto lasted for one hour (*p<0.05, compared with Pre. n=8 rats, Dunn’s post hoc test following Friedman ANOVA). (F) Effect of Prop and Buto on the locomotor performance (Rota-rod test) 30 minutes after Prop and Buto application. Prop at the dose of 2 mg/kg and Buto at the dose of 1.5 mg/kg did not significantly alter the time for rats to stay on the bar (n=8 rats for each group).
REFERENCES


29. Mullady DK, Yadav D, Amann ST, O'Connell MR, Barkmada MM, Elta


Figure 2

A. Response Frequency (%) vs. Filament strength (g) for Suramin.

B. Response Frequency (%) vs. Time after Suramin (0.5h, 1h, 2h, 4h, 8h) for VFF 10 g.

C. Response Frequency (%) vs. Filament strength (g) for Suramin.

D. Response Frequency (%) vs. Filament strength (g) for A317491.

E. Response Frequency (%) vs. Time after A317491 (0.5h, 1h, 2h, 4h, 8h) for VFF 10 g.

F. Response Frequency (%) vs. Filament strength (g) for A317491.

G. Response Frequency (%) vs. Filament strength (g) for NS.

H. Time to fall (s) for Suramin.

I. Time to fall (s) for A317491.
Figure 3

A

<table>
<thead>
<tr>
<th>TNBS</th>
<th>NS</th>
<th>Prop</th>
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<tr>
<td>P2X3</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>GAPDH</td>
<td>![Image]</td>
<td>![Image]</td>
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</tbody>
</table>

Relative densitometry

- NS: 3.0 ± 0.2
- Prop: 2.5 ± 0.1

![Image]

B

<table>
<thead>
<tr>
<th>TNBS</th>
<th>NS</th>
<th>Phen</th>
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<tbody>
<tr>
<td>P2X3</td>
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<tr>
<td>GAPDH</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

Relative densitometry

- NS: 0.8 ± 0.1
- Phen: 0.7 ± 0.1

![Image]

C

NS ATP

- Current density (pA/pF):
  - NS: 100 ± 5 pA/pF
  - Prop: 50 ± 5 pA/pF

![Image]

D

Cells with AP (%)

- NS: 100%
- Prop: 75%

![Image]
Figure 4

(A) A317491

(B) NE2, NE20, NE200

(C) NE, NE+CdCl₂

(D) NE, TNBS

(E) NE, NE+Phen

(F) NE, NE+Prop, NE

Graphs showing ΔRatio (340/380) and % of increase for different conditions.
Figure 7

A. Response Frequency (%) vs. Filament strength (g)

B. Response Frequency (%) vs. Filament strength (g)

C. Response Frequency (%) vs. Time after injection

D. Response Frequency (%) vs. Filament strength (g)

E. Response Frequency (%) vs. Time after injection

F. Time to fall vs. Prop 2 and Buto 1.5