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ABSTRACT

Dental pulpal nerve fibers express ionotropic adenosine triphosphate (ATP) receptors, suggesting that ATP signaling participates in the process of dental nociception. In this study, we investigated if the principal enzymes responsible for extracellular ATP hydrolysis, namely, nucleoside triphosphate diphosphohydrolases (NTPDases), are present in human dental pulp. Immunohistochemical and immunofluorescence experiments showed that NTPDase2 was predominantly expressed in pulpal nerve bundles, Raschkow's nerve plexus, and in the odontoblast layer. NTPDase2 was expressed in pulpal Schwann cells, with processes accompanying the nerve fibers and projecting into the odontoblast layer. Odontoblasts expressed the gap junction protein, connexin43, which can form transmembrane hemichannels for ATP release. NTPDase2 was localized close to connexin43 within the odontoblast layer. These findings provide evidence for the existence of an apparatus for ATP release and degradation in human dental pulp, consistent with the involvement of ATP signaling in the process of dentin sensitivity and dental pain.

KEY WORDS: dentin, neuroscience/neurobiology, pain, pulp biology, ectoATPase, dentin sensitivity.

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Expression of Ecto-ATPase NTPDase2 in Human Dental Pulp

INTRODUCTION

Dentin sensitivity is generally explained by the hydrodynamic theory, which states that external stimuli induce fluid movements that activate the nerve fibers in dentin tubules and cause transient pain (Brannström, 1986). This hypothesis has been challenged by recent observations that fluid movements in dentin tubules are not associated with dentin sensitivity and dental pain in human (Ajcharanukul *et al.*, 2011). Accumulating evidence indicates that odontoblasts may act as sensory cells that mediate dental nociception (Magloire *et al.*, 2009). Mechanical, thermal, and acid-responsive channels have been detected in odontoblasts (Allard *et al.*, 2000; Magloire *et al.*, 2003; Son *et al.*, 2009; El Karim *et al.*, 2010; Sole-Magdalena *et al.*, 2010). Expression of excitable Na⁺ channels and generation of impulses necessary for pain transmission have also been demonstrated in odontoblasts (Allard *et al.*, 2006). As abundant sensory nerve fibers reach the odontoblast layer and extend into dentin tubules, it is plausible that dental pain is initiated by sensory impulses in odontoblasts and subsequently transmitted to adjacent sensory nerve fibers. However, the cellular and molecular mechanisms remain unclear (Magloire *et al.*, 2010).

ATP is widely recognized as a molecule important in peripheral pain transmission (Burnstock and Wood, 1996; Hamilton, 2002; Wirkner *et al.*, 2007). ATP depolarizes the membrane potential and excites sensory neurons by activating ionotropic purinergic (P2X) receptors (Chen *et al.*, 1995; Hamilton, 2002; Wirkner *et al.*, 2007). Ionotropic P2X3 receptors have been detected in trigeminal ganglia neurons and in dental pulp nerve fibers projecting into the odontoblast layer and the dentin tubules (Alavi *et al.*, 2001; Jiang and Gu, 2002; Renton *et al.*, 2003). These observations suggest that ATP signaling might be involved in the process of dental nociception.

ATP signaling is dictated by ATP release and ATP receptor activation and degradation by selective ectonucleotidases (Zimmermann and Braun, 1996; Abbracchio *et al.*, 2009). Gap junction/hemichannel-mediated ATP release has been demonstrated for a diversity of cell types (Gomes *et al.*, 2005; Zhao *et al.*, 2005). The connexin family of gap junction proteins has been detected in developing, secretory, and mature odontoblasts (Pinero *et al.*, 1994; Fried *et al.*, 1996; About *et al.*, 2002). Gap-junction-mediated dye uptake and coupling were also observed in odontoblasts (Ikeda and Suda, 2006).

Figure 1. NTPDase2 is expressed in human dental pulp. Immunohistochemical staining was performed with hN2-D5s in pulp tissues from decalcified teeth. (A) Positive staining of NTPDase2 in nerve bundles, Raschkow's nerve plexus, and the odontoblast layer of coronal pulp. (B) Positive staining along the nerve fibers and in the odontoblast layer at high magnification. (C,D) Strung-bead-like and punctuated staining in the odontoblast layer at high magnification. (E) Specificity of antibodies to human NTPDase2. In Western blot, specific bands appear at the expected molecular weight in samples from NTPDase2-transfected COS-7 cells when probed with the monoclonal antibody hN2-D5s or hN2-H9s. In immunocytochemistry, positive staining is visualized in COS-7 cells transfected with plasmid encoding for human NTPDase2 when detected with hN2-D5s or hN2-H9s, respectively. Staining with the irrelevant IgG2b (control) is devoid of reactions. OB, odontoblast. Scale bars are 50 (A), 40 (B), 30 (C), 20 (D), and 50 (E) μ m, respectively.

Connexin43 expression appeared to be up-regulated in odontoblasts facing caries lesions (About *et al.*, 2002), suggesting the involvement of connexin43 in caries-related pulp pathology.

Extracellular ATP is rapidly inactivated by several types of ectoenzymes, including members of the ecto-NTPDase family. Because of their dynamic catalytic activity in physiological conditions, the ecto-NTPDases play a dominant role in the hydrolysis of extracellular ATP (Zimmermann and Braun, 1996; Robson *et al.*, 2006). Eight members of the E-NTPDase family have been cloned and functionally identified (Bigonnesse *et al.*, 2004; Robson *et al.*, 2006), which include cell-surface (NTPDases1-3,

8), intracellular (NTPDases4, 5, 7), and cell-surface/intracellular (NTPDase6) enzymes. Cell-surface NTPDases1-3 and 8 provide the primary means for extracellular ATP degradation (Zimmermann, 2000; Kukulski *et al.*, 2005). Expression of NTPDase2 in the nervous system and its potential roles have been explored (Braun *et al.*, 2000, 2003, 2004; Shukla *et al.*, 2005; Mishra *et al.*, 2006). However, it is not known if NTPDase2 is present in human dental pulp and provides a mechanism for ATP degradation, a necessary step for ATP signal transmission to occur. The purpose of the present study was to determine the expression profiles of NTPDase2 and connexin43 in human dental pulp and to provide evidence for the involvement of ATP signaling in the process of dental nociception.

MATERIALS & METHODS

Sample Preparation

Extracted third molars were obtained from the oral surgery clinic following guidelines of the Research Subject Review Board of the authors' institution. Details for sample preparation are described in the Appendix.

Generation and Specificity of Human NTPDase Antibodies

Genetic immunization protocol was carried out with plasmids (pcDNA3.1) encoding each protein, human NTPDase1 (GenBank accession no. U87967), human NTPDase2 (NM_203468), and human NTPDase8 (AY430414). For details, see the Appendix.

Immunohistochemical Staining and Immunohistofluorescence

The antibodies used in this study include: NTPDase2 (hN2-D5s, hN2-H9s), NTPDase3 (hN3-H10s), NTPDase8 (hN8-6s), NTPDase1 (hN1-9I), vimentin (Chemicon, 1:1000), connexin43 (Sigma, 1:500), heavy neurofilament (NF-H, Neuromics, 1:2000) or S-100B (Sigma, 1:1000). For details of the experimental process, see the Appendix.

Statistical Analysis

Co-localization analysis for staining signal was performed with Zen software (Zeiss). The quantitative data for staining density were acquired with Image J. All numerical data are presented as mean $\pm$ SD. Student's *t* test was used for statistical analysis.

RESULTS

Expression of NTPDase2 in Human Dental Pulp

The dental pulp sections were obtained from either decalcified tooth or directly isolated pulp tissue (Appendix Figs. 1A, 1B). To confirm that the outermost layer cells with processes were odontoblasts, we performed immunostaining for vimentin, a specific marker for odontoblasts. As expected, both the cell bodies and their processes were vimentin-positive (Appendix Fig. 1C), while the pulpal cells beneath the odontoblast layer were negative for vimentin.

Immunohistochemical staining with antibodies to NTPDases was performed in dental pulp sections, and obvious immunoreactivity for NTPDase2 (with either hN2-D5s or hN2-H9s) was detected. As shown in Fig. 2, positive staining for NTPDase2 (hN2-D5s) was observed in the presumed pulpal nerve bundles (Fig. 1A), Raschkow's nerve plexus (Figs. 1A, 1B), and along the nerve fibers projecting into the odontoblast layer (Figs. 1A-1C). The punctuated staining for NTPDase2 in the nerve bundles suggests that its expression along the nerve fibers is not evenly distributed. Strung-bead-like staining along single nerve fibers (Figs. 1C, 1D) further corroborated the uneven distribution pattern of NTPDase2 expression. Fine punctuated staining for NTPDase2 also appeared in the odontoblast layer and formed a positive band beneath the predentin layer (Figs. 1A-1D), implying a plausible role for NTPDase2 within the odontoblast layer. We also performed immunohistochemical staining for other membrane NTPDases in dental pulp (Appendix Fig. 2). Immunoreactivities were visible in pulpal structures outlining the blood vessels (NTPDase1 and 3), the subodontoblastic nerve plexus (NTPDase1), and the odontoblast layer (NTPDase1 and 3). We did not detect an immunoreactivity signal for NTPDase3 in other parts of the dental pulp.

The specificity of antibodies to human NTPDase1 (hN1-9I), NTPDase2 (hN2-D5s and hN2-H9s), NTPDase8 (hN8-6s), and the secondary antibodies was demonstrated by Western blot and immunocytochemistry (Fig. 1E, Appendix Fig. 2).

NTPDase2 Is Expressed around the Pulp Nerve Fibers

To confirm that NTPDase2 is expressed along the nerve fibers, we performed double-immunostaining for NTPDase2 and neurofilament (NF). As illustrated in Fig. 2A1, NF-positive nerve axonal fibers and their enlargement parts were observed in coronal pulp, including the odontoblast layer. Almost all positive NF staining was accompanied

Figure 2. NTPDase2 is expressed in accompanying pulpal nerve fibers. **(A1)** NF immunostaining revealed the nerve fibers and their bulb-like enlargement portion in the coronal dental pulp. NTPDase2-positive staining processes overlapped the nerve fibers in dental pulp beneath the odontoblast layer (solid triangles). In the odontoblast layer, some of the nerve fibers were not accompanied or overlapped by NTPDase2-positive processes (open triangles). The arrows indicate the nuclei of cells with long processes staining positive for NTPDase2. **(A2)** The ratio of NTPDase2 to NF staining density at NF-positive elements in odontoblast and subodontoblast layers. ** $p < 0.01$, $n = 4$. **(B1)** Topogram of Z-stack scanning sections from NTPDase2 and NF staining in dental pulp. The scanning was acquired at 2048×2048 pixels with a $0.5\text{-}\mu\text{m}$ pinhole and $0.5\text{-}\mu\text{m}$ Z-inter-distance. The red NTPDase2 signal and the green NF signal are not co-localized, but are in a tight enveloping and accompanying relation. **(B2)** Correlation graph of the red NTPDase2 signal and the green NF signal in B1. No correlation was observed between the NTPDase2 and NF signals. **(C)** NTPDase2 and NF immunostaining in trigeminal ganglia. Positive staining for NTPDase2 was not detected in the trigeminal ganglia neurons (stars), but was detected in the surrounding satellite cells and their processes (arrows). OB, odontoblast. Scale bars are 40 (A), 40 (B), 10 (C) μm , respectively.

Figure 3. NTPDase2 is expressed in pulpal Schwann cells. (A) Immunostaining showed that NTPDase2-positive cells displayed small elongated nuclei (open triangles) and long processes projecting to the odontoblast layer (solid triangles) in dental pulp. (B1) Immunostaining showed that NTPDase2 staining was co-localized with S-100B staining in dental pulp. (B2) The ratio of NTPDase2 to S-100B staining density at NTPDase2-positive elements in odontoblast and subodontoblast layers. (B3) Correlation graph of red NTPDase2 signal and the green S-100B signal in B1. High correlation was observed between the NTPDase2 and S-100B signals, with a correlation R of 0.99 and an overlap coefficient of 0.98. Scale bars are 20 (A) and 30 (B1) μm , respectively.

with NTPDase2 staining beneath the odontoblast layer, while in the odontoblast layer, much of the NF staining was not accompanied with NTPDase2 staining (Fig. 2A1). The NF staining revealed the characteristic thin and singular nerve fibers with bulb-like enlargements, while NTPDase2 staining usually displayed thicker cell processes overlapping the nerve fibers. These observations suggest that NTPDase2 may not be expressed in axonal fibers, but most likely in Schwann cells with processes wrapping or accompanying the axonal fibers. We found that the ratio of NTPDase2 to NF staining density (in NF-positive elements) was higher in the subodontoblast layer than in the odontoblast layer (Fig. 2A2), which is in accordance with the phenomenon that nerve fibers lose their myelin as they enter the odontoblast layer. To provide direct evidence that NTPDase2 is not expressed in dental pulp axonal fibers, we performed Z-stack multiphoton confocal scanning at high resolution (2048 x 2048 pixels with a 0.5- μm pinhole and 0.5- μm Z-inter-distance). As illustrated in Fig. 2B1, the topogram derived from the Z-stack sections showed that the red NTPDase2 signal and the green NF signal are not co-localized, but are in a close spatial relationship. We then used Zen software (Zeiss) to analyze their co-localization in the Z-stack sections (Fig. 2B1). As shown in Fig. 2B2, the NTPDase2 signal and the NF signal were not correlated (correlation $R = 0.00$), with an overlap coefficient of 0.00. Furthermore, we demonstrated that the trigeminal ganglia

neurons were NTPDase2-negative, even though positive NTPDase2 staining was detected in the surrounding satellite cells and their processes (Fig. 2C).

NTPDase2 Is Expressed in Pulpal Schwann Cells

We observed NTPDase2-positive staining in pulpal cells with small elongated nuclei and long processes projecting into the odontoblast layer (Fig. 3A). To demonstrate that these NTPDase2-positive cells are Schwann cells, we performed co-immunostaining for Schwann cell marker S-100B. As shown in Fig. 3B1, the NTPDase2 staining overlapped with S-100B staining in the dental pulp. The ratio of NTPDase2 to S-100B staining density (in S-100B-positive elements) displayed a value around 1 in either the subodontoblast or the odontoblast layer (Fig. 3B2), which is different from that of NTPDase2 to NF staining (Fig. 2A2). We further analyzed the co-localization of NTPDase2 and S-100B (Fig. 3B1). As shown in Fig. 3B3, the red NTPDase2 and the green S-100B signals are completely co-localized (correlation $R = 0.99$), with an overlap coefficient of 0.98.

Connexin43 Is Expressed in Human Pulpal Odontoblasts

Since ATP has previously been shown to be released from cells through connexin43 hemichannels, we tested for connexin43 expression in odontoblasts by co-immunostaining for connexin43 and vimentin. As shown in Fig. 4A, connexin43 positive immunoreactivity was detected in human dental pulp. Punctuated and plank-like connexin43 staining, a characteristic of functional gap junction/hemichannel staining, was observed within the odontoblast layer, especially at contact sites between adjacent cells and at the pulp-dentin junctional areas. Unlike vimentin, connexin43 expression in odontoblast processes was sparse compared with that within the odontoblast layer, which is consistent with the lack of gap junctions in isolated odontoblast processes. Connexin43 expression was progressively reduced beneath the odontoblast layer and became essentially undetectable in the lower part of the dental pulp (Fig. 4A), an expression pattern in close agreement with that of vimentin.

NTPDase2 and Connexin43 Are Localized in Proximity within the Odontoblast Layer

To support the potential for ATP signaling in dental nociception, we examined the spatial relationship between NTPDase2 and connexin43 in the odontoblast layer. Similar to that shown in Fig. 1, punctuated NTPDase2-positive immunofluorescence was confirmed in the odontoblast layer and along nerve fibers projecting

into the odontoblast layer (Fig. 4B1). The punctuated NTPDase2 staining was closely localized with connexin43 staining only within the odontoblast layer, while close localization of NTPDase2 and connexin43 was not observed along the nerve fibers under the odontoblast layer (Fig. 4B1). To show that NTPDase2 was closely localized with connexin43 in the odontoblast layer, but not in the subodontoblast layer, we examined the ratio of connexin43 to NTPDase2 staining (in NTPDase2-positive elements) and found that it was close to 1 (0.82) within the odontoblast layer, while it was only 0.10 in the subodontoblast layer ($p < 0.01$, $n = 4$). Co-localization analysis further indicated that the NTPDase2 signal was slightly correlated with the connexin43 signal within the odontoblast layer (correlation $R = 0.64$), with an overlap coefficient of 0.52 (Fig. 4B3). Although NTPDase2 was confirmed to be present in the processes of Schwann cells, these results could not entirely exclude the possibility that NTPDase2 is also expressed in odontoblasts and other cell processes in the odontoblast layer.

DISCUSSION

Previous studies have shown that odontoblasts receive stimuli and participate in dental pain transmission (Magloire *et al.*, 2009). The findings of the present study indicated that ATP signaling is likely involved in sensory signal transmission from odontoblasts to adjacent nerve fibers. We found that NTPDase2 is expressed in Schwann cells along pulp nerve fibers that project into the odontoblast layer, where NTPDase2 is closely localized with connexin43. The intimate spatial relationship between connexin43 and NTPDase2 indicates the existence of molecular mechanisms for ATP release and degradation within the odontoblast layer. Thus, our findings support that ATP signaling may mediate the sensory transmission from odontoblasts to pulpal nerve fibers in dentin sensitivity and dental pain.

The involvement of ATP in peripheral nociception was based on observations that pain was induced by injection of ATP into human blisters, skin, and rat neck muscles (Bleehen and Keele, 1977; Hamilton *et al.*, 2000). Subsequent evidence convincingly demonstrated that ATP acts as a pain mediator *via* activation of purinergic receptors on peripheral

Figure 4. NTPDase2 proteins are localized close to connexin43 in the dental pulp odontoblast layer. **(A)** Double-immunostaining for connexin43 and vimentin in dental pulp. Positive punctuated and plank-like staining for connexin43 was detected in odontoblast layer, especially at the contact region between the adjacent cells (arrows) and at the pulp-dentin junction region (open triangles). The odontoblasts were identified by their positive staining of vimentin. **(B1)** Double-immunostaining for NTPDase2 and connexin43 in dental pulp. Positive NTPDase2 staining was observed along the nerve fibers (open triangles) projecting from the dental pulp to the odontoblast layer. Positive punctuated staining for NTPDase2 was also detected in the odontoblast layer, which was closely localized to connexin43 staining (arrows). **(B2)** The ratio of NTPDase2 to connexin43 staining density at NTPDase2-positive elements in odontoblast and subodontoblast layers. **(B3)** Correlation graph of red NTPDase2 signal and green connexin43 signal in B1. Slight correlation was observed between the NTPDase2 and connexin43 signals, with a correlation R of 0.64 and an overlap coefficient of 0.52. **(C)** Diagram shows the hypothesis for dentin sensitivity. External thermal, mechanical, and chemical stimuli induced ATP release through connexin43 gap junction hemichannels. ATP stimulated adjacent sensory pulp nerve fibers *via* activation of ionotropic P2X3 receptors. ATP signaling and dental nociception were terminated by the activity of NTPDase2 present in the processes of dental pulp Schwann cells. OB, odontoblast. Scale bars are 10 (A and $B1$) μ m.

nerves (Chen *et al.*, 1995; Hamilton, 2002; Wirkner *et al.*, 2007). Ionotropic purinergic P2X3 receptors are expressed in nerve fibers of the dental pulp (Alavi *et al.*, 2001; Jiang and Gu, 2002; Renton *et al.*, 2003), consistent with a role for ATP signaling in dental nociception (Magloire *et al.*, 2010). Millimolar concentrations of ATP are present in cells and can actively be released into the extracellular milieu *via* either exocytosis or through certain ion channels (Fitz, 2007). Among ATP-permeable channels, gap junction hemichannels composed of connexin43 represent a strong potential candidate for ATP release (Gomes *et al.*, 2005; Zhao *et al.*, 2005). Connexin43 forms channel-like hexamers permeable for small molecules including ATP, and are gated by both intracellular and extracellular signals, such as Ca^{2+} , CO_2 , IP_3 , and NO (Retamal *et al.*, 2009; Liu *et al.*, 2010; Nakagawa *et al.*, 2010). We demonstrated that connexin43 was expressed in human odontoblasts, suggesting that, when stimulated, odontoblasts possess the molecular apparatus to release ATP into the dental pulp.

ATP signaling is terminated by a scavenging mechanism that dictates the cellular response duration by stepwise degradation of extracellular nucleotides to nucleosides (Zimmermann and Braun, 1996). The presence of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent ecto-ATPases in mammalian dental pulp has long been demonstrated by enzyme histochemistry (Severson, 1968). Such activity is attributable to 1 or more of the 4 ecto-NTPDase family members, *i.e.*, NTPDases1, 2, 3, and 8 (Zimmermann, 2000). We found that NTPDase2 was expressed in pulpal Schwann cells with processes associated with nerve fibers and projecting into the odontoblast layer. It has been generally accepted that fluid movement in the dentinal tubules results in the activation of myelinated A δ fibers, causing sharp pain (dentin sensitivity). The present findings suggest that NTPDase2 is likely the predominant enzyme to modulate ATP signaling in A δ fibers responsible for dentin sensitivity. Immunoreactivity for NTPDases1 and 3 were also detected in dental pulp around blood vessels, in the subodontoblastic nerve plexus, and in the odontoblast layer, which suggests that other NTPDases might also contribute to the extracellular ATP degradation in dental pulp. Since NTPDase2 hydrolytic activity is considerably stronger for ATP compared with ADP (Zimmermann and Braun, 1996; Kukulski *et al.*, 2005), NTPDase2 activity will significantly change the ratio of extracellular ATP/ADP, which will differentially affect the activation of P2Y (metabotropic, multiple nucleotides) *vs.* P2X (ionotropic, ATP-selective) receptors. Therefore, identification of NTPDase and purinergic receptor expression patterns in normal and inflammatory dental pulp will help to further elucidate the precise role of purinergic signaling in dental nociception.

Based on available evidence and findings of the present study, we propose a hypothesis for dentin sensitivity and dental pain: that odontoblasts receive thermal, mechanical, and chemical stimuli through respective sensory receptors, which subsequently induce ATP release *via* connexin43 gap junction hemichannels, and then stimulate adjacent sensory pulp nerve fibers through activation of ionotropic ATP receptors. ATP signaling and dental nociception are eventually terminated by the activity of NTPDase2 expressed in the processes of dental pulp

Schwann cells and other possible structures, such as the nerve fibers and odontoblasts (Fig. 4C). If supported, this proposed mechanism may reveal several potential therapeutic targets (*e.g.*, connexin43 hemichannels, purinergic receptors, and ecto-ATPase activity) for alleviating dental pain.

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