

## INVOLVEMENT OF P2X<sub>2</sub> AND P2X<sub>3</sub> RECEPTORS IN DORSAL ROOT GANGLION NEURONS OF STREPTOZOTOCIN-INDUCED DIABETIC NEUROPATHY

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**Abstract** Painful diabetic neuropathy causes allodynia and does not respond to commonly used analgesics such as non-steroidal anti-inflammatory drugs or opioids at doses below those producing disruptive side effects. In the present study, we examined the effect of P2X receptor antagonists, which are known to modulate the pain pathway, on mechanical allodynia in streptozotocin (STZ)-induced diabetic mice. The paw withdrawal frequency measured by von Frey filaments, began to significantly increase 5 days after STZ injection and was maintained for more than 14 days. Intrathecal administration of P2X receptor antagonists (PPADS and TNP-ATP) inhibited the mechanical allodynia in diabetic mice. Next, the levels of mRNA for the P2X receptors in DRG in diabetic mice were measured using quantitative real-time PCR. The levels of P2X<sub>2</sub> and P2X<sub>3</sub> receptors mRNA were significantly increased in diabetic mice at 14 days after the intravenous injection of STZ. Furthermore, we investigate the localization of glial cells and neuron in spinal cord of diabetic mice. However, microglia, astrocyte and neuron were not changed in spinal cord. These results suggest that the upregulation of P2X<sub>2</sub>, P2X<sub>3</sub> and/or P2X<sub>2/3</sub> receptor in DRG neurons is associated with mechanical allodynia in STZ-induced diabetic mice.

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**Key words:** diabetic neuropathy; DRG; P2X

### 1. Introduction

Many patients with diabetic neuropathy suffer from various types of aberrant pain<sup>1,2)</sup>. Painful diabetic neuropathy does not always respond to commonly used analgesics such as non-steroidal anti-inflammatory drugs or opioids at doses below those producing disruptive side effects<sup>3-5)</sup>. This painful neuropathy develops in the early stage of diabetes. Although hyperglycemia is considered to be a major pathogenic factor in the development of diabetic neuropathy, the mechanisms are not fully understood.

Previous studies showed that neuropathic pain is caused by injured traumatic nerves in the spinal dorsal horn<sup>6,7)</sup>. Tissue damage triggers the release of neurotransmitters, including

adenosine-5'-triphosphate (ATP)<sup>8,9)</sup>. ATP is recognized as an important neurotransmitters and binds to purinergic receptors that are subdivided into the ionotropic P2X receptors (P2X<sub>1</sub> - P2X<sub>7</sub>) and the G-protein coupled P2Y receptors (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub> - P2Y<sub>14</sub>)<sup>10,11)</sup>. A subtype of ionotropic P2X receptors, the P2X<sub>3</sub> receptor, is highly expressed in small size dorsal root ganglion (DRG) neurons with primary afferent fibers in the pain pathway<sup>12,13)</sup>. In fact, it has been reported that downregulation of P2X<sub>3</sub> receptor protein treated by antisense oligonucleotides or siRNA reduce mechanical allodynia observed after spinal nerve ligation or inflammation<sup>14,15)</sup>. In addition, other P2X receptor subtypes in DRG might also contribute to pain transmission under normal and pathologic conditions. In situ hybridization studies

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have indicated that DRG have mRNA of all P2X receptor subtypes<sup>16,17</sup>. Electrophysiological studies also revealed that small to medium size DRG neurons responded to ATP and that DRG neurons expressed multiple P2X channels with different current kinetics<sup>18,19</sup>. However, there is no evidence that the modulation of P2X receptors expression and function are associated with the mechanisms of painful diabetic neuropathy in DRG. In the present study, we examined streptozotocin (STZ)-induced diabetic mice as a model to investigate the expression and function of P2X receptors that are associated with mechanical allodynia.

## 2. Materials and Methods

### 2.1. Animals

Male ddY mice (Kyudo, Kumamoto, Japan) weighing 25-30 g were used in our experiments. Mice were housed at 22 ± 2°C with a 12/12 h light/dark cycle (lights on at 07:00 h), and were given free access to commercial food and tap water. Experimental procedures were based on the Guidelines of the Committee for Animal Care and Use of Fukuoka University and Hirosaki University.

### 2.2. Diabetic mice

Diabetes was induced by a single intravenous injection of STZ (200 mg/kg body weight) dissolved in 33 mM sodium citrate buffered (pH 4.5) saline into the tail. Age-matched non-diabetic mice were injected with the same volume of citrate buffered saline only. Diabetes was confirmed in experimental animals at 1 or 2 weeks after STZ injection. This was done by measuring urinary glucose with a Test-tape A (Shionogi Pharmaceutical Co., Osaka, Japan) and by measuring the glucose concentration in a blood sample obtained from the tail vein with a GLUTEST E kit (Sanwa Chemical Co., Nagoya, Japan). Mice with blood glucose levels above 300 mg/dL were used as diabetic mice.

### 2.3. Intrathecal (i.t.) injection

For the i.t. injection of drugs, a 28 gauge

needle was connected to a 25 µL Hamilton microsyringe, and inserted into the intervertebral space between lumbar (L) 5 and 6 vertebrae as previously described<sup>20</sup>. Drugs for i.t. injection were given slowly in a total volume of 5 µL. Control mice received the same volume of only artificial cerebrospinal fluid (ACSF), saline or vehicle.

### 2.4. von Frey Test

Mechanical sensitivity was determined with von Frey filaments (Semmes-Weinstein monofilaments, Stoelting, IL, USA) with calibrated bending forces (g), as previously described<sup>21</sup>. In briefly, mice were placed individually in a glass cage with a wire mesh bottom. After mice had adapted to the testing environment for 60 min, a series of von Frey filaments (0.166, 0.407, 0.692, 1.202 and 1.479 g) were pressed perpendicularly against the mid-planter surface of the hind paw from below the mesh floor and held for 3–5 s with it slightly buckled. Lifting of the paw was recorded as a positive response. Filaments were applied to the point of bending six times to the planter surface of the left and right hind paw for a total of 12 times per mouse at intervals of 5 s; the next lightest filament was chosen for each subsequent measurement. In preliminary experiments, the withdrawal response frequency cased in about 7% when 0.166 g von Frey filament was applied 12 times to the planter surface in non-diabetic mice. We therefore were considered an adequate value for the measurement of mechanical allodynia. We used the non-noxious 0.166 g von Frey filament to assess mechanical allodynia.

### 2.5. Quantitation of P2X Receptor mRNA

The methods for quantifying P2X receptor mRNA in the DRG were as described previously<sup>17</sup>. Briefly, the DRG were dissected from L4 to L6 of mice under ether anesthesia and total RNA was isolated. One sample contained a total of 5–6 DRG from two mice. Complementary DNA was synthesized by reverse transcriptase (RT)

reaction using TaqMan RT Reagents and Gene Amp 9600 thermal cycler (Perkin-Elmer). Real-time PCR amplifications were performed using SYBR Green PCR Core Reagents (Perkin-Elmer); SYBR Green buffer, dNTP blended with dUTP, AmpliTaq Gold, AmpErase UNG, MgCl<sub>2</sub>, and the clone-specific primers. The primers for P2X<sub>1-7</sub> and  $\beta$ -actin were as follows: P2X<sub>1</sub>, forward-5'GAGAGTCGGGCCAGGAC-TTC3', reverse-5'GCGAATCCCAAACACCTTGA3'; P2X<sub>2</sub>, forward-5'TCCCTCCCC-CACCTAGT CAC3', reverse-5'CACCACCTGCTCAGTC AGAGC3'; P2X<sub>3</sub>, forward-5'CTGCCTAACCT CACCGACAAG3', reverse-5'AATACCCAGA ACGCCACCC3'; P2X<sub>4</sub>, forward-5'CCCTTTG CCTGCCAGATAT3', reverse-5'CCGTACG CCTTGGT-GAGTGT3'; P2X<sub>5</sub>, forward-5' GGATGCCAATGTTGAGGTTGA3', reverse-5' TCCT-GACGAACCCTCTCCAGT3'; P2X<sub>6</sub>, forward-5'CCCAGAGCATCCTTCTGTTCC3', reverse-5'GGCACCAGCTCCAGATCTCA3'; P2X<sub>7</sub>, forward-5'GCACGAATTATGG-CACCGTC3', reverse-5'CCCCACCCTCTGTGACATTCT3';  $\beta$ -actin, forward-5'ATC-GCTGACAGGATG CAGAA3', reverse-5'CAGGAGGAGCAATGA TCTTGA3'. PCR was done by 15 s denaturation at 95°C, 10 s annealing at 57°C, and 1 min elongation at 72°C for 40 cycles in a Gene Amp 5700 Sequence Detection System (Perkin-Elmer). Fluorescence of the amplified target gene was acquired at the end of each 72°C extension phase. To exclude the contamination of unspecific PCR products such as primer dimers, dissociation curve analysis was applied to all final PCR products after the cycling protocol. The standard curve method was employed for quantifying gene expression of the P2X receptors. Each P2X expression level was normalized by  $\beta$ -actin. The PCR products were run on a 3% (w/v) agarose/TAE gel to confirm the product size and subcloned with pGEM-T vector (Promega) for sequencing with an ABI Prism310 automated sequencer (Perkin-Elmer).

## 2.6. Immunohistochemistry

PSNL and STZ model mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused through the heart with saline, followed by 4% paraformaldehyde made in 0.1 M sodium phosphate buffer (pH 7.4). The spinal cord (SC) was removed, and transverse sections were cut in at 40  $\mu$ m on a freezing microtome. Sections were incubated for 2 h in mouse anti-GFAP (a specific astrocyte marker glial fibrillary acidic protein, 1:1000), anti-NeuN (a neuronal marker, 1:1000) and anti-Ibal (a specific microglial marker, 1:1000) diluted in 5% goat serum and in potassium buffered saline (KPES, pH 7.4) at room temperature. Sections were then washed in KPBS, transferred to Cy3-conjugated goat anti-rabbit (1:200) for 1 h at room temperature, washed again, mounted on gelatin-coated glass slides, dried, and coverslipped with an anti-fade glycerol solution.

## 2.7. Statistical analysis

Statistical analysis of data was performed using Student's t-test for comparison between two groups, and using analysis of variance (ANOVA) followed by Dunnett's test or Turkey's test for multiple comparisons. Results are expressed as means  $\pm$  S.E.M. The level of significance was set at  $P < 0.05$ .

## 2.8. Drugs

2'-O-(trinitrophenyl)adenosine 5'-ribose phosphate, trisodium salt (TNP-ATP) was obtained from Molecular Probes (Molecular Probes, Leiden, Netherlands). Pyridoxal-phosphate-6-azophenyl-2', 4'-disulfonate (PPADS) and other drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

## 3. Results

### Blood glucose level in STZ-induced diabetic mice and non-diabetic (vehicle-treated) mice.

Mice developed hyperglycemia within 3 days of STZ injection (Fig. 1A). The mean blood glucose level at 7 days after STZ injection (479.3

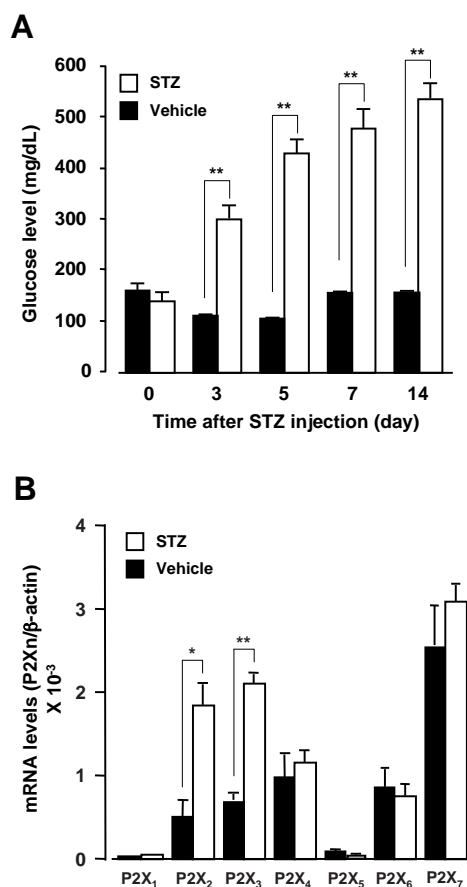
$\pm 37.3$  mg/dL) was significantly higher than that in vehicle-treated mice ( $156.4 \pm 9.4$  mg/dL). The increase of blood glucose level in STZ-induced diabetic mice was maintained for at least 14 days ( $540.9 \pm 27.5$  mg/dL).

#### Changes in P2X receptors mRNA in DRG of PSNL and diabetic mice

The levels of mRNA for the P2X receptors in DRG were measured using quantitative real-time PCR. The level of P2X<sub>3</sub> receptor mRNA was remarkably increased in diabetic mice at 14 days after the injection of STZ (Fig. 1B). The level of P2X<sub>2</sub> receptor mRNA was also increased in diabetic mice at 14 days after STZ injection. However, the expressions of P2X<sub>1</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, P2X<sub>6</sub> and P2X<sub>7</sub> mRNA were not significantly different between the vehicle-treated and diabetic mice.

#### Effect of i.t. injection of P2X receptor antagonists on tactile allodynia in STZ-induced diabetic mice

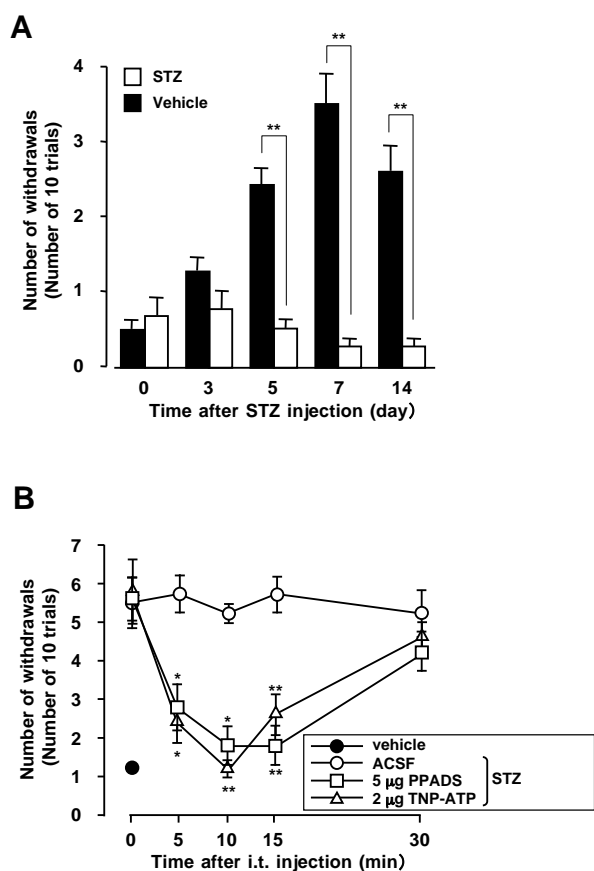
The paw-withdrawal-response (PWR), measured by 0.166 g von Frey filament, began to increase significantly at 5 days after STZ injection, compared with the vehicle group (Fig. 2A). The increase of PWR in diabetic mice persisted for more than 14 days after STZ application. We thus tested the effect of P2X receptor antagonists on PWR in diabetic mice at day 14 (Fig. 2B). First, PPADS (5  $\mu$ g), a non-specific P2X receptor antagonist, was intrathecally administered at 15 min before the von Frey test. PPADS inhibited the mechanical allodynia in diabetic mice to the non-diabetic level. Second, TNP-ATP (2  $\mu$ g), a specific antagonist to P2X<sub>1</sub>, P2X<sub>3</sub> and heteromeric channels of P2X<sub>2/3</sub>, was intrathecally injected at 15 min before the von Frey test. TNP-ATP also significantly reduced the mechanical allodynia to the non-diabetic level.



**Figure 1** Blood glucose level and levels of P2X receptor mRNA in STZ-induced diabetic mice and non-diabetic (vehicle-treated) mice. (A) Blood glucose level. Closed bars indicate the response in non-diabetic mice. Open bars indicate the response in diabetic mice. (B) Changes in levels of P2X receptor mRNA in dorsal root ganglia. Columns and bars represent the mean and S.E.M., respectively (\* $P < 0.05$ , \*\* $P < 0.01$  vs. vehicle,  $N = 4$ ). In each sample, the P2X mRNA expressions were normalized by the respective  $\beta$ -actin level.

#### Changes in GFAP, OX-42 and NeuN immunoreactivities (IRs) in L5 spinal cord of PSNL and diabetic mice

GFAP, Iba 1 and NeuN IRs were detected on the ipsilateral side of the L5 spinal cord of sham-operated mice. NeuN IR was not remarkably changed in PSNL mice (Fig. 3). In contrast, GFAP IR in the ipsilateral side of the L5 spinal cord was slightly increased as compared with that of the contralateral side in PSNL mice. Furthermore, Iba 1 IR on the ipsilateral of the L5 spinal cord was dramatically elevated as

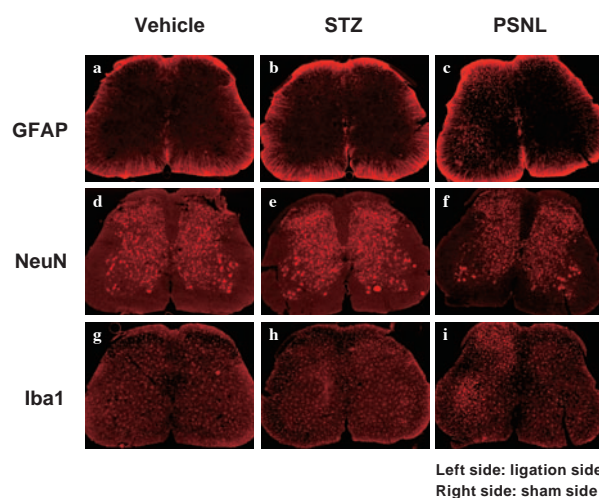


**Figure 2** Characteristic of pain behavior and effect of P2X antagonists in STZ-induced diabetic mice and non-diabetic (vehicle-treated) mice. (A) Number of hindpaw withdrawals (the degree of mechanical allodynia) in response to tactile stimulation with 0.166 g von Frey filament. Each point represents the mean  $\pm$  S.E.M. (\*\* $P < 0.01$  vs. vehicle,  $N = 6-10$ ) (B) Effects of intrathecal administration of P2X receptor antagonists on mechanical allodynia at 14 days after STZ injection. PPADS and TNP-ATP were intrathecally administered at 15 min before the von Frey test. Closed circles indicate the response in non-diabetic mice. Open symbols indicate the response in diabetic mice. Each value represents the mean  $\pm$  S.E.M. (\*\* $P < 0.01$  vs. vehicle,  $N = 8-10$ ).

compared with that on the contralateral side in PSNL mice. However, GFAP, Iba 1, NeuN IRs were not remarkably changed in diabetic mice.

#### 4. Discussion

Pain pathway involve in various neurotransmitters in the terminal of the primary afferent fibers. Previous studies have shown that P2X<sub>3</sub> receptor on DRG neuron is remarkably mediated



**Figure 3** Immunofluorescent staining for GFAP (a-c), NeuN (d-f) and Iba1 (g-i) on the ipsilateral and contralateral sides of the L5 spinal cord at 14 days after sciatic nerve ligation or STZ injection.

in the pain transmission<sup>22,23</sup>. However, there is no report demonstrating the association with P2X receptors in DRG on hyperalgesia caused by diabetic mice. Therefore, we quantified the mRNA levels of P2X receptors in DRG and determined the involvement of P2X receptors in neuropathic pain by the i.t. administration of P2X antagonists in STZ-induced diabetic mice.

In our present study, mechanical allodynia was also observed in diabetic mice<sup>20,25</sup>. The mechanical allodynia was significantly inhibited by i.t. administration of PPADS and TNP-ATP in diabetic mice to the non-diabetic level. Based on the pharmacological aspects of these P2X antagonists, P2X<sub>1</sub>, P2X<sub>3</sub> and P2X<sub>2/3</sub> receptors are probably involved in the mechanical allodynia in diabetic mice. In our real-time quantitative PCR experiment, P2X<sub>3</sub> receptor mRNA was strongly increased in the DRG of diabetic mice at 14 days after the injection of STZ (Fig. 1B). The level of P2X<sub>2</sub> receptor mRNA was also elevated in diabetic mice. However, P2X<sub>1</sub> receptor mRNA did not change. A previous study showed that P2X<sub>2</sub> and P2X<sub>3</sub> subtypes assemble into heteromeric channels of P2X<sub>2/3</sub><sup>13</sup>. Therefore, our results suggest that P2X<sub>2</sub>, P2X<sub>3</sub> or P2X<sub>2/3</sub>



receptors in DRG are involved in the allodynia induced by diabetic mice. Similar results have been observed in the partial sciatic nerve ligation model of DRG neuron<sup>25)</sup>. These findings indicate that P2X<sub>2</sub> or P2X<sub>3</sub> receptors in DRG are involved in the mechanism of neuropathic pain.

Sweitzer *et al.* previously reported that the IR levels of GFAP and OX-42 were elevated under the neuropathic pain-like state caused by L5 spinal nerve transection in rats<sup>26)</sup>. We therefore tested GFAP, NeuN, Iba 1 IRs on the SC in PSNL and diabetic mice. Our result shows that microglia, astrocyte and neuron were not changed in SC of diabetic mice.

These results suggest that microglia in spinal cord is essential for allodynia induced by PSNL, but not by diabetes. The upregulation of not only P2X<sub>3</sub> receptor but also P2X<sub>2</sub> receptor in DRG neurons may facilitate transmission of mechanical stimuli at the presynapse of the spinal level contribute to spinal processing of mechanical allodynia in diabetic neuropathy. Therefore, the formative process of neuropathic pain in spinal cord might be different between PSNL model and STZ model.

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