

Research Article

Overexpression of P2X3 Receptors is Associated with the Painful Dental Pulp Tissue of Human Primary Teeth

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Introduction

Adenosine Triphosphate (ATP) is an important neurotransmitter involved in the conduction of noxious stimuli, which is widely present in peripheral and central nervous systems [1,2]. Earlier studies showed that ATP can be released from various cells of injured tissue or distension viscera and may cause pain via stimulating the local nociceptors [3,4]. An accumulating evidence indicates that extracellular ATP functions as a possible neurotransmitter by acting on purinoceptors expressed by nociceptive nerve terminals [5,6], thus activating peripheral nociceptors [7-9].

ATP exerts its effects via P2 receptors, which are subdivided into two major families, the P2X and P2Y receptors. P2X receptors are ligand-gated ion channels, and P2Y receptors are G-protein-coupled receptors. Seven P2X subunits (P2X1-7) have been identified and cloned so far [10-12]. P2X3 is a subfamily of P2X, which is dominantly found in a subset of predominantly small sensory neurons in human sensory ganglia [13-16], and also in rat sensory ganglia [17].

Evidence is accumulating which supports that P2X3 receptor has been shown in rat and human dental pulp [18,19]. However, our knowledge of whether the distribution of P2X3 receptor changes with dental pulp's types is limited. Therefore, the aim of this study was to

Abstract

ATP-gated P2X3 receptors play a crucial role in neurogenic inflammation and neuronal sensitization, which might be associated with pain. Our study was to investigate whether P2X3 receptors are involved in tooth pain. Dental pulp tissues were obtained from normal (n=14) and painful (n=16) pulps in permanent teeth as well as normal (n=10) and painful (n=14) pulps in primary teeth. Western blot and Real-time fluorescence quantitative PCR were used to detect the expression level of P2X3 receptors in dental pulp tissues. The results showed that P2X3 receptors were upregulated significantly in painful pulp when compared with normal pulp in primary teeth (P<0.05). However, there was no difference of the expression of P2X3 receptors between normal and painful permanent tooth pulp (P>0.05). Interestingly, we found that the intraneural expression of P2X3 receptors of primary tooth pulp was significantly higher than that of permanent tooth pulp (P<0.05). Our study shows for the first time that the P2X3 receptors may be related to primary dental pain, but not to permanent dental pain.

Keywords: P2X3 receptor; Permanent teeth; Primary teeth; Dental pulp; Dental pain

investigate the way of P2X3 receptors' expression in human primary and permanent tooth pulp and study the relationship between dental pain and P2X3 receptors expression level.

Materials and Methods

Ethics statement

The Research and Ethics Committee of Tongji University granted approval for the study and an informed consent was obtained from all the subjects or accompanying guardians.

Pulpal tissue collection

Teeth from subjects were divided into four groups: normal third molars extracted for orthodontic purposes or prophylactic prevention of future problems (n=14), third molars diagnosed with irreversible pulpitis that about to be extracted (n=12), normal primary teeth extracted for prolonged retention or orthodontic purposes (n=10) and primary teeth diagnosed with irreversible pulpitis (n=12). Teeth had deep caries and/or restorations with spontaneous pain were diagnosed with reversible pulpitis. Patients with dental pain which thought was caused by any other disease such as heart attack were excluded from the study; neither were teeth with not closed apices. All primary teeth were free of physiologic root resorption because this process always associated with degenerative neural changes

[20]. Screening of all teeth were judged by radiographic and clinical examination. Immediately following simple forceps extraction, a vertical groove was cut along the buccal surface of each normal tooth and the tooth was split longitudinally using an elevator. The pulp cavity of pain group was open under local anesthesia, then the pulp tissue was pulled out with marrow needle. Pulpal tissues were removed and divided into two groups: one half of the tissues were stored in 4% paraformaldehyde (pH 7.4) for 24h at 4°C for further immunofluorescence investigation; the other half were used to extract RNA for Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and protein for Western blot.

Immunofluorescence

The pulps were placed in 0.1M PBS containing 30% sucrose solution for cryoprotection (24h at 4°C), then embedded in Tissue-Tek OCT compound (Bayer Sakura Tissue-Tek, USA). 12µm longitudinal sections were cut from each pulp and collected, in sets of three, on poly D-lysine-coated glass slides. Slides were stored at -20°C after air-drying for 60min at room temperature.

An indirect immunofluorescence method was used in this study [21]. Slides were left to air-dry at room temperature for 15min after were removed as required from storage. They were then washed in PBS for five minutes (three times). Following this, they were incubated with Quick Antigen Retrieval Solution for Frozen Sections (Weiao Biotech Well, China) for 5min to repair antigen activity. Then, they were washed in PBS containing 0.2% Triton X-100 (PBST).

For P2X3 receptor localization, sections were first incubated with 10% normal goat serum (Vector Laboratories, Peterborough, UK) diluted in PBS for 60min at room temperature, followed by anti-human P2X3 antibody (Abcam, US) diluted 1:800 in Primary Antibody Dilution Buffer (38hrs at 4°C). Slides were then washed in PBS containing 0.05% Tween-20 (PBST) before incubation, for further 60min at room temperature, with Oregon Green conjugated goat anti rabbit IgG (dilution 1:100; Vector).

Slides were then washed again in PBS containing 0.2% Triton X-100 (PBST) and incubated with 0.5ug/ml DAPI at room temperature for 5min. Slides were finally washed in PBS, and sections were carefully dried and mounted in Antifade Solution (Weiao Biotech Well, China). Replacement of the primary antibody with non-specific rabbit IgG or omission of the primary antibody were used to establish negative controls.

Western blot

The total protein of dental pulp tissues was extracted with RIPA buffer (Santa Cruz Biotech, Shanghai). The specific protein was detected by Western blot analysis as previously described [22]. In brief, the tissue lysates were boiled and then electrophoresed in 12% SDS-PAGE acrylamide gels. They were then transferred onto nitrocellulose membranes (Bio-Rad, US) and incubated for 1h in TBS-T containing 5% nonfat milk. The membranes were incubated overnight at 4°C with primary antibodies against P2X3 (Abcam, US). The membranes were incubated for 1h at room temperature with HRP-conjugated anti-rabbit (Santa Cruz Biotech, Shanghai) after they were washed three times in TBS-T. The membranes were photographed on a Kodak 4000 image station (Carestream Molecular Imaging, Canada) after developed with the SuperSignal West Pico

HRP substrate kit (Pierce, US). To control sample loading and protein transfer, we stripped and reprobed the membranes with β-actin (Santa Cruz Biotech, Shanghai).

Real-time fluorescence quantitative PCR

Total RNA was prepared using Trizol reagent (Invitrogen, US) and following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in two steps according to the RT PrimeScript™ reagent Kit with gDNA Eraser (TaKaRa, Japan).

PCR reaction mixture was prepared following the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Japan). Amplification was performed at 40 cycles for GAPDH and P2X3 in a thermal cycle. Each cycle consisted of 30sec of denaturation at 95°C, 34sec of annealing at 60°C (ABI 7900HT PCR, US). The sequences of primers used were as follows: 1) P2X3-Forward: CTACGCCAACAGAGTCATGG; P2X3-Reverse: CACAGCGGTATTTCTCCTCA; 2) GAPDH-Forward: TGACAACAGCCTCAAGATCATCA; GAPDH-Reverse: ACTGTGGTCATGAGTCCTTCCA.

Statistical analysis

All data are expressed as mean ± SE (standard error), and analyzed using a one-way analysis of variance (ANOVA) with the SPSS statistical software program for Microsoft Windows (SPSS Inc., Chicago, IL, USA). P <0.05 was considered statistically significant.

Results

P2X3 receptor expressions in human tooth pulp

P2X3 receptor expressions were detected by immunofluorescence. In permanent tooth pulp, P2X3 immunoreactive (IR) nerve fibers were found mainly in the body of the pulp, the subodontoblastic plexus of Raschkow, and the odontoblastic area (Figure 1A and 1B). In primary tooth pulp, the distribution of P2X3 immunoreactive (IR) nerve fibers is similar to permanent teeth (Figure 1C and 1D). The results showed that P2X3 receptor were expressed in both of permanent and primary tooth pulp.

P2X3 receptors mRNA expressions in normal and painful human tooth pulp

To compare the mRNA levels of P2X3 between clinically healthy pulp and painful pulp tissues, real-time fluorescence quantitative PCR was used to detect the expression level of P2X3 receptors in dental pulp. The results showed that P2X3 mRNA expression in normal permanent pulps (NPer) is similar to painful permanent pulps (PPer) (1.0±0.23 vs. 1.15±0.25, P>0.05). However, the P2X3 mRNA levels are significantly upregulated in painful primary pulps (PPri) when compared with normal primary pulps (NPri) (1.84±0.45 vs. 1.48±0.31, *P<0.05). And the expression of P2X3 receptors in the primary pulp is significantly greater than that in the permanent pulp (*P<0.05) (Figure 2).

P2X3 receptors protein expressions in normal and painful human tooth pulp

The protein levels of P2X3 in pulp tissues were tested with Western blot. Representative immunoblots showed that the bands of primary dental pulp were stronger than that of permanent dental pulp, and the band of primary painful pulp is the strongest. GPDH served as loading control (Figure 3A). The immunoblot band intensity was quantitated and the data were expressed as mean ± SE, ANOVA.

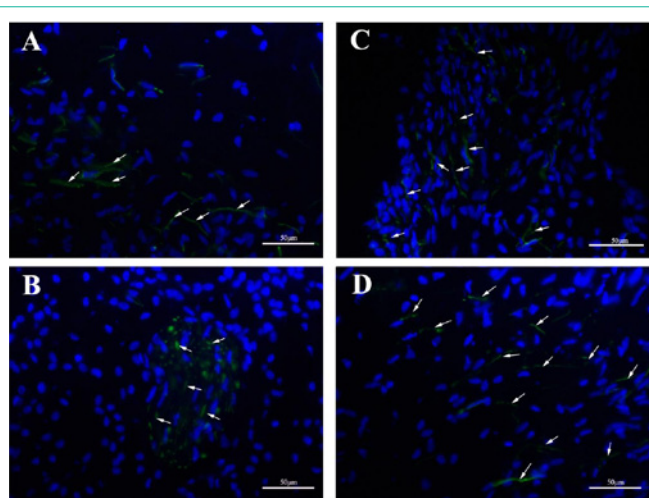


Figure 1: Immunofluorescence detection of P2X3 receptors expressions in normal permanent tooth pulp (A), painful permanent tooth pulp (B), normal primary tooth pulp (C), and painful human primary tooth pulp (D). Figure in blue for the cell nucleus in pulp tissue; Green strips dyeing for P2X3 receptor positive expression (as indicated by the arrows), scale: 50 microns.

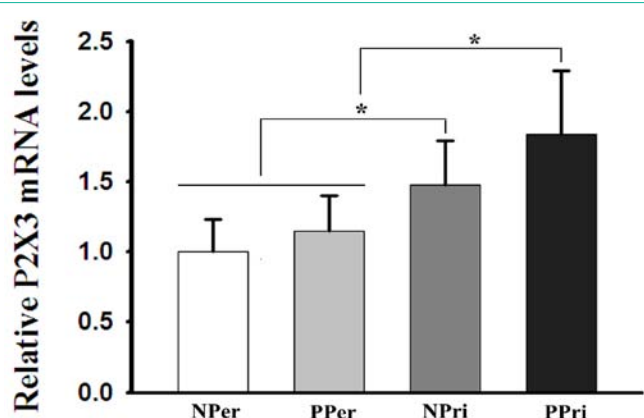


Figure 2: Comparison of the P2X3 expression in mRNA level detected by real-time PCR. The results showed that P2X3 mRNA expression in normal permanent pulps (NPer) is similar to painful permanent pulps (PPer) ($P > 0.05$). However, the P2X3 mRNA levels are significantly upregulated in painful primary pulps (PPri) when compared with normal primary pulps (NPri) ($*P < 0.05$). And the expression of P2X3 receptors in the primary pulp is significantly greater than that in the permanent pulp ($*P < 0.05$).

The results showed that there is no difference between the P2X3 protein expression in normal permanent pulps (NPer) and in painful permanent pulps (PPer) (1.0 ± 0.13 vs. 1.18 ± 0.31 , $P > 0.05$) (Figure 3B). As for primary pulp, the expression of P2X3 is significantly upregulated in painful primary pulps (PPri) when compared with normal primary pulps (NPri) (2.24 ± 0.35 vs. 1.52 ± 0.09 , $*P < 0.05$). And the expression of P2X3 receptors in the primary pulp is significantly greater than that in the permanent pulp ($*P < 0.05$).

The results showed that there is no difference between the P2X3 protein expression in normal permanent pulps (NPer) and in painful permanent pulps (PPer) ($P > 0.05$). As for primary pulp, the expression of P2X3 is significantly upregulated in painful primary pulps (PPri) when compared with normal primary pulps (NPri) ($*P < 0.05$). And the expression of P2X3 receptors in the primary pulp is significantly

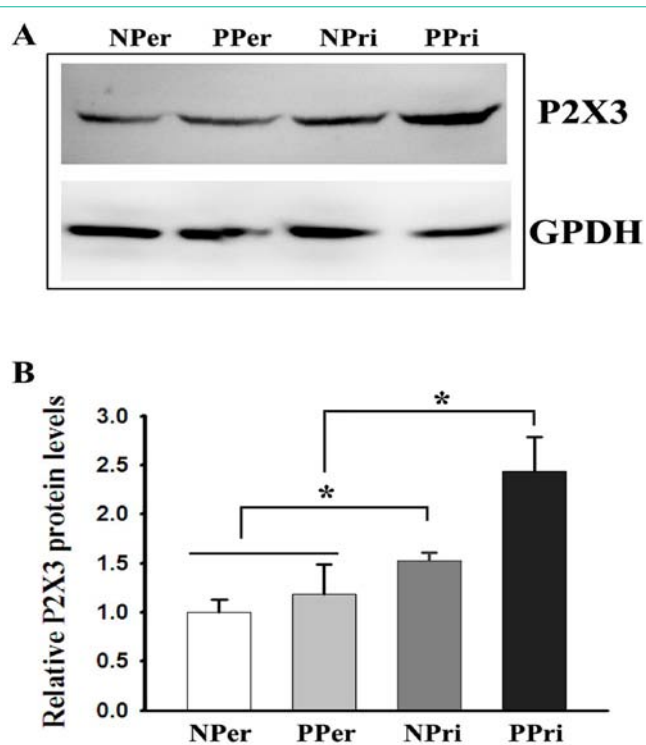


Figure 3: Comparison of P2X3 expression in protein level detected by Western blot. (A) Representative immunoblots showed that the bands of primary dental pulp were stronger than that of permanent dental pulp, and the band of primary painful pulp is the strongest. GPDH served as loading control. (B) The immunoblot band intensity was quantitated and the data were expressed as mean \pm SE, ANOVA.

greater than that in the permanent pulp ($*P < 0.05$).

Discussion

Human dental pulp tissue has rich innervation, which consists of afferent nerve fiber of Trigeminal Ganglion (TG) branch. Stimuli can be swapped into nerve impulses in nerve terminal receptors, once nerve fibers in dental pulp received external stimuli. Then, the nerve impulses transmit to the central nervous system via afferent nerve fibers and caused the feeling of pain. These sensitive dental pulp nerve fibers are pain receptors, can be divided into two categories: a delta of myelin sheath fibers and C unmyelinated fibers [23]. Most of the nerve fibers were detected in the odontoblastic area, and about 40% of the end of the nerve fibers distributed in close to the odontoblasts bumps of dentin tubule [24]. Under normal condition, the pulp tissue was less susceptible to the external stimuli because that was wrapped in complete tooth hard tissue; however, when the tooth tissue damaged to a certain extent, pulp were exquisitely sensitive to mechanical, thermal, and noxious stimuli that can cause the response of nociceptor, thus mediating dental pulp nerve hyperalgesia.

ATP is an important neurotransmitter involved in the conduction of noxious stimuli, which is widely present in peripheral and central nervous systems. ATP exerts its effects *via* P2 receptors. P2X3 is a subfamily of P2X, which is dominantly found in a subset of predominantly small sensory neurons in sensory ganglia. Many acute or chronic pain models, such as experimental rat sciatic nerve chronic injury of oppressive, orthodontic tooth movement and subcutaneous

injection formalin to pain, and other experiments showed that the producing and maintaining of these acute or chronic pain were all associated with P2X3 receptors' expression and activity [25-27]. At the same time, some studies provided direct evidences that P2X3 receptor is associated with pain. Carolina believed that P2X3 receptors contribute to transition from acute to chronic muscle pain [28]. Jarvis found in chronic sciatic nerve oppressive model and spinal nerve ligation model, using P2X3 receptor antagonist A-317491, can completely block the pain and 50% of bitterness in mechanical and heat hyperalgesia [29]. Liveira proved the existence of P2X3 receptor in the rat Temporomandibular Joint (TMJ). Furthermore, through the injection of agonist α, β -meATP into P2X3 receptor on rats TMJ, it could cause harmful reaction enhancement. Whereas, using P2X3 receptor antagonist PPADS, it could alleviate inflammatory pain effectively which caused by injecting carageen glue in rats TMJ [30]. These findings suggested P2X3 receptors might play a role in the process of painful signal's transduction.

Our study found that P2X3 immunoreactive (IR) nerve fibers exist in dental pulp tissue of human primary teeth, and its distribution were similar to permanent teeth. The present study is consistent with the former researches that P2X3 receptors exist in painful and nonpainful human permanent tooth pulp [31]. Moreover, P2X3 receptors were upregulated in the painful dental pulp of primary teeth, whereas there was no difference statistically significant in the normal permanent teeth group compared with the pain group. The results also showed that the expression of P2X3 receptors in primary teeth was higher than the permanent teeth in both normal and painful group. This could provide evidence for clinical primary tooth pain often appeared with chronic pain while permanent teeth performance with acute pain. Our findings established that P2X3 receptors might be associated with primary pulpitis pain; nevertheless, P2X3 receptors may be not associated with permanent pulpitis pain.

As described above, dental pulp neurons can be divided into non-myelinated C-fiber and a delta of myelin sheath fibers. Group of C-fiber neurons is comprised of both peptidergic neurons and non-peptidergic neurons [32]. Helen used immunohistochemical method to establish that intraneural expression of neuropeptides Calcitonin Gene-Related Peptide (CGRP), substance P (SP), Vasoactive Intestinal Polypeptide (VIP) was significantly lower in the primary than in the permanent tooth pulp, and the changes of neuropeptide expression was associated with dental caries [33]. Those findings suggested that the innervation of the neuropeptide in permanent tooth pulp were significantly higher than that of primary teeth. The majority of P2X3-positive neurons belongs to non-peptidergic neurons [34]. Meanwhile, our study showed that the expression of P2X3 receptors in primary teeth was higher than the permanent teeth in both normal and painful group. Based on the above research, we can speculated that the innervation of the non-peptidergic neurons in the primary tooth pulp significantly higher than that of permanent teeth. Interestingly, in the permanent dentition, overall innervation density is also significantly greater than that of primary teeth [35]. In addition, we have recently shown that there is less amount and developed myelin in primary tooth nerve fibers as permanent teeth. In conclusion, the differences of pain neurotransmitter that cause pulpitis pain of permanent and primary teeth may be associated with differences in the amount and proportion of innervations of C-fiber

and A-fiber neurons, or peptidergic and non-peptidergic neurons of C-fiber.

Conclusions

This experiment verified the overexpression of ligand-gated nonselective ion channels P2X3 receptor in human permanent tooth pulp, and for the first time, found that P2X3 immunoreactive (IR) nerve fibers exist in dental pulp tissue of human primary teeth. Meanwhile, it showed that the expression level of P2X3 receptors increased significantly in painful primary dental pulp tissue, suggesting that P2X3 receptors may play a possible role in the development and transmission of primary dental pain. Thus, we speculated that specifically block the expression of P2X3 receptors may inhibited the sensitization process of primary dental pulp pain. However, the concrete means and mechanism of the involvement of P2X3 receptors in tooth pain remains to be further studied.

Declaration

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Author's contributions: Limei Jin, Qi Zhong conceived and coordinated the study and wrote the paper. Beizhan Jiang, Xi Yang analyzed the data shown in Figure 1, 2, 3. Chun Xu, Yumei Zhao provided technical assistance and contributed to the preparation of the data. All authors reviewed the results and approved the final version of the manuscript.

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