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Original Article

Acute Effects of Transforming Growth Factor-β1 on Neuronal Excitability and Involvement in the Pain of Rats with Chronic Pancreatitis

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Background/Aims
This study was to investigate whether transforming growth factor-β1 (TGF-β1) plays a role in hyperalgesia in chronic pancreatitis (CP) and the underlying mechanisms.

Methods
CP was induced in male adult rats by intraductal injection of trinitrobenzene sulfonic acid (TNBS). Abdominal hyperalgesia was assessed by referred somatic behaviors to mechanical stimulation of rat abdomen. Dil dye injected into the pancreas was used to label pancreas-specific dorsal root ganglion (DRG) neurons. Whole cell patch clamp recordings and calcium imaging were performed to examine the effect of TGF-β1 on acutely isolated pancreas-specific DRG neurons. Western blot analysis was carried out to measure the expression of TGF-β1 and its receptors.

Results
TNBS injection significantly upregulated expression of TGF-β1 in the pancreas and DRGs, and TGF-β1 receptors in DRGs (T9-T13) in CP rats. Intrathecal injection of TGF-β receptor I antagonist SB431542 attenuated abdominal hyperalgesia in CP rats. TGF-β1 application depolarized the membrane potential and caused firing activity of DRG neurons. TGF-β1 application also reduced rheobase, hyperpolarized action potential threshold, and increased numbers of action potentials evoked by current injection of pancreas-specific DRG neurons. TGF-β1 application also increased the concentration of intracellular calcium of DRG neurons, which was inhibited by SB431542. Furthermore, intrathecal injection of TGF-β1 produced abdominal hyperalgesia in healthy rats.

Conclusions
These results suggest that TGF-β1 enhances neuronal excitability and increases the concentration of intracellular calcium. TGF-β1 and its receptors are involved in abdominal hyperalgesia in CP. This and future study might identify a potentially novel target for the treatment of abdominal pain in CP.
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Key Words
Abdominal pain; Chronic pancreatitis; Dorsal root ganglion; Transforming growth factor beta 1

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Introduction

Chronic pancreatitis (CP) is a common disease characterized by persistent inflammation, parenchymal fibrosis, and destruction of the glandular pancreas.[1] The most clinically relevant feature of CP is recurrent upper abdominal pain, which is not only intense and persistent but also difficult to treat.[1] The pathobiological mechanisms of pain in CP are incompletely understood, which makes obstacles in developing novel effective therapeutic approaches. Although central sensitization inevitably follows, sensitization of peripheral nociceptors is the critical and initiating event in pain caused by local inflammation.[2] Previous studies demonstrated the sensitization of pancreas-specific dorsal root ganglion (DRG) neurons and its involvement in abdominal pain in CP.[3,4] However, the detailed mechanisms of peripheral sensitization in CP remain largely unknown.

Under pathological conditions, a variety of small molecules, cytokines and enzymes can induce sensitization of sensory neurons, resulting in persistent pain.[5-7] Transforming growth factor-β1 (TGF-β1) is also prominently expressed in such conditions and plays a central role in wound healing and promoting fibrosis in various diseases, including CP.[8-10] However, the role of TGF-β1 in pain processing is controversial. Evidence suggests that TGF-β1 has protective effects against neuropathic pain in the central nervous system,[11,12] while in peripheral nervous system, TGF-β1 is recently reported as an algogenic substance that contributes to peripheral sensitization by downregulation of the *KCNA4* gene,[13] or activation of cyclin-dependent kinase-transient receptor potential vanilloid type 1 (Cdk5-TRPV1) signaling[14,15] and transforming growth factor β activated kinase 1 (TAK1)/protein kinase C (PKC)-TRPV1 signaling.[16] As mentioned in those studies,[13-16] TGF-β1 is a complex modulator of sensory neuronal function, and its signaling pathway in the induction and development of pancreatic pain in rats with chronic pancreatitis was not fully understood. Therefore, the roles of TGF-β1 and its receptors were re-explored in the setting of chronic pancreatitis.

In the present study, we show that TGF-β1 promptly excites primary sensory neurons and increases the concentration of intracellular calcium, both of which have not been reported before. Since TGF-β1 is known to be upregulated in the pancreas in rodents as well as humans with CP,[17,18] we investigated the role of TGF-β1 in the development of abdominal pain. We show that TGF-β1 in vivo promptly induces mechanical hyperalgesia of the abdomen. TGF-β1 and its receptors are up-regulated in pancreatic DRGs in CP rats. Blockade of this signaling attenuates mechanical hyperalgesia. Our results provide a novel mechanism underlying the involvement of TGF-β1 in abdominal pain hypersensitivity.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (200-220 g) were employed in the present study. Care and handling of rats were approved by the Institutional Animal Care and Use Committee of Soochow University. All experiments were performed in accordance with the guidelines of the International Association for the Study of Pain. Abdominal surgery was carried out under anesthesia of ketamine (80 mg/kg, intraperitoneal [i.p.]) plus xylazine (5-10 mg/kg, i.p.). Following tissue harvest, rats were sacrificed by decapitation.

Induction of Chronic Pancreatitis in Rats

Chronic pancreatitis (CP) was induced by an intraductal injection of trinitrobenzene sulfonic acid (TNBS), as described previously.[3,4] Control rats received normal saline (NS). Further intervention and experiments in vivo or in vitro are performed at 3 weeks after TNBS treatment when a robust chronic pancreatitis had developed.

Western Blotting

Proteins were extracted from T9-T13 DRGs from control and CP rats as described previously.[19,20] Protein extracts from DRGs or pancreas were loaded onto a 15% Tris-HCl SDS-PAGE gel (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were electrotransferred onto 0.22 μm polyvinyldifluoride membrane (Millipore, Billerica, MA, USA) at 200 mA for 2 hours at 4°C. Primary antibodies used in the present study were mouse anti-TGF-β1 (1:2000; Abcam ab27969, Cambridge, UK), rabbit anti-TGF-βR I (1:200; Abcam ab31013), rabbit anti-TGF-βR II (1:1000; Abcam ab186838), and rabbit anti-GAPDH or mouse anti-β-actin (1:1000; MultiSciences Biotech Co, Hangzhou, China). Mouse anti-TGF-β1 can recognize both TGF-β1 and latent TGF-β1 complex. The same loading control, GAPDH, was used for analysis of both TGF-β1 and latent TGF-β1 complex (Fig. 1E and 1F). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or rabbit antibody (1:4000; MultiSciences Biotech Co). The immunoreactive proteins were detected by enhanced chemiluminescence (ECL kit; Amersham Biosciences, Arlington Heights, IL, USA). The membranes were

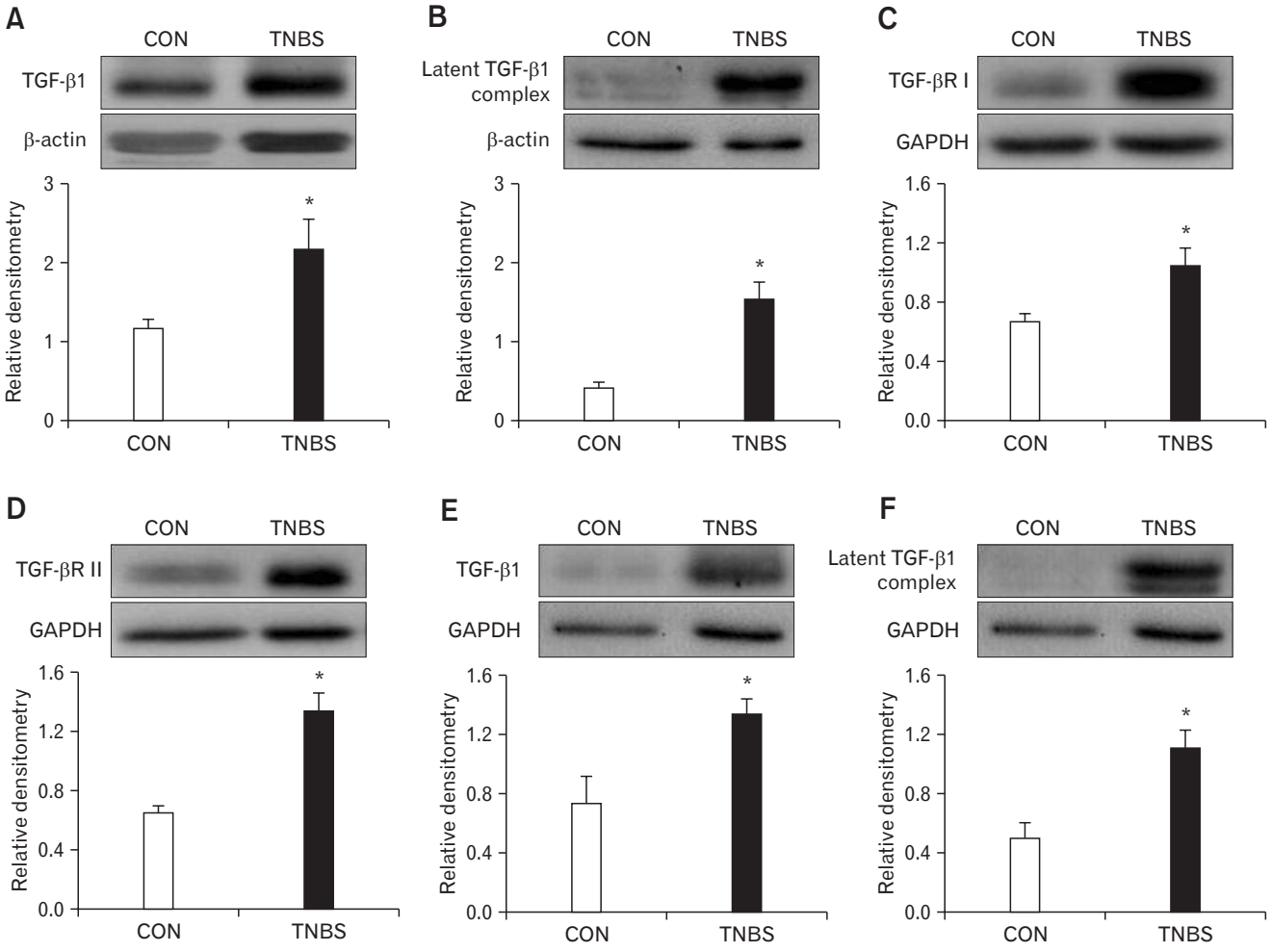

Figure 1. Trinitrobenzene sulfonic acid (TNBS) injection up-regulates expression of transforming growth factor-β1 (TGF-β1) and TGF-β receptors. (A, B) TNBS injection significantly enhanced the expression of TGF-β1 and latent TGF-β1 complex in the pancreas of rats (*$P < 0.05$, n = 4 per group, two-sample *t* test). (C-F) TNBS injection significantly enhanced the expression of TGF-β receptor I and II, TGF-β1, and latent TGF-β1 complex in the pancreatic dorsal root ganglions (DRGs) of rats (*$P < 0.05$, n = 4 per group, two-sample *t* test). CON, control.

scanned and bands were visualized by Bio-Rad ChemiDoc XRS+. Band intensities were measured by Image J software. All samples were normalized to GAPDH or β-actin as a loading control.

Von Frey Filament Measurements

Von Frey filament (VFF) testing was performed as described previously.[4,20] The filament at the force of 10 g (North Coast Medical Inc, San Jose, CA, USA) was applied 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. All behavioral tests were performed in a blinded manner. Behavioral data were expressed as the number of positive responses per 10 times stimulation for each rat.

Drug Application

TGF-β1 (PeproTech, Rocky Hill, NJ, USA) was dissolved in phosphate buffered saline (contain 0.1% bovine serum albumin) as stock solution, and freshly prepared diluted in normal external solution or 0.9% NS. TGF-β receptor I antagonist, SB431542 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) as stock solution, and freshly prepared in normal external solution or 0.9% NS. TRPV1 antagonist, capsazepine (CZP; Sigma-Aldrich) was dissolved in methanol as stock solution, and freshly prepared diluted in normal external solution.

Evaluation of Motor Performance

The effects of SB431542 and TGF-β1 on motor performance of rats were determined by the Rota-rod test as described previously.[21] The length of time for rats to stay on the revolving bar (20 rpm) was recorded and analyzed.

Labeling of Pancreas-specific Dorsal Root Ganglia Neurons

For experiments involving calcium imaging and patch clamp recordings, the lipid-soluble fluorescence dye, 1,19-dioleyl-3, 3, 39, 3-tetramethy-lindocarbocyanine methanesulfonate (DiI; Invitrogen, Carlsbad, CA, USA), 25 mg in 0.5 mL methanol, was injected in 1 μL volume at 8-10 sites on the exposed pancreas under anesthesia, as described previously.[3,4] Three weeks after DiI injection, DRGs (T9-T13) were dissected out for patch clamp recordings and calcium imaging study.

Dissociation of Dorsal Root Ganglion Neurons

Isolation of DRG neurons from adult rats was performed as described previously.[3,4,22] In brief, bilateral T9-T13 DRGs were dissected out and incubated in dissecting solution containing collagenase D (1.5-1.8 mg/mL; Roche, Indianapolis, IN, USA) and trypsin (1.2 mg/mL; Sigma-Aldrich) for 90 minutes at 34.5°C. DRGs were then washed and transferred to the dissecting solution containing DNase (0.5 mg/mL; Sigma-Aldrich). A single cell suspension was obtained by repeated trituration through flame-polished glass pipettes. Cells were then plated onto acid-cleaned glass coverslips for patch-clamp recordings and calcium measurements.

Patch-clamp Recordings

As described previously,[3,21] coverslips containing adherent DRG neurons were put in a chamber (~1 mL volume), attached to the stage of an inverting microscope (IX70 Olympus, Tokyo, Japan), and continuously superfused (1.5 mL/min) at room temperature with normal external solution.[3] Recording pipettes were pulled from the borosilicate glass tubing using a horizontal puller (P-97; Sutter Instruments, Novato, CA, USA) and typically had a resistance of 5-8 MΩ when filled with normal pipette solution.[3] Recordings were performed with an EPC10 amplifier and the program Patchmaster (HEKA Elektronik, Lambrecht, Germany).

Intracellular Calcium Measurements

Fura-2 Ca^{2+} imaging was performed as described previously.[20] Recordings were performed by the program MetaFlour (Molecular Device, Sunnyvale, CA, USA). 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. The percentage of changes in ratio was calculated to measure the drug-induced calcium mobilization.

Statistical Methods

All data in the present study were expressed as mean ± SEM or as a percentage. Statistical analyses were conducted using OriginPro 8 (OriginLab, Northampton, MA, USA). Normality of all data was checked before analyses. Significance of difference was determined by two-sample or paired sample *t* test, one-way repeated measures ANOVA followed by Tukey post hoc test, or two-way ANOVA followed by Tukey post hoc test. A P-value < 0.05 was considered statistically significant.

Results

TNBS Injection Up-regulates Expression of Transforming Growth Factor-β1 and Transforming Growth Factor-β Receptors

Three weeks after TNBS injection, the expression of TGF-β1 and latent TGF-β1 complex (TGF-β1 precursor) in pancreas of rats were significantly increased (*$P < 0.05$ versus control; n = 4 per group; Fig. 1A and 1B), consistent with clinical features of patients with CP described previously.[17] We further examined the expression of TGF-β1 and its receptors in pancreatic DRGs (T9-T13) from control and TNBS-treated rats. The expression of TGF-β1, latent TGF-β1 complex, and TGF-β receptor I and II in pancreatic DRGs were significantly increased (*$P < 0.05$ versus control; n = 4 per group; Fig. 1C-F). These data suggest that TNBS injection leads to up-regulation of TGF-β1 and its receptors in peripheral nervous system, which implicates the possible activation of TGF-β1/TGF-β receptors signaling in pancreatic DRGs of CP rats.

Transforming Growth Factor-β Receptor I Antagonist Attenuates Mechanical Hyperalgesia

Single intrathecal injection (i.t.) of SB431542, a specific and potent TGF-β receptor I antagonist, significantly reduced the response frequency to stimulation force of 10 g compared with the NS group in a dose-dependent manner (**$P < 0.01$, ***$P < 0.001$ versus NS; n = 7 per group; Fig. 2A). The optimized dose of the maximal inhibition effect was 100 μg/kg body weight (P

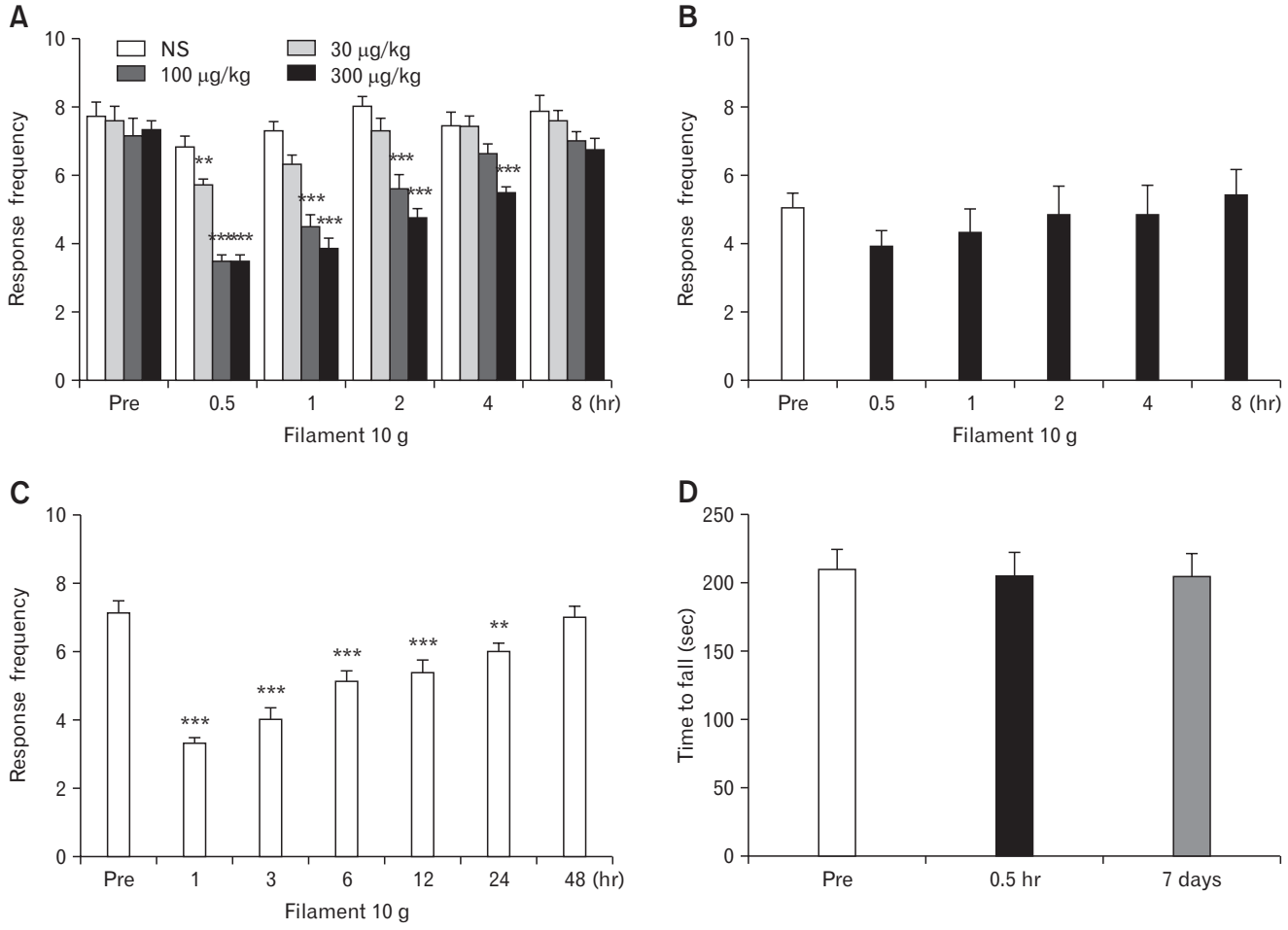

Figure 2. Transforming growth factor-β (TGF-β) receptor I antagonist SB431542 attenuates mechanical hyperalgesia. (A) Intrathecal injection (i.t.) of SB431542 significantly reduced response frequency of chronic pancreatitis (CP) rats to von Frey filament (VFF) stimuli (10 g) (**$P < 0.01$, ***$P < 0.001$ versus normal saline [NS]; n = 7 per group). (B) SB431542 at 100 μg/kg (i.t.) had no significant effect on response frequency of control rats (n = 7). (C) Daily intrathecal injection of SB431542 at 100 μg/kg for 7 days significantly reduced the response frequency of CP rats, which last for 24 hours (**$P < 0.01$, ***$P < 0.001$ versus Pre [before administration]; n = 7). (D) SB431542 at 100 μg/kg (i.t.) did not produce any effect on time for the CP rats to stay on the rotation bar (n = 8). Paired sample *t* test (B-D), two-way ANOVA followed by Tukey post hoc test (A).

> 0.05, 300 μg/kg versus 100 μg/kg), which lasted for 2 hours. Thus, we used this dose in age-matched healthy rats to determine the drug specificity. SB431542 at a dose of 100 μg/kg had no significant effect on response frequency in healthy rats, indicating that SB431542 is not a nonspecific analgesic (compared with Pre [before administration], n = 7; Fig. 2B) and exerts its effect on behavior only in CP rats. Furthermore, SB431542 at 100 μg/kg was administered intrathecally once daily for 7 consecutive days in CP rats. As expected, multiple injections of SB431542 produced a dramatic antinocifensive effect in CP rats, lasting for at least 24 hours (**$P < 0.01$, ***$P < 0.001$ versus Pre; n = 7; Fig. 2C). To exclude the possible effect of SB431542 on motor performance, the Rotarod test was performed. Neither single nor multiple injections of SB431542 at 100 μg/kg altered the time for rats to stay on the rotation bar compared with Pre (n = 8; Fig. 2D). These data suggest that TGF-β1/TGF-β receptor signaling is involved in mechanical hyperalgesia of abdomen in CP.

Transforming Growth Factor-β1 Produces Mechanical Hyperalgesia

To determine whether TGF-β1 produces hyperalgesia, TGF-β1 was injected intrathecally in healthy rats. Administration

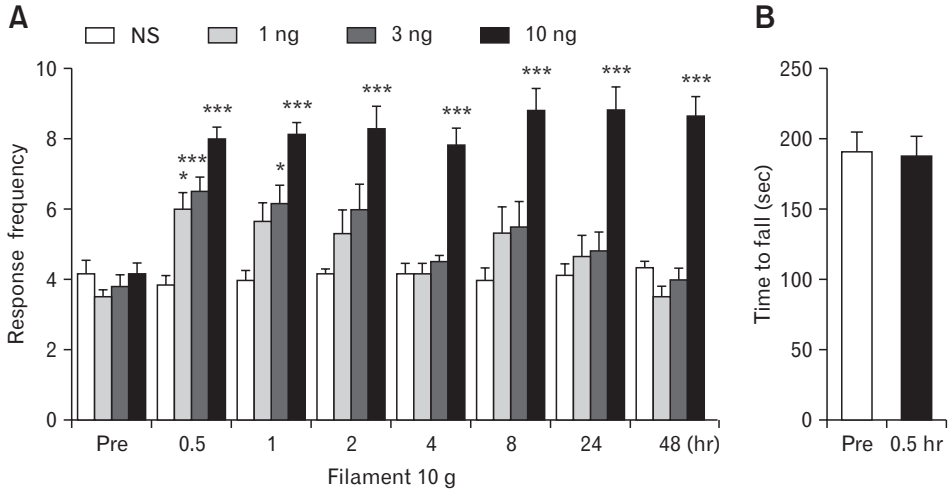

Figure 3. Transforming growth factor-β1 (TGF-β1) produces mechanical hyperalgesia. (A) TGF-β1 (intrathecal injection [i.t.]) produced an increase of response frequency of control rats to von Frey filament (VFF) stimuli (10 g) (*$P < 0.05$, ***$P < 0.001$ versus normal saline [NS]; n = 7 per group; two-way ANOVA followed by Tukey post hoc test). (B) TGF-β1 at 10 ng per rat (i.t.) did not produce any effect on time for the control rats to stay on the rotation bar (n = 8, paired sample *t* test).

of TGF-β1 produced an increase in response frequency to stimulation force of 10 g compared with NS controls in a dose-dependent manner (*$P < 0.05$, ***$P < 0.001$ versus NS; n = 7 per group; Fig. 3A). The maximal hyperalgesic effect was observed at a dose of 10 ng per rat. The effect produced by 1 and 3 ng per rat TGF-β1 lasted for 0.5 and 1 hours, respectively (Fig. 3A). Interestingly, the effect produced by 10 ng per rat TGF-β1 last for at least 48 hours. However, Rota-rod test shows TGF-β1 at 10 ng per rat did not alter the time for rats to stay on the rotation bar compared with Pre (n = 8; Fig. 3B).

Transforming Growth Factor-β1 Induces Depolarization or Firing Activity of Dorsal Root Ganglion Neurons

In order to investigate the potential role of TGF-β1 in the activation of peripheral nociceptors, electrophysiological recordings were performed on small and medium-size DRG neurons that are likely to mediate nociception and pain.[23,24] In our study, Dil-labeled pancreas-specific DRG neurons were recorded (Fig. 4A). Under whole-cell current clamp recordings, bath application of 10 ng/mL TGF-β1 depolarized the resting membrane potential (RP) in a portion of DRG neurons (34 out of 55 neurons recorded; Fig. 4B and 4C). The RP returned to the baseline within 1 minute after washout. Sixteen of these 34 neurons exhibited action potentials (APs) after TGF-β1 application (16 out of 55 neurons recorded; Fig. 4B and 4C). For the remaining 18 activated neurons without firings, the average amplitude of depolarization was 5.9 ± 1.2 mV (Fig. 4D). The other 21 neurons showed no response to TGF-β1 application (Fig. 4B and 4C).

Transforming Growth Factor-β1 Enhances Excitability of Dorsal Root Ganglion Neurons

As the above results suggest that TGF-β1 depolarized the RP in a portion of DRG neurons, we then examined the effect of TGF-β1 (10 ng/mL, bath application for 3 minutes) on provoked excitability of DRG neurons. Rheobase, the minimal stimulation current to evoke APs, was significantly decreased after TGF-β1 application (Pre: 103.2 ± 15.8 pA, Post: 87.4 ± 14.1 pA; *$P < 0.05$; n = 19; Fig. 5A). The AP threshold, the minimal voltage at which the AP was generated, was markedly decreased after TGF-β1 application (Pre: −29.7 ± 0.9 mV, Post: −33.3 ± 0.9 mV; **$P < 0.01$; n = 19; Fig. 5B). In addition, application of TGF-β1 also increased the number of APs evoked by 300-milliseconds 2 times (2×) and 3 times (3×) rheobase current stimulation (2×, Pre: 4.84 ± 0.24, Post: 5.79 ± 0.44, *$P < 0.05$, n = 19; 3×, Pre: 8.00 ± 0.31, Post: 9.26 ± 0.58, **$P < 0.01$, n = 19; Fig. 5C and 5D). Furthermore, application of TGF-β1 significantly increased the number of APs evoked by 1-second ramp current stimulation from 0 to 200 pA or 500 pA (200 pA ramp, Pre: 2.84 ± 0.61, Post: 4.26 ± 0.76, **$P < 0.01$, n = 19; 500 pA ramp, Pre: 6.32 ± 0.85, Post: 7.89 ± 0.96, **$P < 0.01$, n = 19; Fig. 5E and 5F). These results suggest that TGF-β1 treatment promptly enhances the excitability of pancreas-specific DRG neurons.

Transforming Growth Factor-β1 Increases Intracellular Calcium Ion Concentration via Transforming Growth Factor-β Receptor I in Dorsal Root Ganglion Neurons

We then studied the acute effect of TGF-β1 on intracellular calcium mobilization of DRG neurons. Bath application of 10 ng/

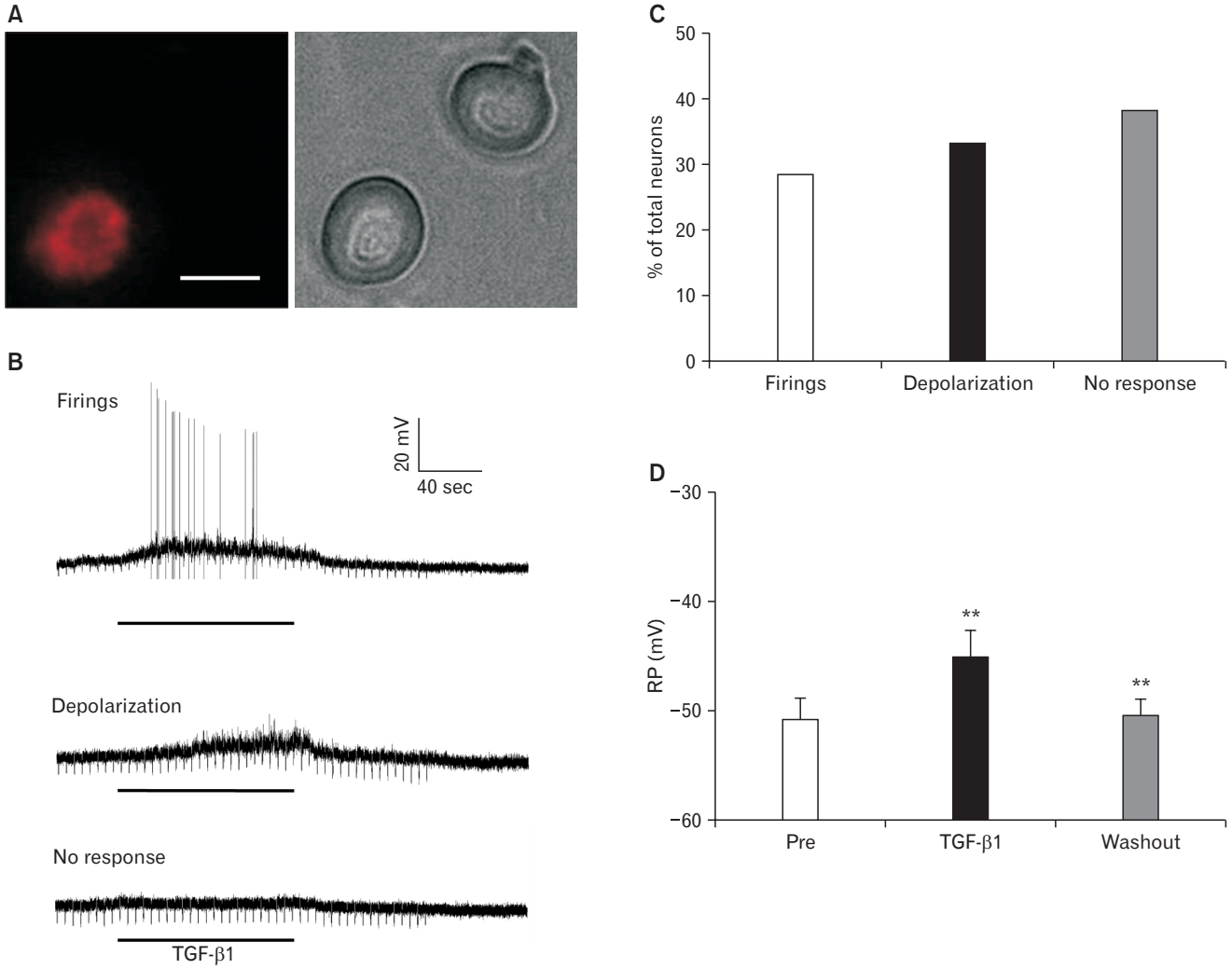

Figure 4. Transforming growth factor-β1 (TGF-β1) induces depolarization and firing activity of dorsal root ganglion (DRG) neurons. (A) DiI-fluorescence (left) and bright-field (right) images of acutely isolated DRG neurons. Pancreas-specific neurons are shown in red in DiI-fluorescence image. Bar = 25 μm. (B) The typical current-clamp recordings of DRG neurons showing firings, depolarization or no response after application of 10 ng/mL TGF-β1. (C) The percentage of DRG neurons showing firings (28.6%), depolarization (33.3%), or no response (38.1%) after application of TGF-β1 (n = 55 neurons in total). (D) Resting membrane potentials (RPs) before TGF-β1 application (Pre), peak value of RPs during TGF-β1 application (TGF-β1) and RPs after washout (Washout) (**$P < 0.01$ for TGF- β1 versus Pre, and Washout versus TGF-β1; n = 18; one-way repeated measures ANOVA followed by Tukey post hoc test).

mL TGF-β1 for 1 minute caused significant intracellular calcium ion concentration ($[Ca^{2+}]_i$) increase in a portion of DRG neurons (32 of 114 neurons recorded; Fig. 6A). The average magnitude of TGF-β1-induced $[Ca^{2+}]_i$ increase was 36.4 ± 6.0% (n = 32; Fig. 6A). The $[Ca^{2+}]_i$ returned to baseline within 1 minute after washout in most neurons tested. When TGF-β1 was applied twice at an interval of 10 minutes, the magnitude of the second $[Ca^{2+}]_i$ increase was 33.7 ± 7.0%, which was not significantly different from that of the first amplitude (n = 32; Fig. 6A), suggesting that no desensitization occurs during repetitive application of TGF-β1.

To examine whether TGF-β receptor I was involved in the TGF-β1-evoked $[Ca^{2+}]_i$ increase, DRG neurons were pretreated with SB431542 (10 μM, 3 minutes). In the presence of SB431542, the $[Ca^{2+}]_i$ increase evoked by TGF-β1 was significantly less than that in the control medium (***$P < 0.001$ versus Pre, n = 43; Fig. 6B). The inhibitory effect of SB431542 on the $[Ca^{2+}]_i$ increase was partly reversed after 10 minute washings (*$P < 0.05$ versus SB431542, n = 43; Fig. 6B). Since previous reports revealed that

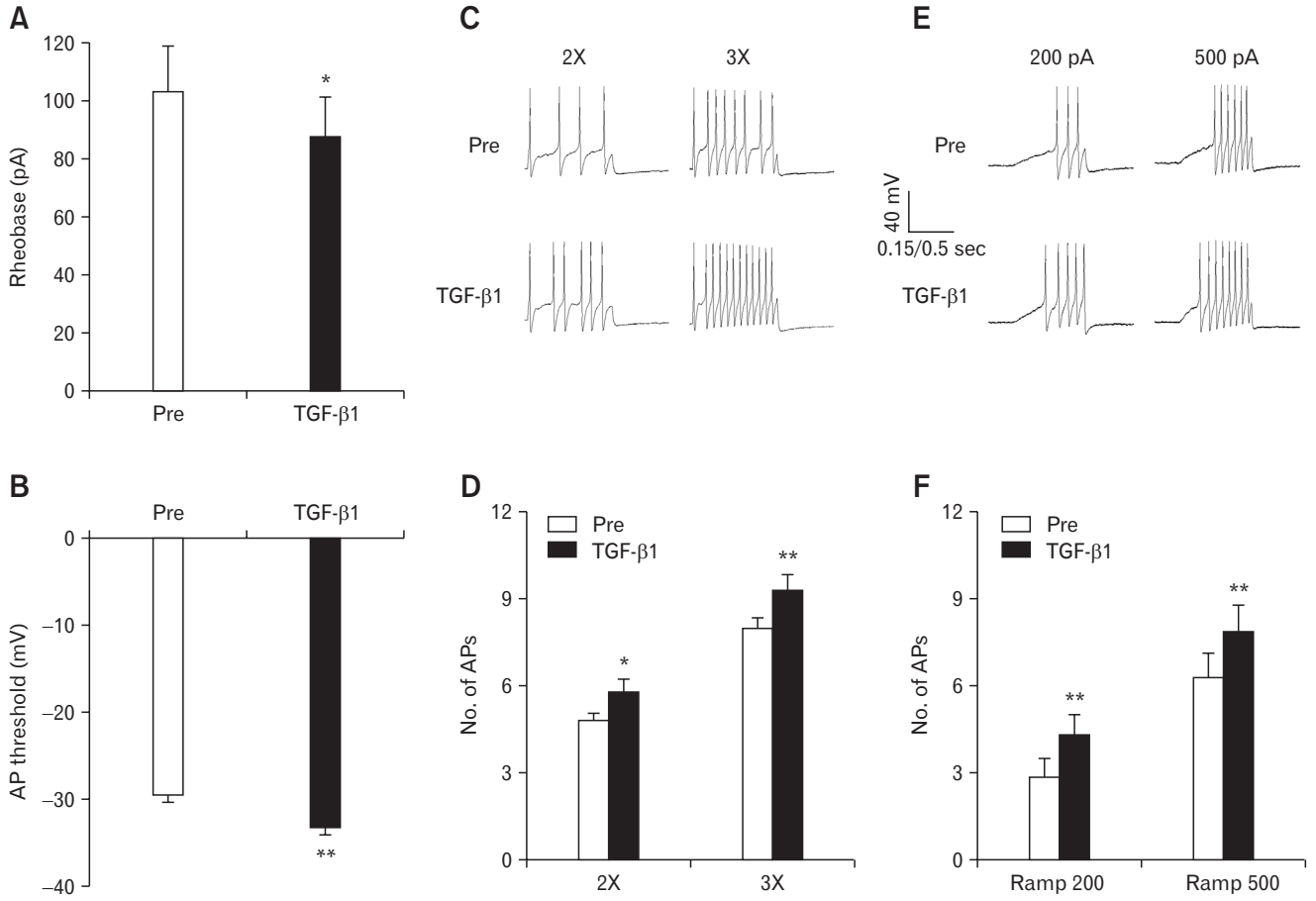

Figure 5. Transforming growth factor-β1 (TGF-β1) enhances excitability of dorsal root ganglion neurons. (A) TGF-β1 application significantly reduced rheobase (*$P < 0.05$, n = 19). (B) TGF-β1 application markedly decreased action potential (AP) threshold (**$P < 0.01$, n = 19). (C) Typical traces of APs evoked by 2 times (2×) and 3 times (3×) rheobase current stimulation. (D) Bar graph shows the numbers of APs evoked by 2× and 3× rheobase current stimulation were significantly increased after TGF-β1 application (*$P < 0.05$, **$P < 0.01$; n = 19). (E) Typical traces of APs evoked by 200 pA and 500 pA ramp current stimulation. (F) Bar graph shows the numbers of AP evoked by 200 pA and 500 pA ramp current stimulation were significantly increased after TGF-β1 application (**$P < 0.01$, n = 19). Paired sample *t* test (A-F). Pre, before TGF-β1 application.

TGF-β1 sensitizes TRPV1 in DRG neurons[14,16,25] we next examined whether TRPV1 was involved in the TGF-β1-induced $[Ca^{2+}]_i$ increase. Pretreatment with TRPV1 antagonist CZP (10 μM, 3 minutes) did not alter the magnitude of TGF-β1-evoked $[Ca^{2+}]_i$ increase (Fig. 6C, n = 28), suggesting that TGF-β1-evoked $[Ca^{2+}]_i$ increase is not mediated by TRPV1.

Discussion

The present study demonstrated that TGF-β1 and its receptors are involved in the mechanical hyperalgesia of abdomen in rats with chronic pancreatitis (CP). It is well known that TGF-β1 is up-regulated in the pancreas of rodents as well as patients with CP[17,18], and plays a dominant role in the development of CP by contributing to local inflammation and promoting pancreatic fibrosis.[10,18,26] In addition to upregulation of TGF-β1 expression in the pancreas of TNBS-treated rats, we furthermore showed that TGF-β1 and it receptors were also up-regulated in pancreatic DRGs of CP rats, which implicated that TGF-β1/TGF-β receptor signaling might be also activated in the peripheral nervous system in CP rats. A recent report shows that administration of TGF-β1 neutralizing antibody significantly attenuated the pancreatic hyperalgesia in rats with CP.[13] In the present study, administration of SB431542, a potent and selective TGF-β receptor I antagonist,[27] significantly attenuated mechanical hyperalgesia of abdomen in CP rats. Together, these data suggest a role for TGF-β1 and its receptors in the develop-

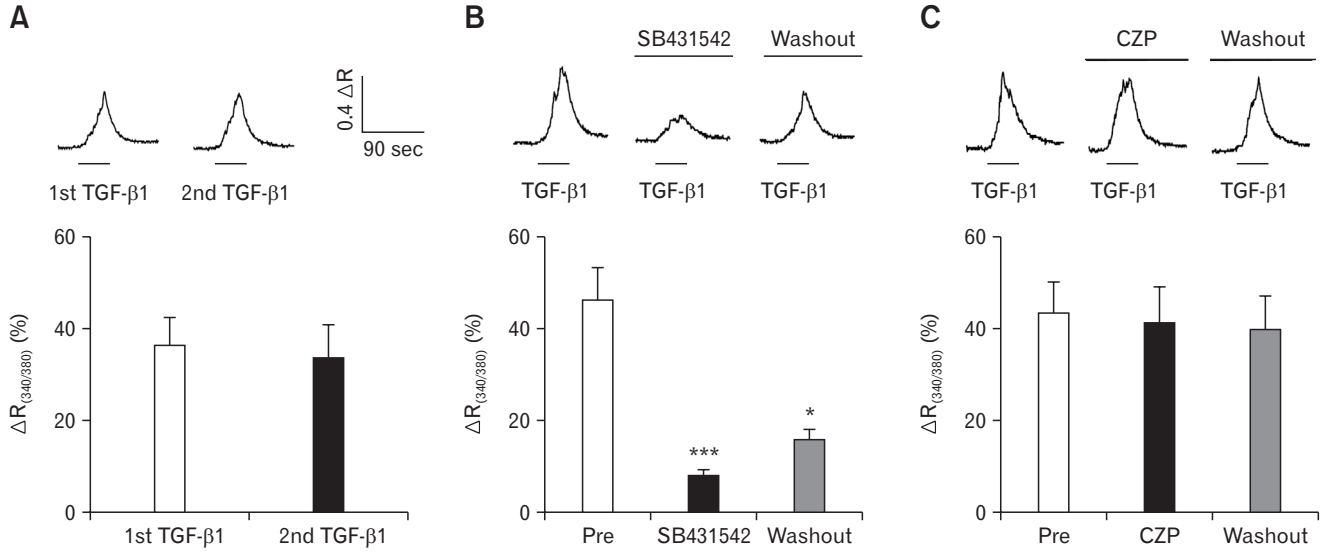

Figure 6. Transforming growth factor-β1 (TGF-β1) increases intracellular calcium ion concentration ($[Ca^{2+}]_i$) via TGF-β receptor I. (A) Representative traces of changes in $[Ca^{2+}]_i$ ($R_{(340/380)}$) in dorsal root ganglion neurons induced by 10 ng/mL TGF-β1 (top) and bar graph (bottom) shows no desensitization of TGF-β1-evokded $[Ca^{2+}]_i$ increase after the second time application with an interval of 10 minutes (n = 32). (B) Representative traces of the inhibitory effect of TGF-β receptor I antagonist, SB431542, at the dose of 10 μM on TGF-β1-evoked $[Ca^{2+}]_i$ increase. Bar graph shows SB431542 inhibited 80% of TGF-β1-evoked $[Ca^{2+}]_i$ increase, which was partly reversed after washout (***$P < 0.001$ versus Pre, *$P < 0.05$ versus SB431542; n = 43). (C) Representative traces (top) and bar graph (bottom) show capsazepine (CZP) at the dose of 10 μM did not alter the TGF-β1-evoked $[Ca^{2+}]_i$ increase (n = 28). One-way repeated measures ANOVA followed by Tukey post hoc test (A-C). Pre, before SB431542 or CZP application; Washout, after washout.

ment of abdominal hyperalgesia in rats with CP. Of particular note is that SB431542 did not affect nociceptive behaviors in control rats in our study, while in the previous report,[13] TGF-β1 neutralization in control rats resulted in pancreatic hyperalgesia. This discrepancy may be due to differences in the administration methods (intrathecal vs intraperitoneal) and in the focus on action time of the drugs (0.5-8 hours after injection vs 1 week after injection), or more probably due to the fact that we used the TGF-β receptor I antagonist which blocks the TGF-β signaling including TGF-β1, TGF-β2 and TGF-β3,[27-29] while TGF-β1 neutralizing antibody does not block the other 2 isoforms (TGF-β2 and TGF-β3) that can also bind to TGF-β receptors and further activate the downstream signaling.

Another important finding is that TGF-β1 plays an acute effect on neuronal excitability. To the best of our knowledge, this is the first report that shows the acute effect of TGF-β1 on neuron excitability. This is supported by the following observations. First, TGF-β1 application depolarized the membrane potential and caused firing activity of pancreas-specific DRG neurons. Second, TGF-β1 application also reduced the rheobase, hyperpolarized action potential threshold, and increased the number of action potentials evoked by current injection of pancreas-specific DRG neurons. In a previous report[13] TGF-β1 sensitization of DRG neurons in vitro, occurring after incubation of TGF-β1 for 24 hours but not earlier (1 hour), was attributed to downregulation of the Kv1.4 gene and decreased voltage-gated A-type K^+ currents (I_A), while TGF-β1 sensitization in our study, occurring after application of TGF-β1 for 3 minutes, was accompanied by decreased AP duration (data not shown), suggesting that the acute sensitized effect of TGF-β1 was due to some factors other than Kv1.4. Although these factors remain to be further investigated, one possibility is that 2-pore domain potassium channels are the effectors in this pathway because inhibition of their activity leads to membrane depolarization[30] similar to that observed in our study. This pathway mediates an immediate but mild depolarization that is not enough to trigger discharges.

In addition to enhancing the neuronal excitability, TGF-β1 quickly increased $[Ca^{2+}]_i$ in pancreas-specific DRG neurons. Pretreatment of SB431542 largely inhibited or even completely blocked TGF-β1-induced $[Ca^{2+}]_i$ increase, indicating the involvement of TGF-β receptor I. Since TGF-β receptors (I and II) are serine-threonine kinase receptors that cannot act as ion channels,[31] downstream signaling and effectors are definitely necessary for TGF-β1-induced $[Ca^{2+}]_i$ increase. Recent studies indicate that TGF-β1

signaling sensitizes TRPV1 in primary sensory neurons.[14-16] However, our result suggests that TGF-β1-induced $[Ca^{2+}]_i$ increase was independent of TRPV1. Of special note is that the proportion (16 of 55 neurons) of neurons displaying TGF-β1-induced firing is very similar to the proportion (32 of 114 neurons) of neurons showing $[Ca^{2+}]_i$ increase to TGF-β1, suggesting that TGF-β1-induced $[Ca^{2+}]_i$ increase might be due to the extracellular calcium influx through voltage-gated calcium channels (VGCCs). Nevertheless, our preliminary experiments show that neither pretreatment of $CdCl_2$ (a none selective VGCC antagonist) nor removal of the extracellular calcium inhibited TGF-β1-induced $[Ca^{2+}]_i$ increase (data not shown), suggesting that calcium release from intracellular calcium pools might be a main source of TGF-β1-induced $[Ca^{2+}]_i$ increase. Thus, another possibility mediating the acute effects of TGF-β1 is that calcium released from intracellular calcium pools as a second message opens one or more non-selective cation channels in direct or indirect ways, thus resulting in robust depolarization and discharges.

Although the detailed mechanisms have yet to be investigated, administration of TGF-β1 induced mechanical hyperalgesia dose- and time- dependently in healthy rats, confirming the involvement of acute effects of TGF-β1 in pancreatic nociception. Surprisingly, a single administration of TGF-β1 at a high dose produced a persistent algogenic effect lasting for at least 48 hours. It is possible that excessive TGF-β1 may trigger some chronic effects. Our study does not exclude the importance of chronic effects of TGF-β1 in peripheral sensitization. Acute effects may be an initiating event and accumulation of acute effects probably contributes to the occurrence and development of chronic effects, and in return, the chronic effects may amplify acute effects. Although the detailed mechanisms by which the acute effects of TGF-β1 leads to chronic pain are unknown, we propose that the acute effects of TGF-β1 might contribute to chronic pain in at least 2 ways. Firstly, TGF-β1 causes discharges and increases the intracellular calcium in DRG neurons. As a second message, Calcium ions may trigger many cascade signal responses and further influences gene transcription or phosphorylation of kinases and receptors. In the present study, this acute effect may lead to the upregulation of TGF-β1/TGF-β receptors in DRGs of the CP model. Secondly, since the expression of TGF-β1/TGF-β is increased, we hypothesize that the acute excitatory effects of TGF-β1 may be amplified and thus cause more discharges and more calcium increase in DRG neurons of the CP model. It is possible that the acute effects of TGF-β1 at normal level is not enough to trigger mechanical hyperalgesia in vivo, whereas the acute effects of TGF-β1 under pathophysiological conditions may cause mechanical hyperalgesia and contribute to chronic pain in CP model. This hypothesis is supported by our results that SB431542 attenuates mechanical hyperalgesia in CP rats but has no effect on responses in healthy rats. Further studies are needed to test the detailed signaling pathways of TGF-β1 effect in the animal model of chronic pancreatitis. The origin of TGF-β1 might be from mononuclear cells located in the fibrotic areas and ducts damaged by fibrosis in the pancreas of CP patients.[17]

In summary, our study demonstrated for the first time that TGF-β1 plays an acute effect on neuronal excitability, which might contribute to the mechanical hyperalgesia of abdomen in rats with chronic pancreatitis.

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